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Woodhead Publishing Series in Food Science, Technology and Nutrition: Number 265

Advances in Fermented Foods and Beverages

Improving Quality, Technologies and Health Benefits

Edited by

Wilhelm Holzapfel

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80 High Street, Sawston, Cambridge, CB22 3HJ, UK
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Langford Lane, Kidlington, OX5 1GB, UK

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Control Number: 2014940218

ISBN 978-1-78242-015-6 (print)
ISBN 978-1-78242-024-8 (online)

Typeset by TNQ Books and Journals
www.tnq.co.in
Printed and bound in the United Kingdom

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Probiotic fermented foods and health promotion

1

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1.1 Introduction

Food fermentation has, throughout much of human history, been the most common way of preserving perishable foods, thereby maintaining and in some cases even improving the nutritional value of these foods. Genesis 18:8 refers to how Abraham serves curds and milk to his guests. Not surprisingly, some of these fermented foods were perceived to be inherently healthy. The mechanism behind this preservation was not clarified until 1857, when Louis Pasteur identified "lactic yeast" as the source of lactic acid fermentation. A first "scientific" promotion of fermented food specifically as a health product came in the early 1900s with Ilya Metchnikoff, who advertised yogurt, fermented with the Bulgarian bacillus, and insisted it would contribute to longevity (Metchnikoff, 1907). In the 1930s, Minoru Shirota specifically isolated a health-promoting microbe and introduced the oldest still-existing probiotic food, Yakult.

Probiotics have been defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Maintaining viability imposes some technological requirements on the manufacturing of the probiotic food product; the minimal counts should be guaranteed until the end of shelf life. The required level of these counts is likely to depend on the probiotic strain and the intended health benefit. As a rule of thumb, a minimum of 10^9 colony-forming units (CFU)/consumption is used (Forssten, Sindelar, & Ouwehand, 2011). A correct approach would be to use a minimum dose according to that used in studies documenting the given health benefit.

Although probiotics are widely consumed as dietary supplements, the focus of the present chapter is on fermented probiotic foods. Most commercially available probiotics belong to the genera *Bifidobacterium* and *Lactobacillus*; strains from other genera are being marketed as well, but these rarely find application in fermented foods and will thus not be discussed here.

1.2 Probiotic fermented foods and health promotion

Probiotics can be included in many different foods, fermented and unfermented. The food matrix is known to have an important role in the stability of probiotics (Forssten et al., 2011). Manufacturers take this into account when developing such foods. However, which role the matrix plays in efficacy is less well understood. For some strains

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00001-3

it does not seem to play a role, while it does for others. This topic requires further investigation and is not discussed here owing to lack of information. It is important to realise that diet is likely to be a bigger cause of variation than the matrix of one or another probiotic food (Kelly, Colgan, & Frank, 2012).

1.2.1 Different types of fermented probiotic foods

Numerous fermented foods exist, but not all of these food classes can be linked with the probiotic concept, such as alcoholic beverages and fermented meats, or foods in which the fermentations merely fulfil a technological function in the processing of the food, such as in coffee, tea and cocoa.

1.2.2 Fermented dairy foods/beverages

Fermented dairy foods are the most widely used carriers of probiotics in Western societies, in particular yogurt and yogurt-type drink products. This may have historic reasons as mentioned above, but it has also practical reasons. Most commercially available probiotics belong to the genera *Bifidobacterium* and *Lactobacillus*. Members of these genera tend to grow well in milk, and it may even be their most common habitat.

In fermented probiotic dairy products, probiotics are usually accompanied by starter cultures such as *Lactobacillus delbrueckii* subsp. *bulgaricus* and/or *Staphylococcus thermophilus*. There are two main reasons for the inclusion of starter cultures in a probiotic product. The first is technological: starter cultures provide structure and flavour to the product. In addition, starter cultures support functionality; some probiotics do not grow well as a pure culture in milk and grow better in symbiosis with a starter culture.

Besides fresh fermented dairy products, probiotics can be included in nonfermented milk such as the so-called "sweet acidophilus milk". The milk is not sweet in the sense of sweet taste, but is referred to as such because it is not sour (Mcdonough, Hitchins, Wong, Wells, & Bodwell, 1987).

Furthermore, probiotics can be included in cheese. Despite the long ripening and shelf life of cheese, probiotic counts appear to be stable in cheese for months. By optimizing fermentation techniques, it is feasible to produce a good-quality cheese with high probiotic counts so that a standard portion of cheese (15 g) provides a dose of at least 10^9 CFU (Ibrahim et al., 2010).

1.2.3 Fermented soy foods/beverages

In Asian societies, fermented soy foods are common. They are, however, not typically used to function as a delivery matrix for probiotics. Probiotic soy products most commonly designed to resemble dairy products are called "soy yogurts". Lactic fermentation of "soy milk" may improve the bioavailability of isoflavones, improve mineral availability and increase the level of B vitamins (Rekha & Vijayalakshmi, 2010). Fermentation has the additional advantage of reducing the bean flavour of soy foods. In general, soy yogurts aim at providing a vegetarian/vegan alternative to regular (probiotic) yogurt and are not developed specifically as carriers for probiotics. Nevertheless,

the stability of probiotics in a fermented soy matrix is usually good and is very similar to that in yogurt.

1.2.4 Fermented plant foods/beverages

Lactic fermented plant foods are common in Asian, African and East European societies. These are fermented vegetables such as sauerkraut and kimchi, which are mainly based on spontaneous fermentations dictated by the storage conditions and ingredients used for this fermentation (Jung et al., 2011).

Lactic fermented cereals are common, such as in sourdough, although obviously subsequent processing (baking) will not allow survival of microbes. Lactic fermentation of cereals otherwise contributes to improved flavour and reduces phytic acid activity, thereby improving biological availability of minerals such as iron. Lactic acid bacteria involved in the fermentation may also produce vitamins, in particular B vitamins (Nout, 2009). Traditionally, fermented foods of vegetable or cereal origin have not been used as carriers for probiotics. However, it cannot be excluded that the microbes involved in these fermentations have a direct influence on health, similar to a probiotic. Specifically designed foods are successfully marketed, usually on the basis of fermented cereals, and have been studied as probiotic carriers. Such products have only sporadically been used as carriers of probiotics (Molin, 2001). The stability of selected probiotics in fermented cereal-based products has been documented to be good.

1.2.5 Other carriers of probiotics

Probiotics have been included successfully in ice cream. Relevant to this book is the production of ice cream based on frozen yogurt, which basically follows the same manufacturing as probiotic yogurt (Davidson, Duncan, Hackney, Eigel, & Boling, 2000). But probiotics can also be included in the ice cream base or in a chocolate coating of the ice cream.

As mentioned earlier, supplements are probably the most common format for probiotic consumption, in addition to dairy products. Furthermore, probiotics have been included in fruit juices, which is particularly challenging because of the low pH (<4) and the presence of various natural antimicrobial components in fruits (such as benzoate and anthocyans). Probiotics have also been included in chocolate (Vriens, 2009), providing extremely long shelf lives of up to 2 years – not so much to produce probiotic chocolate bars, but to provide a probiotic-carrying chocolate coating to various foods. These applications fall outside the focus of this chapter, but indicate that formats for probiotics exist.

1.3 Health benefits deriving from the consumption of probiotics

As discussed above, the role that the food matrix plays in the efficacy of probiotics, if any, has been insufficiently investigated. Many studies have been performed with

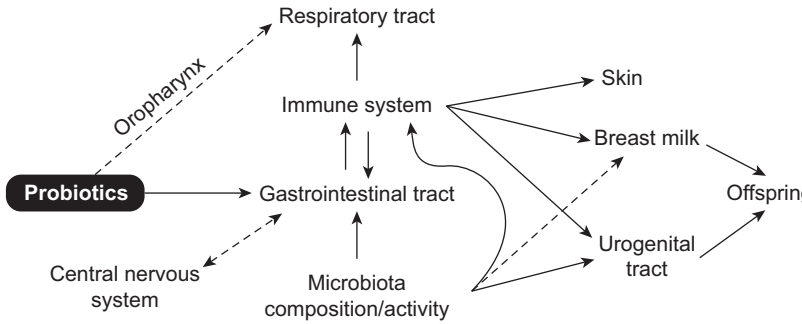

Figure 1.1 Schematic representation of how consumed probiotics may exert their benefits in the intestine and beyond.

probiotics as supplements because it is easier to produce a placebo and the logistics are easier than for a fresh fermented dairy product. We therefore present below the results of probiotic consumption, regardless of the matrix in which it was consumed. Although probiotics are consumed, they can have health benefits beyond the gastrointestinal tract. Potential mechanisms for these extra-intestinal effects are depicted in Figure 1.1.

1.4 Gastrointestinal health

Given that probiotics are consumed, it is not surprising that the prime target for probiotic use has traditionally been various diarrhoeas, with intestinal health as the main benefit.

1.4.1 Modulating microbiota composition

With the advances in molecular biological techniques, such as metagenome sequencing, 16S rDNA pyrosequencing, and quantitative real-time polymerase chain reaction, an unprecedented understanding of the composition and genetic diversity of the intestinal microbiota has been possible. International research consortia have sequenced intestinal microbiomes of an increasing number of subjects. At the same time, metabolomics provides us with an ever deeper insight into the metabolic capacity of the intestinal microbiota. Notwithstanding these advances, it is still not possible to define a "healthy" or "normal" microbiota (Qin et al., 2010).

One of the early targets of probiotics has been the improvement of the composition of the intestinal microbiota. From the above, it is obvious that criteria for "improving" intestinal microbiota composition are at present not clearly defined. Nevertheless, some options exist for improving intestinal microbiota. Keeping under control potential pathogens such as *Clostridium difficile* and *Helicobacter pylori*, which can be present in low numbers in the microbiota of asymptomatic healthy carriers, may be beneficial (see following text). Furthermore, although the composition of a "healthy" or "normal" microbiota is not known, it is assumed that the microbiota a

healthy subject carries is appropriate for that particular host. It is therefore reasonable to assume that allowing a disturbed microbiota (as a consequence of whatever stress) to return to "normal" quicker or reduce the aberrance from "normal" is a health benefit. However, increasing levels of faecal bifidobacteria, hitherto one of the traditional targets, is not sufficient. The proof that increasing *Bifidobacterium* numbers is a health benefit per se is lacking.

1.4.2 Infectious diarrhoea

Infectious diarrhoeas can have many different aetiologies, viz., viral, bacterial, protozoal or chemical. The various causes also make it a challenging target. In children, the effect of probiotics on the duration of rotavirus diarrhoea has been well documented for various strains (Van Niel, Feudtner, Garrison, & Christakis, 2002). Despite this and other documented benefits of probiotics for children, the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) does not encourage the widespread use of probiotics in infant nutrition (Braegger et al., 2011). In adults, travellers' diarrhoea is relatively common, but probiotics have shown inconsistent results (Mcfarland, 2007; Ritchie & Romanuk, 2012). This inconsistency is most likely attributable to the heterogeneity of diarrhoeal causes.

1.4.3 Antibiotic-associated diarrhoea

Antibiotics have provided great medical progress in the treatment of bacterial infectious diseases. However, because of their antimicrobial nature, one of their side effects is a disturbance of the intestinal microbiota. This antibiotic-related disturbance was recognised even in the early days of therapeutic use of antibiotics around 1950, and, as a response, the term "probiotic" was probably first introduced by Kollath in 1953 and Vergio in 1954 (Holzapfel & Schillinger, 2002). This kind of disturbance, or "dysbiosis", may lead to intestinal complaints and, in the worst cases, to antibiotic-associated diarrhoea (AAD). AAD is caused by various pathogens, most notably *C. difficile*, followed by *Staphylococcus aureus* and *Clostridium perfringens*. However, these three pathogens together explain, at most, only half of the reported cases of AAD. The cause of the remaining AAD cases is largely unknown. Many probiotic strains and combinations of probiotic strains have been documented to reduce the incidence and/or duration of AAD. This is among one of the best documented health benefits of probiotics (Hempel et al., 2012). Probiotics do not replace the microbes that are reduced by antibiotic therapy, but support the microbiota in maintaining or returning more quickly to its original composition (Engelbrektson et al., 2009). Probiotics are highly successful in the prevention, shortening and/or relief of AAD, whereby one case of AAD can be prevented for every eight patients treated with probiotics (Avadhani & Miley, 2011).

1.4.4 Necrotising enterocolitis

Premature infants, and in particular very low birth weight infants, are at risk of developing necrotising enterocolitis (NEC). The mortality of NEC is substantial. Probiotics

provide one of the few preventive treatment options. While many probiotics on their own do not provide a significant reduction in NEC incidence, meta-analyses clearly show a reduced incidence of NEC and reduced mortality (Wang, Dong, & Zhu, 2012). Although the aetiology of NEC is poorly understood, it is likely to have an infectious component. By competitive exclusion, probiotics may prevent the establishment of potentially pathogenic microbes. Although preterm infants are a population at risk for infection, administering probiotics has not been reported to lead to adverse events; sepsis has neither been reported to be reduced by probiotic therapy nor been observed to be increased. As almost all tested probiotics have been observed to reduce NEC risk, it is tempting to argue that the often suggested strain specificity of health effects is not valid for NEC and that in this case there is a health benefit of probiotics in general.

1.4.5 Helicobacter pylori *infection*

Helicobacter pylori can be a resident of the gastric mucosa. It is the causative agent of gastric ulcer and is thought to cause gastric cancer. Most probiotics have been selected to survive gastric conditions and many produce antimicrobial substances; probiotics could therefore be effective against *H. pylori* infection. However, this appears not to be the case. Nevertheless, probiotics can play a valuable role in *H. pylori* eradication, which has serious side effects. In analogy with the effects of probiotics on AAD, probiotics have been observed to reduce the side effects of *H. pylori* eradication therapy, thereby improving compliance and also otherwise improving the success rate (Wang, Gao, & Fang, 2013).

The question is whether we should strive for the eradication of *H. pylori*. Carriage or prior exposure to the organism has been found to be associated with reduced risk for allergy and obesity. It is possible that *H. pylori* should only be kept under control, and probiotics could play an important role here.

1.4.6 Intestinal transit

Slow colonic transit is associated with constipation, though not all constipation is caused by slow transit: transit may be normal, but obstruction may cause constipation. Constipation correlates well with hard stools, as measured with the Bristol Stool Scale (Lewis & Heaton, 1997). There are several ways to determine intestinal transit, i.e. scintigraphy, radio-opaque pellets and remote recording capsules. Dyes have also been used, but these are inaccurate as their excretion is strongly correlated with defecation frequency. Slow colonic transit is thought to increase the risk of, among others, colorectal cancer and diverticulitis. Sufficient fluid and fibre intake are recommended but are not always adequate. Shortening of intestinal transit should not lead to diarrhoea. Selected probiotic strains such as *Bifidobacterium animalis* subsp. *lactis* HN019 and *B. animalis* subsp. *lactis* DN-173 010 have been documented to normalise slow intestinal transit (Ibrahim & Ouwehand, 2011; Waller et al., 2011). The mechanism by which probiotics are able to shorten colonic transit remains to be established, although short chain fatty acid production may be one of the contributing factors.

1.4.7 Irritable bowel syndrome

Irritable bowel syndrome (IBS) is one of the most common functional gastrointestinal disorders in industrialised countries, affecting an estimated 2–20% of the population (Rey & Talley, 2009). IBS is usually diagnosed according to the so-called Rome III criteria, which comprise prolonged and recurrent abdominal pain or discomfort along with disturbances in bowel movements (diarrhoea and/or constipation). Along with this, patients usually experience gastrointestinal symptoms such as bloating, abdominal distension and flatulence. Although the exact mechanisms behind IBS are still unclear, visceral sensory and motor disturbances, as well as increased gut permeability and inflammation, may play an important role. IBS patients appear to have an altered microbiota in comparison to healthy subjects; whether this is the cause or the effect of IBS is, however, currently unknown. Probiotics such as *Bifidobacterium infantis* 35624 have shown beneficial effects on symptoms of IBS (Brenner, Moeller, Chey, & Schoenfeld, 2009). However, the beneficial effects are often symptom-specific and seem to depend on the specific strain, dose and duration (Clarke, Cryan, Dinan, & Quigley, 2012). The proposed mechanisms behind the efficacy of probiotics include decreasing pain radiation, as has been shown for *Lactobacillus acidophilus* NCFM (Rousseaux et al., 2007), and colonisation of pathogenic/gas-producing bacteria in the intestine and modulating the host immunity (Clarke et al., 2012).

1.4.8 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is, as the name suggests, a collection of chronic inflammatory conditions of the intestine, such as ulcerative colitis (UC), referring to inflammation of and restricted to the colon; Crohn disease, being an inflammation that can be anywhere from mouth to anus; and pouchitis, and inflammation of an ileoanal pouch that has been constructed after severe UC. The aetiology of the disease is poorly understood but the microbiota is clearly involved, as germ-free animals prone to IBD do not develop the disease until they are colonised with a microbial population. One of the microbiota components involved in IBD has been found to be a lack or low level of *Faecalibacterium prausnitzii* (Sokol et al., 2009). The disease can be brought into remission with anti-inflammatory drugs, but tends to flare up after some time (Hedin, Whelan, & Lindsay, 2007). Probiotics have not been successful in inducing remission, but they have been found to prolong remission. In particular, a multi-strain probiotic product, VSL#3 (consisting of *Bifidobacterium longum*, *B. infantis*, *Bifidobacterium breve*, *L. acidophilus*, *Lactobacillus casei*, *L. delbrueckii* subsp. *bulgaricus*, *Lactobacillus plantarum* and *S. thermophilus*), has been shown to be successful in the treatment of pouchitis (Mimura et al., 2004).

1.5 Immune health

The human gastrointestinal microbiota, as well as microbiota of other mucosal body surfaces, consists of a large number of bacterial species that are living in symbiosis

with the host and are able to cross-talk with the intestinal and adaptive immune system. It has become obvious that different microbes are able to influence the development and function of the immune system, and the balance between the so-called beneficial versus potentially harmful microbes has a pivotal role in either the maintenance or disruption of the inflammatory control. Therefore, the protective and immunomodulatory role of probiotic microbes has been investigated in several aspects of the immune system function and different infectious diseases (Purchiaroni et al., 2013).

1.5.1 Respiratory tract infections

The common cold or upper respiratory tract infection (URTI) is a frequently occurring disease in all age groups. URTI are usually viral infections and are the reason for the largest proportion of school and work absenteeism. None of the current interventions (vitamins, remedies and antibiotics) are very effective in preventing colds. The effect of probiotics is still less well studied, and reviews and meta-analyses on the topic show a slightly positive effect but variable results, mainly owing to the diverse strains and doses used and different age groups tested (Hao, Lu, Dong, Huang, & Wu, 2011; Kang, Kim, Hwang, & Ji, 2013). Thus, also in this application, the effects seem to be strain dependent. In a double-blind, placebo-controlled study with young children (3–5 years of age) attending daycare, *L. acidophilus* NCFM alone or in combination with *B. animalis* subsp. *lactis* Bi-07 was effective in reducing fever, rhinorrhoea and cough duration and the incidence of antibiotic prescription, as well as the number of missed daycare days. The supplementation continued for 6 months over the winter period (Leyer, Li, Mubasher, Reifer, & Ouwehand, 2009). In another study with older children (aged between 8 and 13 years), a two-strain probiotic product with *L. acidophilus* and *Bifidobacterium bifidum* consumed twice a day for 3 months was effective in reducing the risk of fever, cough, rhinorrhoea or school absence in comparison to placebo (Rerksuppaphol & Rerksuppaphol, 2012). Also, college students living on campus (aged between 18 and 24) gained benefits from consuming *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis animalis* subsp. Bb-12 during the spring semester; the duration of URTI was reduced by 2 days in the probiotic group and they also recorded lower disease severity scores in comparison to placebo group. Moreover, the number of missed school days attributable to illness was less in the group receiving probiotics compared to the placebo group (Smith, Rigassio-Radler, Denmark, Haley, & Touger-Decker, 2013). Furthermore, in highly active adult athletes at higher risk to develop URTI because of increased endurance-based physical activity, the use of *L. casei* Shirota for 16 weeks reduced the frequency of URTI episodes, although the severity and duration of the illness were not affected (Gleeson, Bishop, Oliveira, & Tauler, 2011). Although the results from studies on adult populations are mixed, it is tempting to conclude that probiotics would be an effective method to reduce the incidence of this very common disease in children and in adults that causes a huge economic burden owing to school and work absenteeism. The precise mechanism of protective effects remains to be elucidated, but it has been speculated that probiotics are able to compete against pathogens, enhance the barrier function in respiratory epithelia, and stimulate the cellular

immunity in airways to confer their protective effects against URTI (Ahanchian, Jones, Chen, & Sly, 2012).

1.5.2 Allergies, asthma and atopic dermatitis

An ever-increasing number of children in developed countries suffer from allergies, asthma and atopic dermatitis (AD), which are regarded as a failure in the development of a balanced immune response. The highly increased prevalence of these diseases indicates that environmental factors play a significant role in the disease development, as the escalation has been too fast for a shift in the genetic constitution to occur. The lack of microbial exposure early in life attributable to changed living conditions and diet are thought to lead to a dysbiosis in the microbiota composition and skew the immune response against common environmental antigens, leading to loss of oral tolerance and development of clinical symptoms (Fujimura, Slusher, Cabana, & Lynch, 2010). Reduced bacterial diversity in the infant's gut has been associated with an increased risk of allergic sensitisation and allergic rhinitis (Bisgaard et al., 2011), as well as atopic dermatitis (Nylund et al., 2013). Therefore, the protective role of probiotics has been investigated in these diseases, and probiotic supplementation early in life and also prenatally (used by the mother during pregnancy) seems to have a role in preventing AD and IgE-associated AD (Doege et al., 2012; Pelucchi et al., 2012; Toh, Anzela, Tang, & Licciardi, 2012). In initial randomised human studies using *L. rhamnosus* GG, it was shown that with probiotic supplementation in the mothers' diet before delivery and in infants after birth, a 50% reduction in AD prevalence in a high-risk population was achieved (Kalliomäki et al., 2001), while the protective effect persisted at least up to 7 years of age (Kalliomäki et al., 2007). Very similar results have also been demonstrated for another *L. rhamnosus* strain (HN001) (Wickens et al., 2008; Wickens et al., 2012). The key protective mechanism of *L. rhamnosus* against eczema may be related to its ability to promote the growth of other beneficial microbial species in the gut, resulting in a more stable and functionally redundant gastrointestinal community of the infant (Cox et al., 2010). In allergic diseases, the evidence of protective effects of probiotics is more limited (Toh et al., 2012), although some recent data point to reduced severity of seasonal pollen allergies in children and in adults. *L. acidophilus* NCFM and *B. animalis* subsp. *lactis* Bl-04 alleviated the symptoms of allergic rhinitis in children during the birch pollen season, and this effect was shown to derive from the prevention of pollen-induced infiltration of eosinophilic cells into the nasal mucosa during probiotic supplementation (Ouwehand et al., 2009). In adults, *B. animalis* subsp. *lactis* NCC2818 has also been shown to impact the function of blood lymphocytes and reduce concentrations of proinflammatory cytokines, and thus mitigate allergic symptoms during grass pollen season (Singh et al., 2013). The anti-inflammatory effect may derive from the same mechanisms as in eczema: the probiotic microbes improve the balance of the normal microbial composition and thus impact the function of the immune cells and the clinical symptoms of allergies. Intriguingly, the microbiota composition of allergic individuals has indeed been shown to fluctuate during the allergy seasons, and probiotic

intervention reduces this fluctuation (Odamaki et al., 2007; Ouwehand et al., 2009). This seems to hold true also in food allergies, where altered microbiota composition is seen between allergic and healthy individuals (Canani & Di Costanzo, 2013; Noval Rivas et al., 2013), although no evidence on the protective effects of probiotics exists to date. However, many studies have failed to demonstrate the protective effects of probiotics against allergies and eczema (Koyama et al., 2010; Kukkonen et al., 2007; Taylor, Dunstan, & Prescott, 2007), and their use in clinical practice is still not officially encouraged (Fiocchi et al., 2012). Therefore, although some data are promising thus far (Isolauri, Rautava, & Salminen, 2012), more research is still needed to demonstrate strain-specific benefits in the prevention of a range of allergic diseases (Prescott & Nowak-Wegrzyn, 2011; Toh et al., 2012).

1.5.3 Urogenital infections

The human vagina is also colonised by over 250 different species of bacteria comprising the vaginal microbiota, and it is dominated particularly by *Lactobacillus* species. Similarly to the colonic microbiota, dysbiosis in the vaginal microbiota has been linked with diseases such as bacterial vaginosis and aerobic vaginitis, where increased levels of pathogenic bacteria in the microbial community cause inflammation (Li, McCormick, Bocking, & Reid, 2012). Probiotic treatment with different *Lactobacillus* species holds much promise in the treatment of urogenital infections. This may also have even more long-lasting effects besides reduced risk of infections, since the vaginal microbiota is associated with conception and pregnancy and it is the first microbial community that the newborn faces during labour (Reid, Bisanz, Monachese, Burton, & Reid, 2013). However, to date very few studies have explored probiotic effects in this area of human health.

1.5.4 Rheumatic diseases

Evidence exists that dysbiosis of colonic microbiota is also contributing to initiation and perpetuation of inflammatory joint diseases, such as rheumatoid arthritis (RA) and spondyloarthritis. Theories of the mechanisms through which the microbiota may influence the pathogenesis of these diseases include increased permeability of the gastrointestinal epithelia, loss of tolerance towards indigenous microbiota members, and trafficking of activated proinflammatory immune cells and cytokines and antigenic material between the gastrointestinal tract and joints (Yeoh, Burton, Suppiah, Reid, & Stebbings, 2013). The potential of probiotics to influence the inflammatory processes in these diseases has been investigated to some extent in animals and to a very limited extent in humans. Two human studies on RA patients have not been able to demonstrate any benefits attributable to probiotic use on the clinical symptoms of the disease, but the reported quality of life (Pineda Mde et al., 2011) and wellbeing of the RA patients (Hatakka et al., 2003) were improved during probiotic therapy. Probiotic supplementation did not interfere with the medical therapy of RA, when probiotics were used simultaneously with sulfasalazine therapy (Lee et al., 2010).

1.6 Metabolic health

In addition to the evidence on the impact on human immunity, evidence has accumulated during the past decade on the capability of the intestinal microbiota to participate in and also influence the metabolism of its human host. The effects of probiotics are still little studied in this area, but considering the global epidemic of obesity and metabolic diseases, this application holds huge possibilities if probiotic efficacy can be scientifically demonstrated and the epidemic of "Western diseases" ameliorated.

1.6.1 Obesity

Obesity is a metabolic disorder that is impacting humans at an increasing pace all over the world, and it predisposes people to diseases such as increased blood pressure, atherosclerosis, metabolic syndrome and diabetes. The role of intestinal microbiota composition in obesity began to be uncovered when an altered ratio of two dominant colonic microbial phyla, *Bacteroidetes* and *Firmicutes*, was demonstrated between lean and obese rodents (Bäckhed et al., 2004; Ley et al., 2005) and humans (Turnbaugh et al., 2009). Furthermore, decreased levels of bifidobacteria and increased levels of *S. aureus* in infant faeces have been shown to precede increased adiposity of children at 7 years of age (Kalliomäki et al., 2008). The obese microbiome has an increased capacity to harvest energy from the diet and it impacts the deposition of fat into adipose tissue (Bäckhed, Manchester, Semenkovich, & Gordon, 2007; Samuel et al., 2008). On the other hand, the diet that humans typically consume influences the microbiota structure, and thus the role of microbes in energy balance together with dietary factors and genotype all seem to contribute to increased adiposity and development of obesity (Sanz, Rastmanesh, & Agostonic, 2013). Owing to the partial contribution of colonic microbes to the obesity epidemic, intervention trials have investigated the possibility of probiotics to impact adiposity. Few studies have demonstrated a beneficial effect of probiotic microbes on adiposity: in an intervention trial with *Lactobacillus gasseri* SBT2055, the consumption of fermented milk with this probiotic for 3 months was shown to significantly reduce abdominal visceral and subcutaneous fat, weight and body mass index (BMI) of subjects with elevated BMI at the beginning of the trial (Kadooka et al., 2010). In another intervention with pregnant women, probiotic *L. rhamnosus* GG, together with *B. animalis* subsp. *lactis* Bb-12 and dietary counselling, was shown to reduce central adiposity in the postpartum period (Ilmonen, Isolauri, Poussa, & Laitinen, 2011). In a 10-year follow-up study on children, pre- and postnatal consumption of *L. rhamnosus* GG inhibited excessive weight gain in children (Luoto, Kalliomaki, Laitinen, & Isolauri, 2010). It goes without saying that more research is needed in this area before definite answers on the protective mechanisms of probiotics against obesity can be given (Sanz et al., 2013).

1.6.2 Metabolic syndrome and diabetes

In the same study in which *L. rhamnosus* GG and *B. animalis* subsp. *lactis* Bb-12 were shown to impact central adiposity, the probiotic intervention also improved glucose tolerance and insulin sensitivity during pregnancy and 12 months postpartum

(Laitinen, Poussa, Isolauri, Nutrition, & Mucosal Immunology and Intestinal Microbiota Group, 2009). Furthermore, the risk of the mothers to develop gestational diabetes was decreased in the probiotic groups (Luoto, Laitinen, Nermes, & Isolauri, 2010). Thus, in addition to impacting the energy balance of humans, gut microbiota and probiotics seem to participate in the regulation of glucose and insulin metabolism and impact the risk of developing metabolic diseases.

Several other studies have investigated the link between colonic microbes, probiotics and onset of diabetes, although mainly in animal models. An inverse correlation between the numbers of colonic bifidobacteria and serum concentrations of lipopolysaccharide (LPS), a component of the bacterial cell wall, has been reported in mice fed a high-fat diet (Cani et al., 2007). These studies have led to the hypothesis of metabolic endotoxaemia, where a high-fat diet causes dysbiosis in the colonic microbiota composition and leads to increased permeability of the intestine, further contributing to increased concentrations of LPS and systemic low-grade inflammation. Chronic inflammation impairs the function of several organs and contributes to the development of metabolic syndrome and diabetes (Cani et al., 2008). These results have been, to some extent, seen also in humans, since endotoxaemia (i.e. increased LPS in blood) was significantly associated with increased risk for prevalent and incident diabetes, and the LPS activity was linearly related to several components of metabolic syndrome (Pussinen, Havulinna, Lehto, Sundvall, & Salomaa, 2011). Nevertheless, although the link between microbiota modulation, metabolic health, and development of metabolic syndrome and diabetes has been demonstrated, the proof of beneficial effects deriving from probiotics is still mostly lacking. In animals, the antidiabetic effects of *B. animalis* subsp. *lactis* 420 (Amar et al., 2011) and probiotic dahi containing *L. acidophilus* and *L. casei* (Yadav, Jain, & Sinha, 2007) have been demonstrated, but in humans the results have been inconsistent; neither *L. casei* Shirota (Leber et al., 2012) nor *L. salivarius* Ls-33 (Gobel, Larsen, Jakobsen, Molgaard, & Michaelsen, 2012) was able to influence the parameters of metabolic syndrome or inflammatory markers in adolescents and adults.

1.6.3 Hypercholesterolaemia

Elevated serum cholesterol level is an acknowledged risk factor for atherosclerosis and coronary heart disease, which are still among the leading causes of mortality in the world. Lactic acid bacteria have been recognised as potential mediators that would have a beneficial impact on hypercholesterolaemia; lactobacilli possess enzymes that participate in the deconjugation of bile salts to form primary bile acids, thereby inhibiting micelle formation, cholesterol absorption from the intestine and the enterohepatic circulation of cholesterol. Also, systemic effects are thought to derive from the metabolic activity of probiotic microbes and increased concentrations of fatty acids (acetic, propionic and butyric acids), which may impact the cholesterol synthesis and lipid metabolism systemically (Aggarwal, Swami, & Kumar, 2013). Animal studies have shown quite consistent cholesterol-lowering effects of various probiotic microbes (Huang, Wang, et al., 2013; Huang, Wu, et al., 2013), but the data from human studies are very inconsistent (Aggarwal et al., 2013).

1.7 Summary

Probiotics in fermented foods have the potential to modify the composition of the intestinal microbiota, which has been shown to be altered in several diseases of the human host. *Lactobacillus* and *Bifidobacterium* are the two probiotic microbial genera that have been investigated by far the most, perhaps because of their dominance in the traditionally used fermented foods and (except for *Bifidobacterium dentium*) the absence of typical pathogenic microbes within these genera. Through restoring the intestinal microbiota balance and impacting on the function of the immune system, probiotics may be effective in preventing the development of chronic immune-mediated diseases such as metabolic, inflammatory and bowel diseases. Strong scientific evidence suggests that different probiotic microbes may reduce infants' risk of developing atopic diseases or necrotizing colitis and may protect children and adults against antibiotic-associated diarrhoea and upper respiratory tract infections. There are also implications that probiotics may play a role in reducing the risk of gastrointestinal inflammatory diseases and may also regulate the metabolism of humans. Progress in understanding the mechanisms by which the colonic microbiota impact other mucosal surfaces of the body and the function of the immune system has shown that the influence of probiotic microbes reaches way beyond the gastrointestinal tract. Nevertheless, much more research is needed to confirm these benefits in humans. It is also important to bear in mind that the demonstrated health effects are strain-specific and cannot be extrapolated to other probiotic microbes.

1.8 Future trends

New applications for probiotic delivery will certainly be found. However, it is also likely that the current mainstream vehicles for probiotic consumption, i.e. yogurt-type dairy products and supplements (capsules), will remain the leading carriers of probiotics in the near future. This is not so much a technological issue, given the options of other possible carriers that are available, as discussed. It is, however, a matter of consumer acceptance.

New strains will likely be introduced into the market. In addition to strains from the genera *Lactobacillus* and *Bifidobacterium*, strains from other genera, such as *Propionibacterium* and *Lactococcus*, commonly found in fermented foods, are likely to receive more attention. But strains from "new" probiotic genera are also likely to emerge, such as butyrate-producing *Roseburia* and *Clostridium*, or strains from the anti-inflammatory species *F. prausnitzii*.

Furthermore, genetically modified probiotics should be anticipated. Although genetically modified organisms meet resistance in certain parts of the world, they will probably be accepted eventually, provided that they have clear benefits to the user, and they may even target diseases for which there is currently no cure. An example of this is the interleukin-10-producing *Lactococcus lactis* for treatment of Crohn disease (Braat et al., 2006). In the more distant future, individualised strains and combinations

of strains may become available; this may depend on one's own microbiota (Wu et al., 2011) or blood group (Mäkivuokko et al., 2012).

Existing health targets, as described above, will be further investigated and either confirmed or refuted for probiotic efficacy. New targets will also certainly emerge. Many of these targets will be indicated by the substantial research efforts towards understanding of the intestinal and other microbiota. The influence of the intestinal microbiota on, for instance, the gut–brain axis may hint at possibilities for probiotics and stress or mental awareness.

To what extent strains from such new probiotic species can and will be incorporated into fermented foods is uncertain. Likewise, it is uncertain whether these and other new health targets are suitable for fermented foods. What is certain, however, is that the "one-strain-suits-all" approach that has been common until now is going to change. A single strain is simply not able to perform the varied functions that are required to tackle various health risks; the emergence of health benefit-specific strains should be anticipated. Similarly, one could anticipate that more complex mixtures might be more efficacious, as they could provide a multitude of functions to microbiota in disarray.

1.9 Sources of further information and advice

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- Binns, N. (2013). Probiotics, prebiotics and the gut microbiota, ILSI Europe.
  Reference monograph especially for the interested lay person; download for free from: http://www.ilsi.org/Europe/Pages/ConciseMonographSeries.aspx.
- Fredricks, D. N. (Ed.). (2013) *The human microbiota: How microbial communities affect health and disease* (1st ed.). Hoboken, NJ: John Wiley & Sons, Inc.
  Reference book describing the microbiota occupying different parts of the human body.
- ISAPP (International Scientific Association for Probiotics and Prebiotics) webpages: www.isapp.net.
  Up-to-date information on probiotics and human microbiota from an international nonprofit organisation of scientists.
- International Probiotics Association (IPA) webpages: http://internationalprobiotics.org/home/.
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Exopolysaccharides from fermented dairy products and health promotion

2

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2.1 Introduction

Can lactic acid bacteria (LAB) exopolysaccharides (EPSs) play a role in improving gastrointestinal health? More specifically, can the EPSs serve another role by interacting with the human immune system or even the human microbiome itself? More recent gains in knowledge of the mechanisms by which EPS interacts with the intestinal tract, the microbiome and immune function seem to present an additional nutritional role that is offered by fermented dairy products.

A number of reviews and studies have been published dealing with the potential health and nutritional benefits of EPSs from LAB in fermented dairy products: for example, EPSs from LAB have been associated with various health benefits, such as the lowering of cholesterol (Liu et al., 2006; Maeda, Zhu, Omura, Suzuki, & Kitamura, 2004; Nakajima, Suzuki, & Hirota, 1992), anti-hypertensive effects (Maeda, Zhu, Suzuki, Suzuki, & Kitamura, 2004), anticarcinogenic effects (Furukawa, Takahashi, & Yamanaka, 1996; Kitazawa et al., 1991) and immunomodulatory activity (Chabot et al., 2001; Nishimura-Uemura et al., 2003; Vinderola, Matar, Palacios, & Perdigón, 2007). Apart from these effects, there also appears be a complex web of interactions between LAB EPSs and human gut microbiota, some enteric pathogens and toxins, and gut epithelial cells and the immune system; the discussion that follows presents evidence for health and nutritional benefits that are potentially derived from these relationships.

2.2 Exopolysaccharides (EPSs) from fermented dairy products

The commercial exploitation of EPSs from LAB in the area of fermented dairy foods has conventionally been aimed at producing unique physical characteristics (Cerning, 1995) such as enhanced viscosity and water binding; the result of this is that the consumer enjoys an improved "mouthfeel", textural qualities and a longer residence time in the mouth (Duboc & Mollet, 2001; Font de Valdez, Ines Torino, De Vuyst, & Mozzi, 2003). Consumers have been attracted to smooth and creamy textures, characteristics that are conventionally achieved through the addition of fats, sugars and proteins such as pectin or gelatin (Jolly, Vincent, Duboc, & Neeser, 2002). The evolution of advertising towards "healthy" messages to meet a growing consumer demand for healthy

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00002-5

features, e.g. foods that are low in additives (Van der Meulen et al., 2007) or ones that are "natural" or low in fat and sugar, has in fact created room for the use of dairy cultures that produce EPSs that impart these textural attributes – in some cases, claims of up to 20% increases in viscosity are claimed for yogurt. Regulatory provisions (particularly in Europe) that prohibit natural fermented dairy products from containing artificial stabilisers have further strengthened this situation, and hence indirectly, the most apparent health attribute imparted by EPSs is that of reducing the inclusion of sugars and fats associated with obesity. Other benefits are the longer residence time in the gastrointestinal tract, which is thought to favour the transient colonisation by probiotic bacteria, as well as the generation of short-chain fatty acids through colonic degradation.

EPSs are a natural product of some LAB and are long-chain polysaccharides consisting of branched, repeating units of sugars, substituted sugars or derivatives of sugars, as well as other substituents such as phosphate and acetyl groups. The sugar units are most commonly glucose, galactose and rhamnose, and are found in varying ratios of individual sugar monomers (De Vuyst & Degeest, 1999; De Vuyst, De Vin, Vaningelgem, & Degeest, 2001). LAB EPSs typically produce heteropolysaccharides, which have dissimilar monosaccharide units, linked together at different positions, to form multiple copies of "oligo-units", between three and eight monomers in length. The result of these combinations are structures that vary compositionally, structurally and in size. These heteropolysaccharides are between 4×10^4 and 6×10^6 Da in size, and vary in the volume of space that they occupy. The heteropolysaccharides are secreted to the exterior of the cell and either form a capsular EPS (attached to the cell) or are released unattached into the surrounding medium (or both), generating, in most instances, a ropy characteristic (Hassan, 2008).

Despite their GRAS (Generally Recognised as Safe) status and relatively large effect on functionality in dairy products such as yogurt and fermented milks, LAB EPSs are not an article of commerce in an isolated form, mainly owing to their low yield (mostly less than 1 g/l in culture, although higher titres have been reported). Rather, they are introduced in the form of starter dairy cultures or as adjuncts, as this is the most cost-effective way of introducing these attributes. The LAB organisms that are most commonly found in dairy cultures are listed in Table 2.1. Most of these cultures can, depending upon the strain involved, produce some form of EPS, either free or capsular, which contributes to the physical functionality of the product. These functional improvements have a multifactorial origin and have been related to the physical space that the EPS molecule occupies (Tuinier et al., 2001), interaction between EPS and protein, the presence of bacterial cells with EPS at their surface, and hydration water, which is trapped by the polymers (Duboc & Mollet, 2001). The physical effects caused by the structural identity of these EPSs have allowed them to be used widely in dairy applications, imparting thick, smooth and creamy textures to yogurts (as well as controlling syneresis) and water-binding effects in cheeses, allowing for fat reduction and improved textures.

Table 2.1 Lactic acid bacteria associated with fermented dairy products

Fermented dairy product	Associated lactic acid bacteria
Cheese	*Lactococcus lactis* subsp. *lactis*
	Lactococcus lactis subsp. *lactis* var. *diacetylactis*
	Lactococcus lactis subsp. *cremoris*
	Leuconostoc mesenteroides subsp. *cremoris*
	Lactobacillus delbrueckii subsp. *lactis*
	Lactobacillus helveticus
	Lactobacillus casei
	Lactobacillus delbrueckii subsp. *bulgaricus*
	Streptococcus thermophilus
	Propionibacterium freudenreichii
Butter and buttermilk	*L. lactis* subsp. *lactis*
	L. lactis subsp. *lactis var. diacetylactis*
	L. lactis subsp. *cremoris*
	Leuc. mesenteroides subsp. *cremoris*
Yogurt	*Lb. delbrueckii* subsp. *bulgaricus*
	S. thermophilus
Fermented, probiotic milk	*Lactobacillus casei*
	Lactobacillus acidophilus
	Lactobacillus rhamnosus
	Lactobacillus johnsonii
	Lactobacillus plantarum
	Lactobacillus delbrueckii subsp. *delbrueckii*
	Lactobacillus paracasei subsp. *paracasei*
	Lb. delbrueckii subsp. *lactis*
	Bifidobacterium lactis
	Bifidobacterium bifidum
	Bifidobacterium breve
Kefir	*Lactobacillus kefir*
	Lactobacillus kefiranofaciens
	Lactobacillus brevis
	Lactobacillus reuteri
	Lb. rhamnosus
	Lb. plantarum
	Lactobacillus fermentum
	Lactobacillus mesenteroides subsp. *mesenteroides*
	Lactobacillus mesenteroides subsp. *cremoris*
	Lactobacillus mesenteroides subsp. *dextranicum*
	Lb. helveticus
	Lb. acidophilus

2.3 Interaction with the human intestinal microbiome

EPSs produced by starter cultures in fermented dairy products represent a complex matrix of polymers that provide a protective function to the fermenting strains against phages and toxins. After ingestion, these EPSs will ultimately come into contact with the host intestinal microbial population; the question is therefore raised: is there a relationship that emerges between this EPS and the microbiome?

The evolution of human food consumption happened alongside the development of a vast bacterial ecosystem resident within the human body; this gut "microbiome" comprises a cargo of commensals and symbionts that has collectively evolved to survive in the distal part of the human intestine. This community of microorganisms is thought to harbour more than 100-fold the number of genes within the human genome (Bäckhed et al., 2004). This genetic "payload" is carried by an excess of 10^{14} microbial cells with over 1000 bacterial types (Wallace et al., 2011) – the evolution of which probably happened in response to the particular diet available to humans at a particular time.

This very diverse microbial population has evolved to survive in a niche by providing useful benefits for the host, such as protection against pathogenic enteric bacteria (Stecher & Hardt, 2011). The population of gut organisms shows a large degree of diversity, both within individuals and between different persons (Yang, Karr, Watrous, & Dorrestein, 2011). However, although there is a wide variety of different species within the microbiome, large numbers of genes are shared within this diverse population, resulting in a type of metabolic cooperation that benefits the host. A good example of this is the breakdown of complex molecules such as polysaccharides, which otherwise would remain indigestible; this breakdown is achieved by a "glycobiome" containing a large pool of CAZymes (carbohydrate-active enzymes). These enzymes can collectively degrade polymeric carbohydrate structures that are not able to be catabolised by the human metabolism, thereby yielding products of beneficial use to the human body (and to other gut commensals through cross-feeding), such as monosaccharides, vitamins and organic acids. For example, in a study on a human gut microbiome, a combined total of 156 CAZy families were found (Turnbaugh et al., 2008, 2009).

The gut microbiome populations harbouring these enzymes shift in composition depending upon diet, and in turn, have an involvement on disease states and disorders (Dutton & Turnbaugh, 2012). Interestingly, from a study that compared the gut microbial populations of lean and obese mice, investigators (Turnbaugh et al., 2006) determined that a particular microbiota could be associated with obesity, contributing to this condition – the obese mice had a 50% higher proportion of Firmicutes to Bacteroidetes relative to the lean mice. Hence the particular gut populations affected the energy balance by influencing the efficiency of utilisation of energy from the diet.

Given that this complex microbial ecosystem has the means to digest complex carbohydrates in a way that endogenous human enzymes cannot, the question arises as

to whether EPSs (from fermented dairy foods) can play a beneficial role in the gut, similar to that of prebiotics.

2.3.1 Exopolysaccharides from LAB – potential as a prebiotic

An area that has not received much attention is that of the prebiotic potential of EPSs from LAB or yeasts – particularly those found in fermented dairy products. Can EPSs in fermented dairy products act in a similar way to conventional prebiotics? A prebiotic is presently defined as "a non-digestible selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (Roberfroid, 2007). They have assumed commercial significance, as they enhance specific gut bacteria such as bifidobacteria and LAB. The most typical prebiotics are galacto-oligosaccharides (GOS) and fructo-oligosaccharides, and inulin; however, these are not derived by fermentative means, but rather by enzymatic methods. "Next-generation" prebiotics such as human milk oligosaccharides are likely to be very specific for particular gut bacteria that are more likely to be associated with the normal microbiota of an infant. Although the actual amounts of EPS found in these products are very low, there may exist an exclusive prebiotic value for specific bacterial strains coexistent in the microbiome.

Research in the area of biodegradability of EPS by colonic bacteria is scarce; conventional biochemistry would suggest that this is an energetically unfeasible strategy for storing and using energy, although it would appear that there are exceptions. Ruijssenaars et al. (2000) investigated the susceptibility of a number of EPSs from LAB to biodegradability by a consortium of bacteria filtered from faecal slurry. Interestingly, EPSs produced by *Streptococcus thermophilus* SFi12 and SFi39 (both from yogurt starter cultures) were able to be degraded by gastrointestinal microorganisms; conversely, EPSs from *Lactococcus lactis* subsp. *cremoris* B40 (from Finnish ropy milk viili'), *Lactobacillus sakei* 0–1 (non-dairy sourced), *S. thermophilus* SFi20 and *Lactobacillus helveticus* Lh59 strains (from yogurt starter cultures) were not broken down by this community of gut bacteria. The authors then related the EPS structure to susceptibility of degradation; the EPSs from *S. thermophilus* SFi12 and SFi39 were shown to possess a single β-galactosyl side-chain in comparison to the EPSs from *L. lactis* subsp. *cremoris* B40, *Lb. sakei* 0–1, which had two residues. This suggested that, in the latter case, the degradation enzymes were blocked.

Tsuda and Miyamoto (2010) compared the prebiotic activity of EPS from a mutant strain of *Lactobacillus plantarum* isolated from traditional home-made cheese with the prebiotic activities of GOS and inulin against 37 LAB strains. For the seven strains that were positive for prebiotic utilisation, the EPS scored highly for prebiotic activity in comparison to the GOS and inulin prebiotics.

Further evidence that complex carbohydrates such as EPS can be used preferentially to simpler sugars by gut microbes was provided by Semjonovs, Jasko, Auzina, and Zikmanis (2008): in this study, an enhancement was reported in the growth and acidification power of bifidobacteria and other typical dairy starter

cultures such as *Lactobacillus acidophilus*, *S. thermophilus* and *Lactobacillus bulgaricus* when EPS was added. In addition, the strains were able to utilise EPS as a sole carbon source; however, this was not true for all EPSs and all LAB strains, as *S. thermophilus*, for example, was not affected by EPS from two different *Lactobacillus reuteri* strains.

Salazar, Gueimonde, Hernández-Barranco, Ruas-Madiedo, and Clara (2008) compared 11 EPS types isolated from human intestinal *Bifidobacterium* strains with glucose and inulin, as to their potential as fermentable substrates for intestinal bacteria. Short-chain fatty acid levels were markedly higher when these carbohydrates were included in the culture media. EPSs from *Bifidobacterium pseudocatenulatum* obtained from faecal cultures promoted *Desulfovibrio* and *Faecalibacterium prausnitzii*, whereas incubation with EPSs from *Bifidobacterium longum* supported populations such as *Anaerostipes*, *Prevotella* and/or *Oscillospira*. It can therefore be deduced that EPS produced by intestinal bifidobacteria can serve as fermentable substrates for other microorganisms in the human microbiome, changing the interactions amongst intestinal populations; it is hence not inconceivable that EPSs from some dairy starter bifidobacteria may serve as substrates for human microbiota, as well. More recently, *Bifidobacterium bifidum* (strain WBIN03) EPS has been reported to significantly increase the growth of lactobacilli and total anaerobic bacteria, whilst exerting an inhibitory effect on the growth of enterobacteria, enterococci and *Bacteroides fragilis* (Li, Chen, Xu, Dong, Xu, Xiong, & Wei, 2014). Using denaturing gradient gel electrophoresis analysis, it was demonstrated that the EPS enhanced the diversity of total bacteria and lactobacilli but diminished the diversity of the enterobacterial population.

Because of the relatively larger amount of energy needed to degrade EPSs to monomeric sugar units (the heteropolysaccharides consist of structures of up to 1×10^6 Da), it seems paradoxical that some strains within a complex microbiota would use them as a source of energy, even under very limiting carbon availability. However, a few strains of LAB can indeed break down their own EPS after prolonged culture (Pham, Dupont, Roy, Lapointe, & Cerning, 2000), possibly by glycohydrolase enzymes (Degeest, Vaningelgem, & De Vuyst, 2001; Mozzi, de Giori, Oliver, & de Valdez, 1996; Pham et al., 2000) – from this point of view, it would seem that they could also serve as a reserve energy store. Within a complex gut microbiotic environment, the presence of these EPSs may provide a niche whereby the hydrolase-possessing microorganisms can use other organisms' EPSs as a source of energy when carbon supply is limiting. EPSs may also be broken down to oligosaccharides, which are functionally useful to the gut microbial community as a whole, such as in the regulation of polysaccharide molecular weight for biofilm regulation (Badel, Bernardi, & Michaud, 2011).

It can be concluded that the LAB EPSs from fermented dairy foods indeed have the potential to act as prebiotics for specific microbial strains found in the human gut; however, significantly more work is required to identify the specific strains that might be promoted and the beneficial health benefits that may be derived, as well as the EPS compositional structures that would be most beneficial for these purposes. For further reading, in Harutoshi (2013), the potential for EPSs to act as prebiotics is discussed.

From the point of view of dairy foods, therefore, this is an area where further work may yield more data on the health benefits arising from LAB EPSs.

2.4 Interaction with the immune system

A significant amount of evidence has arisen regarding the role that EPSs from fermented dairy foods may play in interacting with the human gut mucosal immune system.

Probably one of the most studied fermented drinks is kefir, which is manufactured from various types of milk on both an artisanal and industrial basis. The milk is inoculated with kefir grains or kefir cultures, and fermented; the cultures are a consortium of homofermentative LAB, yeasts, and acetic acid bacteria. A number of the constituent lactobacilli, including *Lactobacillus kefiranofaciens* and *Lactobacillus kefir* (Table 2.1), produce an EPS known as kefiran (Toba, Arihara, & Adachi, 1987). Vinderola, Perdigon, Duarte, Farnworth, and Matar (2006) were able to show that the EPS from a kefiran source could elicit a gut mucosal response: IgA levels in the small and large intestine in BALB/c mice were increased by oral administration of the EPS; in the case of the cytokines, the levels of IL-10+, IL-6+ and IL-12+ cells were marginally raised relative to control values, and those of IFN gamma+ and TNF alpha+ cells were not changed compared to control values. They deduced that because the increase in the number of IgA+ cells was not accompanied by an increment in IL-4+ cells in the small intestine, this EPS was acting in the same way that polysaccharide antigens are viewed to generally act, i.e. that they elicit a T cell-independent immune response. From this study, they concluded that the EPS was able to up- and down-regulate the gut mucosal response for protective immunity at the level of the small and large intestine, as well as enhance systemic immunity via the release of cytokines into the blood. Medrano, Racedo, Rolny, Abraham, and Pérez (2011) set out to gain an understanding of the effect of oral administration of an extracted kefiran on the balance of immune cells in a murine model. The kefiran was reported to elicit an increase in the number of IgA+ cells after a period of two and seven days. The percentage of B220+/MCH^{High} cells in mesenteric lymph nodes (after two days) and in Peyer's patches (after seven days) was raised relative to untreated mice. They also observed an increment in macrophages (F40/80+ cells) observed in the lamina propria and peritoneal cavity after two and seven days. After day seven, however, the macrophage population in Peyer's patches decreased. The results confirmed the ability of kefiran to change the balance of immune cells in the intestinal mucosa. In addition, these results confirm prior results indicating that these EPSs act as T cell-independent antigens – no changes in T-cell populations were detected.

Lactococcus lactis subsp. *cremoris* is a lactic acid bacterium commonly associated with some cheeses and buttermilk (Leroy & De Vuyst, 2004). *L. lactis* subsp. *cremoris* (strain KVS20) isolated from a starter culture of Scandinavian ropy sour milk (viili), previously shown to elicit an immune response in mice, produces a phosphopolysaccharide (molecular mass of 1.884×10^6 Da) comprising the sugars rhamnose, glucose and galactose. This polysaccharide was shown to induce IFN-1 and IL-la production

in spleen macrophages from C57BL/6 mice (Kitazawa, Itoh, Tomioka, Mizugaki, & Yamaguchi, 1996), prompting the authors to suggest a possible role for this particular polysaccharide as a biological response modifier – an agent that can raise the body's immune response. The presence of a constituent phosphate group on the EPS has been thought to play a role in eliciting these immune responses. For example, the phosphate-containing acidic polysaccharide of *Lactobacillus delbrueckii* subsp. *bulgaricus* (strain OLL1073R-1) has been shown to have mitogenic activity (Kitazawa et al., 1998); when the isolated polysaccharide was dephosphorylated, the mitogenic activity in lymphocytes was reduced, leading the investigators to conclude that the phosphate groups played a role as a "trigger" for mitogenesis (Nishimura-Uemura et al., 2003).

Lactobacillus rhamnosus, although more commonly regarded as a "probiotic", is becoming increasingly included in "lactic beverages", made from yogurt and whey (Oliveira, Sodini, Remeuf, Tissier, & Corrieu, 2002). Chabot et al. (2001) tested *Lb. rhamnosus* RW-9595M EPS fractions for immunomodulating properties on mouse splenocytes and on peripheral blood mononuclear cells (PBMC) from a cohort (14) of healthy human donors. A cytokine response was caused by both the total (unfractionated) EPS isolate and the isolate corresponding to the largest molecular-weight size fraction. This result adds to the growing body of evidence supporting the immunostimulatory potential of microbial EPS from fermented dairy ingredients. Interestingly, EPS from another strain of *Lb. rhamnosus* (KL37C) inhibited the humoral response to ovalbumin (OVA), diminishing the production of anti-OVA IgG, IgG1 and IgG2a antibodies – an immunosuppressive effect (Ciszek-Lenda et al., 2012).

This immunomodulatory activity seems to be variable amongst LAB strains. Ciszek-Lenda et al. (2011) discovered different abilities amongst three LAB strains (*Lb. reuteri*, *Lactobacillus johnsonii and Lactobacillus animalis/murinus*) to stimulate the *in vitro* production of cytokines by murine peritoneal macrophages – in this case, the ability to stimulate cytokine production was correlated with the strength of interaction between the EPS and macrophage receptors. It has been proposed that the actual size and charge of the EPS polymer play a role in determining whether it is immunostimulatory or immunosuppressive. In a *meta*-review of studies on the immunomodulatory properties of EPS from lactobacilli and bifidobacteria (Hidalgo-Cantabrana et al., 2012), it has been proposed that EPSs from these genera that have a negative charge and/or small size (molecular weight), are able to perform as mild stimulators of immune cells, whereas those EPSs that are non-charged and that are larger have a profile of suppressive effects.

A study by Lopez et al. (2012) has provided some new insights: 10 EPS fractions isolated from three different *Bifidobacterium* species (*Bifidobacterium animalis*, *B. longum* and *Bifidobacterium pseudocatenulatum*) were shown to elicit a marginally increased production of PBMCs, but this seemed to be dependent upon the type of EPS tested. On the whole, an EPS that was of a neutral charge and a higher molecular mass caused a decrease in the immune response, whilst acidic, smaller EPSs were able to cause an increased response.

EPS produced by *Lactobacillus paraplantarum* BGCG11 (isolated from a Serbian artisanal cheese) was found to down-regulate cytokine production by PBMCs (Nikolic et al., 2012). Analysis of EPS–CG11 revealed that it is a larger-size polymer (approx.

2×10^6 Da); previous work showed that it was mainly composed of glucose (75.7%) and rhamnose (20.5%), with traces of galactose (2.1%), and having a neutral character (Cerning et al., 1994). The authors suggested a role for the BGCG11 strain in the diet of patients with an increased inflammatory status, e.g. inflammatory bowel disease, or in functional foods for the elderly.

Clearly, it would appear that the compositional and conformational structure of EPSs plays an influencing role in their immunomodulatory effects, i.e. the presence of phosphate groups, molecular size and structure and overall charge on the polymer. The evidence at hand strongly supports the contention that there is indeed a direct interaction between the EPS from fermented dairy products and the immune system, both at a gut epithelial and systemic level. However, there is little to suggest that EPSs act like vaccines, owing to their inability to activate T cells, thereby creating an immunological memory.

2.5 Interaction with enteric pathogens and toxins

The first major line of defence that the body has against enteric pathogens is the mucosal surface lining of the gastrointestinal tract – it is at this layer that pathogenic bacteria succeed in binding to the gut surface.

One of the mechanisms by which EPSs and glycan structures can inhibit enteric pathogens is either by attaching to the gut wall surface, thereby blocking adherence of the pathogen (a competitive binding mechanism), or by binding to the pathogen itself, as a decoy. An example of the first instance is that of *Bifidobacterium breve*, found in fermented milks and cheese. A human isolate of *B. breve* (EPS-producing strain – EPS^+) was demonstrated to be able to evade the B-cell system of mice and colonise the gut, subsequently significantly reducing the infection of a model pathogen, *Citrobacter rodentium* (Fanning et al., 2012), in this case by blocking colonisation by the pathogen. The same effect could not be achieved using a corresponding EPS^- strain.

Wang, Gänzle, and Schwab (2010), using a model system employing enterotoxigenic *Escherichia coli* (ETEC), induced haemagglutination of erythrocytes and found that EPS produced by *Lb. reuteri* could inhibit this binding. EPS isolated from three different *Lb. reuteri* strains (reuteran from strain TMW1.656, levan from strain LTH5794 and glucan from strain FUA3048) all inhibited ETEC-induced agglutination of porcine red blood cells. In this instance, the fimbriae of K88-positive ETEC bacteria known to bind to cell surface receptors that contain carbohydrate structures were inhibited by competitive binding.

Lb. reuteri is a member of the consortium of bacteria involved in kefir production (Heller, 2001). Amongst 58 strains of *Lactobacillus* species isolated from kefir, approximately 19% of the isolates showed the ability to bind to Caco-2 cells by competitive binding with *Salmonella typhimurium* (Santos, San Mauro, Sanchez, Torres, & Marquina, 2003). This area still remains unexplored for determining whether EPS, known to be secreted by some of these strains, is indeed the causative agent. *Lactobacillus plantarum* is not often associated with fermented dairy products, but it has been isolated as one of the major species from a traditional milk of the Maasai (Mathara et al., 2008); *Lb. plantarum* (strain LP6) was determined to bind to rat intestinal mucus

via mannose-specific adhesins, with an isolated EPS (EPS-b) being associated with the binding (Sun, Le, Shi, & Su, 2007). On the basis of similarity in adhesion structure, these authors speculated that the *Lb. plantarum* could competitively bind with pathogens such as *S. typhimurium* or *E. coli* in the small intestine of rats or humans.

In contrast, some EPSs from LAB have not been shown to be successful in this regard. Ruas-Madiedo, Gueimonde, de los Reyes-Gavilan, and Salminen (2006) investigated the potential for EPS isolated from the Scandinavian fermented milk viili to diminish the adhesion of both commercial probiotic strains and some enteric pathogens to intestinal mucus. The EPS in viili is produced by lactococcal strains and confers a pronounced ropy character to the fermented milk. Although the EPS caused a decrease in the adhesion of the commercial probiotic strains, there was no effect on the binding of the enteric pathogens *Listeria monocytogenes* ATCC 15313, *Clostridium difficile* ATCC 9689, *Enterobacter sakazakii* ATCC 29544, *Salmonella enterica* biovar. *typhimurium* ATCC 29631 and *E. coli* NCTC 8603.

Some evidence is available to support the direct binding of EPS to enteric bacterial pathogens. Wu et al. (2010) found EPS from *B. longum* BCRC 14634 (a probiotic), in a dose-dependent manner (at 80 ppm), to directly inhibit the growth of *E. coli* (BCRC10239), *S. typhimurium* (BCRC10747) (ATCC14028), *Pseudomonas aeruginosa* (BCRC10261) (IFO3898), *Vibrio parahaemolyticus* ATCC17802, *Staphylococcus aureus* BCRC10451 (ATCC6538P), *Bacillus subtilis* BCRC10029 and *Bacillus cereus* ATCC10361.

EPSs have been reported to elicit an immune response against viruses, as well. The acidic fraction of EPS isolated from *L. delbrueckii* subsp. *bulgaricus* (OLL1073R-1) was shown to demonstrate antiviral activity against the influenza virus A/PR/8/34 (H1N1) in BALB/c mice via its immunopotentiating activity (Nagai, Makino, Ikegami, Itoh, & Yamada, 2011).

However, a direct or indirect interaction with pathogens is not the only mechanism that has been reported: EPS synthesised by the human and dairy origin strains *Lb. rhamnosus* GG, *B. longum* NB667 and *B. animalis* subsp. *lactis* A1 (and its bile-adapted derivative IPLA-R1) are able to exert an antagonistic effect against the cytopathic effect on Caco-2 cells triggered by *B. cereus* extracellular factors, as well as against the haemolytic activity of a toxin (such as streptolysin-O), on rabbit erythrocytes (Ruas-Madiedo, Medrano, Salazar, De Los Reyes-Gavilán, & Pérez, 2010).

EPS produced from some fermented milk products, although in relatively small quantities, influences the rheology of their matrix, and has also been hypothesised to provide a barrier that slows down the movement of phages (Moineau et al.,1996) and antimicrobial compounds.

It seems, then, that through a variety of mechanisms, some EPSs from LAB have the ability to retard or diminish the effects of gut pathogens, either through a direct interaction with them, acting as decoys, or by binding to the epithelial surface of the gut.

2.6 Diverse interactions and potential health benefits

The following research findings related to the potential health benefits conferred by EPSs from fermented dairy foods may serve as new areas for further investigation.

An interesting finding related to the compositional structure of dairy lactic acid bacteria is that involving *Streptococcus macedonicus* (Strain Sc136), which has a trisaccharide sequence that is the same as the internal backbone of lacto-*N*-tetraose and lacto-*N*-neotetraose, entities that are the structural core of a large number of human milk oligosaccharides (Vincent, Faber, Neeser, Stingele, and Kamerling, 2001). Considering past studies on EPS structure (De Vuyst et al., 2001), this trisaccharide sequence (GlcNAc) is not particularly common in LAB, but has also been identified in *L. acidophilus* LMG 9433 (Robijn et al., 1996) and *Lb. helveticus* TY1-2 (Yamamoto, Murosaki, Yamauchi, Kato, & Sone, 1994). Speculatively, if carbohydrate-degrading enzymes in the "glycobiome" were able to release trisaccharides (or even other oligosaccharide units) with a known antimicrobial activity, significant anti-infective benefits may be transferred to the host.

Another anti-pathogen effect that has been reported for EPSs from LAB is the potential of an EPS to act as a type of interspecies signalling molecule. Kim, Oh, and Kim (2009) demonstrated that *Lb. acidophilus* A4 polysaccharide caused down-regulation of a number of *E. coli* O157:H7 genes involved with the formation of biofilms. This ability to reduce the levels of biofilm could have a significant effect on the treatment of infections resistant to antibiotic therapy because of biofilms; it could be used as an adjuvant to antibiotic therapy, in this instance. In summary, although there is some emerging evidence that EPSs have an anti-infective role to play in dairy foods, more specific evidence is needed to evaluate the benefits. Another interesting aspect lies in the transfer of genetic characteristics – the ability of EPS to aggregate bacteria will decrease the distances between cells, and thereby facilitate the transfer of genetic characters (Badel et al., 2011). This "cross-talk" may afford the sharing of a number of characteristics that would be beneficial not only to both strains or species, but to the host as well.

Oxidative stress is known to play a role in the initiation and development of inflammatory bowel disease and manipulation of this pathway may attenuate disease progress. Şengül, Işık, Aslım, Uçar, and Demirbağ (2011) studied the effect of EPS from two (probiotic) strains of *Lb. delbrueckii* subsp. *bulgaricus* on experimentally induced colitis in a rat model. A significantly greater improvement in oxidative stress parameters was found with the high-EPS group than in the low-EPS group. Li, Jin, Meng, Gao, and Lu (2013), using a rat pheochromocytoma line (PC 12), demonstrated that a purified EPS (EPS-3) from the fermentation of *Lb. plantarum* LP6 could protect PC12 cells from oxidative damage induced by H_2O_2. EPS isolated from *Lactococcus lactis* subsp. *lactis* showed both *in vitro* and *in vivo* superoxide anion, hydroxyl radical and 2,2-diphenyl-1-picrylhydrazyl scavenging activity (Guo et al., 2013). Similarly, EPS from *Lactobacillus paracasei* subsp. *paracasei* (strain NTU 101) and *Lb. plantarum* (strain NTU 102) also demonstrated antioxidant activity (Liu et al., 2011).

2.7 Conclusions

From the evidence available, it is clear that EPSs from LAB are not inert molecules in the gut, and can play a positive role within the complex network of interactions associated with the human microbiome, offering a mutual benefit to the EPS-producing

LAB strains and to the resident gut ecology through a variety of mechanisms. One of the most important could be that of a prebiotic action, sponsoring gut bacterial species that themselves have beneficial health effects, such as the production of specific organic acids.

LAB EPSs also interact and associate with intestinal epithelial cells; however, the way in which they do this is not entirely clear (i.e. how the EPSs interact directly with specific receptors on the epithelial cells' surface). In doing so, they seem in some cases to induce an immunogenic or immunosuppressive response, or can simply block the adherence of gut pathogens. In addition, LAB EPSs may have, wholly or in a partially degraded form, the ability to directly adhere to gut pathogens, thus blocking their ability to bind to the cell wall.

In summary, there is a growing collection of evidence that supports the role of EPSs from fermented dairy foods as health factors in helping to prevent intestinal infection, and possibly serving as aids to the digestive system during periods of antibiotic therapy.

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Bioactive peptides from fermented foods and health promotion

3

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3.1 Introduction

Amongst fermented foods, fermented dairy products have a long history and a good reputation as foodstuffs with a high nutritional value and many health-promoting effects, such as improvement of lactose metabolism, reduction of serum cholesterol and reduction of cancer risk (Kim & Oh, 2013; Vasiljevic & Shah, 2007). The beneficial health effects associated with some fermented dairy products may, in part, be attributed to the release of bioactive peptide sequences during the fermentation process (Korhonen & Pihlanto, 2003). Since the 1990s, numerous peptides and peptide fractions with bioactive properties have been identified in different fermented dairy products. These activities include immunomodulatory, cytomodulatory, hypocholesterolemic, antioxidative, antimicrobial, mineral-binding, opioid and bone formation activities (see reviews by Fitzgerald and Murray (2006), Korhonen and Pihlanto (2006), Korhonen (2009), Hernández-Ledesma, del Mar Contreras, and Recio (2011), Nagpal et al. (2011), Choi, Sabikhi, Hassan, and Anand (2012), Rutherfurd-Markwick (2012)). Many industrially utilized dairy starter cultures are highly proteolytic. The production of bioactive peptides through fermentation by starter and non-starter bacteria can aid development of novel fermented dairy products with specific health benefits. The proteolytic system of lactic acid bacteria (LAB), e.g. *Lactococcus lactis, Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, is already well characterized (Kunji, Mierau, Hagting, Poolman, & Konings, 1996; Savijoki, Ingmer, & Varmanen, 2006). This system consists of a cell wall-bound proteinase and a number of distinct intracellular peptidases, including endopeptidases, aminopeptidases, tripeptidases and dipeptidases (Christensen, Dudley, Pederson & Steele, 1999). *Lb. helveticus* is known to have high proteolytic activities (Luoma et al., 2001) causing the release of oligopeptides from digestion of milk proteins (Foucaud & Juillard, 2000). These oligopeptides can be a direct source of bioactive peptides following hydrolysis by gastrointestinal enzymes. Rapid progress has been made in recent years to elucidate the biochemical and genetic characterization of these enzymes. The fact that the activities of peptidases are affected by growth conditions makes it possible to manipulate the formation of peptides to a certain extent (Kilpi, Kahala, Steele, Pihlanto, & Joutsjoki, 2007; Williams, Noble, Tammam, Lloyd, & Banks, 2002).

Soybeans are a rich source of protein (about 40%) and have a wide range of uses as foods, livestock feeds, oil and health care products. Soy proteins contain

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00003-7

all the essential amino acids and other nutrients, as well as bioactive components, including isoflavonoids (Hassan, 2003; Yang, Park, Pak, Chung, & Kwon, 2011). Fermentation is one of the major processes used in the production of food from soybeans. Fermented soy products are indigenous to the cuisines of East and Southeast Asia. Soybean is considered as an important source of bioactive peptides released during fermentation. A variety of bioactive peptides have been identified in soy protein subunits glycinin and β-conglycinin, as well as in Kunitz and Bowman-Birk inhibitors (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998; Yang et al., 2011).

Cardiovascular disease (CVD) is the single leading cause of death for both males and females in technologically advanced countries in the world. In lesser-developed countries it generally ranks among the top five causes of death. The World Health Organization estimates that by 2020, heart disease and stroke will have surpassed infectious diseases to become the leading cause of death and disability worldwide. Consequently, there has been an increased focus on improving diet and lifestyle as a strategy for CVD risk reduction. Elevated blood pressure is one of the major independent risk factors for CVD (Harris, Cook, Kannel, Schatzkin, & Goldman, 1985). Angiotensin I-converting enzyme (ACE) plays a crucial role in the regulation of blood pressure as it promotes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II and inactivates the vasodilator bradykinin. By inhibiting these processes, synthetic ACE inhibitors have long been used as anti-hypertensive agents. During the last decade, many food proteins have been identified as a source of ACE-inhibiting peptides, which have become the best-known class of bioactive peptides (Gibbs, Zougmanb, Massea, & Mulligan, 2004; Hernández-Ledesma et al., 2011; Martinez-Villaluenga et al., 2012; Pihlanto & Korhonen, 2003). These nutritional peptides have received considerable attention for their effectiveness in both the prevention and the treatment of hypertension (Phelan & Kerins, 2011; Ricci-Cabello, Olalla Herrera, & Artacho, 2011).

Oxidative stress, resulting from an increased production of reactive oxygen species (ROS) in combination with outstripping endogenous antioxidant defense mechanisms, is another significant causative factor for the initiation or progression of several vascular diseases. ROS can cause extensive damage to biological macromolecules like DNA, proteins and lipids. Specifically, the oxidative modification of low-density lipoprotein (LDL) results in the increased atherogenicity of oxidized LDL. Therefore, prolonged production of ROS is thought to contribute to the development of severe tissue injury (Rahman, Hosen, Towhidul Islam, & Shekbar, 2012). Some peptides derived from hydrolysed food proteins exert antioxidant activities against enzymatic (lipoxygenase-mediated) and nonenzymatic peroxidation of lipids and essential fatty acids (Pihlanto, 2006) The antioxidant properties of these peptides have been suggested to be attributable to metal ion chelation, free radical scavenging and singlet oxygen quenching.

This review focuses on release of bioactive peptides during fermentation of milk and soybeans. A particular emphasis is given to peptides with properties relevant to cardiovascular health, including the effects on blood pressure and oxidative stress.

3.2 Release of bioactive peptides during microbial fermentation

3.2.1 Microbes

3.2.1.1 Formation of ACE-inhibition during fermentation of milk

During the fermentation process, LAB hydrolyse milk proteins, mainly caseins, into peptides and amino acids, which are used as nitrogen sources necessary for their growth. Hence, bioactive peptides can be generated by starter and non-starter bacteria used in the manufacture of fermented dairy products. The proteolytic system of *Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus, L. lactis* subsp. *diacetylactis, L. lactis* subsp. *cremoris, Streptococcus salivarius* subsp. *thermophilus* and *Enterococcus faecalis* strains has been demonstrated to hydrolyse milk proteins and release bioactive peptides (Rokka et al., 1997). The synergistic effects of different peptidases are necessary to achieve a reasonable degree of hydrolysis to liberate bioactive peptides. Among LAB, *Lb. helveticus* has a high extracellular proteinase activity and the ability to release large amounts of peptides in fermented milk. The best-characterized ACE-inhibitory and antihypertensive peptides liberated by *Lb. helveticus* alone or in combination with *Saccharomyces cerevisiae* are the lactotripeptides Ile-Pro-Pro and Val-Pro-Pro (Fitzgerald & Murray, 2006; Hernández-Ledesma et al., 2011; Korhonen & Pihlanto, 2006).

In the case of some starter bacteria, bioactive peptides are not formed to any detectable level during fermentation unless further hydrolysed with digestive or other proteolytic enzymes. In addition to microbial strain, the formation of ACE-inhibitory and other peptides from milk proteins has been shown to be affected by many conditional factors, e.g. fermentation time and temperature, pH and calcium ionic concentration in medium (Nielsen, Martinussen, Flambard, Sørensen, & Otte, 2009; Otte, Shalaby, Zakora, Pripp, & El-Shabrawy, 2007; Pan & Guo, 2010). Pihlanto-Leppälä, Rokka, and Korhonen (1998) studied the potential formation of ACE-inhibitory peptides from cheese whey and caseins during fermentation with various commercial dairy starters used in the manufacture of yogurt, ropy milk and sour milk. No ACE-inhibitory activity was observed in these hydrolysates, but their further digestion with pepsin and trypsin resulted in the release of several strong ACE-inhibitory peptides derived primarily from αs1-casein and β-casein. Gobbetti, Ferranti, Smacchi, Goffredi, and Addeo (2000) demonstrated the formation of ACE-inhibitory peptides with two dairy strains, *Lb. delbrueckii* subsp. *bulgaricus* and *L. lactis* ssp. *cremoris*, after fermentation of milk separately with each strain for 72 h. The most inhibitory fractions of the fermented milk mainly contained β-asein-derived peptides with inhibitory concentration (IC_{50}) values ranging from 8.0 to 11.2 μg/ml. Yamamoto, Maeno, and Takano (1999) identified an ACE-inhibitory dipeptide (Tyr–Pro) from a yogurt-like product fermented with *Lb. helveticus* CPN4 strain. This peptide sequence is present in all major casein fractions, and its concentration was found to increase during fermentation, reaching a maximum concentration of 8.1 μg/ml in the final product. Fuglsang, Rattray, Nilsson, and Nyborg (2003) tested a total of 26 strains of wild-type LAB strains, mainly belonging to *L. lactis*

and *Lb. helveticus,* for their ability to produce a fermented milk with ACE-inhibitory activity. All tested strains produced ACE-inhibitory substances in varying amounts, and two of the strains exhibited high ACE inhibition and a high ophthaldialdehyde (OPA) index, which correlates well with peptide formation.

In another study, by Pihlanto, Virtanen, and Korhonen (2010), a total of 25 strains of *Lactobacillus*, *Lactococccus* and *Leuconostoc* were used to investigate their potential to develop ACE inhibition during milk fermentation. The strains were tested alone or in combination and the highest activities were observed with *Lb. jensenii*, *Lb. acidophilus* and *Leuc. mesenteroides.* All strains showed correlation between ACE inhibition and degree of proteolysis. Two β-casein-derived peptides were identified from the water-soluble fraction of milk fermented with *Lb. jensenii.* The identified peptides were Leu-Val-Try-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser-Leu-Pro-Gln-Asn and Leu-Val-Try-Pro-Phe-Pro-Gly-Pro-Ile-His. In a recent study, milk was fermented to defined pH values with 13 LAB strains. Nielsen et al. (2009) observed that the highest ACE-inhibitory activity was obtained with two highly proteolytic strains of *Lb. helveticus* and the *Lactococcus* strains. Fermentation from pH 4.6 to 4.3 with these strains slightly increased the ACE-inhibitory activity, whilst fermentation to pH 3.5 with *Lb. helveticus* reduced the ACE-inhibitory activity. Ashar and Chand (2004) identified an ACE-inhibitory peptide from milk fermented with *Lb. delbrueckii* subsp. *bulgaricus*. The peptide showed the sequence Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile from β-casein with an IC_{50} value of 1.7 mg/ml. In combination with *Str. salivarius* subsp. *thermophilus* and *L. lactis* biovar. *diacetylactis*, a peptide structure with a sequence of Ser-Lys-Val-Tyr-Pro was obtained from β-casein with an IC_{50} value of 1.4 mg/ml. Both peptides were markedly stable to digestive enzymes and acidic and alkaline pH, as well as during storage at 5 and 10 °C for four days. In a recent study it was found that *L. lactis* strains isolated from artisanal dairy starters or commercial starter cultures show potential for the production of fermented dairy products with ACE-inhibitory properties. Especially, a strain isolated from artisanal cheese presented the highest activity with an IC_{50} value of 13 μg/ml (Muguerza et al., 2006; Rodríguez-Figueroa et al., 2010). Rodríguez-Figueroa, González-Córdova, Torres-Llanez, Garcia, and Vallejo-Cordoba (2012) studied the potential of two wild *L. lactis* to release peptides with ACE-inhibitory activity during milk fermentation. The peptide profiles in both strains were similar and 21 novel peptide sequences with potent ACE-inhibitory activity were identified not only from caseins but also from whey proteins.

In another study with wild strains isolated from raw milk, four different *E. faecalis* strains were shown to express ACE-inhibitory activity (IC_{50} values 34–59 mg/ml) in fermented milk (Muguerza et al., 2006). Quiros et al. (2007) identified several peptides in milk fermented with *E. faecalis* CECT 5727, and two of them, Leu-His-Leu-Pro-Leu-Pro [β-casein f(133–138)] and Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro [β-casein f(58–76)], showed IC_{50} values as low as 5 μM. ACE-inhibitory activity has been documented also in some yeast species, namely, *S. cerevisiae* and *Candida parapsilosis* (Hamme, Sannier, Piot, Didelot, & Bordenave-Juchereau, 2009; Kuwabara et al., 1995). Eighteen yeast strains produced fermented milk with ACE-inhibitory values ranging from 8.7% to 88.2%. The

best ACE-inhibitory peptide production was observed in skim milk fermented by the strains *Clavispora lusitaine, Galactomyces geotrichum* and *Pichia kudriavzevii* (Chaves-López et al., 2012). In another study, Didelot et al. (2006) observed that a co-culture of *C. parapsilosis* and *Lb. paracasei* efficiently fermented unsupplemented goat whey and produced ACE-inhibitory peptides. The peptide exhibiting the highest activity was characterized as Trp-Leu-Ala-His-Lys (α-la f[104–108]). In a study by Hamme et al. (2009), the microbiota extracted from Bamalou des Pyrénées cheese most efficiently hydrolysed goat whey proteins and generated ACE-inhibitory activity that was concomitant with high peptide production with an IC_{50} value of 72 mg/ml. The microbiota extracted from this cheese variety consisted of only one yeast strain and one *lactobacillus* strain, *Kluyveromyces marxianus* and *Lb. rhamnosus*, respectively.

Until now, most studies on production of bioactive peptides derived from milk by fermentation have focused on LAB used in production of cheese or yogurt, and a few studies have been carried out with probiotic bacteria so far. Rokka, Syväoja, Tuominen, and Korhonen (1997) found, in milk fermented with a probiotic LAB strain (*Lactobacillus* GG) and subsequently hydrolysed with digestive enzymes, peptides that were identified in previous studies as having ACE-inhibitory, immunostimulating and opioid activities. Recently, Gonzalez-Gonzalez, Tuohy, and Jauregi (2011) screened eight probiotic bacterial strains for the development of ACE-inhibitory activity in fermented skim milk and studied the effects of calcium and pH on this activity. The strains included *Lb. rhamnosus* GG, *Lb. reuteri* NCIMB 11951, *Lb. casei* YIT 9029, *Lb. salivarius* subsp. *salivarius* NCIMB 11975, *Lb. plantarum* NCIMB 8826, *Bifidobacterium bifidum* MF 20/5, *Bifidobacterium longum* subsp. *infantis* CCUG 52486 and *Lb. helveticus* DSM 13137 as a reference strain known to produce ACE-inhibitory peptides IPP and VPP. *Lb. casei* YIT 9029 and *B. bifidum* MF 20/5 induced the strongest ACE-inhibitory activity, which correlated positively with the degree of hydrolysis. Also, it was found that ionic calcium released in milk during fermentation may activate or inhibit the ACE-inhibitory activity depending on its concentration. The authors suggested that ACE-inhibitory activity and ionic calcium concentration may be used as selection criteria for strains with potential to produce fermented mlk products with anti-hypertensive activity. Rojas-Ronquillo et al. (2012) observed that the probiotic strain *Lb. casei* Shirota and *Str. thermophilus* strain released ACE-inhibitory peptides during milk fermentation. The *Lb. casei* Shirota produced also antithrombotic activity. Seven active peptides were purified and tested for resistance to digestive enzymes pepsin and trypsin. The most active ACE-inhibitory peptide was identified as fragment f193–f209 of beta-casein.

The ACE-inhibitory activity in fermented milk can be increased by adding a proteolytic enzyme into milk at the beginning of fermentation. Tsai, Chen, Pan, Gong, and Chung (2008) observed that fermentation of milk by yogurt culture in combination with Flavorzyme protease accelerated the production of ACE-inhibitory peptides from 5.8 mg/g to 32.8 mg/g of whey powder. The bioactivity was contributed mainly by the peptide Tyr-Pro-Tyr-Tyr, which remained active during in vitro gastrointestinal digestion. The blood pressure-reducing activity of this peptide was confirmed in vivo in a spontaneously hypertensive rat (SHR) model.

3.2.1.2 Formation of antioxidant peptides during fermentation of milk

The in vitro release of antioxidant peptides from milk proteins by digestive enzymes has been demonstrated in many studies, as reviewed by Pihlanto (2006) and Ricci-Cabello, Olalla Herrera, and Artacho (2012). Similarly, substances with antioxidant activity can be formed in milk during fermentation by LAB. Such substances can be metabolic compounds produced by bacteria or degradation products of milk proteins. The studies carried out have demonstrated that the antioxidant production is more common and stronger within the group of obligately homofermentative lactobacilli than within the facultatively or obligately heterofermentative strain groups. Also, heterofermentative *Lactobacillus* strains have been reported to exhibit antioxidative activity. *Lb. acidophilus*, *Lb. bulgaricus*, *Str. thermophilus* and *Bifidobacterium longum* exhibited antioxidative activity by various mechanisms, like metal ion chelating capacity, scavenging of ROS and reducing activity of oxidative enzymes (Lin & Yen, 1999; Ou et al., 2009). Hernández-Ledesma, Miralles, Amigo, Ramos, and Recio (2005) found a moderate 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging capacity in commercial European fermented milk products. Virtanen, Pihlanto, Akkanen, and Korhonen (2007) observed that fermentation with *Leuc. mesenteroides* subsp. *cremoris*, *Lb. jensenii* and *Lb. acidophilus* strains produce compounds that showed both radical scavenging activity and inhibition of lipid peroxidation. Farvin, Baron, Nielsen, and Jacobsen (2010) evaluated the antioxidant activity of commercial yogurt with different in vitro assays, including inhibition of oxidation in a liposome model, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity, iron-chelating activity and reducing power. The lower-molecular-weight protein fractions were found to possess more antioxidative capacity than higher-molecular-weight protein fractions. Further studies indicated that the 3–10 kDa fraction revealed many peptide sequences and some of the peptides contained Leu or Val at the N-terminal and Pro and Tyr in their sequence that correlated with antioxidant peptides reported by others (Farvin, Baron, Nielsen, Otte, & Jacobsen, 2010). Kudoh, Matsuda, Igoshi, and Oki (2001) detected an antioxidative peptide derived from κ-casein after milk fermentation with *Lb. delbrueckii* subsp. *bulgaricus.*

3.2.1.3 Other bioactive peptide activities in fermented milk

Inflammation plays a key role in the development of cardiovascular disease. It often develops with inflammatory changes in the endothelium, which begins to express the vascular cell adhesion molecule VCAM-1. VCAM-1 attracts monocytes, which then migrate through the endothelial layer under the influence of various proinflammatory chemoattractants (Libby, 2006). Accordingly, fermentation by LAB may be able to release peptides that possess immunomodulatory properties. Most of the studies on such compounds have been performed with synthetic peptides derived from enzymatic treatment of milk proteins using different in vitro models. LeBlanc, Matar, Valdéz, LeBlanc, and Perdigon (2002) investigated the effect of peptides released during the fermentation of milk by *Lb. helveticus* on the humoral immune system and on the growth of

fibrosarcomas. The permeate fraction of the ultrafiltrated fermented milk was able to modulate the in vitro proliferation of lymphocytes by acting on the production of cytokines (Laffineur, Genetet, & Leonil, 1996). Tompa et al. (2011) found that the peptide fractions from *Lb. helveticus* BGRA43 fermented milk exhibited anti-inflammatory potential. Matar, Valdez, Medina, Rachid, and Perdigon (2001) fed milk fermented with a *Lb. helveticus* strain to mice for three days and detected significantly higher numbers of IgA-secreting cells in their intestinal mucosa, compared with control mice fed with similar milk incubated with a non-proteolytic variant of the same strain. The immunostimulatory effect of fermented milk was attributed to peptides released from the casein fraction.

3.2.1.4 Formation of bioactive peptides during soy fermentation

Many authors have investigated the anti-hypertensive activity of soybean fermented with different strains under different conditions. The proteases and peptidases of *Bacillus* and *Rhizopus* strains have been shown to hydrolyse soy proteins into active peptides. Wang, Mejia, and Gonzalez (2005) identified several ACE peptides, such as Val-Ala-His-Ile-Asn-Val-Gly-Lys or Tyr-Val-Trp-Lys, from soybean meal fermented with *Bifidobacterium natto* or *Bacillus subtilis* strains. Tsai, Lin, Pan, and Chen (2006) and Tsai et al. (2008) fermented soy milk with LAB strains (*Lb. casei, Lb. acidophilus, Lb. bulgaricus, Str. thermophilus* and *B. longum*) and observed an IC_{50} value of 2.89 mg/ml after 30 h fermentation. When a protease (Prozyme 6) was added after 5 h fermentation, a much lower IC_{50} value (0.66 mg/ml) was obtained, showing increase in the ACE-inhibitory activity. Nakahara et al. (2010) made a peptide-enriched soy sauce-like seasoning called fermented soybean seasoning (FSS) by modifying the process of soy sauce brewing. FSS had a 2.7-fold higher concentration of total peptides than the regular soy sauce, and the concentrations of ACE-inhibitory peptides were higher than levels found in the regular soy sauce. Dipeptides Ser-Tyr and Gly-Tyr were identified from FSS. Also, Nakahara, Yamaguchi, and Uchida (2012) noticed that the content of the same dipeptides, Ser-Tyr and Gly-Tyr, were significantly higher when fermentation was done at 45 °C as compared to 15 °C. In another study, Rho, Lee, Chung, Kim, and Lee (2009) observed that a fermented soybean extract prepared through rapid fermentation at elevated temperature produced a pentapeptide (Leu-Val-Gln-Gly-Ser) with an IC_{50} value of 43.7 μM. Zhu et al. (2008) isolated two ACE-inhibitory peptides from the traditionally fermented soy sauce and the IC_{50} values were 165.3 and 65.8 μM, respectively. During miso paste fermentation by *Aspergillus oryzae* casein was added in order to release ACE-inhibitory activity and tripeptides Val-Pro-Pro and Ile-Pro-Pro (Inoue et al., 2009). Comparison of the IC_{50} values of ACE-inhibitory peptides prepared by an enzymatic hydrolysis of soy proteins using commercial proteases has revealed higher inhibition potencies, with an IC_{50} value of 1.69 μM (Mallikarjun Gouda, Gowda, Rao, & Prakash, 2006).

Fermentation of soybean has also yielded peptides with antioxidant properties in two soy-fermented products, natto and tempeh (Wang et al., 2008). Also, natto fermented by *B. subtilis* has been shown to exhibit radical scavenging activity and inhibitory effect on the oxidation of rat plasma LDL in vitro (Iwai, Nakaya, Kawasaki, & Matsue, 2002). A low-molecular-weight viscous substance (molecular weight < 100 kilo Daltons) had

the strongest radical scavenging activity for hydroxyl and superoxide anion radicals, as assessed by electron spin resonance. The increase of conjugated dienes in LDL oxidized by copper and an azo pigment was depressed by the addition of low-molecular-weight substances and soybean water extract. The aqueous extracts of another fermented soybean product, douchi, showed radical scavenging activities and chelating ability of ferrous ions. The radical scavenging activities were higher than that of Trolox, an analogue of vitamin E used as a standard (Wang et al., 2008). Sufu, a Chinese traditional fermented soybean product, fermented by *A. oryzae*, showed DPPH radical-scavenging activity, which was higher than in the non-fermented tofu extract. The ripening temperature and the duration of ripening period affected the antioxidant activity of the sufu extracts. The most profound enhancement of antioxidant capacity was noted in the sufu product ripened at 45 °C for 16 days (Huang, Lai, & Chou, 2011). It was not possible to confirm whether these antioxidant capacities were attributable only to peptides or to a combination of peptides and isoflavones.

Fermentation is not enough to fully hydrolyse soybean proteins. Glycoproteins, phosphoproteins and other post-translationally modified species or domains that contain a higher number of disulfide bridges are more difficult to hydrolyse. The proteases of *Bacillus* and *Rhizopus* strains can only cleave soybean proteins into large peptides. Further enzymatic degradations, such as pronase, trypsin, Glu C protease, plasma proteases and kidney membrane proteases hydrolysis, are needed to produce peptides with high activities (Gibbs et al., 2004; Wang et al., 2005). Fermentation may also synthesize new peptide sequences. In enzymatic hydrolysates of soy-fermented foods, a precursor of the peptide sequence Glu-Leu-Leu-Val-Tyr-Leu-Leu with good surface active properties could not be identified and it was believed to be synthesized during fermentation (Gibbs et al., 2004).

Chen et al. (1995) and Chen, Muramoto, Yamauchi, and Nokihara (1996) isolated six antioxidative peptides from the proteolytic digests of soybean protein. The segment His-His of the smallest active peptide Leu-Leu-Pro-His-His was found to play a major role in the antioxidative activity of the peptide. Histidine-containing peptides are known to be antioxidative, and this was confirmed by Chen et al. (1998) when using synthetic, histidine-containing peptides that were designed on the basis of the antioxidative peptide Leu-Leu-Pro-His-His.

3.2.2 Microbial enzymes

Many different proteolytic enzymes have been identified in LAB (Savijoki et al., 2006). The components of the proteolytic systems of LAB are divided into three groups: (1) the extracellular proteinase that catalyses casein breakdown to peptides, (2) peptidases that hydrolyse peptides to amino acids and (3) a peptide transport system. Specialized peptidases are capable of hydrolysing proline-containing sequences and are important for the degradation of casein-derived peptides because of their high proline content (Christensen, Dudley, Pederson, & Steele, 1999; Kunji et al., 1996). The extracellular proteinase activity has been correlated closely with ACE-inhibitory activity in the fermented milk, suggesting that the proteolysis of casein by the extracellular proteinase is the most important parameter in the production of bioactive components from milk

proteins (Minervini et al., 2003). The importance of the proteinase is also supported by the fact that a proteinase-negative mutant was not able to generate anti-hypertensive peptides in the fermented milk, whereas the wild-type strain had the ability to release strong anti-hypertensive peptides in the fermented milk (Yamamoto et al., 1999). The enzymatic process generating the anti-hypertensive peptides VPP and IPP from milk proteins by *Lb. helveticus* has been elucidated. The proteolytic action of the extracellular proteinase generates a long peptide with amino acid residue including VPP and IPP sequences. Next, the long peptide is hydrolysed to shorter peptides by intracellular peptidases. A key enzyme that can catalyse C-terminal processing of Val-Pro-Pro-Phe-Leu and Ile-Pro-Pro-Leu-Thr to VPP and IPP has been purified from *Lb. helveticus* CM4. The endopeptidase has sequence homology in the amino terminal sequence to a previously reported pepO-gene product (Ueno, Mizuno, & Yamamoto, 2004). A mixture of intracellular peptidases from *Lb. helveticus* ATCC 15009 produced several ACE-inhibitory peptides. X-Pro dipeptides reached the maximum concentration after 24 h of hydrolysis, whereas the maximum concentration of tripeptides VPP and IPP was observed after 72 h (Stressler, Eisele, & Fischer, 2013). Kilpi et al. (2007) found a higher ACE inhibition in milk fermentation using peptidase-deletion mutants compared to the wild-type of *Lb. helveticus* strain. Unlike with the wild-type strain, ACE inhibition remained constant during the course of fermentation with the proline-specific peptidase mutant. The mutant strains also had different peptide profiles than the wild-type strain.

Pan and Guo (2010) investigated the effect of fermentation conditions on the production of ACE-inhibitory peptide in sour milk fermented by *Lb. helveticus* LB10 using response-surface methodology. Optimal conditions to yield the maximum production of ACE-inhibitory peptides were found to be 4% (v/w) inoculums, 7.5 initial pH of medium and 39 °C. The cell-envelope proteinase, assisted by X-prolyldipeptidyl aminopeptidase of *Lb. helveticus* LB10, produced the ACE-inhibitory peptides. A novel ACE-inhibitory peptide from whey protein hydrolysate was purified and identified as beta-lactoglobulin fraction f48–f53 with a peptide sequence of Arg-Leu-Phe-Asn-Pro and an IC_{50} value of 177.4 μM. Proteinases from micro-organisms such as *Mucor* sp. *A. oryzae* and *B. subtilis* 1389 may also be exploited to hydrolyse proteins and generate short-chain peptides (Wang et al., 2005).

3.3 Bioactive peptides in fermented dairy and soy products

3.3.1 Dairy products

Proteolysis in cheese has been linked to its importance for texture, taste and flavour development during ripening. Changes of the cheese texture occur owing to the breakdown of the protein network. Proteolysis contributes directly to taste and flavour by the formation of peptides and free amino acids, as well as by liberation of substrates for further catabolic changes and, thereby, formation of volatile flavour compounds. Besides sensory quality aspects of proteolysis, formation of bioactive peptides as a

result of proteolysis during cheese ripening has been reported in many studies since the late 1990s (Fitzgerald & Murray, 2006; Yang et al., 2011). Cheese contains phosphopeptides as natural constituents (Roudot-Algaron, Bars, Kerhoas, Einhorn, & Gripon, 1994; Singh, Fox, & Healy, 1997) and secondary proteolysis during cheese ripening leads to the formation of other bioactive peptides, such as those with ACE-inhibitory activity. Meisel, Goepfert, and Günter (1997) showed that the inhibitory activity increased as proteolysis developed; however, the bioactivity decreased when proteolysis during ripening exceeded a certain level. Another link between potential anti-hypertensive peptides and proteolysis was found in Parmesan cheese (Addeo et al., 1992). ACE-inhibitory peptide fractions expressing different potencies have been isolated from various Italian cheeses, e.g. Crescenza (37% inhibition), Mozzarella (59% inhibition), Gorgonzola (80% inhibition) and Italico (82% inhibition) (Smacchi & Gobbetti, 1998). ACE-inhibitory peptides have also been found in enzyme-modified cheeses (Haileselassie, Lee, & Gibbs, 1999), in a low-fat cheese produced in Finland (Ryhänen, Pihlanto-Leppala, & Pahkala, 2001), in Manchego cheeses manufactured with different starter cultures (Gomez-Ruiz, Ramos, & Recio, 2002) and in Asiago cheeses (Lignitto et al., 2010). Mexican fresco cheese manufactured with *Enterococcus faecium* or an *L. lactis* subsp. *lactis-E. faecium* mixture showed the largest number of fractions with ACE-inhibitory activity among tested LAB strains (Torres-Llanez, González-Córdova, Hernandez-Mendoza, Garcia, & Vallejo-Cordoba, 2011). Pripp, Sørensen, Stepaniak, and Sørhaug (2006) investigated the relationship between proteolysis and ACE inhibition in Gamalost, Castello, Brie, Pultost, Norvegia, Port Salut and Kesam. The traditional Norwegian cheese Gamalost had, per unit cheese weight, a higher ACE inhibition potential than Brie, Roquefort and Gouda-type cheese. However, the ACE inhibition, expressed as IC_{50} per unit peptide concentration from ethanol soluble fraction assessed by the OPA assay, was highest for Kesam, a Quark-type cheese with a low degree of proteolysis. Gomez-Ruiz, Taborda, Amigo, Recio, and Ramos (2006) studied six Spanish traditional cheese varieties for the presence of bioactive peptides and found 41 ACE-inhibitory peptides, mainly derived from beta- and α_{s1}-casein. Cabrales cheese, which had the highest proteolysis index, also expressed the highest ACE-inhibitory activity, while Mahòn showed the lowest.

When the presence of β-casomorphins was examined in commercial cheese products, no peptides were found or their concentration in the cheese extract was below 2 μg/ml (Muehlenkamp & Warthesen, 1996). β-casomorphins 5, -6, -7 and their precursors were identified in various types of cheeses, e.g. Swiss, Elsberg, Cheddar, Gouda, Feta, Blue, Brie, Parmesan and Crescenza (Addeo et al., 1992; Jarmołowska, Kostyra, Krawczuk, & Kostyra, 1999; Sabikhi & Mathur, 2001; Smacchi & Gobbetti, 1998).

β-casomorphins were found in the mould cheeses at higher levels (166–648 mg/100 g) compared to semi-hard cheeses where opioid peptides (casoxin-6) with antagonistic activity were identified at higher levels (136–276 mg/100 g) than β-casomorphins (4–100 mg/100 g) (Sienkiewicz-Szłapka et al., 2009). Also, immunomodulating properties have been found in water-soluble extracts of traditional French Alps cheeses, Abondance and Tomme de Savioe (Durrieu, Degraeve, Chappaz, & Martial-Gros, 2006).

As shown in Table 3.1, a number of bioactive peptides have been isolated and identified in different types of fermented cheese. Several ACE-inhibitory peptides have been

Table 3.1 Examples of ACEI-inhibitory peptides identified in different cheese varieties

Cheese variety	Milk protein fragment	Peptide sequence	ACE-inhibition IC_{50} (μM)	References
Gouda	α_{s1}-cn f(1–9)	RPKHPIKHQ	13.4	Saito et al. (2000)
	α_{s1}-cn f(1–13)	RPKHPIKHQGLPQ	ND	
	β-cn f(68–66)	YPFPGPIPN	14.8	
	β-cn f(109–119)	MPFPKYPVQPF	ND	
Manchego	Ovine α_{s1}-cn f(102–109)	KKYNVPQL	77.2	Gomez-Ruiz et al. (2002)
	Ovine α_{s1}-cn f(205–208)	VRYL	24.1	
Cheddar (with probiotics)	α_{s1}-cn f(1–9)	RPKHPIKHQ	ND	Ong & Shah (2008)
	α_{s1}-cn f(1–7)	RPKHPIK		
	α_{s1}-cn f(1–6)	RPKHPI		
	α_{s1}-cn f(24–32)	FVAPFPEVFGK		
	β-cn f(193–209)	YQEPVLGPVRGPFPIIV		
Different Swiss cheese varieties	β-cn, f(84–86)	VPP	9	Meyer et al. (2009), Bütikofer et al. (2007), Bütikofer, Meyer, Sieber, Walther, and Wechsler (2008)
	β-cn, f(74–76) and κ-cn, f(108–110)	IPP	5	
Fresco cheese	α_{s1}-cn f(1–15)	RPKHPIKHQGLPQEV	ND	Torres-Llanez et al. (2011)
	α_{s1}-cn f(1–22)	RPKHPIKHQGLPQEVLNENLLR		
	α_{s1}-cn f(14–23)	EVLNENLLRF		
	α_{s1}-cn f(24–34)	FVAPFPEVFGK		
	β-cn f(193–205)	YQEPVLGPVRGPF		
	β-cn f(193–207)	YQEPVLGPVRGPFPI		
	β-cn f(193–209)	YQEPVLGPVRGPFPIIV		

ACE, angiotensin I-converting enzyme; ND, not described; IC_{50}, peptide concentration that shows 50% inhibition of ACE activity. One-letter amino acid codes used.

identified in N-terminal of α_{s1}-casein of Gouda, Festivo, Cheddar and fresco cheeses (Ong, Henriksson, & Shah, 2007; Ryhänen, et al., 2001; Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000; Torres-Llanez et al., 2011). Novel ACE-inhibitory peptides with high in vitro activity (IC_{50} from 4 to 32 μM) have been identified using a combination of proteomic techniques and quantitative structure–activity relationship analysis (Sagardia, Iloro, Elortza, & Bald, 2013). Anti-hypertensive peptides VPP and IPP have also been identified and quantified in different Swiss cheese varieties (Bütikofer, Meyer, Sieber, Walther, & Wechsler, 2008; Bütikofer, Meyer, Sieber, & Wechsler., 2007; Meyer, Bütikofer, Walther, Wechsler, & Sieber, 2009). In some varieties physiologically relevant amounts were observed. However, a large variation seems to exist between samples of the same cheese variety, as well as between different varieties. The concentrations of VPP and IPP were in the range of 0–224 mg/kg and 0–95 mg/kg, respectively, indicating that some cheese varieties contain concentrations of VPP and IPP comparable to fermented milk products. Highest amounts of these two peptides were found in hard and semi-hard cheese varieties, such as Hobelkäse, Emmental, Gruyere and Gouda. Milk pretreatment, cultures, scalding conditions and ripening time were identified as the key factors influencing the concentration of these two naturally occurring bioactive peptides in cheese. Thus, it is necessary to develop a standardized cheese-making process with selected cultures to secure production of higher amounts of specific biopeptides that could be used for clinical trials and, if proven effective, for commercial production. Proteolytic probiotic bacteria may increase the release of active peptides in fermented dairy products. Ong et al. (2007) made Cheddar cheese added with two probiotic strains, *Lb. casei* 279 and *Lb. casei* L26, and observed increased ACE-inhibitory activity as compared to cheese made without an addition of probiotic cultures. The activity increased during ripening at 4 °C, possibly owing to enhanced proteolysis. Various ACE-inhibitory peptides derived from α_s-casein and β-casein were identified in probiotic cheese.

Peptides with different bioactivities have been identified in many fermented milks, such as sour milk, yogurt, kefir and dahi, as reviewed by Fitzgerald and Murray (2006), Korhonen (2009) and Hernández-Ledesma et al. (2011). In addition to anti-hypertensive peptides, casein phosphopetides, antimicrobial, antioxidative and immunomodulatory peptides have been found depending on the origin of milk, dairy cultures and technology applied in production.

3.3.2 Soy products

Fermented soybean products such as natto and tempeh (Gibbs et al., 2004), douchi (Zhang, Tatsumi, Ding, & Li, 2006) and soybean paste (Shin et al., 2001) have been shown to contain bioactive peptides, such as antioxidative and ACE-inhibitory peptides, mainly owing to the action of fungal proteases. In two separate studies (Okamoto et al., 1995; Kuba, Tanaka, Tawata, Takeda, & Yasuda, 2003) different fermented soybean foods showed IC_{50} values of 0.51 mg/ml for tempeh, 1.77 mg/ml for tofuyo, 3.44 and 0.71–17.80 mg/ml for soy sauce, 5.35 and 1.27 mg/ml for miso paste, and 0.16 and 0.19 for natto, respectively. Commercial Chinese-style soy paste exhibited ACEI-inhibitory activity with the lowest and the highest IC_{50} values of 0.012 and 3.24 mg/ml, respectively.

The results indicated that the processing technique impacts largely on the ACEI-inhibitory activity of this fermented soy product (Li, Yin, Cheng, & Saito, 2010). Liu, Chen, and Lin (2005) demonstrated that soymilk–kefir possesses significant anti-mutagenic and antioxidant activity and suggested that fermented soymilk may be considered among the most promising food components in terms of preventing mutagenic and oxidative damage. More research is, however, needed to demonstrate if the peptides produced during fermentation may play an important role in this biological activity.

3.4 Bioactive peptides in health promotion

3.4.1 Animal studies

The in vivo assay of ACE-inhibitory activity is generally conducted by measuring the blood pressure response in SHRs following intravenous injection and intraperitoneal or oral administration of the peptide fractions or peptides. Some of the orally administered ACE-inhibitory peptides have demonstrated strong anti-hypertensive effects in SHR. A great number of studies have addressed the effects of both short-term and long-term administration of potential anti-hypertensive peptides using this animal model (see reviews by Murray and FitzGerald (2007), Saito (2008), Jäkälä & Vapaatalo (2010), Hernández-Ledesma et al. (2011), Urista, Fernandez, Rodriguez, Cuenca, & Jurado (2011)). Fermented milks with different IC_{50} values ranging from 0.08 to 1.88 mg/ml have been shown to decrease blood pressure in SHR from 10 to 32 mmHg (Table 3.2). Several animal studies have been conducted with lactotripeptides VPP and IPP, produced from milk fermentation with *Lb. helveticus*. In an early study, single oral administration of sour milk (5 ml/kg body weight) containing 0.6 mg/kg VPP and 0.3 mg/kg IPP significantly decreased systolic blood pressure (SBP) between 4 and 8 h after administration (Nakamura, Yamamoto, Sakai, & Takano, 1995). Furthermore, a dose-dependent anti-hypertensive effect of these lactotripeptides was observed from 0.1 to 10 mg/kg of body weight. Long-term administration of fermented milk or lactotripeptides has also been demonstrated to be effective in reducing SBP in SHR (Jauhiainen, Collin, et al., 2005). These peptides also prevented the development of hypertension in SHR after long-term (12, 13 and 9 week) oral feeding. At the end of the 12 week treatment period, SBP was 17 mmHg lower in the group receiving *Lb. helveticus* LBK-16H fermented milk containing IPP and VPP than in the control group receiving water and 12 mmHg lower in the group receiving the tripeptides in water than in the control group in SHR. Attenuation in SBP was 10 mmHg in high salt-fed GK rats and 19 mmHg in dTGR rats in comparison to control (Jäkälä, Hakala, Turpeinen, Korpela, & Vapaatalo, 2009; Jauhiainen, Collin, et al., 2005; Sipola, Finckenberg, Korpela, Vapaatalo, & Nurminen, 2002; Sipola et al., 2001). Pure tripeptides did not produce as strong an anti-hypertensive effect as the milk products containing them. In addition, minerals alone did not attenuate the development of blood pressure as much as the fermented milk products (Jauhiainen, Collin, et al., 2005). These studies indicate that the bioavailability of peptides may be better from milk in comparison to water or is improved by other milk components.

Table 3.2 ACE-inhibitory and anti-hypertensive activity in spontaneously hypertensive rats of peptides produced by fermentation of milk and soybean

Micro-organism	ACE-inhibition	Identified peptides		Dose	Response (ΔSBP mmHg)	References
	IC_{50} (mg/ml)	Sequence	IC_{50} (μM)			
Milk fermentation						
Lactobacillus helveticus and *Streptococcus thermophilus*	ND	VPP IPP	9 5	5 ml/kg	−21.8 ± 4.2 After 6 h	Nakamura et al. (1995)
Lb. helveticus		VPP IPP	9 5	27 ml/day	−21 after 4 week	Sipola, Finckenberg, Korpela, Vapaatalo, and Nurminen (2002)
Lb.helveticus CPN4	ND	YP	720	10 ml/kg	32.1 ± 7.4 after 6 h	Yamamoto et al. (1999)
Lb. helveticus CHCC637	0.16	Not identified	ND	10 ml/kg	−12 after 4–8 h	Fuglsang et al. (2003)
Lb. helveticus CHCC641	0.26				−11 after 4–8 h	
Lactobacillus delbrueckii ssp. *bulgaricus* *Streptococcus salivarius* ssp. *thermophilus* and *Lactococcus lactis biovar. diacetylactis*	ND	SKVYPFPGPI SKVYP	1.7 mg/ml 1.5 mg/ml	ND	ND	Ashar & Chand (2004)
Lactobacillus jensenii	0.52	LVYPFPG-PIHNSLPQN LVYPFPGPIH	71 89	0.2 kg/kg	Approx. −12 after 2 h	Pihlanto et al. (2010)
Enterococcus faecalis CECT 5727	0.053	LHLPLP LVYPFPG-PIPNSLPQNIPP	5.5 5.2	2 mg/kg 6 mg/kg	−21.87 ± 4.51 After 4 h Approx. −15 After 4 h	Quiros et al. (2007)
Lb. delbrueckii subsp. *bulgaricus* SS1 *Lc. lactis* subsp. *cremoris* FT4	ND	NIPPLTQTPV LNVPGEIVE DKIHPF	173.3 300.1 256.8	ND	ND	Gobbetti et al. (2000)

Mixed lactic acid bacteria (*Lactobacillus casei, Lacidophilus acidophilus, Lactobacillus bulcaricus, Str. thermophilus, Bifido-bacterium*) and protease	0.24	GTW GVW	464.4 240.0	5 mg/ml	−22 after 8 weeks	Chen et al. (2007)
Lc. lactis NRRL B-50571 *Lc. lactis* NRRL B-50572B	0.034 0.041	TVQVTSTAV (k-cn f 161–f169) HPHPHLSFMAIPP (k-cn f98–f110) SLPQPNIPPL (b-cn f69–f77) YPSYGL (k-cn f35–f40)	ND	ND	ND	Rodríguez-Figueroa et al. (2012)
Soybean fermentation						
Aspergillus sojae (fermented soybean seasoning, FSS)	0.45	AW GW AY SY VG	10 µg/ml 30 48 67 1100	FSS 10% v/w	−20 after 7 weeks	Nakahara et al. (2010)
Soybean paste	ND	HHL	2.2 µM	5 mg/kg	−61	Shin et al. (2001)
Fermented soy milk	2.89	ND	ND	ND	−19 after 8 weeks	Tsai et al. (2006)
Soybean protease from *Bacillus subtilis*	ND	PGTAVFK	26.5 µM	ND	ND	Kitts and Weiler (2003)
Soy flour *Aspergillus oryzae*	1.46	LVQGS	43.7 µM	ND	ND	Rho et al. (2009)
Soybean *A. oryzae*	ND	AF IF	165.3 µM 65.8 µM	ND	ND	Zhu et al. (2008)
Tofoyu	1.77	IFL WL	44.8 µM 29.9 µM	ND	ND	Kuba et al. (2003)

ACE, Angiotensin I-converting enzyme; ND, not described. One-letter amino acid codes used.

Besides the most extensively studied lactotripeptides, other fermented milk products produced with different strains of LAB, such as *Lb. helveticus* CPN4, *Lb. bulgaricus*, *Lb. jensenii* and *Str. thermophilus*, have also been shown to provoke liberation of peptides with anti-hypertensive activity in SHR (Fuglsang et al., 2003; Pihlanto et al., 2010; Yamamoto et al., 1999). The milk fermented with *Lb. jensenii* caused a transient reduction of blood pressure in SHR and peptides corresponding to β-casein f(11–26) and f(11–20) were identified in the fermented milk (Pihlanto et al., 2010). Two peptides, corresponding to β-cn fragments Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro and Leu-His-Leu-Pro-Leu-Pro, have been isolated in the milk fermented with *E. faecalis* and the anti-hypertensive effect of these peptides in SHR, after acute and long-term administration, has been proved. The administration of 2 mg/kg of peptide Leu-His-Leu-Pro-Leu-Pro resulted in a significant decrease of the SBP in SHR 4 h post-administration (Quiros et al., 2007). Fermentation of milk with one or more LAB strains followed by hydrolysis using food-grade enzymes liberated tripeptides Gly-Thr-Trp and Gly-Val-Trp. Oral administration of this fermented whey significantly lowered SBP in SHR from 9 to 15 weeks of age. It has also been shown that other bioactive substances contained in fermented milks, such as γ-aminobutyric acid (GABA), can contribute to lowering blood pressure of SHR (Chen, Tsai, & Sun Pan, 2007; Tsai et al., 2006).

Some of the ACE-inhibitory peptide fractions from cheese have shown in vivo activities in SHR. A water-soluble peptide preparation isolated from Gouda ripened for 8 months was found to have the most potent anti-hypertensive activity (maximum decrease in SBP = 24.7 (±0.3) mmHg ($P \leq 0.01$) after 6 h) when administered to SHR by gastric intubation at doses between 6.1 and 7.5 mg/kg body weight. Three peptide fractions were isolated from water-soluble extract by hydrophobic chromatography using different concentrations of acetonitrile (Saito et al., 2000).

Several sequences have been proposed as responsible for the anti-hypertensive activity of soy protein hydrolysates and fermented products. In the study of Shin et al. (2001) the peptide His-His-Leu derived from fermented soy paste was assayed in pure form in SHR and a decrease of 32 mmHg of SBP was reached at a dose of 100 mg/kg. Moreover, the synthetic tripeptide His-His-Leu resulted in a significant decrease of ACE activity in the aorta of SHR. Soybean-derived products contain isoflavones, which are thought to possess a favourable effect in reducing cardiovascular risk factors as well as vascular function (Sacks, 2006). However, on the basis of in vitro results and literature review, Wu and Muir (2008) suggested that the contribution of isoflavones to a blood pressure-lowering effect by soybean ACE-inhibitory peptides may be negligible. Similarly, it has been reported that the reduction of hypertension by a fermented soy milk product was contributed mainly by peptides of 800–900 Da but it could also be attributable to GABA (Tsai et al., 2006). Moreover, the fermented soy product miso with added tripeptides (VPP and IPP) from casein was reported to act as an anti-hypertensive agent in SHR (Inoue et al., 2009). A peptide-enriched FSS gradually decreased the SBP in SHR during 7 weeks of treatment. Moreover, Dahl salt-sensitive rats, as a model of salt-sensitive hypertension, were used to evaluate the anti-hypertensive effect of a peptide-enriched soy sauce-like seasoning. These studies indicated that FSS suppressed the elevation of SBP in a dose-dependent manner

after 4 weeks, and FSS had a more potent anti-hypertensive effect than regular sauce (Nakahara et al., 2010).

Some of the results described in the above studies highlight an important lack of correlation between the in vitro ACE-inhibitory activity and the in vivo action. This fact has provided doubts on the use of the in vitro ACE-inhibitory activity as the exclusive criterion for potential anti-hypertensive substances, since physiological transformations may occur in vivo, and because mechanisms of action other than ACE inhibition might be responsible for the anti-hypertensive effect.

3.4.2 Human studies

Evidence of the beneficial effects of bioactive peptides has to be based on studies performed in humans. So far, most of the clinical research has focused on lactotripeptides and their anti-hypertensive properties. More than 20 human studies have been published linking the consumption of products containing lactotripeptides with significant reductions in both SBP and diastolic blood pressure (DBP). Oral administration of these tripeptides included different formulas, such as fermented milk, tablets, capsules, fruit juice and other products. Most clinical trials have assessed blood pressure (BP)-lowering effects at multiple points over time. The human studies with lactotripeptides have been done mainly in Japanese subjects and some in Finnish subjects (Aihara, Kajimoto, Hirata, Takahashi, & Nakamura, 2005; Hata et al.,1996; Jauhiainen, Vapaatalo, et al., 2005; Kajimoto, Aihara, Hirata, Takahashi, & Nakamura, 2001; Kajimoto, Nakamura, et al., 2001; Nakamura et al., 2004; Seppo, Jauhiainen, Poussa, & Korpela, 2003; Seppo, Kerojoki, Suomalainen, & Korpela, 2002). Generally, maximum duration of treatment has been 8 weeks at doses between 3 and 52 mg/day (Table 3.3). From these data, it becomes apparent that the largest part of the total BP reduction takes place in the first 1–2 weeks of treatment. Thereafter, a further gradual lowering is seen, but to a lesser extent than in the first period (Kajimoto, Aihara, et al., 2001; Seppo et al., 2003).

The first significant effects of lactotripeptides on BP in hypertensive subjects have been observed after 1–2 weeks of treatment with dosages as low as 3.8 mg/day. For example, Aihara et al. (2005) observed that lactotripeptides (VPP and IPP) induced a gradual lowering of SBP compared to a control treatment by 7.8, 10.5, 10.6 and 11.2 mmHg after 1, 2, 3 and 4 weeks of active treatment, respectively. Kajimoto et al. (2002) demonstrated a comparable profile; SBP decreased by 7.6 mmHg after 1 week intake of lactotripeptides compared with placebo and gradually thereafter to 12 mmHg after 8 weeks. Maximum BP-lowering effects of lactotripeptides approximated 13 mmHg SBP and 8 mmHg DBP active treatment versus placebo, and were likely to reach the maximum after 8–12 weeks of treatment. Consumption of fermented milk containing 15.9 mg/l IPP and 18.7 mg/l VPP decreased SBP during 8 h after ingestion. This effect on BP was more pronounced in subjects with higher baseline SBP (Van der Zander, Jäkel, Bianco, & Koning, 2008). De Leeuw, van der Zander, Kroon, Rennenberg, and Koning (2009) found that administration of yogurt drinks containing IPP and VPP at a daily dose of 2.3, 4.6 or 9 mg for 8 weeks demonstrated a modest dose-dependent effect on office BP in mildly hypertensive subjects, although this could

Table 3.3 Human studies on hypotensive effects of milk-based products containing bioactive peptides IPP and VPP

Study design	Duration (weeks)	Study population	Treatment		Source of production of peptides	Formula and dose per day	Effect on blood pressure (mmHg)		References
			IPP (mg/day)	VPP (mg/day)			SBP	DBP	
R, p-c, s-bld, parallel*	8	30 Asian elderly hypertensive patients	1.1	1.5	*Lactobacillus, Saccha-romyces helveticus & Saccharomyces cerevisiae*	1 × 95 ml milk drink	−14.1	−6.9	Hata et al. (1996)
R, p-c, d-bld, parallel*	8	64 Asian subjects with SBP 140–159 and DBP 90–99 mmHg	1.58	2.24	*Lb. helveticus & S. cerevisiae*	2 × 150 g milk drink	−13	−8.4	Kajimoto, Nakamura, et al. (2001)
R, p-c, d-bld, parallel*	8	32 Asian subjects with SBP 140–180 and DBP 90–105 mmHg	1·60	2·66	*Lb. helveticus & S. cerevisiae*	1 × 120 g milk drink	−12.1	−5.8	Hirata, Nakamura, Yada, et al. (2002)
R, p-c, d-bld, parallel*	8	18 Asian hyper-tensive and 26 normotensive subjects	1.1	1.5	*Lb. helveticus & S. cerevisiae*	2 × 100 g milk drink	−7.6	−2	Itakura, Ikemoto, Terada et al. (2001)

R, p-c, d-bld, parallel*	8	30 Asian subjects with SBP 140–180 and DBP 90–105 mmHg	1.52	2.53	*Lb. helveticus* & *S. cerevisiae*	2×160 g milk drink	−13.2	−7.8	Kajimoto, Nakamura, et al. (2001)
R, p-c, d-bld, parallel*	12	144 Asian subjects with high-normal or mild hypertension	1.60	1.47	Casein hydrolyzed by *A. oryzae*	1×200 ml vegetable and fruit juice	−4.1	−2.5	Sano et al. (2005)
R, p-c, d-bld, parallel*	21	39 Finnish subjects with SBP 133–176 and DBP 86–108 mmHg	2.25	3.0	*Lb. helveticus* LBK-16H	2×150 ml milk drink	−6.7	−3.6	Seppo et al. (2003)
R, p-c, d-bld, parallel, cross-over†	10 7	60 Finnish subjects with SBP 140–180 and DBP 90–110 mmHg	2.4–2.7	2.4–2.7	*Lb. helveticus* LBK-16H	1×150 ml milk drink	−2.3 −12.3	−0.5 −3.7	Tuomilehto et al. (2004)
R, p-c, d-bld, parallel*	10	94 Finnish hypertensive patients	30	22.5	*Lb. helveticus* LBK-16H	2×150 ml milk drink	−4.1	−1.8	Jauhiainen, Vapaatalo, et al. (2005)
R, p-c, d-bld, parallel*	1	20 Asian healthy volunteers with normal blood pressure (<130 mmHg SBP and <85 mmHg DPB)	11.5	17.7	*Lb. helveticus* CM4 proteinase	1×14 tablets	−2.6	−2	Yasuda et al. (2001)

Continued

Table 3.3 Continued

Study design	Duration (weeks)	Study population	Treatment		Source of production of peptides	Formula and dose per day	Effect on blood pressure (mmHg)		References
			IPP (mg/day)	VPP (mg/day)			SBP	DBP	
R, p-c, d-bld, parallel*	8	275 Dutch subjects with mild hypertension	10.2	(VPP + IPP)	Casein hydrolysed by *Aspergillus oryzae*	1 × 200 g	−2.3	−0.7	Van der Zander et al. (2008)
R, p-c, d-bld, parallel*	8	135 Dutch subjects with untreated high-normal BP or mild hypertension	4.2–5.4	5.0–5.8	Fermentation	1 × 200 ml yoghurt drink	−0.5	−1.2	Engberink et al. (2008)
R, p-c, d-bld, parallel, cross-over†	4	70 Caucasian subjects with pre-hypertension or stage 1 hypertension	15	–	Hydrolysis by endopeptidase	2 × 7.5 mg capsules	−3.8	−2.3	Boelsma and Kloek (2010)

IPP, Isoleucine-proline-proline; VPP, valine-proline-proline; SBP, systolic blood pressure; DBP, diastolic blood pressure; R, p-c, d-bld, randomized, placebo-controlled, double-blinded.
*Results reported as changes in SBP and DBP after each month of treatment for all subjects (intention-to-treat analysis), and as mean changes over the total intervention period among subjects who had BP measurements for each month (per protocol analysis).
†First part of the study was carried out in parallel design and second part of the study was carried out in crossover design.

not be confirmed with ambulatory examination. The highest effective dosage of lactotripeptides was evaluated in a safety study, and consisted of 52.5 mg/day (Jauhiainen, Vapaatalo, et al., 2005). No major adverse effects were observed and neither did they affect serum clinical chemistry, substantiating the safety of consumption of lactotripeptides (Aihara et al., 2005; Yasuda, Aihara, Komazaki, Mochii, & Nakamura, 2001).

A few interventions evaluated BP-lowering effects after treatment periods that lasted longer than the generally applied 8 weeks. It is speculated that the blood pressure-lowering effect is already present at the first days of consumption and that this effect is repeated on every new day that the lactotripeptide-containing product is consumed, with only a very small additional chronic effect after at least 6 weeks of intervention. Sano et al. (2005) demonstrated a decrease of SBP by 3.3 mmHg after 8 weeks of intake of 3.1 mg/day lactotripeptides compared with placebo and a slight additional decrease by 4.4 and 4.1 mmHg after 10 and 12 weeks, respectively, in subjects with high-normal BP and subjects with moderate hypertension. In none of the trials with normotensives have statistically significant BP changes been found (Kajimoto, Nakamura, et al., 2001). Even at the highest dosage of lactotripeptides used in normotensives, which included a total of 29.2 mg/day during a period of 7 days, no BP-lowering effects by lactotripeptides were observed (Yasuda et al., 2001). Thus, lactotripeptides only seem to be active at elevated BP values.

Not all human intervention studies with lactotripeptides VPP and IPP have reported a statistically significant reduction on BP in hypertensive subjects, as compared to either to the control group or initial BP (Engberink et al., 2008; van Mierlo et al., 2009; Usinger, Jensen, Flambard, Linneberg, & Ibsen, 2010; Van der Zander et al., 2008). Several possible reasons have been proposed for the discrepancy between studies conducted in Europe and elsewhere: size (weight) of study subjects, differences in habitual diet, inclusion criteria, selection of the control product, length of intervention period and different methods used in blood pressure measurement (Boelsma & Kloek, 2010; Jäkälä & Vapaatalo, 2010).

The conflicting results have been analysed in five published *meta*-analyses.In four *meta*-analyses, decreases around 5 mmHg for SBP and 2.3 mmHg for DBP have been reported (Cicero, Gerocarni, Laghi, & Borghi, 2010; Pripp, 2008; Turpeinen, Järvenpää, Kautiainen, Korpela, & Vapaatalo, 2013; Xu, Qin, Wang, Li, & Chang, 2008). The *meta*-analysis of Xu et al. (2008) included nine studies (12 trials) with 585 subjects who consumed 2.6–52.5 mg of lactotripeptides daily for 4–12 weeks and a significant decrease both in SBP (–4.8 mmHg) and DBP (–2.2 mmHg) was observed. Cicero et al. (2010) conducted a *meta*-analysis of 18 randomized, placebo-controlled trials with a total of 1691 subjects. The length of interventions ranged from 4 to 21 weeks, with a mean duration of 6.8 weeks, and peptide doses of 2–52 mg/day were consumed in fermented milk products, juice or tablets. The pooled effect of lactotripeptides was a reduction of 3.73 mmHg for SBP and 1.97 mmHg for DBP; however, it was highlighted that the effect was more evident in Asian subjects than in Caucasians. In another recent *meta*-analysis of Turpeinen et al. (2013) a random-effects model was used on 19 randomized, placebo-controlled clinical intervention trials (published between 1996 and October 2010) consisting of about 1500 pre-hypertensive or mildly hypertensive subjects. Small daily doses (2.0–10.2 mg) of lactotripepides showed an overall lowering of SBP (4.0 mmHg)

and DBP (1.9 mmHg) in mildly hypertensive subjects. In the most recent *meta*-analysis by Cicero, Aubin, Azais-Braesco, and Borghi (2013) the effect of IPP and VPP on SBP in Europeans was assessed in particular. The analysis included 14 trials with a total of 1306 subjects. The combination of all data for the two peptides yielded a statistically significantly greater effect for IPP/VPP than for placebo. The decrease in SPB with IPP/VPP was 1.28 mmHg and the decrease in DPB was 0.59 mmHg. The authors concluded that the peptides IPP and VPP are effective in moderately reducing SBP in European subjects, as is known for Asian populations.

Hypertension is a complex multifactor disorder that is thought to result from an interaction between environmental factors and genetic background. Subject characteristics such as age and race/ethnicity can affect BP, including the BP response to specific anti-hypertensive medication. For certain anti-hypertensive drugs, it has been reported that a polymorphism found in humans can affect the clinical effectiveness, and, similarly, these differences could be also affecting clinical trials of functional ingredients (Arsenault et al., 2010). Although ACE inhibition has been postulated as the underlying mechanism of these lactotripeptides, results about the inhibition of this enzyme are not conclusive in humans. Several studies have shown that rennin or ACE activity was not affected by the oral administration of the tripeptides (Boelsma and Kloek, 2009). Fermented milk did not significantly affect plasma levels of angiotensin I or angiotensin II, ACE activity or active plasma renin concentration, but it significantly decreased the angiotensin II/angiotensin I ratio by 29% (Van der Zander et al., 2008). Therefore, other mechanisms could be implicated in the observed blood pressure reduction. It has been found that the intake of fermented milk containing these peptides may decrease sympathetic activity, leading to a diminished heart rate variability, heart rate and total peripheral resistance, although differences did not reach statistical significance (Usinger, Reimer, & Ibsen, 2012). Other potential mechanisms of action for the anti-hypertensive effect of lactotripeptides could be production of vasodilators, such as nitric oxide (Hirota et al., 2007).

3.4.3 Bioavailability

Bioavailability is an important means of approach to establish the relationship between in vitro and in vivo activities of bioactive peptides. The likelihood of any bioactive peptide released during fermentation mediating a physiological response is dependent on the ability of that peptide to reach an appropriate target site. Therefore, peptides may need to be resistant to further degradation by proteolytic and peptidolytic enzymes in the digestive tract. Thereafter peptides should be absorbed and enter systemic circulation. Resistance to hydrolysis is one of the main factors influencing the bioavailability of bioactive peptides. The effects of digestive enzymes on bioactive peptides, in particular ACEI-inhibitory peptides derived from different food matrices, have been evaluated in in vitro gastrointestinal simulated systems. These studies have shown that the ACE inhibition is low after fermentation by LAB but increases during subsequent hydrolysis by digestive enzymes that simulate gastrointestinal digestion (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004; Pihlanto-Leppala et al., 1998). Proline- and hydroxyproline-containing peptides are

usually resistant to degradation by digestive enzymes pepsin and trypsin. Tripeptides containing C-terminal proline–proline are generally resistant to proline-specific peptidases (Vermeirssen, Van Camp, & Verstraete, 2004). Some of the peptides isolated from Manchego cheese have been shown to be resistant to the incubation with pepsin followed by hydrolysis with a pancreatic extract. The ACE-inhibitory activity after simulated digestion increased for peptide αs2-CN f(195–204) (Thr-Gln-Pro-Lys-Thr-Asn-Ala-Ile-Pro-Tyr) sixfold while the activities of peptides Val-Arg-Tyr-Leu and Lys-Lys-Tyr-Asn-Val-Pro-Gln-Leu decreased (Gómez-Ruiz, Ramos, & Recio, 2004). The ACE-inhibitory activity of Trp-Leu peptide, isolated from tofuyo, was completely preserved after pepsin–trypsin or chymotrypsin treatment, whereas another peptide, Ile-Leu-Phe, was completely preserved after pepsin treatment, but the activity decreased by 62% and 75% of the original value following chymotrypsin and trypsin treatment, respectively (Kuba et al., 2003). Different peptides with ACE-inhibitory activities have been produced by in vitro pepsin–pancreatin digestion of milk and soy protein isolates and this had led to the speculation that the physiological gastrointestinal digestion could also yield ACE-inhibitory peptides, not only from protein isolates but also from fermented products (Hernández-Ledesma et al., 2004; Lo & Li-Chan, 2005).

Peptides have been reported to have poor permeation across biological barriers (e.g. intestinal mucosa) (Pauletti et al., 1996). Peptides can be transported by active transcellular transport or by passive processes. Although substantial amino acid absorption occurs in the form of di- and tripeptides at the apical side of enterocytes, efflux of intact peptides via the basolateral membrane into the general circulation seems to be negligible. The intestinal absorption of peptides has been performed using in vitro tests with monolayer of intestinal cell lines, simulating intestinal epithelium, as well as analysis of peptides and derivatives in blood samples after animal and clinical studies (Pauletti, Gangwar, Siahaan, Aubé, & Borchardt, 1997; Yang, Dantzig, & Pidgeon, 1999). Foltz et al. (2007, 2008) investigated the transport of IPP and VPP by using three different absorption models and demonstrated that these tripeptides are transported in small amounts intact across the barrier of the intestinal epithelium. The major transport mechanisms of IPP and VPP were demonstrated to be paracellular transport and passive diffusion (Satake et al., 2002). Caco-2 cell monolayers were used to study the transport of three dipeptides, Ala-Phe, Phe-Ile and Ile-Phe, identified from the salt-free soy sauce. Kinetic studies revealed that Ile-Phe exhibited greater affinity towards the transport compared with Ala-Phe and Phe-Ile (Zhu et al., 2008).

Vascular endothelial tissue peptidases and soluble plasma peptidases further contribute to peptide hydrolysis. As a consequence, the plasma half-life of most peptides is limited to minutes, as shown for endogenous peptides, such as angiotensin II and glucagon-like peptide 1 (Deacon et al., 1995; Werle & Bernkop-Schnürch, 2006). In order to exert anti-hypertensive effect the ACE-inhibitory peptides need to resist different peptidases, such as ACE. In this regard ACE-inhibitory peptides can be classified into three groups: the inhibitor type, of which the IC_{50} value is not affected by pre-incubation with ACE; the substrate type, peptides that are hydrolysed by ACE to yield peptides with a weaker activity; the pro-drug type inhibitor, peptides that are converted to true inhibitors by ACE or other proteases/peptidases. Only peptides

belonging to pro-drug or inhibitor type exert anti-hypertensive properties after oral administration. Soy peptides Ile-Leu-Phe and Trp-Leu are noncompetetive inhibitors, whereas captopril is a competitive inhibitor (Kuba et al., 2003).

There are some examples showing that peptides are absorbed in animals and humans and can exert in vivo activities. Jauhiainen et al. (2007) used a radio-labelled Ile-Pro-Pro and showed that this tripeptide absorbed partly intact from the gastrointestinal tract after a single oral dose to Sprague–Dawley rats. Considerable amounts of radioactivity were found in several tissues, e.g. liver, kidney and aorta. The excretion of IPP was slow; even after 48 h the radio-labelled peptide had not been completely excreted. IPP did not bind to albumin or other plasma proteins in vitro. Considering this and the long-lasting retention of the radioactivity in the tissues, accumulation of IPP may occur in sufficient concentrations to cause BP-lowering effects, e.g. by ACE inhibition in the vascular wall. In another animal study, the absolute bioavailability of the tripeptides in pigs was found to be below 0.1%, with an extremely short elimination half-life ranging from 5 to 20 min (van der Pijl, Kies, Ten Have, Duchateau, & Deutz, 2008). In a placebo-controlled, full-crossover study on healthy subjects Foltz et al. (2007) showed that IPP was absorbed intact from a fermented milk drink into circulation, but maximal plasma concentration of IPP did not exceed picomolar amounts.

The improvement of limited absorption and stability of peptides has been a goal when evaluating their effectiveness. For example, some carriers interact with the peptide molecule to create an insoluble entity at low pH that later dissolves and facilitates intestinal uptake by enhancing peptide transport over the nonpolar biological membrane (Kumar Malik, Baboota, Ahuja, Hasan, & Ali, 2007). Bioavailability of bioactive tripeptides (VPP, IPP, LPP) was improved by administering them with a meal containing insoluble dietary fibre (Kies & Pijl Van Der, 2012). Ko, Cheng, Hsu, and Hwang (2006) applied emulsification, microencapsulation and lipophilization to enhance the anti-hypertensive activity of a hydrolysate of tuna cooking juice. Among these treatments, lipophilization was the most effective, followed by microencapsulation and lecithin emulsification, gaining for each of them a stronger effect than that obtained with the double untreated dosage. Emulsions have long been used to enhance oral bioavailability or promote absorption through mucosal surfaces of peptides and proteins (Kumar Malik et al., 2007).

3.4.4 Regulatory aspects

Since the beginning of the 21st century, increasing emphasis has been placed worldwide on the safety aspects and legal regulation of health claims attached to food products. This development has been prompted by many global or regional health catastrophes caused by fraudulent or unfit foodstuffs on the markets. Authorities around the world have developed systematic approaches for review and assessment of scientific data. Evidence on the beneficial effects of a functional food product should be detailed, extensive and conclusive enough for the use of a health claim in the product labelling and marketing. Besides being based on generally accepted scientific evidence, the claims should be well understood by the average consumer. First, it is necessary to identify and quantify the active sequences of biologically active peptides in the food consumed.

Anti-hypertensive peptides are only minor constituents in highly complex food matrices and, therefore, a rigorous monitoring of the large-scale production utilizing hydrolytic or fermentative industrial processes is mandatory. Second, extensive investigations to prove the anti-hypertensive effect in humans, as well as the minimal dose to show this effect, are necessary to fulfil the requirements of the legislation concerning health claims that can be approved in the European Union (EU). A common regulation on nutrition and health claims on foods was adopted by EU in 2006 and the evaluation of such claims was mandated to the European Food Safety Authority (EFSA). Recently, the EFSA concluded that the evidence is insufficient to establish a cause-and-effect relationship between the consumption of the tripeptides VPP and IPP and the maintenance of normal blood pressure (EFSA, 2012). Consequently, more clinical data with consistent results are required so as to provide convincing evidence for substantiation of a health claim on these peptides for approval by the EU. Until that time, products containing such peptides have to be marketed in EU countries without any health claim related to blood pressure.

3.5 Conclusions and future trends

The generation of bioactive peptides during microbial fermentation of milk and soybean is now well documented. Such peptides have been shown to exert several bioactivities that have been demonstrated in animal and human studies. Potential health benefits associated with bioactive peptides have been subject of growing commercial interest, especially in the context of functional foods. Milk proteins are the best-known source of bioactive peptides, but peptides with similar properties have also been identified in soybean. Anti-hypertensive peptides derived from milk proteins have already found commercial applications both in Japan and in Europe. This trend is likely to continue, although controversial results in clinical trials and global differences in health claim regulations call for further scientific and clinical research.

A systematic screening of industrial dairy starter bacteria for formation of certain bioactive peptides with specific properties has produced a great number of anti-hypertensive peptides. However, just a few of these peptides have been tested clinically for health benefits, so far. The new knowledge about the frequent occurrence of different active peptides, e.g. anti-hypertensive, immunomodulatory and mineral-binding, in traditional fermented dairy products and also in fermented soy products could be appreciated more by industrial manufacturers. It should encourage the industry to invest more in the added-value products with scientific evidence of health benefits. To this end, novel technologies are available to standardize and stabilize the concentrations of active peptides in the fermented products by means of membrane separation techniques and encapsulation. Important research topics will continue to be the bioavailability and safety of bioactive peptides. In this field, more in-depth topics include the stability of the biological activity of peptides, both in the food matrix where released or incorporated and in vivo in the body before being absorbed and transported to the target site. Also, the integrity of the chemical structure of peptides, their interactions with food matrix components and organoleptic properties are important attributes to be considered when formulating novel products containing bioactive peptides. Furthermore, molecular studies

are needed to assess the mechanisms by which bioactive peptides exert their activities in the body. To this end, it may be necessary to employ proteomic and metabolomic methods. By means of these novel nutrigenomic approaches, it is possible and, in future, perhaps essential to investigate the impact of peptides on the expression of genes and hence endeavour to optimize the nutritional and health effects delivered by peptides.

Many studies have shown it to be a wrong approach that peptides are screened first in vitro for potential targets and then in vivo to confirm efficacy. According to Foltz, van der Pijl, and Duchateau (2010), it appears that it is only valid to propose efficacy once the peptide exhibits reasonable proteolytic stability and physiologically relevant absorption, distribution, metabolism and excretion profiles.

Moreover, the safety of all novel peptides intended for food or pharmaceutical uses should be tested in accordance with international and national food safety regulations. In cases of products intended to be marketed in the EU member states, the novel food legislation has to be observed. Until now, no toxicological safety concerns have been reported about bioactive peptides derived from fermented dairy or soy products. On the other hand, potential allergenic properties of milk and soy proteins and their hydrolysates are well known and should be taken into account when formulating novel products based on these compounds (Cordle, 2004; Eigenmann, 2007). Other challenges with dietary bioactive peptides are posed by health claims, which in the EU countries are strictly regulated and require science-based documentation before approval by the European Commission. At present, there are worldwide efforts to harmonize these regulations so as to develop fair global food marketing and protect consumers against false or misleading product information.

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Conjugated linoleic acid production in fermented foods

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4.1 Introduction

In the last two decades numerous studies have been published on conjugated linoleic acid (CLA) isomers. The most important motivation for related research investigations is the possible positive health effects of these "unusual" fatty acids; therefore, the authors prepared a brief outline on the health amelioration potential of CLA, presented at the end of this chapter. Nevertheless, the main part of the chapter is a summary of published data relating to the presence of CLA in fermented milk and meat products, including functional foods, and, moreover, the study of factors that can influence the CLA content of these food items. The CLA content of raw materials is an important issue because it can vary over a broad range, depending on several factors. The products of ruminants usually contain higher amounts of CLA than those of monogastric animals, and the effect of feeding is also an important influencing factor. Several successful attempts have been made through which the CLA content of raw milk and meat was increased with appropriate feeding regimens. A possible means of production of fermented foods with enhanced CLA levels is the incorporation of these initial materials into the product. Another promising approach is the accomplishment of CLA increment during fermentation by the selection of CLA-forming strains and application of appropriate external conditions. These issues are discussed in detail, following a short introduction to the chemistry and synthesis of CLA in Section 4.2.

4.2 Basic knowledge of conjugated linoleic acid (CLA)

Polyunsaturated fatty acids of natural origin have double bonds, mostly in isolated positions. The collective term "conjugated linoleic acid" refers to a group of positional and geometric isomers of linoleic acid having double bonds in a conjugated position ranging from 2,4 to 15,17. Each positional isomer has four geometric isomers, those being *cis, trans*; *trans, cis*; *cis, cis*; or *trans, trans* – that is, the term CLA may refer to a total of 56 possible isomers (Buccioni, Decandia, Minieri, Molle, & Cabiddu, 2012). From these, numerous CLA isomers have been shown to be present in food. In milk fat, one of the most important sources of CLA, the positions of double bonds range from 6,8 to 13,15; therefore the term "total CLA content" includes a total of 32 isomers (Bessa, Santos-Silva, Ribeiro, & Portugal, 2000; Kramer et al., 2004;

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00004-9

Shingfield et al., 2008). The predominant isomer originally present in food was shown to be *cis*-9,*trans*-11 CLA that occurs together with *trans*-10,*cis*-12 and *trans*-7,*cis*-9 CLA, which are also present in minor but significant amounts. In contrast, commercial CLA preparations used for functional foods can contain similar concentrations of both enantiomers (Or-Rashid, AlZahal, & McBride, 2011; Park, 2009; Wahle, Heys, & Rotondo, 2004).

The term "conjugated linolenic acid" (CLnA) encompasses a collection of positional and geometric isomers of linolenic acid (*cis*-9,*cis*-12,*cis*-15 18:3) in which two or three double bonds have a conjugated arrangement (conjugated diene CLnA and fully conjugated CLnA, respectively) instead of methylene interruption. Conjugated diene CLnA is mostly present in the fats of ruminants (Collomb, Schmid, Sieber, Wechsler, & Ryhänen, 2006; Plourde, Destaillats, Chouinard, & Angers, 2007), whereas fully conjugated CLnA is present in plant seeds (Hopkins & Chisholm, 1968).

One of the most important pathways of CLA formation in nature is the biohydrogenation of polyunsaturated fatty acids (Figure 4.1). This bacterial enzymatic process is achieved mostly in the rumen, but it cannot be excluded that bacteria in the gastrointestinal tract of nonruminants are also able to convert unsaturated fatty acids to CLA to some extent, as higher linoleic acid intake caused an increment of CLA level in tissues of rats (Buccioni et al., 2012; Chin, Liu, Albright, & Pariza, 1992; Shorland, Weenink, & Johns, 1955). Formation of a very limited amount of CLA has also been shown by gastric bacterial biohydrogenation in the pig (Dugan, Aalhus, & Kramer, 2004).

The first discovered CLA precursor is linoleic acid (*cis*-9,*cis*-12 18:2). The double bond of linoleic acid present further for the carboxyl group can be translocated by *cis*-12,*trans*-11 isomerase (Kepler & Tove, 1967) and the resulting rumenic acid (*cis*-9,*trans*-11 CLA) is then hydrogenated by the reductase of *Butyrivibrio fibrisolvens* to vaccenic acid (*trans*-11 18:1) (Hughes, Hunter, & Tove, 1982). These steps occur relatively fast, whereas the hydrogenation of vaccenic acid to stearic acid is

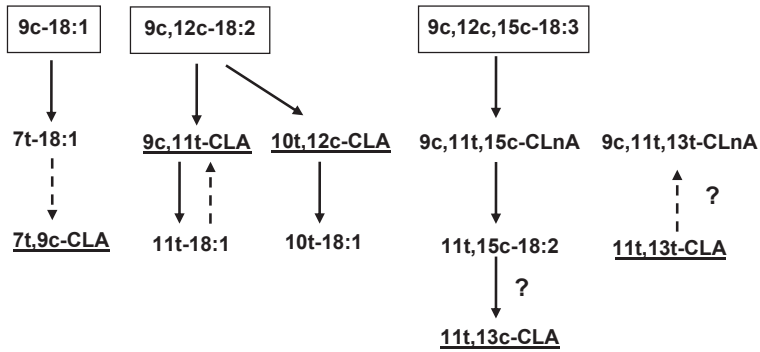

Figure 4.1 Biosynthetic pathways of those CLA isomers present in the most abundant quantities in human foods. Ruminal formation is marked with solid arrow, while formation in other organs by Δ^9-desaturase reaction is shown with dotted arrows (according to Collomb et al. (2006), Buccioni et al. (2012) and Lerch et al. (2012)). Question marks indicate that the exact mechanism is unclear. The underlined compounds are the conjugated linoleic acid isomers. The compounds without underline are the intermediaries of conjugated linoleic acid synthesis.

much slower, as it involves different groups of organisms (Griinari, Chouinard, & Bauman, 1997; Harfoot & Hazelwood, 1997). As the decomposition of vaccenic acid is the rate-limiting step, this intermediate can accumulate in the rumen and following absorption it can be a precursor for the endogenous synthesis of *cis*-9,*trans*-11 CLA in the mammary gland by Δ^9-desaturase (Corl, Barbano, Bauman, & Ip, 2003; Griinari et al., 2000; Kay, Mackle, Auldist, Thomson, & Bauman, 2004; Piperova et al., 2002). The majority of *cis*-9,*trans*-11 CLA present in bovine milk was reported to be synthesised in this pathway in the mammary gland and not in the rumen (Griinari et al., 2000; Lerch, Shingfield, Ferlay, Vanhatalo, & Chilliard, 2012). Moreover, CLA is also synthesised – also from vaccenic acid – in muscle adipose tissues both in ruminants (Bauman, Baumgard, Corl, & Griinari, 2007; Raes et al., 2004) and in monogastric animals (Gläser, Wenk, & Scheeder, 2002).

The other pathway of the isomerisation of linoleic acid is provided by the action of *cis*-9,*trans*-10 isomerase in the rumen (Griinari & Bauman, 2006). The resulting *trans*-10,*cis*-12 CLA can be reduced to *trans*-10 18:1 by *Butyrivibrio fibrisolvens* JW11 (McKain, Shingfield, & Wallace, 2010).

Some part of oleic acid (*cis*-9 18:1) originates from fodder and can be converted further via pathways other than hydrogenation to stearic acid in the rumen. Oleic acid has been proposed as a precursor of the formation of *trans*-7,*cis*-9 CLA. The first step is the action of microbial isomerases resulting in positional isomers of 18:1, ranging from 6 to 16 (Mosley, Powell, Riley, & Jenkins, 2002). These *trans*-octadecenoic acids escape the rumen together with linoleic acid-origin vaccenic acid. In the mammalian tissues the dehydrogenation of *trans*-7 18:1 isomer via Δ^9-desaturase results in the formation of *trans*-7,*cis*-9 CLA (Corl et al., 2002). This pathway was proposed as the main process for the formation of *trans*-7,*cis*-9 CLA (Lerch et al., 2012). Irrespective of the diet composition, the formation of *trans*-7,*cis*-9 was not observed in the rumen (Shingfield et al., 2010).

The CLA formation pathway from α-linolenic acid precursors (*cis*-9,*cis*-12,*cis*-15 18:3) has not been completely elucidated. Kepler and Tove (1967) suggested first that *cis*-12,*trans*-11 isomerase might be active also on α-linolenic acid in the rumen. This first step results in a conjugated diene CLnA (*cis*-9,*trans*-11,*cis*-15 CLnA) via microbial isomerases in the rumen. The following reduction steps do not yield *cis*-9,*trans*-11 CLA, but *trans*-11,*cis*-15 18:2 is formed. This fatty acid has been assumed to be a precursor for *trans*-11,*cis*-13 CLA (Kraft, Collomb, Möckel, Sieber, & Jahreis, 2003), but the exact mechanisms of the double bond translocation is yet unclear. The concentration of conjugated linolenic acids *cis*-9,*trans*-11,*trans*-13 CLnA and *cis*-9,*trans*-11,*trans*-15 CLnA possessed strong correlation with *trans*-11,*trans*-13 CLA and *trans*-11,*trans*-15 18:2 CLA, respectively, in milk fat. These results may indicate that CLnA in milk fat are synthesised endogenously via the action by Δ^9-desaturase in the mammary gland (Lerch et al., 2012).

CLA can also be synthesised in the human colon. Some bacterial strains normally present in the human large intestine (*Lactobacillus*, *Propionibacterium* and *Bifidobacterium* species and some *Clostridium*-like bacteria) have been found to metabolise linoleic acid and form CLA that is converted further to *trans*-11 18:1 in vitro. Vaccenic acid may be also a precursor of rumenic acid (*cis*-9,*trans*-11 CLA) in human tissues (Devillard, McIntosh, Duncan, & Wallace, 2007).

4.3 CLA content of unprocessed food ingredients

A possible means of production of fermented foods rich in CLA is the selection of raw materials with high CLA content and the preservation of their elevated CLA levels during fermentation. The main natural sources of CLA in human nutrition are, undisputedly, the products of ruminants, including their milk, meat and adipose tissue. The enzymatic processes responsible for the formation of CLA proceed much more intensively in the tissues and organs of ruminants than those of monogastric animals. Vegetable oils usually contain only negligible amounts of CLA. In this section the CLA levels of raw materials and the factors affecting their formation are summarised.

4.3.1 Raw milk

The discovery of anti-carcinogenic properties of CLA (Pariza & Hargraves, 1985) and other biological activities led to the implementation of surveys in several countries to assess the CLA supply from food and daily intake of these fatty acids. Cows' milk fat proved to be one of the richest sources of CLA. The total CLA value of raw milk samples has been shown to extend over a wide range, from 2 to 37 mg/g CLA in fat (Jiang, Björck, Fondén, & Emanuelson, 1996; Lin, Boylston, Chang, Luedecke, & Schultz, 1995; Parodi, 1999; Stanton, Murphy, McGrath, & Devery, 2003). The main isomer, rumenic acid (*cis*-9,*trans*-11 CLA), was reported to represent more than 80% of total CLA content (Chin, Liu, Storkson, Ha, & Pariza, 1992; Fritsche & Steinhart, 1998; Parodi, 1994). Precht and Molkentin (2000) determined the amount of CLA in 2110 milk samples originating from 14 European Union countries. The average value for *cis*-9,*trans*-11 CLA was 7.6 mg/g in fatty acid methyl esters (FAME) and the extreme values were 1.3 and 18.9 mg/g, respectively. The *trans* 18:1, *trans* 18:2 and total *trans* fatty acid content was 36.7 mg/g (12.9–71.7 mg/g), 11.2 mg/g (3.0–20.4 mg/g) and 49.2 mg/g (17.1–87.0 mg/g), respectively. Surpassing values, i.e. 53.7 and 51.5 mg/g of fatty acids, were obtained for rumenic acid, respectively, by Shingfield et al. (2006) and Bell, Griinari, and Kennelly (2006). The large differences in CLA and vaccenic acid content of cows' milk fat can be attributed to several factors, of which the feeding regimen was noted to have a major influence (Wahle et al., 2004). These topics are discussed in detail in Section 4.4.

4.3.2 Meats and seafoods

Meats of ruminant origin have markedly higher CLA concentrations than those from monogastric animals (Chin, Liu, Strokson, et al., 1992). In addition, there can be significant variation in the CLA content of meat samples within the same ruminant species owing to factors such as feeding.

The highest amount of CLA was found in lamb and in beef (19.0 and 13.0 mg/g fat, respectively), and the published results on these two meats are almost overlapping. The CLA content of pork, rabbit, chicken and meat of horses is around 1 mg/g fat, while that of turkey is slightly higher. The difference in CLA content

between the meats of ruminants and nonruminants can reach one order of magnitude in some cases. Nevertheless, the lowest values for ruminants are very close to those of monogastric animals (Table 4.1). The main influencing factors are breed, feeding regimen, seasonal variations, production practices, part of the carcass and muscle type; moreover, large animal-to-animal variations have also been observed (Ma, Wierzbicki, Field, & Clandinin, 1999; Raes et al., 2003; Shantha, Crum, & Decker, 1994). The tissues of ruminants are provided with higher amounts of CLA

Table 4.1 Total CLA content of meats and eggs

Products	CLA content (mg/g)	CLA content as:	References
Beef	13.0	Fat	Fogerty et al. (1988)
	3.7	Fat	Chin, Liu, Strokson, et al. (1992)
	3.1–8.5	Fat	Shantha et al. (1994)
	6.5	FAME	Fritsche and Steinhart (1998)
	3.6–6.2	Fat	Dufey (1999)
	1.2–3.0	Fat	Ma et al. (1999)
	2.7–5.6	FAME	Rule, Broughton, Shellito, and Maiorano (2002)
	4.0–10.0	Fat	Raes et al. (2003)
Veal	2.7	Fat	Chin, Liu, Strokson, et al. (1992)
Lamb	14.9	Fat	Fogerty et al. (1988)
	12	FAME	Fritsche and Steinhart (1998)
	5.6	Fat	Chin, Liu, Strokson, et al. (1992)
	11.0	Fat	Dufey (1999)
	0.64–3.13	FAME	Mir, Rushfeldt, Mir, Paterson, and Weselake (2000)
	8.8–10.8	FAME	Wachira et al. (2002)
	4.32	Fat	Badiani et al. (2004)
	19.0	FAME	Knight, Knowles, Death, Cummings, and Muir (2004)
Pork	1.2; 1.5	FAME	Fritsche and Steinhart (1998)
	1.4	Fat	Fogerty et al. (1988)
	0.6	Fat	Chin, Liu, Strokson, et al. (1992)
Rabbit	1.1	FAME	Fritsche and Steinhart (1998)
Chicken	1.5	FAME	Fritsche and Steinhart (1998)
	1.8	Fat	Fogerty et al. (1988)
	0.9	Fat	Chin, Liu, Strokson, et al. (1992)
	0.7	FAME	Rule et al. (2002)
Turkey	2.5	Fat	Chin, Liu, Strokson, et al. (1992)
	2.0	FAME	Fritsche and Steinhart (1998)
Horse	0.6	Fat	Dufey (1999)
Eggs	0–2.4	Fat	Fogerty et al. (1988)
	0.6	Fat	Chin, Liu, Strokson, et al. (1992)
	0.2 (yolk)	FAME	Fritsche and Steinhart (1998)

CLA, conjugated linoleic acid; FAME, fatty acid methyl esters.

for incorporation into glycerolipids than those of monogastric animals, if feeding conditions are appropriate. CLA and its precursors can also originate from microbiological synthesis in the rumen, and probably also other parts of the gastrointestinal tract, as well as from de novo synthesis in the mammary gland or other tissues, as was described in the Introduction. The main cause of variation within the products of one breed has been assigned to the differences in feeding regimens (Dufey, 1999; Parodi, 1994).

The CLA level of common marine foods proved to be lower than that of meats and raw milk. The total CLA content was measured to be approximately only 0.5 mg/g; moreover, the most abundant isomer in continental food products (*cis*-9,*trans*-11 CLA) was not detected in food of marine source (Chin, Liu, Strokson, et al., 1992). The CLA content of fishes varied from 0.1 (pike perch) to 0.9 mg/g (carp) (Fritsche & Steinhart, 1998).

4.3.3 Plant oils

Fatty acids with conjugated double bonds do occur in different seed oils, but CLA is often not found in oils from a number of plant species or its level is negligible in relation to dairy products. Some studies led to the conclusion that CLA contents of edible oils (olive, soybean, sunflower, refined or unrefined walnut, safflower, grapeseed, avocado, cashew and peanut oil, and coconut fat) and margarines are below 0.1 mg/g (Fogerty, Ford, & Svoronos, 1988; Fritsche & Steinhart, 1998), while other workers have found larger quantities of CLA in similar products. CLA was quantified in exotic oils originating from Brazil (Spitzer, Marx, Maia, & Pfeilsticker, 1991a, 1991b) and was also detected in low amounts in commercial edible oils (corn, peanut, soybean and palm oil) in Canada (Ackmann, Eaton, Sipos, & Crewe, 1981). Corn oil, olive oil and coconut fat were reported to contain approximately 0.2 mg/g CLA. In samples obtained from stores the *cis*-9,*trans*-11 CLA isomer accounted for 45% of the total amount of CLA, while corn oil processed in the laboratory did not have this isomer in detectable amounts. The total CLA content of the home-made corn oil was almost the same (0.2 mg/g), as in the case of the commercial product (Chin, Liu, Strokson, et al., 1992).

The observed differences in the CLA content of vegetable oils may suggest that isomer distribution and the amount of total CLA of plant oils can be notably influenced by processing conditions in which heat and/or chemical treatment are applied (Fritsche & Steinhart, 1998).

4.4 Factors influencing the CLA content of raw materials, and the effect of animal diet on CLA content of milk and meat

Several factors have already been observed that may influence the CLA content of animal products, i.e. animal genetics, production practices and seasonal variation.

The animal diet is probably the most important among these factors because it provides substrates for CLA formation (Wahle et al., 2004). Besides the intake of unsaturated fatty acid precursors, other characteristics of feeding may also be important, e.g. energy values and fibre content, the number of feedings per day and the form of the oil source (free or present in intact seeds or in crushed seeds, etc.). Owing to the formation mechanisms of CLA, the main target group is the group of ruminants, as feeding them with CLA precursors can lead to the formation of significant amounts of CLA in the rumen. By contrast, the products of monogastric animals could be efficiently enriched in CLA, if the feed itself contains CLA or vaccenic acid, as Δ^9-desaturase reaction also occurs in nonruminants. As these feed additives are more expensive than oil seeds or vegetable oils, production of CLA-enriched meat with direct addition of CLA to the feed is questionable from an economic point of view.

Since the mid-1990s a large number of studies have repeatedly shown the advantage of pasture feeding over concentrate-based diet from the viewpoint of CLA content in ruminant products and also for other significant fatty acid parameters in the fat of these products, i.e. the n3/n6 polyunsaturated fatty acid (PUFA) ratio and PUFA/saturated fatty acid ratio (see reviews by Collomb et al. (2006), Schmid, Collomb, Sieber, and Bee (2006), van Wijlen & Colombani (2010)). Seasonal variations have been observed in the CLA content of milk between winter and summer. These differences could be attributed to the change in diet: indoor winter feeding versus pasture feeding. A switch from concentrate-based diet to pasture increased the CLA content of milk (Dhiman, Helmink, McMahon, Fife, & Pariza, 1999; Kelly, Kolver, Bauman, van Amburgh, & Muller, 1998; Lock & Garnsworthy, 2003; Precht & Molkentin, 2000; Salamon, Varga-Visi, et al., 2009; Stanton et al., 1997). A possible explanation for these observations is that provision of increasing proportions of fresh green forage resulted in higher intake of PUFA, especially α-linolenic acid, which could be a possible precursor of *trans*-11 18:1 in the rumen, following subsequent desaturation to *cis*-9,*trans*-11 CLA in the mammary gland (Bauman, Corl, & Peterson, 2003). However, the enhanced PUFA intake alone cannot explain why the milk fat of cows fed fresh green forage had more CLA than milk fat from silage-fed cows (Elgersma, Tamminga, & Ellen, 2006). The lower "CLA-generating" effect of silages related to fresh grass may be attributed to changes in nutrients during the ensiling process, that is, the sugar and soluble fibre contents of the material decreases and thereby generates a different ruminal environment related to grasses (French et al., 2000; Schmid et al., 2006).

Pasture feeding has also been reported to exert an advantageous effect on the CLA content of the meat of ruminants. The amount of CLA in intramuscular fat of pasture-raised steers (French et al., 2000; Poulson, Dhiman, Ure, Cornforth, & Olson, 2004; Realini, Duckett, Brito, Dalla Rizza, & DeMattos, 2004), bulls and lambs (Aurousseau, Bauchart, Calichon, Micol, & Priolo, 2004; Nuernberg et al., 2004; Santos-Silva, Bessa, & Santos-Silva, 2002) was significantly higher than in those fed with concentrate diet. Grass silage enhanced the level of CLA in the *longissimus dorsi* muscle of steers, but to a lesser extent than fresh grass (French et al., 2000).

In summary, pasture feeding seems to be an effective tool to produce raw materials of outstanding biological value. However, with respect to CLA, its efficiency seems to

be a little higher for milk than for meat, as the CLA content of milk fat was observed to be higher than that achieved in the fat of ruminant meat (Woods & Fearon, 2009).

Following the discovery of the favourable properties of CLA, several attempts have been made to increase its level in animal products with the addition of different plant oils and marine oils. Studies conducted on the CLA enrichment of the milk and meat ruminants have been reviewed by Collomb et al. (2006) and Schmid et al. (2006), respectively. Several types of plant oils (sunflower, peanut, soybean, canola and linseed oil) were used successfully to achieve significant increase in the CLA content of milk. Sunflower oil was found to be more effective than peanut or linseed oil (Kelly, Berry, et al., 1998; Collomb, Sieber, & Bütikofer, 2004; Collomb, Sollberger, et al., 2004) and the use of soybean oil as a supplement also has been shown to be more advantageous than feeding linseed oil (Dhiman et al., 2000). The addition of fish oil was more effective than addition of plant oils (Chilliard, Ferlay, Mansbridge, & Doreau, 2000).

In the case of CLA-rich meat production, feed complemented with sunflower seed increased the CLA content of subcutaneous fat of bulls, while linseed and rapeseed seemed not to be efficient (Casutt et al., 2000). Linseed addition exerted only a minor influence on the CLA levels in the *longissimus dorsi* muscle in bulls (Stasiniewicz, Strzetelski, Kowalczyk, Osieglowski, & Pustkowiak, 2000; Strzetelski et al., 2001). Sunflower seed also increased the CLA content of the *longissimus thoractis* muscle of lambs (Santos-Silva, Bessa, & Mendes, 2003), and, contrary to most of the experiments carried out with bulls, Wachira et al. (2002) and Demirel, Wood, and Enser (2004) managed to increase the CLA content of the *longissimus dorsi* muscle of lambs with linseed feeding. Inclusion of other oilseeds in the diet also proved to be an effective tool to increase the CLA content of meat. Safflower seed in lambs (Bolte, Hess, Means, Moss, & Rule, 2002; Kott et al., 2003), extruded full-fat soybeans in steers (Madron et al., 2002) and whole crushed soybeans with crushed raw flax in bull calves (Aharoni, Orlov, & Brosh, 2004) proved to be efficient. Among vegetable oils, applied in the free form, sunflower oil can be used to increase CLA in the *longissimus* muscle of beef cattle (Mir et al., 2003; Mir et al., 2002) and heifers (Noci, O'Kiely, Monahan, Stanton, & Moloney, 2005), and intramuscular CLA content in lambs (Ivan et al., 2001). Conversely, rapeseed oil did not exert any positive effect on the *longissimus dorsi* muscle of beef cattle or lambs (Stasiniewicz et al., 2000; Strzetelski et al., 2001; Szumacher-Strabel, Potkanski, Cieslak, Kowalczyk, & Czauderna, 2001). Results regarding soybean oil were inconsistent, as the effective dose of this type of oil was observed to differ (Beaulieu, Drackley, & Merchen, 2002; Griswold et al., 2003; Santos-Silva, Mendes, Portugal, & Bessa, 2004). Using oil in the free form means that at higher doses the adverse effect of milk fat depression has to be considered. The CLA content of meat parts was affected differently by soybean oil addition (Griswold et al., 2003). This finding draws attention to the possibility that the same situation could have been observed in the case of the other oil sources if other parts of the body had been analysed. That is, CLA enrichment with feeding could lead to different results for different parts of the carcass.

Obviously, one cannot expect any effect on CLA content when ruminally protected lipid supplements were added in order to improve the energy balance of milking cows (Scollan, Enser, Gulati, Richardson, & Wood, 2003).

The CLA-enhancing effect of a given forage supplementation (e.g. sunflower seed or soybean) has been shown to be different for milk fat and for the lipids of meat. With the application of these feed additives, the increase in CLA content of the fat in meat was relatively lower than that of milk fat (Collomb, Sieber, & Bütikofer, 2004; Dhiman et al., 2000; Lawless, Murphy, Harrington, Devery, & Stanton, 1998). Similar advantages were reported for milk fat over meat fat when pasture feeding versus concentrate-based diet was applied (see earlier). These findings may refer to differences between formation of milk and meat lipids with respect to the intensity of Δ^9-desaturase reaction or incorporation of CLA into glycerides.

The three main precursors for CLA formation in milk were denominated as oleic acid, linoleic acid and linolenic acid (Collomb et al., 2006). There were strong positive correlations between the intake of these fatty acids and the amount of several CLA isomers in milk fat (Collomb, Sieber, & Bütikofer, 2004). In part these relationships have been illuminated (Figure 4.1), but the biochemical explanations for many of them have not been completely revealed yet. The explanation of the mechanism of fish oil is also unclear. The direct formation via precursors is unlikely because these oils contain less linoleic and linolenic acids than plant oils, and long-chain PUFAs are not direct precursors of rumenic or vaccenic acid. However, supplementation of the diet with fish oil increased the amount of *trans*-11 18:1 leaving the rumen (Shingfield et al., 2003). One possible explanation may be the inhibitory effect of docosahexanoic acid (DHA, *cis*-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:6), because DHA hampers the reduction of vaccenic acid to stearic acid in vitro (AbuGhazaleh & Jenkins, 2004). However, additional experiments are needed to provide an explanation.

The level of inclusion of oils is limited by milk fat depression, that is, the fat content of milk decreases if the free oil content of the feed exceeds a certain limit (Banks, Clapperton, Kelly, Wilson, & Crawford, 1980; Jenkins, 1993). The decrease of protein content of milk has also been reported (Grummer, 1988), as free oil being present in too high quantities in the rumen can hamper both fermentation and protein synthesis by microbiota. Therefore the limit of levels that can be applied for different oil sources should be considered. If oil sources are intact or seeds are only partially disintegrated, the rate of oil liberation is slower in the rumen and therefore milk fat depression can be avoided (DePeters, Taylor, Franke, & Aguirre, 1985; Dhiman et al., 2000; Mohamed, Satter, Grummer, & Ehle, 1988). Nevertheless, a rise in CLA level in products cannot be obtained without overflowing the rumen with fatty acid substrates, because high levels of these could prevent the completion of the biohydrogenation, and by-products rumenic and vaccenic acid may escape the rumen in significant amounts.

Besides the application of shortenings, other diet ingredients that influence the biohydrogenation process can also be applied. The last step of biohydrogenation, i.e. the reduction of oleic acid to stearic acid, can be hampered with increasing starch and decreasing fibre content in the diet (Gerson, Jihn, & King, 1985; Jiang et al., 1996; Kelly & Bauman, 1996; Palmquist & Schanbacher, 1991; Varga-Visi & Csapó, 2003). Moreover, some authors were led to the assumption that there may be synergism between the high-forage concentration and dietary supplementation with PUFA (Aharoni et al., 2004).

The frequency of feeding might also influence the CLA content of the product. However, Banks et al. (1980) found that the effect of feeding frequency on the vaccenic acid content of milk was minor.

One can come to the conclusion that feeding is a possible way to produce CLA-rich animal products, for example, raw materials for fermented milk and meat products with enhanced biological value. However, several factors have to be considered in order to avoid possible negative changes that may lower fat and protein content. Moreover, the CLA increment has been reported as a transient phenomenon, likely owing to the adaptation of microbiota. The CLA level of milk decreased after a few weeks following the administration of the experimental diet (Bauman, Barbano, Dwyer, & Griinari, 2000; Shingfield et al., 2006). Therefore, it is hard to imagine how to maintain the production of milk rich in CLA during the whole lactation period. In the case of meats of ruminants, the transient feature of the blocking of biohydrogenation can be resolved if these diets are applied in the finishing period. However, a more substantial increase in the CLA content of beef cattle has been reported when sunflower oil was added to both growing and finishing diets (Mir et al., 2002), that is, the transient feature of CLA increment may follow different patterns in the case of milk and meat fat, probably owing to the different mechanisms and speed of lipid incorporation. Nevertheless, in order to establish the appropriate feeding regimen, additional studies are needed to clarify the effect of elapsed time on the CLA content of fat in animal products.

4.5 CLA content of fermented food products

One may expect that the evaluation of factors having an effect on CLA level of processed food products is more difficult than doing the same for non-processed foods. Besides the factors influencing the CLA level of raw materials, food processing steps may also have significant effects on the level of these constituents. Fermentation and application of heating can change the CLA content of food originating from unprocessed raw materials.

4.5.1 Fermented milk products

The CLA content of milk products ranged from 4.6 to 7.1 in Sweden (Jiang, Björck, & Fondén, 1997), from 3.6 to 7.0 in the United States (Chin, Liu, Strokson, et al., 1992; Ha, Grimm, & Pariza, 1989; Lin et al., 1995; Shantha, Deckeer, & Ustunol, 1992; Shantha, Ram, O'Leary, Hicks, & Decker, 1995) and from 4.0 to 11.7 in Germany (Fritsche & Steinhart, 1998). The magnitude of the differences in CLA content seems to be smaller for different milk products (Table 4.2) than for unprocessed milk.

Based on the data in Table 4.2 it can be seen that none of the groups of milk products contain notably more CLA than another, but values within a group can cover a wide range. In the case of cheeses, length of aging has been suggested as a possible factor of variance, as CLA level of Parmesan was measured to be higher than that of the other cheeses with shorter ripening periods (Ha et al., 1989). Moreover, hard cheeses with long aging time had higher CLA levels than hard cheeses with short maturation

Table 4.2 Total CLA content of fermented milk products and processed cheese

Products	CLA content (mg/g)	CLA content as:	References
Yogurt	6.9 ± 3.0	FAME	Fritsche and Steinhart (1998)
	4.8	Fat	Chin, Liu, Strokson, et al. (1992)
	3.8	Fat	Lin et al. (1995)
	4.3 ± 1.3	Fat	Prandini, Sigolo, Tansini, Brogna, and Piva (2007)
Cheese	8.4 ± 3.8	FAME	Fritsche and Steinhart (1998)
	5.0–7.1	Fat	Jiang et al. (1997)
	2.9–7.1	Fat	Chin, Liu, Strokson, et al. (1992)
	5.1–5.4	Fat	Werner et al. (1992)
	4.0–4.9	Fat	Nunes and Torres (2010)
	3.9–8.1	Fat	Prandini et al. (2007)
	0.6–1.9	Product	Ha et al. (1989)
Processed cheese	5.0	Fat	Chin, Liu, Strokson, et al. (1992)
	3.2–8.9	Fat	Shantha et al. (1992)
	1.9–8.8	Product	Ha et al. (1989)

CLA, conjugated linoleic acid; FAME, fatty acid methyl esters.

time (Zlatanos, Laskaridis, Feist, & Sagredos, 2002). Another possible explanation for the observed differences is the use of different starter cultures. On a theoretical basis it could be assumed that Roquefort, blue cheese and Emmental cheese are possible candidates for an enhanced CLA level, owing to the strong lipolysis during ripening resulting in a high free fatty acid content, of which linoleic acid could be a good substrate for the formation of CLA by lactic acid bacteria and propionibacteria during ripening (Sieber, Collomb, Aeschlimann, Jelen, & Eyer, 2004). Nevertheless, studies in which the whole process of cheese making was monitored did not verify this assumption because there were only slight variations in the CLA content related to fat content during ripening (Jiang et al., 1997; Lin et al., 1995; Werner, Luedecke, & Schultz, 1992) or when different starter cultures were used (Werner et al., 1992). Emmental cheese produced with strains of *Propionibacterium* sp. with different lipolytic activities gave almost the same results (Gnädig, 2002). In a comparative study conducted in Italy and France, the authors observed that the CLA content of cheeses produced from milk of the same ruminant species but with different technologies did not differ significantly (Prandini, Sigolo, & Piva, 2011). In a literature survey on the published

CLA results of cheeses between 1998 and 2011, no differences were found between the groups of hard, semi-hard, soft, mouldy and processed products (Abd El-Salam & El-Shibiny, 2014). Analysing the total amount of CLA and that of the individual isomers during ripening of Spanish cheeses protected with designation of origin, the authors found that differences that can be attributed to ripening were negligible (Luna, Juárez, & de la Fuente, 2007). Pasteurisation and ripening did not change the sum of CLA significantly in Pecorino Toscano (protected denomination of origin) cheese made from sheep milk (Buccioni et al., 2010); however, contrary to the observation of the previous group, significant differences have been observed in the distribution of the isomers.

The CLA content of dairy products is largely dependent on the CLA content of the milk from which they originate. Processing appears to have either no or only little effect on the amount of CLA in the final product (Wahle et al., 2004). However, heat processing of cheeses seems to be an exception, causing an increase in the CLA content. The level of increase was more pronounced when whey protein concentrate was added before heating, and, parallel with the increment, the ratio of *cis*-9,*trans*-11 CLA has been reported to remain the same (Ha et al., 1989; Shantha et al., 1992). The presence or absence of oxygen may also exert an effect on CLA during this process, as cheddar cheese following processing at 90 °C had 10% higher levels of CLA than before heating, while the same treatment in nitrogen atmosphere caused no change in the CLA level of the product (Shantha et al., 1992). This finding may support the hypothesis that heat accelerates the autoxidation of linoleic acid and therefore the formation of conjugated positional isomers.

Another possible means of formation of CLA is via enzymatic processes of fermentation. Elevated CLA levels were reported several times in fermented milk products related to raw milk (Aneja & Murthy, 1990; Jiang et al., 1997; Shantha, Ram, O'Leary, Hicks, & Decker, 1995), whereas others found no differences (Boylston & Beitz, 2002; Lin et al., 1995; Shantha et al., 1995) in fat basis. However, it seems that commercially used dairy starter bacteria have only a minor contribution to the CLA level of the fermented product (Salamon, Lóki, Csapó-Kiss, & Csapó, 2009; Sieber et al., 2004), while the use of suitable strains may lead to favourable results.

4.5.2 Dairy-based functional foods

The inclusion of certain microbial cultures in the fermentation during food processing may result in health benefits of the product and therefore can lead to functional attributes. The key functions of the body can be beneficially affected by the delivery of probiotics or alteration of biotransformation reactions during fermentation, including the elimination of unwanted compounds, formation of health-beneficial molecules or enhancement of their bioavailability (van Hylckama Vlieg, Veiga, Zhang, Derrien, & Zhao, 2011; Stanton, Ross, Fitzgerald, & Van Sinderen, 2005).

The improvement of the CLA supply of humans can be achieved via CLA-producing strains. Formation of CLA can be accomplished during the preparation of the food product via dairy cultures, but this process could also be amplified in the human gastrointestinal tract with the administration of probiotics. Recently, a number of

bacteria have been proved to form CLA, including strains of lactobacilli, bifidobacteria, propionibacteria, pediococci, enterococci, streptococci and lactococci (Hennessy, Ross, Stanton, Devery, & Murphy, 2007; Oh et al., 2003; Owaga et al., 2005). The CLA-producing capacity of these strains using different growth media, skim milk and whole milk was reviewed by Sieber et al. (2004). The CLA formation follows similar mechanisms as in the case of the ruminal bacterium *Butyrivibrio fibrisolvens* via the action of linoleic acid isomerase (Lin, Lin, & Wang, 2002), while the CLA metabolism may differ among strains.

CLA formation intensity of strains, as in the case of the other metabolites, is influenced by external conditions as well as by genetic traits. Optimization of fermentation to achieve the highest yield and to maintain this level, as far as possible, until the product is consumed is an indispensable task when CLA-enriched fermented functional foods are to be produced. In the late 1990s Lin, Lin, and Lee (1999) discovered that addition of linoleic acid significantly increased the CLA content of skim milk incubated with six lactic cultures, while incubation with cultures without the inclusion of linoleic acid did not result any changes in the CLA level of the fermented product. In a later experiment, addition of linoleic acid did not increase the amount of CLA when yogurt was fermented by the commercial starter culture alone (Xu, Boylston, & Glatz, 2005) but did enhance the CLA level of yogurt when the starter culture was applied together with probiotic strains.

Kim and Liu (2002) concluded that the CLA production of *Lactococcus lactis* I-01 in milk depends on several factors, i.e. substrate (sunflower oil) concentration, pH, incubation time and culture conditions. When sunflower oil was added initially, growing cells produced more CLA than cells in the stationary phase; however, stationary cells were capable of producing more CLA when sunflower oil was added shortly before the end of the incubation period.

The dose dependence of the addition of sunflower oil on the CLA content of fermented milk was evaluated by Salamon, Lóki, Varga-Visi, Mándoki, and Csapó (2009). Two stains of lactobacilli produced the maximum amount of CLA when 100–150 µl sunflower oil was added to 100 cm^3 milk. The authors observed a severe decline in CLA when addition exceeded the "200 µl/100 cm^3" value. In the samples fermented by *Lactobacillus plantarum* the CLA level dropped to the level of raw milk when 400 µl or more oil was added, while for *Lactobacillus acidophilus* significantly lower CLA levels were measured than those of the initial material when 600 µl or more sunflower oil was added to 100 cm^3 milk. A promising candidate for CLA formation, when rigorous optimization of CLA addition may not be required, is *Lactobacillus casei*. Using this species for fermentation, the level of CLA also increased significantly when sunflower oil was added; nevertheless, dose-dependent decline was not observed in the case of this strain.

Lin (2003) reported that mixed cultures of *L. acidophilus* CCRC 14079 and yogurt bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) significantly enhanced the CLA content of nonfat set yogurt when linoleic acid alone or linoleic acid and fructo-oligosaccharides were added. Obviously, the inclusion of fructo-oligosaccharides alone did not prove to be an effective tool for the enhancement of CLA level in yogurts, but the application of these prebiotics

together with the probiotic bacteria *L. acidophilus* (La-5) and *Bifidobacterium animalis* resulted in significantly higher levels of CLA. The level of CLA was higher when probiotic yogurt was prepared with the addition of fructo-oligosaccharides related to yogurt without prebiotics (Akalin et al., 2007). The influence of other commercially available polysaccharides such as maltodextrin, oligofructose and polydextrose was also studied on the CLA content of milks fermented by *S. thermophilus* and a strain of either *Lactobacillus* or *Bifidobacterium animalis* subsp. *lactis* (Oliveira et al., 2009).

The latest trend is the addition of by-products from fruits to enhance the dietary fibre intake and to improve the viability of bacteria in yogurt. These fruit pulps are cheap and have complex ingredients, and may be valuable from both an economic and nutritional point of view. However, the application of these fruit materials in fermentation is a challenge because of their acidity and the antimicrobial compounds they may contain. do Espírito Santo et al. (2010) used açai pulp as a prebiotic to improve the fatty acid profile of yogurt. Later, the same research group investigated the use of total dietetic fibre prepared from apple, banana and passion fruit pressing by-products in the processing of probiotic yogurt and, among others, the fatty acid composition and counts of viable microorganisms were evaluated (do Espírito Santo et al., 2012). Different probiotic strains were used together with the traditional yogurt starter culture composed of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. The effect of probiotic strains on the CLA content was different, with moderate increase or about the same value as was observed for the control. Moreover, in the case of fermentation with *L. acidophilus* L10, the CLA content was significantly lower in yogurt than in the initial milk, that is, some part of CLA present in the raw milk was converted into other fatty acids. In fibre yogurts inclusion of passion fruit during fermentation enhanced the CLA level in all of the examined probiotic strains. The influence of the other types of total dietetic fibres was not uniform, but it also depended on the probiotic strain that was applied. Banana fibre had a negative effect on the CLA content of yogurt produced with *Bifidobacterium animalis* subsp. *lactis* B104, B94 and HN019, but a positive effect was observed for *L. acidophilus* L10.

In cases where significant CLA formation can be achieved, it is very important to maintain its level until the product is consumed. The concentration of *cis*-9,*trans*-11 CLA remained at the same level until the 28th day of storage in probiotic yogurt at 4 °C but declined after 35 days, which was attributed to oxidative destruction of the conjugated double bond system (Akalın, Tokuşoğlu, Gönc, & Aycan, 2007). Some researchers also reported at least 1 week stability (Shantha et al., 1995; Boylston & Beitz, 2002) while others observed a slight decline during this period (Florence et al., 2012). The "storage stability" of isomers was reported to be different: while *cis*-9,*trans*-11 remained at its initial level, *trans*-10,*cis*-12 CLA declined rapidly and could not be detected in any yogurt after 35 days of storage (Akalın et al., 2007).

The cited findings verify that the advantage of higher CLA levels can also be included in the health-enhancing properties of probiotic yogurts in certain cases. The CLA content of the product can be increased by particular strains of probiotic bacteria if they are provided with adequate substrates such as linoleic acid, fructo-oligosaccharides or other prebiotics, or even fruit by-products. But one should be aware of the varying CLA-producing capability of *Lactobacillus*, *Lactococcus* and *Bifidobacterium* species

and strains during fermentation of milk (Ekinci, Okur, Ertekin, & Guzel-Seydim, 2008; do Espírito Santo et al., 2010; do Espírito Santo et al., 2012; Oliveira et al., 2009). It depends to a great extent on the added prebiotics (do Espírito Santo et al., 2012); therefore, the appropriate selection of strains and substrates is an indispensable task. Moreover, the CLA level of each dairy product notably depends on that of the raw milk. The main factor that determined the CLA content of probiotic fermented milk products was the type of milk (organic and conventional) and not the fermentation conditions, i.e. the applied strains or duration of time (Florence et al., 2012), and the CLA content of raw milk was mainly influenced by the feeding of dairy ruminants.

4.5.3 Fermented meat products

Sparse data are available on the CLA content of meat products, and the reported values ranged from 0.8 to 6.6 mg CLA/g fat. Fermented meat products ranked in the upper middle part of this range, i.e. different types of sausages contained 3.3–4.4 mg CLA/g fat (Chin, Liu, Strokson, et al., 1992; Fritsche & Steinhart, 1998). The CLA content of commercial meat products was reported to be similar to that of the raw material, that is, fermentation and other processing steps did not exert an important effect on its level (Fritsche & Steinhart, 1998).

4.5.4 Functional meat products

Meat is a highly nutritious food with great potential for delivering very valuable nutrients such as fatty acids, minerals, antioxidants and bioactive peptides into the human diet. Consumers have a high degree of preference for its organoleptic properties, although its intake is associated with health risks (e.g. the elevated risk of cardiovascular diseases). Among other factors, the presence of nitrites and nitrates and its saturated fatty acid profiles have been attributed to health problems. The amount of these so-called "negative nutrients" can be decreased to some extent. Several attempts have been made to resolve this matter, among them modification of the fatty acid profile. Besides the enhancement of the ratio of unsaturated fatty acids and changing the omega 6/omega 3 ratio, fatty acids with health amelioration potential, i.e. CLA, were also at the centre of interest. The supplementation of meat with bioactive, health-preserving essential or non-essential ingredients can be accomplished by feed or postmortem modification (Decker & Park, 2010; Khan et al., 2011). Several studies have been conducted towards the production of CLA-enriched meat in which the forage of ruminants was complemented with vegetable oil, oilseed oil or fish oil, as described earlier in Section 4.4. Researchers also managed to increase the CLA content of pork by dietary supplementation of pigs with synthetic CLA (Bee, 2001; Eggert, Belury, Kempa-Steczko, Mills, & Schinckel, 2001; Joo, Lee, Ha, & Park, 2002; Lauridsen, Mu, & Henckel, 2005). Although synthetic CLA is more expensive than oil seeds or vegetable oils that can be applied in ruminants to achieve CLA increases in meat, in the present context the importance of this finding is enhanced by the fact that in the field of fermented meat products – in particular in Europe and North

America – pork as a raw material is much more important for sausages than the meat of ruminants (Leroy, Verluyten, & De Vuyst, 2006). These products with improved nutritional profiles can be categorized as functional foods, as their health-preserving and nutritive values exceed those of conventional products (Decker & Park, 2010).

On the one hand, fermented meat products can be enriched with CLA by the application of raw materials derived from animals reared with appropriate feeding techniques. On the other hand, synthetic CLA can be added to meats during processing. CLA has been added directly to pork patties (Joo, Lee, Hah, Ha, & Park, 2000) and beef patties (Chae, Keeton, & Smith, 2004; Hur et al., 2004) and injected into beef strip loin (Baublits et al., 2007). Similar addition could be practised during the production of fermented meat products. Another prospective avenue for the modification of CLA content might be the inclusion of CLA-forming strains in meat starters. As pork is the most important initial material for sausages and its CLA content is low, finding the appropriate strains for the enhancement of CLA via fermentation is a promising prospect.

Fermented sausages can be processed by co-fermentation of probiotics and commercial starter culture. Probiotic bacteria are usually applied in dry (fermented) sausages, which are prepared without the application of heat; therefore their presence in the product in appropriate numbers can enable them to exert desirable properties such as adherence, colonization, the production of antimicrobial substances, antagonism against pathogenic bacteria and immune modulation (Brassart & Schiffrin, 2000; Molly, Demeyer, Civera, & Verplaetse, 1996). However, the application of probiotic starter cultures in the production of functional meat products is still rare (Toldrá & Reig, 2011; Zhang, Xiao, Samaraweera, Lee, & Ahn, 2010). The application of probiotics in meat products that, besides the above effects, contribute to the improvement of the CLA supply of humans is a challenge for the future.

4.6 Health effects of CLA

CLA present in the fats of ruminants has been reported to have health amelioration potential. Its discovery is dated in 1979 when Pariza, Ashoor, Chu, and Lund (1979) discovered anti-mutagenic substances instead of mutagens in pan-fried beef burgers that inhibited the initiation of mouse epidermal tumours (Pariza & Hargraves, 1985) and were identified as CLA (Ha, Grimm, & Pariza, 1987). Nowadays, in general, numerous health benefits are attributed to CLA, encompassing the range of common diseases in Westernised populations such as cancer, diabetes and cardiovascular diseases. Positive effects on body composition, the immune system and bone health have also been observed (Jahreis, Kraft, Tischendorf, Schöne, & von Loeffelholz, 2000; Khanal, 2004; Kraft & Jahreis, 2004; Larsen, Toubro, & Astrup, 2003; Martin & Valeille, 2002; O'Shea, Bassaganya-Riera, & Mohede, 2004; Pariza, 2004; Terpstra, 2004; Wahle et al., 2004; Wang & Jones, 2004; Watkins, Li, Lippman, Reinwald, & Seifert, 2004). However, most experiments were carried out using animal trials and are not inevitably conclusive for humans; moreover, results obtained in many areas

of health-effect investigations are contradictory. Physiological actions of CLA have been shown to be isomer specific, and the two isomers that have most frequently been examined are rumenic acid (*cis*,9-*trans*,11 CLA) and *trans*-10,*cis*-12 CLA. Isomer distribution of natural food sources significantly differs from synthetic CLA supplementations. In food, rumenic acid dominates, accounting for approximately 90% of CLA intake in the diet (Mooney, McCarthy, & Belton, 2012), while *trans*-10,*cis*-12 CLA is present only in minor quantities. In synthetic supplements the amount of *trans*-10,*cis*-12 CLA may be significant (Or-Rashid et al., 2011; Park, 2009; Wahle et al., 2004). These facts should be considered when assessing the significance of CLAs impact on health.

The most frequently studied area within the health effects of CLA is probably carcinogenesis. Rumenic acid has been shown to have inhibitory properties in several kinds of cancer occurring in skin, breast, colon, liver, lung and kidney (Banni, Heys, & Wahle, 2003; Belury, 2002; Bhattacharya, Banu, Rahman, Causey, & Fernandes, 2006; Ip, Masso-Welch, & Ip, 2003; Kelley, Hubbard, & Erickson, 2007; Lee, Lee, Cho, & Kim, 2005; Parodi, 1994; Park, 2009). In most experiments animal cancer models were applied, and only a small number of human studies involving CLA have been reported. In some epidemiological studies inverse relationships were observed between milk consumption and incidence of breast cancer (Aro et al., 2000; Knekt, Jarvinen, Seppanen, Pukkala, & Aromaa, 1996), while others failed to detect any association between these factors (Chajes et al., 2002; Chajes et al., 2003; Larsson, Bergkvist, & Wolk, 2009; McCann et al., 2004; Moorman & Terry, 2004; Rissanen, Knekt, Jarvinen, Salminen, & Hakulinen, 2003; Voorrips et al., 2002). In one study, milk consumption was assessed as having an adverse effect, increasing the risk of breast cancer (Talamini et al., 1984). It is assumed that CLA can influence the initiation, promotion and progression of cancer. The effect of CLA on these three steps of carcinogenesis may also depend on the type of isomer and the type and site of the cell or organ (Lee et al., 2005). CLA incorporates phospholipids into the membrane; thus, cell metabolism and signal transduction may be modified in several ways: influence on eicosanoid synthesis, regulation of gene expression, modulation of antioxidative mechanisms, cell proliferation and apoptosis (Kraft & Jahreis, 2004).

Observations in animal trials pointed out that differences exist between mammalian species regarding their response to CLA. It can be assumed that not all the impacts that were proved for animals also pertain to humans (Wahle et al., 2004). In order to clarify the effect of dietary CLA intake on the process of human carcinogenesis, more clinical studies are needed.

The anti-diabetic effect of CLA may depend on both species and types of isomer. Rumenic acid seemed to be inactive (Martin & Valeille, 2002), but the role of *trans*-10,*cis*-12 isomer is controversial: some studies verified decreasing glucose levels and increased insulin sensitivity with increased intake of *trans*-10,*cis*-12 CLA (see the review by Khanal, 2004), while others reported opposite effects – that is, the promotion of insulin resistance (Khanal, 2004; Moloney, Yeow, Mullen, Nolan, & Roche, 2004; Wang & Jones, 2004).

Several human studies were conducted in order to assess the effect of dietary CLA on blood parameters for cardiovascular diseases. In most experiments there were no

changes found regarding low-density lipoprotein, triacylglycerol and cholesterol content in blood by CLA, while some studies led to negative conclusions as enhanced CLA intake decreased high-density lipoprotein, associated with negative health impact (see the review by Park, 2009). In rodents CLA was shown to exert a positive effect on blood parameters by lowering serum cholesterol and triacylglycerol concentrations (Lock, Horne, Bauman, & Salter, 2005). The anti-atherogenic effect of CLA is also a controversial issue. The severity of cholesterol-induced atherosclerotic lesions was reduced with increasing doses of fed CLA in rabbits (Kritchevsky, Tepper, Wright, & Czarnecki, 2002; Kritchevsky, Tepper, Wright, Tso, & Czarnecki, 2000), while CLA also induced regression of pre-established atherosclerosis in ApoE−/− mice (Toomey, Harhen, Roche, Fitzgerald, & Belton, 2006; Toomey, Roche, Fitzgerald, & Belton, 2003). However, other workers either did not observe any amelioration of fatty streak lesions (Lee, Kritchevsky, & Pariza, 1994) or found CLA having a downright potential pro-atherogenic effect (Munday, Thompson, & James, 1999). Current studies focus on the cellular targets and mechanisms through which CLA exerts its effects regarding atherogenesis. In the context of atherosclerosis, the influence of CLA on smooth muscle and endothelial cells was comprehensively examined by Eder and Ringseis (2010), while the results of in vitro studies regarding functionality of monocytes and macrophages were reviewed by Mooney et al. (2012).

Animal trials with ad libitum dietary regimens strongly supported the observation that inclusion of CLA in the diet lowers the body weight and the fat mass while increasing the lean body mass (see the review by Roche, Noone, Nugent, and Gibney (2001), Collomb et al. (2006)). Human studies, however, did not prove such strong evidence of the weight loss and body fat reduction-inducing effects of CLA (Bhattacharya et al., 2006; Park & Pariza, 2007; Terpstra, 2004; Wang & Jones, 2004). Trying to resolve this contradiction, Park (2009) observed that CLA in animal trials that were carried out using diet resulting in a negative energy balance proved to be ineffective on body weight or body fat, similar to human studies in which calorie restriction was applied, suggesting that CLA may be effective in cases of positive energy balance in the reduction of the ratio of fat within weight gain. The body composition effect of CLA has been attributed to *trans*-10,*cis*-12 CLA, while *cis*-9,*trans*-11 CLA isomer seems to be neutral (Martin & Valeille, 2002; Terpstra, 2004). The possible mechanism by which CLA reduces adiposity was recently reviewed by Kennedy et al. (2010).

The enumeration of possible effects of CLA on human health has not been completed yet. CLA intake may have an influence on bone mass. The mechanism of this action, however, is unambiguous (see the review by Park (2009)). Another possibility is the utilization of the impact of CLA on inflammation and immune function for asthma therapy (MacRedmond & Dorscheid, 2011).

4.7 Future trends

Producing food enriched in CLA can be accomplished with appropriate feeding regimens; however, to achieve this purpose, several factors have to be considered in order to avoid negative changes to other parameters of the product – for instance, reducing

the fat and protein content of milk. Moreover, the time-dependent accumulation of CLA in the fats of ruminants by feeding is not completely clarified. Thus, future research is needed to study the pattern of this phenomenon for each animal product.

Fermentation with commercial starter cultures has been shown to result in only a minor contribution to the CLA level of food items, though some strains of lactobacilli, bifidobacteria, propionibacteria, pediococci, enterococci, streptococci and lactococci can produce CLA if external conditions are appropriate. Some probiotic strains are also capable of forming CLA, while the presence of prebiotics can also influence CLA formation. The main challenges are the development of complete product recipes through which significant increase of CLA may occur by fermentation while maintaining the level of CLA until the product is consumed. This area may be a promising future approach, especially for fermented meat products, as the CLA content of pork – being an important raw material – is very low. Therefore, finding appropriate stains to enhance the CLA level during ripening would be a worthwhile task.

CLA participates in or influences several cellular mechanisms in the microworld of the body, resulting in varying effects with encouraging results. However, it has to be considered that these fatty acids cannot be regarded as a general panacea; their applicability in health preservation or amelioration depends on several factors, among which the most important are probably the type of the isomer, the differences between species and initial state of health. Changes in health status are the result of multiple mechanisms. The elucidation of the exact means of action of CLA beyond the observed phenomena might help us to resolve the contradictory results of experiments that were observed in cases of several health effects, and present an opportunity to determine the suitable diet for therapy and prevention of different diseases.

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Effect of fermentation on the phytochemical contents and antioxidant properties of plant foods

5

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5.1 Introduction

Free radical-mediated damage may play a role in many disorders, such as chronic heart disease, diabetes and cancer. Free radicals are atoms or molecules with an unpaired electron. This contributes to their highly reactive nature, and thus they can easily interact with cell membranes and with macromolecules such as lipids, DNA and proteins, which causes disruption, leading to permanent damage and, finally, cell death (Hu et al., 2004). Because humans live in an oxygen-rich atmosphere, oxidative stress is an unavoidable consequence of human life. Nevertheless, the oxidative free radicals can be neutralised by enzymatic activity or by natural antioxidants. Antioxidant properties such as radical-scavenging activities are important to counteract the deleterious role of free radicals in foods and in biological systems. Therefore, the generation of free radicals is harmless in cells as long as a balance between oxidant species production and antioxidant defences is achieved, which thus releases the cells from oxidative stress.

Plant foods provide natural sources of functional phytochemicals and their consumption has long been associated with physical well-being. Phytochemicals are non-nutritive plant chemicals that have protective or disease-preventive properties. It is widely accepted that predominantly plant-based diets that include fruits, vegetables, cereals, legumes and seeds reduce the risk of oxidative stress-induced diseases (Birt, 2006; Hertog et al., 1995). Amino acids, peptides, proteins, flavonoids and other phenolic compounds are the antioxidants derived from plant foods that play a significant role as physiological and dietary antioxidants, which thus enhance the body's natural resistance to oxidative damage (Shahidi, 2000). The levels and bioavailability of phytochemicals can remarkably be influenced by processing such as fermentation, compared to what is taking place in the gut, where some of the food composition is further degraded by the gut microbes to release the bioactive components. This chapter encompasses the effect of fermentation on phytochemical contents and antioxidant properties of plant foods, illustrated by using several groups of daily foods such as legumes, cereals, fruits and vegetables. In addition, the health impacts of the

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00005-0

fermented plant foods with phytochemicals and antioxidant properties on humans are also discussed.

5.2 Effect of fermentation on phytochemical profiles of plant foods and the bioavailability of nutrients

The phytochemicals that exist naturally in plant foods are mostly in bound form and less bioavailable than the free form. Bio-processing methods such as fermentation have long been adapted to improve the nutritive value of plant foods. With the aid of microorganisms capable of modifying plant constituents, such as releasing the chemically bound compound during fermentation, the fermented plant foods thereby are enriched with phytochemical contents with improved bioavailability and bioactivity, in addition to the altered ratio of nutritive and anti-nutritive components of plants, as well as improved texture and organoleptic characteristics.

5.2.1 Legumes, seeds and nuts

Legumes belong to the Fabaceae (or Leguminosae) family, which includes peas, beans, lentils, peanuts and other podded plants. Food legumes have played a crucial role in the traditional diets in many regions throughout the world. Apart from providing an excellent source of macronutrients such as protein, dietary fibre, fatty acids and carbohydrates, legumes are also rich in micronutrients and phytochemicals.

Fermentation of leguminous seeds can modify their phytochemical composition. Soybeans are unique among the legumes because they are a concentrated source of isoflavones, one of the highly researched phytochemicals that possesses antioxidant activity (Kao & Chen, 2006). Examples of traditional fermented soybean products are miso, natto, Korean fermented soybean (Chungkookjang) and douchi (Chen et al., 2005; Hirota, Taki, Kawaii, Yano, & Abe, 2000; Iwai, Nakaya, Kawasaki, & Matsue, 2002; Kim, Song, Kwon, Kim, & Heo, 2008). In a study performed by Chien, Huang, and Chou (2006) on the transformation of isoflavone phytoestrogens during soymilk fermentation, the concentration of isoflavone aglycones (daidzein, glycitein and genistein) increased 100%, while a reduction of 50–90% in the concentration of glycoside counterparts upon fermentation by *Streptococcus thermophilus* and *Bifidobacterium longum* was observed. It is the microbial β-glucosidase activity that is responsible for the conversion of isoflavone glycosides in soybean into their aglycone counterparts that are bioactive and bioavailable during fermentation (Ewe, Wan Abdullah, & Liong, 2011). The bioavailability of isoflavones is largely attributed to their chemical structures; the aglycone form is more readily absorbed and bioavailable than the highly polar conjugated glucosides (Kano, Takayanagi, Harada, Sawada, & Ishikawa, 2006).

Fermentation often increases the bioavailability of phytochemicals by releasing the esterified compounds to free form. Only the free and conjugated phenolic acid forms were found to be bio-accessible (Patel, 2012). Oboh, Ademiluyi, and Akindahunsi

(2009) demonstrated that fermentation could improve the bioavailability of phytochemicals by increasing the free soluble phenolic content of pigeon peas (*Cajanus cajan*), Bambara groundnut (*Vigna subterranean*), African yam bean (*Sphenostylis stenocarpa* Harms) and kidney bean (*Phaseolus vulgaris*). The increase in the free phenolic content and the decrease in the bound phenolic content after fermentation are suggested to be indicative of microbial secreted enzyme hydrolysis of the glycosidic bonds of bound phenolics (Oboh & Rocha, 2007). Fermentation of chickpea seeds (*Cicer arietinum cv. Blanco lechoso*) with natural microbiota and with *Lactobacillus plantarum* modified the content of antioxidant vitamins and total phenolic and antioxidation compounds. Tocopherol isomers and vitamin E content were found to be decreased in both naturally fermented and lactobacilli-fermented chickpeas. On the other hand, fermentation could increase the total phenolic compounds, suggesting that fermentation is an adequate and effective process for increasing nutritional and biological quality of the product. During fermentation, the concentrations of reduced glutathione decreased while oxidised glutathione increased after both natural and *L. plantarum* fermentation of chickpeas (Fernandez-Orozco et al., 2009). The same observation in changes of reduced and oxidised glutathione is also reported by Dueñas, Fernandez, Hernández, Estrella, and Muñoz (2005) in cowpea (*Vigna sinensis* L.) fermentation. In the natural fermentation of lentils, the *p*-hydroxybenzoic and protocatechuic acids, as well as (+)-catechin, increased, whereas hydroxycinnamic acids and procyanidin dimers decreased (Bartolomé, Estrella, & Hernández, 1997). Apart from legumes and seeds, nuts also contain polyphenols and carotenoids with health-improving effects. Although fermentation reduced concentration of phytochemicals in nuts (α-tocopherol in almonds and hazelnuts, γ- and δ-tocopherol in pistachios and walnuts) compared to the native nuts, extracts of both products showed a strong antioxidant potential (Lux, Scharlau, Schlörmann, Birringer, & Glei, 2012).

5.2.2 Cereals

Cereal foods are important components of the daily diet, providing carbohydrates, proteins, dietary fibre and vitamins. Cereals are also sources of many phytochemicals, including phytoestrogens, phenolic compounds, saponins, phytic acid and sterols.

Fermentation of cereals has long been associated with the production of bread and beer (Poutanen, Flander, & Katina, 2009), which involves the modification of wheat and/or grain constituents by enzymes and microorganisms that are present naturally or that are being added. Sourdough fermentation has a well-known role in improving the nutritional properties of wheat, rye and oat baked goods via increasing the levels of bioactive compounds such as phytochemicals in cereals (Katina et al., 2005). These health-beneficial phytochemicals includes lignan, phenolic acids, phytosterols, tocos, folates and other vitamins that are found concentrated in the germ and the outer layer of kernel (Glitsø & Bach Knudsen, 1999). It is suggested that the increase or decrease of levels of bioactive compounds during fermentation of cereals depends on the nature of the compound and the type of microbes involved (Katina et al., 2005). Yeast fermentation has repeatedly been shown to increase the folate content in the baking of both wheat (Kariluoto et al., 2004) and rye (Kariluoto et al., 2004, 2006; Katina, Laitila,

et al., 2007; Liukkonen et al., 2003), but the level of folate detected in the fermentation of rye was more than doubled (Liukkonen et al., 2003). Additionally, the level of folate in rye meal upon fermentation by yeast was 2.5-fold higher than that fermented by lactobacilli (Katina, Liukkonen, et al., 2007). In spite of this, mixed fermentation (both yeast and lactic acid bacteria) has also been reported to promote higher levels of folates, sterols, lignans, free ferulic acids and alkylresorcinols during the fermentation of both native and germinated rye. This was credited to the optimum pH provided for the action of cell wall-degrading enzymes derived from the grain itself or from indigenous microbes by the higher acidity (pH 4.5–6.0) upon yeast fermentation (Katina, Liukkonen, et al., 2007). In a recent study performed by Đorđević, Šiler-Marinković, and Dimitrijević-Branković (2010), the total phenolic content of cereals such as buckwheat, wheat germ, barley and rye increased upon fermentation by both *Lactobacillus rhamnosus* and *Saccharomyces cerevisiae*. Enzymes such as amylases, xylanases and proteases derived from grain and microbes contributed to the modification of grain composition, which thus released the bioactive compounds and improved the nutritional value of the fermented cereals.

5.2.3 Vegetables and herbs

Vegetables provide essential nutrients needed not only for life but also for health promotion and disease prevention. Consumption of vegetables, especially cruciferous vegetables, has been strongly associated with reduced risk of cardiovascular disease and cancer (Temple, 2000). A popular explanation among scientists has been that vegetables possess compounds that can exert antioxidant properties (including vitamins C and E, selenium, flavonoids and β-carotene). Such properties may prevent the process involved in the development of cancer, including prevention of oxidative stress and protection of DNA from oxidative damage (Temple, 2000). These antioxidant properties of vegetables may be affected by processing technology (Oszmianski, Wolniak, Wojdylo, & Wawer, 2007). In reality, only a small amount of vegetables are consumed in their raw state, whilst most of them need to be processed for safety, quality and economic reasons. Processing such as fermentation has been shown to improve original activity and bioavailability of naturally occurring phytochemicals.

White cabbage fermented with *L. plantarum* CECT 748 (LP), *Leuconostoc mesenteroides* CECT 219 (LM) or a mixed culture of both strains at a 1:1 ratio (LP:LM) has been reported to exert higher ascorbigen (ABG) content (12-fold higher) than their unfermented counterparts (Martinez-Villaluenga et al., 2012). This was probably attributable to the presence of myrosinase-like activity in the starter culture, the enzymes being involved in ABG formation (Tolonen et al., 2004). ABG is a phytochemical that acts as a moderate free radical scavenger in vitro and as a potent inhibitor of chemical-induced lipid peroxidation in human keratinocytes (Wagner et al., 2008). In another study, Penas, Pihlaya, Vidal-Valverde, and Frias (2012) evaluated the effect of fermentation on the production of glucosinolate (GLS) hydrolysis products in cabbage. The authors found that fermentation of cabbage cv. Megaton can give rise to the production of GLS hydrolysis products such as iberin, iberin nitrile, allyl cyanide, allylisothiocyanate and sulforaphane, whereas such GLS hydrolysis products

were not detected in raw cabbage (Penas et al., 2012). GLS derivatives, particularly isothiocyanates, nitriles and indoles, have attracted much attention as potential chemopreventive agents against certain types of cancer. Glucosinolates are inactive in the intact raw vegetable but they can be hydrolysed to a broad range of bioactive breakdown products, including volatile compounds such as isothiocyanates, nitriles and thiocyanates, upon cellular disruption by the endogenous enzyme myrosinase (thioglucoside glucohydrolase E.C. 3.2.3.1.). Thus, the production of GLS hydrolysis products is attributable to myrosinase-like activity exerted by the endogenous microbiota during fermentation of cabbage.

Plant-based herbs such as red ginseng are also a rich source of various phytochemicals. Red ginseng extracts contain approximately 13.20 µg/mg of total flavonoid; fermentation by *Lactobacillus fermentum* (0.1% inoculum) for 12 h at 40 °C could increase the total flavonoids in red ginseng extract by approximately 39% compared to unfermented red ginseng (Kim, Lee, et al., 2011). Additionally, fermentation of red ginseng could alter the herb's ginsenoside profiles. Ginsenosides are important phytochemicals that can exert various health-beneficial effects. including anti-carcinogenic and antidiabetic effects (Kim et al., 2010). In a study conducted by Kim, Lee, et al. (2011), the authors reported that ginsenosides such as Rb_1 and Rh_2, which are known to have antioxidant activities, were not present in unfermented red ginseng but were detected in fermented red ginseng. Fermentation also significantly increased the concentrations of antidiabetic ginsenosides (Rg_3) by threefold compared to unfermented red ginseng, indicating that fermentation could improve the profile of bioactives in red ginseng.

Wu, Su, and Cheng (2011) determined the effect of fermentation on another medicinal tropical/subtropical plant, *Graptopetalum paraguayense* E. Walther. *Graptopetalum paraguayense* E. Walther has been used in ancient Chinese prescriptions for treatment of several disorders and diseases, including reduction of blood pressure, antimutagenic effect and prevention of oxidative damage and lipid peroxidation (Chung, Chen, Hsu, Chang, & Chou, 2005). Fermentation of *G. paraguayense* E. Walther juice with *L. plantarum* BCRC 10357 (10^5 CFU/ml; 37 °C for 72 h) can significantly increase the level of flavonoids and total phenolics, including quercetin and gallic acid, and thus may further increase the antioxidant properties of this plant (Wu et al., 2011).

5.2.4 Fruits

Fruit juices or fruit-related products are a rich source of polyphenolic compounds. Polyphenols play a critical role in sensory properties and health benefits as antioxidants, antitumoral agents and preventers of coronary heart disease. Red wine, a fermented beverage from red grapes, contains a large number of polyphenolic compounds and is a good resource for flavonols and flavones. Most of the phenolic compounds, including gallic acid, syringic acid, ethyl gallate, caftaric acid, coutaric acid, caffeic acid, coumaric acid, catechin and quercetin, showed the highest concentrations during the malolactic fermentation stage of red wine compared to other stages of wine processing (Gimjom et al., 2011). In another study, Lee and Kwak (2011) also

demonstrated that the content of total phenolic compounds and flavonoids in grape *yakju* (a Korean fermented alcoholic beverage) increased greatly with fermentation time (25 °C for 20 days). In particular, anthocyanin content in grape *yakju* was significantly higher upon fermentation for 20 days at 25 °C as compared to content before fermentation ($t=0$).

Increase of anthocyanin level was also observed in fermentation of strawberries (*Fragaria ananassa* cv. Shikinari) (Zhang, Seki, Furusaki, & Middelberg, 1998). The concentration of anthocyanin increased continuously to a maximum value of 0.86 mg/g fresh cell weight after fermentation for 6 days at 27 °C by cell suspension culture (200 g/l inoculums), and the synthesis of anthocyanin was partially growth-associated. It has been suggested that some materials such as sucrose and other thermostable compounds were secreted from cells in the medium during culturing, which might stimulate the anthocyanin synthesis and accumulation (Sakurai & Moru, 1996).

5.3 Effect of fermentation on antioxidant properties of plant foods

Plant foods contain a number of bioactive substances, including phenolics. Phenolics such as flavonoids, phenolic acids, lignans and tannins have antioxidant properties, whilst the fermentation process has been demonstrated to modify the amount of these contents. 1,1-Diphenyl-2-picryldrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) are tests commonly used to determine the free radical-scavenging effectiveness of compounds in evaluating the antioxidant activity of food systems.

5.3.1 Legumes, seeds and nuts

During microbial fermentation, changes may occur in plant phytochemicals, thereby affecting the antioxidant property of plant foods. Soy germ fermented by lactic acid bacteria has been found to contain higher amounts of phytochemicals with antioxidant properties, such as isoflavones, saponins, phytosterols and tocopherols, than that in the raw materials. The fermented soy germ extracts exhibited higher inhibition against the superoxide anion radical and significant ferric-reducing and DPPH radical-scavenging effects compared with raw soy germ (Hubert, Monique, Françoise, Françoise, & Jean, 2008). Additionally, soybean koji that was produced upon fermentation by *Rhizopus* sp. showed 5.2-fold higher scavenging effects than those of non-fermented soybean extract for DPPHc-free radicals (Lin, Wei, & Chou, 2006). As compared with unfermented soybean, the DPPH and ABTS radical-scavenging activity of douchi (a traditional *Bacillus subtilis* fermented soybean product in China) was doubled and increased 4.5-fold, respectively, during the primary fermentation process (Fan, Zhang, Chang, Saito, & Li, 2009). Chungkukjang (a fermented soybean product fermented with *B. subtilis*) extract and its isoflavone constituents (genistein and daidzein) also

showed significant antioxidant activity. The extract exhibited free radical-scavenging effects against DPPH radical generation and had an inhibitory effect on low-density lipoprotein (LDL) oxidation. In this study, the isoflavone glycones in soy, such as genistein and daidzein, were suggested to play a role in this antioxidative activity by inhibiting H_2O_2-induced DNA damage from NIH/3T3 fibroblasts (Kim et al., 2008). Isoflavone genistein that was hydrolysed further by yeast to 2′-hydroxygenistein showed higher antioxidant activity against DPPH and ABTS radicals than genistein (Choi et al., 2009).

Fermentation of cowpeas either with natural microbiota or with *L. plantarum* significantly increased the antioxidant activity of phenolic compounds in seeds, as assayed by the DPPH test (Dueňas et al., 2005). Oboh et al. (2009) demonstrated that fermentation of the free soluble phenol from the fermented legumes (Bambara groundnut, African yam bean and kidney bean) had a higher reducing power, free radical-scavenging ability and inhibition of lipid peroxidation than free soluble phenols from the unfermented beans. Fernandez-Orozco et al. (2009) also reported that both natural and *L. plantarum* fermentation of chickpeas resulted in a drastic reduction in antioxidant capacity, such as superoxide dismutase (SOD)-like activity (determination of superoxide radicals). The fermentation processes in chickpeas were also accompanied by a gradual increase in peroxyl-radical trapping capacity, trolox equivalent antioxidant capacity and lipid peroxidation inhibition. An increase in antioxidant activity of phenolic compounds present in oat tempeh was reported by Berghofer, Grzeskowiak, Mundigler, Sentall, and Walcak (1998). In a study comparing a microbial fermented bean product with commercial antioxidants, the *B. subtilis* and *Aspergillus oryzae* fermented red bean extract showed lower DPPH radical-scavenging effects compared to commercial antioxidants, as well as a weaker reducing power compared to that of α-tocopherol and butylated hydroxytoluene. However, the extracts showed a better Fe^{2+} chelating capacity than the commercial antioxidant (Chou, Chang, Chao, & Chung, 2002; Chung, Chang, Chao, Lin, & Chou, 2002).

5.3.2 Cereals

The presence of microorganisms is important for enhanced levels of antioxidant activity in cereals (Đorđević et al., 2010). The phenolic contents in buckwheat, wheat germs, barley and rye showed an increase in DPPH radical-scavenging activities upon fermentation by *L. rhamnosus* but were not significantly influenced upon fermentation by yeast. *L. rhamnosus*-fermented barley has shown powerful antioxidant properties, as the phenolics appear to have enhanced ferric-reducing antioxidant power, while all fermented cereals showed higher lipid peroxidation ability than the unfermented samples. Sourdough fermented wheat germ with *L. plantarum* and *L. rossiae* contained higher concentrations of total phenols than the raw wheat germ, with the phenols showing higher DPPH and ABTS radical-scavenging activities than the unfermented samples (Rizzello, Nionelli, Coda, De Angelis, & Gobbetti, 2010). A fungus-fermented wheat has been proposed as a health food, whereby its antioxidant activity measured by DPPH and ferrous ion chelating ability were higher in the fermented form than unfermented (Zhang et al., 2012).

5.3.3 Vegetables and herbs

The increased awareness of health and nutrition has tremendously increased the consumption of vegetables owing to the presence of antioxidants in vegetables. Processing such as fermentation has been shown to increase the antioxidant activity of plant-based food, including vegetables. One of the most commonly consumed fermented vegetables is fermented cabbage, better known as sauerkraut. Sauerkraut is produced by lactic acid fermentation of shredded and brined cabbage. Fermentation of white and red cabbage has been shown to increase the antioxidative capacity of the vegetables (Hunaefi et al., 2013; Kusznierewicz, Śmiechowska, Bartoszek, & Namieśnik, 2008). ABTS and DPPH free radical-scavenging activity of white cabbage has been shown to increase gradually with fermentation time (spontaneous fermentation) and reaches a plateau after 10 days. Likewise, Martinez-Villaluenga et al. (2012) also reported that fermentation of white cabbage with *L. plantarum* CECT 748 (LP), *Leuconostoc mesenteroides* CECT 219 (LM) or mixed culture (1:1; LP:LM) increased the oxygen radical absorbance capacity (ORAC) values by up to twofold compared to unfermented cabbage. The elevated antioxidant capacity of sauerkraut is probably attributable to the effects of wounding (owing to cutting) and chemical processes incurred by lactic acid bacteria (Kusznierewicz et al., 2008).

Fermentation of carrot juice by *L. bulgaricus* and *L. rhamnosus* (5×10^9 CFU after 48 h fermentation) also increased the DPPH radical-scavenging activity compared to unfermented carrot juice (Nazzaro, Fratianni, Sada, & Orlando, 2008). In another study, Wu et al. (2011) evaluated the antioxidant properties of fermented *G. paraguayense* E. Walther juice and found that fermentation with *L. plantarum* BCRC 10357 (10^5 CFU/ml; 37 °C for 72 h) significantly increased the level of antioxidative activities, including DPPH, ABTS and superoxide anion-scavenging activities, and reducing power. Additionally, the antioxidant activity, including catalase (CAT), gluthathione peroxidase (GPx), gluthathione reductase (GR) and SOD, in mouse normal liver cell line FL83B treated with fermented *G. paraguayense* E. Walther juice was significantly higher than in those treated with the unfermented juice.

5.3.4 Fruits

Fresh fruits are strongly recommended in the human diet since they are rich in vitamins, dietary fibres, minerals and antioxidants. In particular, sweet cherries contain remarkable contents of polyphenols, such as anthocyanins, which give them the characteristic colour and antioxidant properties. Fermentation can further enhance the antioxidant properties of sweet cherries. Sweet cherry (*Prunusavium* L.) puree fermented by selected autochthonous lactic acid bacteria (*L. plantarum*, *Pediococcus acidilactici*, *Pediococcus pentosaceus* and *Leuconostoc mesenteroides* subsp. *mesenteroides*) at 25 °C for 36 h has been reported to exhibit significantly higher DPPH radical-scavenging capacity compared to unfermented sweet cherry puree (Cagno et al., 2011).

Prus and Fibach (2012) demonstrated that fermented papaya preparation (FPP) exhibits reactive oxygen species (ROS) scavenging effect on blood cells in both an in vitro and in vivo model (in thalassemic patients and experimental animals). Fermented papaya effectively reduces ROS and it was suggested that its antioxidant mechanism is related, at least in part, to iron chelation.

In another study involving fermentation of grape and rice paste with *Aspergillus oryzae* (25 °C for 20 h), the resultant beverage (grape *yakju*) has been reported to possess higher antioxidant activities, as determined by ABST free radical-scavenging activity, hydroxyl free radical-scavenging activity and reducing power, than before fermentation. The increased antioxidant activity of grape *yakju* was correlated with the increased total phenolics and flavonoids of the beverage upon fermentation. In agreement, Davalos, Bartolome, and Gomez-Corddoves (2005) also demonstrated that antioxidant capacity, as evaluated by oxygen radical absorbance capacity (ORAC-FL) of grape wine (fermented grape beverage), was higher compared to unfermented grape juice. Interestingly, although lactic fermentation of grape juice to produce wine can significantly increase the antioxidant capacity of the beverage, acetic fermentation of wine in vinegar production may decrease the content of high-antioxidant-activity phenolic compounds, leading to new vinegar phenolic compounds with lower antioxidant activity than those originally present in wine.

5.4 Health-promoting effects of fermented plant foods: a case of phytochemical and antioxidant property changes

It has been suggested that antioxidants may contribute to the health benefits of plant foods by reducing the incidence of aging-related chronic diseases, including heart diseases and certain types of cancer. Experimental investigations involving in vitro and in vivo trials have produced positive results on various oxidative stress-related diseases. A recent study investigating chemo-preventive effects on cancer cells of five types of nuts (almonds, macadamias, hazelnuts, pistachios, walnuts) after in vitro fermentation showed that the fermentation extracts of nuts exhibited higher total tocopherol contents and antioxidant activity than native nuts. In addition, all fermentation supernatants (except pistachios) reduced growth of HT29 adenocarcinoma cells, whilst only fermentation supernatants of walnuts significantly reduced DNA damage induced by H_2O_2 after 15 min co-incubation of HT29 cells (Lux et al., 2012). Thus, consumption of fermented nuts could provoke anti-carcinogenic effects. Choi et al. (2009) demonstrated that 2′-hydroxygenistein (2′-HG, bio-converted from genistein by yeast hydrolase) showed higher antioxidant activity against DPPH and ABTS radicals than genistein. The 2′-HG also exhibited greater anti-proliferative effects in MCF-7 human breast cancer cells than did genistein. Studies have also been done on traditional soy-based fermented foods, such as natto, miso and douchi, that are believed to have caused longevity of people in the East. In one of the studies conducted by

Chang et al. (2010), it is shown that a diet of natto leads to a low prevalence of cardiovascular disease, attributed to natto's antioxidant activity. The occurrence of apoptosis of laser-induced endothelial cells was significantly reduced in the presence of natto extract. Additionally, both the natto extract and natto kinase suppressed intimal thickening in rats with endothelial injury upon 28 days administration. On the other hand, *L. rhamnosus* and *S. cerevisiae* fermented rice bran extracts showed an inhibitory effect on melanogenesis through down-regulation of microphthalmia-associated transcription factor, a key regulator of melanogenesis, along with reduced cytotoxicity (Chung et al., 2009). Apart from that, lactic acid bacteria and yeast co-fermented rice bran extracts also showed antioxidative effect on UVB-damaged skin. The bran extract has been found to be able to protect fibroblast cultures against UVB-induced damage in vitro, preventing cancer risks (Seo, Jung, Song, Park, & Park, 2010). Table 5.1 summarises some of the in vivo studies of fermented plant foods exhibiting antioxidant properties.

Numerous studies have indicated that the consumption of fermented fruits and vegetables could exert health-protective effects. Most of the fermented fruits and vegetables are rich sources of antioxidants, which are beneficial for prevention of LDL oxidation. In a prospective, crossover, randomised and controlled cross-sectional study evaluating the effects of red wine on antioxidant status and risk of cardiovascular disease, 30 healthy adults (30–50 years old) were randomly assigned to two groups for an intervention of 30 days (Estruch, 2000). One group received wine (30 g of ethanol) as the first intervention and hard liquor with a very low polyphenolic content as the second intervention (30 g of ethanol), and another group received the hard liquor with a very low polyphenolic content as the first intervention and red wine as the second intervention. The author demonstrated that consumption of red wine decreased serum oxidation parameters and the tendency of LDL to undergo lipid peroxidation, and increased high-density lipoprotein cholesterol. Thus, consumption of red wine could delay the early onset of atherosclerosis. One of the proposed mechanisms is the antioxidant properties of wine. Wine contains antioxidants such as quercetin, resveratrol and epicatechin, which are effective in preventing oxidation of LDL, the major cholesterol-carrying lipoprotein, leading to prevention of atherosclerosis.

Consumption of fermented vegetables such as kimchi (fermented cabbage) has been associated with health-protective effects including reduction or elimination of free radicals, which might be direct or indirect causes for certain cancers and cardiovascular disease. Kim, An, et al. (2011) evaluated the effects of fresh and fermented kimchi on metabolic parameters related to cardiovascular disease and metabolic syndrome risks in overweight and obese subjects. Twenty-two overweight and obese subjects (7 male, 15 female; body mass index: >25 kg/m^2) were recruited and randomly assigned to two experimental groups based on a crossover design: 4 weeks of fresh kimchi or 4 weeks of fermented kimchi. The consumption of fermented kimchi greatly improved metabolic syndrome, including systolic and diastolic blood pressures, percent body fat, fasting glucose, fasting insulin and total cholesterol, compared with the fresh kimchi. These results indicate that fermented kimchi may favourably affect obesity and lipid metabolism processes.

Table 5.1 In vivo studies of antioxidant extracts from some fermented plant foods

Products	Subjects and experimental design	Antioxidant-scavenging effect	References
Chungkookjang (Korean fermented soybean)	Oral administration of Chungkookjang extract (800 mg/kg/day) to SPF mice (male, 5 weeks old) for 2 weeks ($n = 6$)	Chungkookjang extract inhibited the formation of malondialdehyde, damage of DNA and formation of micronucleated reticulocytes in $KBrO_3$-treated mice.	Kim et al. (2008)
Fermented red bean	Oral administration of ethanol extract of non-fermented (RBE) and *Bacillus subtilis* fermented red bean (RBNE) to 12-month-old ICR mouse	RBNE raised vitamin E content in the liver and brain of aged ICR mouse while RBE showed no significant influence. RBNE enhanced superoxide dismutase activity in the brain of aged ICR mouse	Chou, Chung, Peng, and Hsu (2013)
Fermented mung bean	Oral administration of fermented and germinated mung bean extracts to Balb/c mice (male, 8–10 weeks old) at different doses for 14 days ($n = 8$)	Fermented mung bean caused improvement in antioxidant levels (increase in SOD and FRAP activities; reduced MDA and NO level) in the liver of mice compared to unfermented mung bean extract	Ali et al. (2013)
Fermented barley extract (prepared from water-soluble fraction of barley-*shochu* distillery by-products) (FBEP)	Oral administration of unfermented and FBEP to ICR mice (male, 5 weeks old) for 3 months ($n = 8$)	TBARS concentrations in plasma and liver were significantly lower in mice fed with FBEP than in the control; erythrocyte glutathione peroxidase activity and liver glutathione content were higher in the FBEP group	Hokazono, Omori, Suzuki, and Ono (2010)
Fermented wheat germ extract (FWGE) and in combination with selenium nanoparticles (FWGE-nano-Se mixture)	Oral administration of FWGE and FWGE-nano-Se mixture to Swiss albino tumour-bearing mice (female) for 6 weeks; mice received no treatment as control	FWGE-nano-Se mixture group showed significant reduction in tumour volume compared to positive control and FWGE group; FWGE-nano-Se mixture group increased SOD, GSH, GPx and CAT and decreased $NO_{(x)}$ and MDA and improved liver and kidney function test; FWGE-nano-Se mixture had anti-metastatic effect and induced apoptosis in Ehrlich carcinoma cells	El-Batal, Omayma, Eman, and Effat (2012)

ICR, imprinting control region; SPF, specific pathogen-free; $KBrO_3$, potassium bromate; SOD, superoxide dismutase; FRAP, ferric-reducing ability of plasma; MDA, malondialdehyde; NO, nitric oxide; TBARS, thiobarbituric acid reactive substances; GSH, glutathione; GP_x, glutathione peroxidase; CAT, catalase activity.

5.5 Conclusions

The levels and bioavailability of phytochemicals in plant foods such as legumes, cereals, vegetables, herbs and fruits can be greatly affected by processing such as fermentation. This subsequently leads to increased antioxidant properties, which may be beneficial for treatment and/or prevention of diseases such as atherosclerosis and cancer. Thus fermentation, especially with probiotics (functional microbes), can serve as an important process not only for preservation but also for production of functional foods with enhanced bioactive and antioxidant properties.

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Traditional cereal fermented foods as sources of functional microorganisms

6

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6.1 Introduction

For centuries, human civilisation has used different approaches to preserve and to prepare food products. Probably the longest part of this history was based on empirical knowledge, gained by experience without former knowledge either of mechanisms or the scientific basis of fermentation. However, the decisive issue was that fermentation proved to be a successful method of preservation. If we re-evaluate our experience in food biopreservation and traditional medicine, we will be surprised to discover that only since the last century have we tried to find answers to the questions about the scientific bases of these phenomena. If we look back in history, we can find the preparation of alcoholic beverages by ancient Egyptians; the preparation of yoghurt, kumis, kefir by the nomadic peoples from central Asia; fermentation of olives by the Greeks and Romans; fermentation of meat by the Germanic tribes and fish by the Eskimos; preparation of boza by the ancient Persians; or fermenting corn (maize) by the native tribes in pre-Columbian America. All of these peoples most probably did not have any knowledge of microbiology from the perspective of nineteenth century scientists, but they were convinced by their personal experience that using specific technical means of preparing these products succeeded in preserving meat, fish, milk, fruits and vegetables.

Based on empiric experience, some of these fermented products have been used in traditional medicine; moreover, traditional medicine quite frequently made use of fermentation processes to prepare medication. Empiric knowledge, frequently transferred from one generation to the next, was the only basis for preparation of these products. Our great-grandmothers knew that, when preparing yoghurt, you need to use the milk at a temperature that cannot burn yourself, and to add a portion of the yoghurt from the previous day's preparation. Preparation of kefir, differing from yoghurt, was traditionally associated with kefir grains.

6.2 Food fermentation processes

Fermentation of food is a very old technology, and the earliest records may date back to 6000 BC (Fox, Lucey, & Cogan, 1993). These methodologies and knowledge associated

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00006-2

with the manufacturing of fermented products were handed down from generation to generation within local communities (Caplice & Fitzgerald, 1999). Frequently, these communities needed products to be produced in small quantities for distribution in or around the immediate area. However, the population increase in towns and cities, due to the industrial revolution by the middle of the nineteenth century, resulted in a need for these products to be produced in larger quantities, and this led to commercial production of fermented food. Furthermore, developments in science (in general) and particularly in microbiology exponentially increased from the middle of the nineteenth century, and the development of pasteurisation by Louis Pasteur toward the end of the nineteenth century had a major impact on our understanding of the biological basis of fermentation. According to Caplice and Fitzgerald (1999), milk, meat, cucumbers and cabbage are the main substrates used in the production of the most familiar fermented food products.

Even when the scientific basic of fermentation processes and modern microbiology was established by Louis Pasteur, Ilja Metchnikov and his collaborator, Stamen Grigorov, first suggested the concept basic to modern understanding of functional food products and probiotics. At the end of the nineteenth century, it was still not well known how and why probiotics works; the term "probiotics" was not even used. Only much later, Fuller and Gibson (1997) defined this term as live microorganisms that, when administrated in adequate amounts, confer health benefits to the host such as reduction of gastrointestinal infections and inflammatory bowel disease, and modulation of the immune system (Fuller & Gibson, 1997).

Large-scale production required products with consistent quality. Characterisation of microorganisms responsible for the fermentation of various fermented products led to the isolation of starter cultures, which could be produced on a large scale to supply factories involved in the manufacturing of these products. Defined starter cultures replaced undefined starters traditionally used in manufacturing, and ensured reliable fermentation and consistent product quality (Caplice & Fitzgerald, 1999). The digestibility, nutritional value, organoleptic qualities, and shelf-life of industrial food products are increased by controlled fermentation processes (Hancioglu & Karapinar, 1997). However, a number of lactic acid bacteria (LAB) used as starter cultures in fermented food have probiotic properties and may confer potential health benefits to the consumer.

6.2.1 Lactic acid bacteria in cereal-based fermented products

Cereal and cereal-legume–based fermented products are consumed in almost all parts of the world. Cereals are cultivated on more than 73% of agricultural soil and contribute to more than 60% of the world's food production, providing proteins, vitamins, dietary fibre, energy and minerals (Charalampopoulos, Wang, Pandiella, & Webb, 2002). It is therefore important to study the nutritional value and basic composition of their products. Many cereal-based products are heat treated (boiled or steamed), for example, porridges, rice, pasta and cookies. In many cases, the same product is before or after heat treatment subject to fermentation processes, for example, pancakes and flatbreads in Asia, sourdough bread in Europe, and a variety of fermented dumplings, porridges, and alcoholic and nonalcoholic beers in Europe, Asia and Africa (Salovaara, 2004).

By definition, fermentation is the process by which a substrate is subjected to biochemical modification resulting from the activity of microorganisms and their enzymes (Gotcheva, Pandiella, Angelov, Roshkova, & Webb, 2000). Yeasts, LAB, fungi, or mixtures of these are mainly responsible for natural cereal-based fermentation. Yeasts mainly perform carbohydrate metabolism, whereas bacteria show proteolytic activity (Chavan & Kadam, 1989). Both yeasts and bacteria contribute to the sensory and safety aspects of the final products. Fermentations by yeasts and lactobacilli change the biochemical composition of fats, minerals and vitamins in the cereal. Yeasts are predominantly responsible for the production of ethanol (e.g. beers and wines), whereas lactic acid bacteria produce mainly lactic acid (e.g. cereals and fermented milk products). Acetic acid formation, most typically by the aerobic conversion of alcohol to acetic acid in the presence of excess oxygen, is mainly conducted by *Acetobacter* and *Gluconobacter* spp. (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003). Alkaline fermentation is commonly associated with the fermentation of fish and seeds, widely used as condiment (McKay & Baldwin, 1990).

6.2.2 History of boza, a cereal-based fermented beverage

The origin of boza dates back to the ancient peoples who lived in Anatolia and Mesopotamia. The preparation formula were taken by the Ottomans and distributed throughout the countries that they conquered. The Greek historian Xenophon recorded that boza was made in eastern Anatolia in 401 BC and stored in clay jars that were buried beneath the ground. The local speciality remained confined to the region until the arrival of the Turks, who took this nourishing drink and spread it far and wide under the name boza, a word derived from the Persian word “buze”, meaning millet. Boza enjoyed its golden age under the Ottomans, and its preparation became one of the principal trade items in towns and cities from the early Ottoman period (Todorov, 2010).

Beer is supposed to have originated from boza, a drink, probably dating back 4500 years. Although the alcohol and acid content of boza was not known at that time, boza was described in clay tablets as a stimulant and also as a medicine. From perspective of twentyfirst century science, we have sufficient evidence to relate some of the properties to the probiotic characteristics carried by LAB, part of the boza microbiota (Todorov et al., 2008). Boza consumption was initially widespread in the Islamic countries, but production was prohibited in the eighteenth century because of its high alcohol content. However, it is still produced and consumed widely in Anatolia, southern Russia, eastern European countries, the Middle East and northern Persia (Arici & Daglioglu, 2002).

Boza is a low-alcohol beverage produced by the fermentation of barley, oats, millet, maize, wheat or rice. The cooked cereal is strained to remove most of the solids; sugar is added to taste and inoculated with a starter culture, consisting of either yogurt or sourdough. The sludge is fermented at 30 °C for 24 h, cooled and kept refrigerated for 3–5 days (Hancioglu & Karapinar, 1997; Zorba, Hancioglu, Genc, Karapinar, & Ova, 2003). Boza contains about 0.50–1.61% protein, 12.3% carbohydrate and 75–85% moisture (Yegin & Uren, 2008; Zorba et al., 2003). In general, the pH of the boza samples ranges from 3.16 to 4.02 (Yegin & Uren, 2008), and the average alcohol content is 0.13% (w/v) (Köse & Yücel, 2003).

Boza can be classified as either sweet or sour, depending on its acid content. Besides improving organoleptic quality and digestibility by fermentation, it is a nutritious food because of its lactic acid, fat, protein, carbohydrate, fibre and vitamin contents, and thus is a valuable fermented food that beneficially contributes to human nutrition (Morcos, Hegazi, & El-Damhoughy, 1973). The chemical properties of boza during the fermentation and storage time are significantly affected by the raw material ($p < 0.01$) (Akpinar-Bayizit, Yilmaz-Ersan, & Ozcan, 2010). The compositional differences of boza samples may result from the use of different cereals as raw material and their amounts in the recipe (Hammes et al., 2005; Hammes & Gänzle, 1998). The raw material affects the amount and quality of carbohydrates available as primary fermentation substrates, nitrogen sources and growth factors for microbial activity.

6.2.3 Physicochemical characteristics of boza

Total titratable acidity in terms of lactic acid was found to be the lowest in millet, with 0.32 ± 0.04% and the highest in wheat boza (0.61 ± 0.07%) (Akpinar-Bayizit et al., 2010), probably due to the high fermentable carbohydrate content of wheat compared to other raw materials. The pH varied between 3.43 ± 0.08 and 3.86 ± 0.17 (Akpinar-Bayizit et al., 2010). The acidity of the samples increased during storage (being highest after 192 h, with 0.68 ± 0.06%) concomitantly with a decrease in the pH. Furthermore, the alcohol content was lower in wheat boza (0.46 ± 0.04%) (Akpinar-Bayizit et al., 2010) and showed fluctuations during storage, depending on microbial and enzymatic activities. The acidity and alcohol content depended mainly on the fermentation period; it was demonstrated that, with longer fermentation periods, the acidity increases, as does the concentration of alcohol (Akpinar-Bayizit et al., 2010).

The history of boza and similar beverages dates back several thousand years. However, the original boza was probably different from that which is produced nowadays, having high alcohol content (up to 7% by volume). In Egypt, a traditional beverage called bouza is still produced. In the South African region, boza production has become an important section of the beverage industry. Boza and similar beverages are produced with different recipes and methods in various countries (Table 6.1). Boza is called bousa or bouza in Nigeria and some other African countries, and it is similar to beer due to its high alcohol content (Sanni, 1993). In Bulgaria, boza is produced either plain or with cocoa, either in winter or in summer (Enikova, Kozereva, Ivanova, & Yang'ozova, 1985). In the Balkan region of Europe, this beverage is also called boza. In Turkey, boza is mostly produced and consumed in winter, although, because of the refreshing cooling effect of lactic acid, it can also be consumed in summer; however, high temperatures in the summer season can lead to the growth of yeasts and acetic acid bacteria (Arici & Daglioglu, 2002). Thus, sensory qualities of the product can change rapidly, causing a dramatic decrease in the shelf-life (Arici & Daglioglu, 2002).

The steps for boza production can be summarised as: (1) preparation of the raw materials, (2) boiling, (3) cooling and straining, (4) addition of sugar and (5) fermentation. Boza (2–3%) from a previous batch is usually used as a starter culture. The mixture is left to ferment in wooden barrels. The ratio of the starter culture depends on the season and temperature at which it is produced. The inoculated

Table 6.1 Some traditional fermented cereal products consumed in Europe, Africa and Latin America that may be consumed in an uncooked or partially cooked state and contain live microbes

Product	Raw material/ substrate	Sensory property and nature	Microorganisms	Country (references)
Abreh	Sorghum	Solid state and submerged	*Lactobacillus plantarum*	Sudan (Odunfa & Oyewole, 1997)
Ben-saalga	Pearl millet	Weaning food	*Lactobacillus* sp., *Pediococcus* sp., *Leuconostoc* sp., *Weissella* sp., yeasts	Burkina Faso, Ghana (Tou et al., 2006)
Boza	Cereals (barley, oats, millet, maize, wheat, rice)	Sour refreshing liquid	*Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus coprophilus,*[*] *Lactobacillus coryniformis, Lactobacillus fermentum, Lactobacillus paracasei, Lactobacillus pentosus, Lb. plantarum, Lactobacillus rhamnosus, Lactobacillus sanfrancisco,*[§] *Lactococcus lactis* subsp. *lactis, Leuconostoc lactis, Leuconostoc mesenteroides, Leuconostoc oenos* (reclassified as *Oenococcus oeni*), *Leuconostoc raffinolactis, Pediococcus pentosaceus, Weissella confusa, Weissella paramesenteroides, Saccharomyces cerevisiae*	Bulgaria, Balkan peninsula (Arici & Daglioglu, 2002; Botes et al., 2006; Gotcheva et al., 2000; Hancioglu & Karapinar, 1997; Ivanova et al., 2000; Kabadjova et al., 2000; Sahingil et al., 2011; Todorov, 2010; Todorov & Dicks, 2004; Todorov & Dicks, 2005; Todorov & Dicks, 2006; Tuncer & Ozden, 2010; Von Mollendorff et al., 2006; Zorba et al., 2003)
Burukutu	Sorghum and cassava	Creamy, liquid	*Sacch. cerevisiae, Saccharomyces chavelieri, Leuc. mesenteroides, Candida* sp., *Acetobacter* sp.	Nigeria (Odunfa & Oyewole, 1997)
Busa	Maize, sorghum, millet	Submerged	*Sacch. cerevisiae*, *Schizosaccharomyces pombe*, *Lb. plantarum, Lactobacillus helveticus, Lactobacillus salivarius, Lactobacillus casei, Lb. brevis, Lactobacillus buchneri, Leuc. mesenteroides, Pediococcus damnosus*	East Africa, Kenya (Blandino et al., 2003; Odunfa & Oyewole, 1997)

Continued

Table 6.1 Continued

Product	Raw material/ substrate	Sensory property and nature	Microorganisms	Country (references)
Chicha de jora	Maize	Mildly alcoholic drink	*Lb. plantarum*, *Lb. fermentum* , *Sacch. cerevisiae*	Latin America (Quillama, 1993, 1998; Quillama et al., 1995)
Dégué	Millet	Condiment	*Lactobacillus gasseri, Lb. fermentum, Lb. brevis, Lb. casei, Enterococcus* sp.	Burkina Faso (Abriouel et al., 2006)
Enjera	Tef flour, wheat	Acidic, sourdough, leavened, pancake-like bread, staple	*Lactobacillus pontis, Lb. plantarum, Leuc. mesenteroides, Pediococcus cerevisiae, Sacch. cerevisiae, Candida glabrata*	Ethiopia (Lee, 1997)
Hussuwa	Sorghum	Cooked dough	*Lb. fermentum, Pediococcus acidilactici, Ped. pentosaceus*, yeast	Sudan (Yousif et al., 2010)
Kenkey	Maize	Acidic, solid, steamed dumpling, staple, Aflata	*Lb. plantarum, Lb. brevis, Enterobacter cloacae, Acinetobacter* sp., *Sacch. cerevisiae, Candida mycoderma*	Ghana (Odunfa & Oyewole, 1997; Oguntoyinbo, Tourlomousis, Gasson, & Narbad, 2011)
Kishk	Wheat, milk	Refreshing beverage	*Lb. plantarum, Lb. brevis, Lb. casei, Bacillus subtilis*, yeasts	Egypt (Blandino et al., 2003)
Kisra	Sorghum	Thin pancake bread, staple	*Ped. pentosaceus, Lactobacillus confusus,*[¶] *Lb. brevis, Lactobacillus* sp., *Erwinia ananas, Klebsiella pneumoniae, Enterobacter cloacae, Candida intermedia, Debaryomyces hansenii, Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Rhizopus* sp	Sudan (Mohammed, Steenson, & Kirleis, 1991)
Koko	Maize	Porridge	*Enterobacter cloacae, Acinetobacter* sp., *Lb. plantarum, Lb. brevis, Sacch. cerevisiae, Cand. mycoderma*	Ghana (Andah & Muller, 1973; Blandino et al., 2003)

Maheu	Maize, sorghum, millet	Refreshing beverage	*Lactobacillus delbrueckii*	South Africa (Steinkraus, 1979)
Mahewu	Maize	Refreshing beverage	*Lb. delbrueckii, Lactobacillus lactis*	South Africa (Blandino et al., 2003)
Mawè	Maize	Intermediate product used to prepare beverages, porridges	*Lb. fermentum, Lactobacillus reuteri, Lb. brevis, Lactobacillus confusus,*¶ *Lactobacillus curvatus, Lactobacillus buchneri, Lactobacillus salivarius, Lactobacillus. lactis, Ped. pentosaceus, Ped. acidilactici, Leuc. mesenteroides*	Benin, Togo (Hounhouigan, Nout, Nago, Houben, & Rombouts, 1993)
Mbege	Maize, sorghum, millet	Submerged	*Sacch. cerevisiae*, *Schizosaccharomyces pombe*, *Lb. plantarum, Leuc. mesenteroides*	Tanzania (Odunfa & Oywole, 1997)
Ogi	Maize, sorghum, millet	Mildly acidic, viscous porridge, staple	*Lb. plantarum, Lactobacillus pantheris, Lactobacillus vaccinostercus, Corynebacterium* sp., *Aerobacter* sp., *Cand. mycoderma, Sacch. cerevisiae, Rhodotorula* sp., *Cephalosporium* sp., *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp.	Nigeria (Banigo & Akinrele, 1977; Odunfa & Oyewole, 1997)
Pito	Maize, sorghum, millet	Submerged	*Geotrichum candidum, Lactobacillus* sp., *Candida* sp.	West Africa (Odunfa & Oywole, 1997)
Poto poto (Gruel)	Maize	Slurry	*Lb. gasseri, Lb. plantarum/paraplantarum, Lb. acidophilus, Lb. delbrueckii, Lb. reuteri, Lb. casei, Bacillus* sp., *Enterococcus* sp., yeasts	Congo (Abriouel et al., 2006; Louembé et al., 1996)
Pozol	Maize	Mildly acidic, thick, viscous porridge, staple	*Lb. plantarum, Lb. fermentum, Lb. casei, Lb. delbrueckii, Leuconostoc* sp., *Bifidobacterium* sp., *Streptococcus* sp., *Candida parapsilosis*, *Trichosporon cutaneum*, *Geotrichum candidum*, *Aspergillus flavus*, *Enterobacteriaceae*, *Bacillus cereus*, *Paracolobactrum aerogenoides*, *Agrobacterium azotophilum*, *Alkaligenes pozolis*, *Escherichia coli* var. *napolitana*, *Pseudomonas mexicana*, *Klebsiella pneumoniae*, *Saccharomyces* sp., *moulds*	Mexico and Latin America (Ben Omar & Ampe, 2000; Jiménez Vera et al., 2010; Nuraida et al., 1995; Ulloa et al., 1987; Wacher et al., 2000, 1993)

Continued

Table 6.1 Continued

Product	Raw material/ substrate	Sensory property and nature	Microorganisms	Country (references)
Tepache	Maize, pineapple, apple, orange		*B. subtilis*, *Bacillus graveolus* and the yeasts *Torulopsis insconspicna*, *Sacch. cerevisiae* and *Candida queretana*	Mexico (FAO, 1998)
Togwa	Cassava, maize, sorghum, millet	Fermented gruel or beverage	*Lb. brevis, Lactobacillus cellobiosus, Lb. fermentum, Lb. plantarum* and *Ped. pentosaceus, Candida pelliculosa, Candida tropicalis, Issatchenkia orientalis, Sacch. cerevisiae*	Tanzania (Kingamkono et al., 1998; Lorri & Svanberg, 1995; Mugula et al., 2003)
Uji	Maize, sorghum, millet, cassava flour	Acidic, sour porridge, staple	*Leuc. mesenteroides, Lb. plantarum*	Kenya, Uganda, Tanzania (Odunfa & Oyewole, 1997)

*Probably syn. of "*Lactobacillus confusus*", which was later renamed *W. confusa*.
§*Lactobacillus sanfranciscensis.*
¶Renamed *W. confusa*.

mixture is incubated at 15–25 °C for nearly 24 h before it is ready for use (Arici & Daglioglu, 2002).

Two different types of fermentation occur simultaneously during boza fermentation. The first is the alcoholic fermentation that produces carbon dioxide bubbles and increases the volume; the second, lactic acid fermentation, produces lactic acid and gives the acidic character to boza. Due to the increase in volume during fermentation, the wooden barrels should not be fully filled. After production, boza should be consumed within a couple of days to prevent an excessively sour taste. In practice, the fermentation rate is reduced by cold storage to extend the shelf life of boza. In the first boza production of the season, sourdough or yoghurt is used as a starter culture, since fresh boza is not available. When using sourdough, which is less viscous, a more acidic product is obtained when compared to the product that is inoculated with a previous boza batch. If yoghurt is preferred, a viscous but more acidic product is obtained, and the characteristic yoghurt taste is easily detected (Arici & Daglioglu, 2002).

6.2.4 Microbiology of boza

Countries in the Balkan region in Europe are famous for their production of food and beverages fermented with LAB. Boza is one such traditional drink; however, only a few reports have been published on the microbial composition of boza. Most of the LAB that have been isolated belong to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, *Oenococcus* and *Weissella* (Arici & Daglioglu, 2002; Botes, Todorov, von Mollendorff, Botha, & Dicks, 2006; Gotcheva et al., 2000; Hancioglu & Karapinar, 1997; Kabadjova, Gotcheva, Ivanova, & Dousset, 2000; Todorov, 2010; Todorov & Dicks, 2004, 2005, 2006; Von Mollendorff, Todorov, & Dicks, 2006; Zorba et al., 2003). Only one study (Zorba et al., 2003) addressed the selection of boza starter cultures. LAB and presumably yeasts produce a number of vitamins (Leroy & De Vuyst, 2004), and the adequate selection of starter cultures can increase the nutritional value of fermented products (LeBlanc, Taranto, Molina, & Sesma, 2010).

Several studies have reported on the isolation and identification of LAB and yeasts in boza; however, to our knowledge, only the studies of Botes et al. (2006), Todorov and Dicks (2006) and Todorov (2010) used biomolecular approaches to identify these microorganisms. In these studies, the numbers of LAB isolated from three boza samples ranged from 9×10^6 to 5×10^7 CFU/ml. Carbohydrate fermentation reactions and PCR with species-specific primers classified the isolates as *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, *Leuconostoc lactis* and *Enterococcus faecium*.

Only a few papers have reported the isolation of yeasts and moulds from boza. Most of the yeasts were identified as strains of *Candida glabrata*, *Candida tropicalis*, *Geotrichum candidum*, *Geotrichum penicilatum*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae* and *Saccharomyces uvarum* (Arici & Daglioglu, 2002; Gotcheva et al., 2000). The latter identifications were based on morphological, physiological and biochemical characteristics. Boza harbors a diverse population of LAB

that include strains of *Lactobacillus acidophilus*, *Lb. brevis*, *Lactobacillus "coprophilus"* (Syn.: *Weissella confusa*?), *Lactobacillus coryniformis*, *Lb. fermentum*, *Lb. paracasei*, *Lb. pentosus*, *Lb. plantarum*, *Lb. rhamnosus*, *Lactobacillus sanfranciscensis*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc raffinolactis*, *Leuc. lactis*, *Ent. faecium*, *Pediococcus pentosaceus*, *Leuconostoc oenos* (reclassified to *Oenococcus oeni*), *W. confusa* and *Weissella paramesenteroides* (Arici & Daglioglu, 2002; Gotcheva et al., 2000; Todorov, 2010; Todorov & Dicks, 2006; Von Mollendorff et al., 2006).

Yeasts were isolated from two-thirds of the boza samples, with viable numbers ranging from 1.3×10^2 to 1.8×10^3 CFU/ml. Results obtained from sequencing of the D1/D2 rDNA region identified the yeasts as *Candida diversa*, *Candida inconspicua*, *Candida pararugosa*, *Issatchenkia orientalis*, *Pichia fermentans*, *Pichia guilliermondii*, *Pichia norvegensis*, *Rhodotorula mucilaginosa* and *Torulaspora delbrueckii*. *Saccharomyces cerevisiae*, commonly associated with fermented beverages, has not been detected in any of the boza samples (Botes et al., 2006). The absence of *Sacch. cerevisiae* suggests that the species was either not present in the inoculum (at least not in high numbers) or that it was inhibited by the LAB and other yeasts toward the end of fermentation. *Candida inconspicua* has been isolated from human sputum and tongue and is an opportunistic pathogen. *Rhodotorula mucilaginosa* is also an opportunistic pathogen implicated in fungaemia, endocarditis and meningitis. *Pichia norvegensis* has been associated with septicemia in humans (Gomez-Lopez, Mellado, Rodriguez-Tudela, & Cuenca-Estrella, 2005; Maxwell et al., 2003). The presence of potential pathogens emphasises the importance of developing starter cultures with GRAS status for the commercial production of boza and the necessity of introducing novel methods to prevent pathogen propagation.

6.3 Antimicrobial proteins isolated from boza-related lactic acid bacteria

6.3.1 Definition of a bacteriocin

LAB are known for the production of antimicrobial compounds, including bacteriocins or bacteriocin-like peptides (De Vuyst & Vandamme, 1994). Bacteriocins of LAB are defined as ribosomally synthesised proteins or protein complexes usually antagonistic to genetically closely related organisms (De Vuyst & Vandamme, 1994), and are generally low-molecular-weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanism varies and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA and inhibition of peptidoglycan synthesis (De Vuyst & Vandamme, 1994; Heu et al., 2001). In recent studies, specific environmental conditions, including those found in food, have been examined to determine their effect on the production of bacteriocins (Leroy & De Vuyst, 2004). Bacteriocin production is dramatically affected by changes in environmental conditions, and optimal production may require a specific

combination of parameters (Leal-Sánchez, Jiménez-Díaz, Maldonado-Barragán, Garrido-Fernández, & Ruiz-Barba, 2002). Little is known about the interactions of these factors with the production of bacteriocins, especially in a complex food environment.

6.3.2 Boza-associated bacteriocin producers

Kabadjova et al. (2000) and Ivanova, Kabadjova, Pantev, Danova, and Dousset (2000) first reported the isolated of bacteriocin-producing strains from boza (produced in Sofia, Bulgaria). Of the 80 isolated strains of LAB, a group of 33 showed antibacterial activity against different test microorganisms (*Listeria innocua* F, *Lb. plantarum* 73, *Lactococcus cremoris* 117, and even against Gram-negative bacteria such as *Escherichia coli*). The strain defined as *Lact. lactis* subsp. *lactis* 14 (based on biochemical identification tests) was chosen for future tests, and its growth curve and ability to produce bacteriocin under different conditions of cultivation have been studied. An attempt has been made for initial purification of the bacteriocin by means of a classical method (Kabadjova et al., 2000).

Todorov and Dicks (2004) reported on the bacteriocin (mesentericin ST99) produced by *Leuc. mesenteroides* subsp. *dextranicum* ST99 isolated from boza originating from Belogratchik (northwest of Bulgaria). The cell-free supernatant of this strain inhibited the growth of *Bacillus subtilis*, *Enterococcus faecalis*, several *Lactobacillus* spp., *Lactococcus lactis* subsp. *cremoris*, *Li. innocua*, *Listeria monocytogenes*, *Ped. pentosaceus*, *Staphylococcus aureus* and *Streptococcus thermophilus*. However, *Clostridium* spp., *Carnobacterium* spp., *Leuc. mesenteroides* and Gram-negative bacteria were not inhibited (Todorov & Dicks, 2004). Maximum antimicrobial activity, that is, 6400 AU/ml, was recorded in MRS broth after 24 h growth at 30 °C. Metabolically active cells of *Li. innocua* treated with mesentericin ST99 did not undergo lysis, and mesentericin ST99 did not adhere to the cell surface of strain ST99. Mesentericin ST99 were partially purified by precipitation with ammonium sulfate (70% saturation), followed by Sep-Pack C18 chromatography and reverse-phase high-performance liquid chromatography (HPLC) on a C18 Nucleosil column, and yielded one antimicrobial peptide (Todorov & Dicks, 2004).

In 2005, the same authors (Todorov & Dicks, 2005) reported that 13 of the 52 strains isolated from boza produced in Belogratchik, northwest of Bulgaria, inhibited the growth of *Pediococcus* spp., *Li. innocua* and *Lb. plantarum*. The population of LAB recorded in boza was c. 2×10^8 CFU/ml. One of the strains, identified as *Ped. pentosaceus* ST18 (based on biochemical identification tests), produced pediocin ST18 at 3200 AU/ml in MRS broth at the end of logarithmic growth (i.e. after 24 h). Pediocin ST18 was active against all tested strains of *Pediococcus* spp. included in this study, and revealed an important antilisterial activity, including that against *Li. monocytogenes*. From the 54 bacterial strains tested, 29 were sensitive to pediocin ST18. No activity was recorded against Gram-negative bacteria in this study.

Pediocin ST18 have been partially purified by ammonium sulphate precipitation, followed by separation in a Sep-Pack C18 column and reverse-phase HPLC on a C18 Nucleosil column, and yielded in two active antimicrobial peptides, which suggests that pediocin ST18 may be a two-peptide bacteriocin. The peptide had bacteriostatic

action toward *Li. innocua* but did not cause cellular lyses. Pediocin ST18 does not adhere to the cell surface of the producer strain (Todorov & Dicks, 2005).

The study of Todorov and Dicks (2006) is the first report on the isolation of LAB from boza and their identification based on genetic approaches. Boza samples were obtained from the same town in Bulgaria previously reported by Todorov and Dicks (2004, 2005). The population of LAB recorded in Boza was approximately 5×10^7 CFU/ml. *Lactobacillus plantarum* (strains ST194BZ, ST414BZ and ST664BZ), *Lb. pentosus* (strain ST712BZ), *Lb. rhamnosus* (strains ST461BZ and ST462BZ) and *Lb. paracasei* (strains ST242BZ and ST284BZ), isolated from boza, produced bacteriocins active against *Lactobacillus casei*, *E. coli*, *Pseudomonas aeruginosa* and *Ent. faecalis*. Thus far, only a few bacteriocins with activity against Gram-negative bacteria have been reported, but, for the second time, a bacteriocin isolated from LAB originated from boza was reported (Todorov & Dicks, 2006). All eight bacteriocins inhibited *Lb. casei*, *E. coli*, *Ps. aeruginosa* and *Ent. faecalis*. Bacteriocin ST242BZ inhibited the growth of *Acinetobacter baumanii* and bacteriocins ST194BZ, ST242BZ, ST284BZ and ST414BZ were active against *Enterobacter cloacae* (Todorov & Dicks, 2006). All bacteriocins acted bactericidal. The bacteriocins did not adhere to the surface of the producer cells. Production occurred throughout logarithmic growth, with the highest activity recorded at the end of logarithmic and during stationary growth. Based on tricine SDS-PAGE, the bacteriocins ranged from 2.8 to 14.0 kDa. No plasmids were recorded, suggesting that the genes encoding the bacteriocins are located on the genomes (Todorov & Dicks, 2006).

Von Mollendorff et al. (2006) reported on bacteriocins produced by LAB isolated from boza. Four isolates (JW3BZ, JW6BZ, JW11BZ and JW15BZ) produced bacteriocins active against a broad spectrum of Gram-positive bacteria. In addition, bacteriocin JW15BZ was shown to inhibit the growth of *Klebsiella pneumoniae*. The producer strains were identified as *Lb. plantarum* (strains JW3BZ and JW6BZ) and *Lb. fermentum* (strains JW11BZ and JW15BZ) based on biochemical and biomolecular approaches and 16S rDNA sequencing. The spectrum of antimicrobial activity, biochemical characteristics, and mode of action of these bacteriocins were compared with bacteriocins previously described for LAB isolated from boza (Von Mollendorff et al., 2006). The highest level of bacteriocin JW3BZ activity (25,600 AU/ml) was recorded after 18 h of growth in MRS broth (30 °C), and it remained at this level for the duration of fermentation. The same level of bacteriocin JW6BZ activity (25,600 AU/ml) was recorded after 15 h of growth, which was followed by a decrease to 12,800 AU/ml during the next 6 h. Bacteriocins JW11BZ and JW15BZ were produced at lower levels (12,800 AU/ml) and only after 15 and 12 h, respectively. Bacteriocin JW11BZ production decreased to 6400 AU/ml after 18 h of fermentation, and bacteriocin JW15BZ to 3200 AU/ml after 21 h of fermentation (Von Mollendorff et al., 2006). The molecular size of bacteriocins JW3BZ, JW6BZ, JW11BZ and JW15BZ ranged from approximately 2.3 to 3.0 kDa.

Lactococcus lactis subsp. *lactis* YBD11 was isolated from boza, produced in Turkey (Tuncer & Ozden, 2010). The bacteriocin produced by *Lact. lactis* subsp. *lactis* YBD11 inhibited the growth of *Lb. plantarum*, *Lactobacillus sakei*, *Lact. lactis* subsp. *lactis*, *Lact. lactis* subsp. *cremoris*, *Micrococcus luteus*, *Li. innocua*, *Enterococcus*

feacalis, *Staphylococcus aureus*, *Staphylococcus carnosus*, *Ped. pentosaceus* and *Bacillus cereus*; however, the nisin-producing strain *Lactococcus lactis* SIK83 and Gram-negative bacteria were not inhibited. Maximum antimicrobial activity, 10,240 AU/ml, was recorded in glucose-M17 broth after 18 h at 30 °C. Based on cell lysis treatments, bacteriocin of *Lact. lactis* subsp. *lactis* YBD11 was determined to have a bactericidal activity against *M. luteus*. Bacteriocin production occurred throughout the exponential phase, with the highest activity recorded at the end of this phase. Tricine-SDS-PAGE of partially purified bacteriocin gave the same molecular weight as nisin (3.5 kDa). These results indicate that the antimicrobial compound produced by *Lact. lactis* subsp. *lactis* YBD11 is a nisin-like bacteriocin (Tuncer & Ozden, 2010).

A different bacterial strain isolated from boza produced in Turkey was also identified, and the physicochemical and microbiological properties of its inhibitory compounds were characterised (Sahingil, Isleroglu, Yildirim, Akcelik, & Yildirim, 2011). The isolate was identified as *Lact. lactis* subsp. *lactis*, based on morphology, physiology, carbohydrate fermentation, the fatty acid profile and 16S rDNA gene sequence homology. Lactococcin BZ was active against several Gram-positive and Gram-negative foodborne pathogens and food spoilage bacteria. Lactococcin BZ was produced at the maximum level in MRS broth with an inoculum volume of 0.1%, an initial pH of 7.0, and an incubation temperature of 25 °C. Bacteriocin production began during the logarithmic phase and reached the maximal level during the early stationary phase. Its mode of action against *Li. monocytogenes* was bactericidal and its molecular weight was ca. 5.5 kDa, as determined using tricine SDS-PAGE. *Lactococcus lactis* subsp. *lactis* BZ or its bacteriocin, which has a wide inhibitory spectrum, has the potential for use as a biopreservative in food products (Sahingil et al., 2011).

Five bacteriocin-producing LAB strains (*Lb. plantarum* ST69BZ, *Ent. faecium* ST62BZ and *Leuc. lactis* ST63BZ, ST611BZ and ST612BZ) were isolated from boza originating from Belogratchik, Bulgaria (Todorov, 2010). The bacteriocins of all five isolates inhibited the growth of *Enterococcus* spp., *E. coli*, *K. pneumoniae*, *Lactobacillus* spp., *Lact. lactis* subsp. *lactis*, *Listeria* spp., *Ps. aeruginosa*, *Staphylococcus* spp. and *Streptococcus caprinus*. The mode of activity of bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ is bactericidal, as determined against *Ent. faecium* HKLHS and *Lb. sakei* DSM 20017, respectively. Two of the five studied bacteriocinogenic strains (ST69BZ and ST612BZ) exhibited activity against some fungal cultures. In addition, a synergistic effect between bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ and ciprofloxacin were registered (Todorov, 2010).

The highest level of bacteriocin ST69BZ activity (12,800 AU/ml) was recorded after 18 h of growth in MRS broth (30 °C), and it remained at this level for the duration of fermentation. The highest level of bacteriocin ST62BZ activity (25,600 AU/ml) was recorded after 21 h of growth, followed by stable production for the next 3 h. Similar results were recorded for bacteriocin ST63BZ. Maximum levels of bacteriocin ST63BZ (1600 AU/ml) were recorded after 24 h, at the end of stationary growth. Bacteriocin ST611BZ reached 52,600 AU/ml after 15 h, but decreased to 25,600 AU/ml during the following 9 h. Similar results were recorded for strain ST612BZ. Optimum levels of bacteriocin ST612BZ was recorded at 15 h (6400 AU/ml), followed by a decrease to 3200 AU/ml during the following 9 h (Todorov, 2010). The size of

bacteriocins ST69BZ (3.2 kDa), ST62BZ (10.0 kDa), ST63BZ (10.0 kDa), ST611BZ (3.2 kDa) and ST612BZ (6.5 kDa) (Todorov, 2010) was similar to that described for other bacteriocins produced by LAB isolated from boza. Bacteriocins JW3BZ, JW6BZ, JW11BZ and JW15BZ are between 2.3 and 3.3 kDa (Von Mollendorff et al., 2006). Similar results were reported for bacteriocins ST194BZ (3.0 and 14.0 kDa), ST242BZ (10.0 kDa), ST284BZ (3.5 kDa), ST414BZ (3.7 kDa), ST461BZ (2.8 kDa), ST462BZ (8.0 kDa), ST664BZ (6.5 kDa) and ST712BZ (14.0 kDa) (Todorov & Dicks, 2006). The sizes recorded for the five bacteriocins (ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ) are within the range reported for most bacteriocins produced by *Lactobacillus* spp. and *Enterococcus* spp. (De Vuyst & Vandamme, 1994).

Complete inactivation of the bacteriocins was observed after treatment of the cell-free supernatants with proteolytic enzymes, confirming the proteinaceous nature of the antimicrobial compounds (Todorov, 2010). Treatment of cell-free supernatants of the four strains with catalase and α-amylase did not result in activity changes, except for bacteriocin ST63BZ, suggesting that the inhibition factor recorded was not hydrogen peroxide and that carbohydrate moieties were not required for antimicrobial activity (Todorov, 2010). Inhibition of bacteriocin ST63BZ activity by α-amylase suggests that the bacteriocin is glycosylated and belongs to group IV according to the classification of Klaenhammer (1988). Stability of the other four bacteriocins in the presence of α-amylase is not unusual, and similar results have also been reported for other bacteriocins isolated from boza (Todorov & Dicks, 2006; Von Mollendorff et al., 2006). Leuconocin S (Keppler, Geiser, & Holzapfel, 1994) and carnocin 54 (Lewus, Sun, & Montville, 1992) are sensitive to α-amylase, suggesting that their activity is associated with glycosylation of the active peptide.

Thermostability at 100 °C has also been reported for most other bacteriocins (Todorov & Dicks, 2004, 2005, 2006; Von Mollendorff et al., 2006) isolated from boza. The sensitivity of bacteriocins ST63BZ and ST612BZ to 100 °C after 120 min and 121 °C after 20 min may be a result of their molecular mass (10.0 and 6.5 kDa, respectively), although the stability of bacteriocin ST62BZ, a 10.0 kDa peptide was not affected by treatment for 120 min at 100 °C. A difference in the structures of bacteriocin ST62BZ and ST63BZ may be a reason for these results. Bozacin B14 was inactivated after 10 min at 90–121 °C (Ivanova et al., 2000).

The mode of activity of bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ is bactericidal, as determined against *Ent. faecium* HKLHS and *Lb. sakei* DSM 20017, respectively (Todorov, 2010). The results obtained regarding the leakage of DNA, RNA, proteins and β-galactosidase confirm that bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ destabilise the permeability of the cell membrane (Todorov, 2010).

Cell-free supernatants from 24-h-old cultures of *Lb. plantarum* ST69BZ, *Leuc. lactis* ST63BZ, ST611BZ, ST612BZ and *Ent. faecium* ST62BZ (pH neutralised) inhibited the growth of several bacterial strains such as *Li. monocytogenes*, *Ent. faecalis*, and *E. coli* (Meinken & Todorov, 2009). Early log phase cells of test microorganisms treated with bacteriocins resulted in immediate and complete growth inhibition for at least 10 h (Meinken & Todorov, 2009), suggesting that the mode of action is bactericidal. Cells of test microorganisms treated with bacteriocins ST69BZ, ST62BZ,

ST63BZ, ST611BZ, and ST612BZ were clearly deformed or vesiculated as visualised by AFM (Meinken & Todorov, 2009). Sensitive strains treated with bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ, and ST612BZ resulted in leakage of DNA, RNA, proteins, and β-galactosidase. The results obtained by AFM shown by Meinken and Todorov (2009) and leakage of DNA, RNA, proteins, and β-galactosidase confirm that bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ, and ST612BZ destabilise the permeability of the cell membrane. Vesiculation was clearly visible on cells of *Lb. sakei* DSM20017 after treatment with bacteriocin ST62BZ produced by *Ent. faecium* ST62BZ, and bacteriocins ST63BZ, ST611BZ, and ST612BZ produced by *Leuc. lactis* ST63BZ, ST611BZ, and ST612BZ, respectively. Changes in morphology, such as collapse and formation of pores of *Ent. faecium* HKLHS, were observed after treatment with bacteriocin ST69BZ produced by *Lb. plantarum* ST69BZ. The resulting images clearly showed changes in cell morphology, such as collapse of the apical ends or the cell center, signs of cytoplasm leakage or vesiculation. Differences observed between the bacteriocins suggest different modes of action, such as the barrel stave model and the toroidal model, which describe the formation of pores in the cell membrane or the carpet model, which leads to a vesiculation of the outer cell membrane (Meinken & Todorov, 2009).

It was interesting to find that two of these five strains exhibited antifungal activity. *Lactobacillus plantarum* ST69BZ culture showed activity against *Absidia* spp., *Aspergillus niger*, *Epicoccum nigrum*, and *Penicillium* spp., whereas *Leuc. lactis* ST612BZ produced an antifungal substance active against *Botrytis* spp. (Todorov, 2010). As these bacterial species are present in boza, we can presume that they could be an important part of the starter cultures and may therefore contribute to the antifungal stability of the product.

Todorov (2010) reported that, in the combined application of the sublethal levels of clinical antibiotic (ciprofloxacin) and five bacteriocins (bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ), antibacterial activity was strongly increased. These results indicate that the mechanism by which the cationic peptide increases the effectiveness of these antibiotics would be through the dissipation of the proton gradient responsible for the extrusion of these compounds. The synergism between antibiotics, particularly ciprofloxacin and bacteriocins, is important to reduce the level of the MIC of the antibiotics. Similar synergetic effects may be important for the development of treatments against multi-drug-resistant strains.

6.4 Fermented cereal-based food from Africa and Latin America

In Africa, numerous traditional fermented cereal-based foodstuffs are produced in small-scale production units in urban areas (Chavan & Kadam, 1989). Some examples of African traditional fermented cereal-based products are summarised in Table 6.1. Cereal grains including sorghum, maize and millet are common substrates for lactic acid–fermented gruels and beverages known by different names such as, for

example, poto poto in the Congo (Louembé, Brauman, Tchicaya, & Kobawila, 1996), ben-saalga (Tou et al., 2006) and dégué in Burkina Faso, ogi in Nigeria (Banigo & Akinrele, 1977), koko in Ghana (Andah & Muller, 1973) and togwa in Tanzania (Lorri & Svanberg, 1995; Mugula, Nnko, & Sorhaug, 2001). Poto poto is a traditional fermented maize dough prepared in Congo homes for direct use as a weaning food. The process includes a soaking step of the grains (first fermentation step), which on average lasts 55 h, followed by milling and settling of the paste in water (second fermentation step, 10–11 h), and cooking to produce a gruel (Ampe & Miambi, 2000; Louembé, Kéléke, Kobawila, & Nzouzi, 2003). The fermented maize paste is dewatered and modelled into poto poto balls, which can be sold as such or diluted and cooked in water with added sugar (poto poto gruel). Dégué is a pearl millet–based fermented food from Burkina Faso. Dégué has a very different processing diagram resulting after dehulling, grinding of the millet grains, modelling with water and steam cooking in the production of gelatinous balls, which are further fermented during storage (24 h) and afterward consumed mixed with dairy products. In both cases, fermentation is spontaneous and uncontrolled, thus resulting in products of variable quality (Abriuel et al., 2006). In a taxonomic study of poto-poto and dégué, African traditional fermented food products by sequencing of DNA bands from TTGE gels revealed the presence of *Lactobacillus gasseri*, *Enterococcus* sp., *E. coli*, *Lb. plantarum/paraplantarum*, *Lb. acidophilus*, *Lactobacillus delbrueckii*, *Bacillus* sp., *Lactobacillus reuteri*, and *Lb. casei* in poto-poto and *Lb. gasseri*, *Enterococcus* sp., *E. coli*, *Lb. fermentum*, *Lb. brevis*, and *Lb. casei* in dégué (Abriuel et al., 2006).

In some regions of Latin America, fermented products are still manufactured traditionally using very simple equipment. Some examples of Latin American traditional fermented cereal-based products are presented in Table 6.1. In specialised markets throughout the world, these fermented products are highly appreciated and are considered to be of premium value because of their flavour characteristics and uniqueness. Indigenous fermented foods have special organoleptic qualities, and some even possess health-promoting properties. This is principally due to the presence of a biologically diverse microbiota present in the raw material or as a contaminant from either the producers or instruments used for preparation. These microorganisms are important genetic reservoirs and hold great biotechnological and health-improving potential that should be exploited. Those adapted to harsh environmental conditions were able to survive, whereas others had the ability to produce enzymes and compounds necessary for their growth on raw food materials.

Chicha de jora is one of the oldest fermented beverages in South America. It is believed that its origin dates back to the fifteenth century. Maize was first chewed to pulp, since saliva helps to convert starches to fermentable sugars. This pulp was then sun dried, mixed with warm water and left to ferment for a few days, converting the corn into a mildly alcoholic drink. In ancient Japan, sake (rice wine) was also produced by chewing the grains as a source of amylase to convert the starch to monosaccharides and disaccharides (Yoshizawa & Ishikawa, 1979). Later it was discovered that rice overgrown with *Aspergillus*, *Rhizopus* or *Mucor* moulds also became sweet and could be fermented to produce sake (Steinkraus, 1979).

Nowadays, Chicha de jora is prepared by first soaking the maize grains in water for 24–48 h, after which the water is drained and the corn allowed to germinate for 8–15 days, covered in dampened plant leaves and finally sun dried. During the germination process, the starches are converted into fermentable sugars (mono- and disaccharides) by the action of amylases, whereas proteins are broken down by proteases, both activities being necessary for yeast growth. The germinated maize is then boiled for 6–24 h, during which different herbs and spices are added such as cinnamon, allspice and cloves. The liquid is allowed to cool, is strained through a clean cloth, unrefined sugar is added, and the mixture is allowed to ferment for 1–2 weeks in clay pots. The final fermented product has a final pH of 4.0–4.5 and an alcohol content of 4–6%. However, although Chicha de jora is a product known since ancient times, very few studies have been carried out on the fermentation process. It was shown that *Sacch. cerevisiae* is the predominant native yeast species present (Quillama, 1993), and that LAB (such as *Lb. plantarum* and *Lb. fermentum*) are also present in large quantities (Quillama et al., 1995). It was shown that *Lb. plantarum* E2, a strain isolated from Chicha de jora, is capable of producing a bacteriocin that limits the growth of *Lb. fermentum* Chj4C, another strain isolated from the native beverage (Quillama, 1998).

Pozol is a nonalcoholic beverage made from fermented maize that has been produced by the Maya Indians and Mestizo populations in southeastern Mexico and other Central American countries since pre-Hispanic times (Wacher et al., 2000). Today it still forms part of the basic daily nutrition of urban and rural populations of different ethnic groups that reside in this region (Jiménez Vera, González Cortés, Magaña Contreras, & Corona Cruz, 2010). The first step in pozol preparation is called nixtamalisation, where maize kernels (white, yellow or black) are boiled for 1.5 h in a 1% lime solution. These are then dehulled and rinsed using tap water (the discard is called nexayote). The maize is then coarsely ground to make dough (called nixtamal) that is shaped into balls, wrapped in banana leaves, and normally left to ferment at ambient temperatures for 2–7 days but sometimes up to 1 month. The fermented dough is then suspended in water and drunk during meals, at work, or anytime during the day as a refreshing beverage. Traditionally, pozol was produced primarily for family consumption, the drink being consumed by adults, children and infants (Ulloa, Herrera, & Lappe, 1987), although sometimes slightly larger-scale producers have made pozol for sale. Some fibrous components are not completely solubilised by nixtamalisation, and a sediment is present in the beverage when the dough is suspended in water. The Mestizo population has apparently modified the Indian process by adding a second boiling of the grains in water before grinding to reduce sediment formation (Wacher et al., 2000).

In the first complete study of the microbiota of pozol, it was shown that freshly prepared pozol contained 10^4–10^6 CFU/g of LAB, 10^4–10^5 aerobic mesophiles, 10–10^3 *Enterobacteriaceae*, 10–10^4 yeasts, and $<10^3$ mould propagules (Wacher, Cañas, Cook, Barzan, & Owens, 1993). After 30 h at 28 °C, the numbers were 10^9, 7×10^6, 5×10^5, 10^6 and 10^4, respectively. Soaking alkali-treated grains overnight allowed LAB, aerobic mesophiles and *Enterobacteriaceae* to grow, and these then constituted the primary microbiota of the pozol dough. It was shown that, although the additional cooking stage added in the Mestizo process significantly modified the physical

properties of the dough, no significant differences were observed in the microbial composition of Mestizo compared to traditionally prepared pozol (Wacher et al., 2000).

The main soluble sugar of maize is sucrose, which is present at a concentration of 2% (w/w) of the whole kernel on a dry-weight basis. This concentration is reduced to 0.1–0.7% (w/w) of dry dough after alkaline cooking, soaking and washing to produce nixtamal (Diaz-Ruiz, Guyot, Ruiz-Teran, Morlon-Guyot, & Wacher, 2003). This concentration would be insufficient to maintain microbial diversity and the high bacterial concentration reported in pozol (Diaz-Ruiz et al., 2003). Although LAB are the dominant group during all stages of pozol fermentation, as has been shown previously by classical culture methods (Nuraida, Wacher, & Owens, 1995; Wacher et al., 2000, 1993) and culture-independent methods (Ampe, Ben Omar, Moizan, Wacher, & Guyot, 1999; Escalante, Wacher, & Farres, 2001), significant changes in the population dynamics occur throughout pozol production. For example, it was shown that high concentrations of amylolytic LAB (ALAB) were detected at the beginning of the fermentation process, and that a relatively high number of nonamylolytic LAB were observed at the end of fermentation (Diaz-Ruiz et al., 2003). These results suggest a symbiosis in that ALAB would first degrade starch at the beginning of the fermentation, decreasing the pH of the dough and liberating sugars that could be used for the growth of nonamylolytic microorganisms.

LAB (such as *Lb. plantarum* and *Lb. fermentum*, together with members of the genera *Leuconostoc* and *Weissella*) accounted for 90–97% of the total active microbiota of pozol; no streptococci were isolated, although members of the genus *Streptococcus* accounted for 25–50% of the microbiota (Ampe et al., 1999). The presence of *Bifidobacterium*, *Enterococcus* and enterobacteria suggests a fecal origin of some important pozol microorganisms (Ben Omar & Ampe, 2000).

A most comprehensive study on microbial community dynamics during the production of pozol was performed using traditional and culture-independent techniques (Ben Omar & Ampe, 2000). It was shown that *Streptococcus* species dominated the fermentation and accounted for between 25% and 75% of the total microbiota throughout the process. The initial aerobic microbiota was replaced in the first 2 days by heterofermentative LAB (closely related to *Lb. fermentum*), and this heterolactic microbiota was then progressively replaced by homofermentative LAB (mainly by genetically close relatives of *Lb. plantarum*, *Lb. casei* and *Lb. delbrueckii*), which continued acidification of the maize dough. At the same time, an extremely diverse community of yeasts and fungi developed, mainly at the periphery of the dough. This study demonstrated that a relatively high number of species, at least six to eight, is needed to perform pozol fermentation. Overall, the results obtained with different culture-dependent or culture-independent techniques clearly confirmed the importance of developing a polyphasic approach to study the ecology of fermented foods.

Since little sanitary measures are taken during pozol preparation, microbial contamination of the maize dough is inevitable. Potentially pathogenic fungi such as *Candida parapsilosis*, *Trichosporon cutaneum*, *Geotrichum candidum* and *Aspergillus flavus* have been recovered from pozol during the first few hours of fermentation (Ulloa et al., 1987). Although most of these pathogens are killed off by the decrease in pH during the fermentation process, serious health problems can still arise, especially if fresh

underfermented pozol is consumed. It was recently shown that 19% of pozol samples were contaminated with aflatoxin B2 (AFB2) and traces of aflatoxin B1 (AFB1), but only one sample contained aflatoxin concentrations >20 ppb (Mendez-Albores, Arambula-Villa, Preciado-Ortiz, & Moreno-Martinez, 2004). In this study, it was also shown that pozol prepared with white maize and samples prepared with cacao showed the highest ranges of contamination, whereas, when yellow kernels were used, the presence of aflatoxins was not detected. Amerindians consume equal amounts of pozol prepared using white, yellow and black maize, although popular belief has been that the yellow and black varieties contained higher vitamin levels. Mestizos normally use white maize for pozol preparation and also consume chorote, a pozol derivative, in which ground cocoa beans are added to the maize mixture. The former population would thus be less likely to consume aflatoxin containing pozol than the latter; however, in Mestizo preparation, the additional boiling step would be helpful in eliminating aflatoxin-producing microbes.

6.5 Starter cultures and cereal-based fermented food

The use of probiotic LAB, especially *Lactobacillus* and *Bifidobacterium* spp., as starter cultures, alone or in combination with traditional starter cultures in various fermentation processes, has been explored for different fermentation food products. Formulated fermented probiotic food may present consumers with a healthy dietary component at a considerabley low cost (Goldin, 1998). Furthermore, it was reported that LAB may contribute to microbiological safety and/or provide one or more technological, nutritional and organoleptic advantages to a fermented product, through production of ethanol, acetic acid, aroma compounds, exopolysaccharides, bacteriocins and several enzymes (Leroy & De Vuyst, 2004).

Various developments over the years have led to the concept of starter cultures. The earliest fermented food products relied on natural fermentation through microbiota present in the raw material. The load and spectrum of microorganisms populating the raw material have a definite effect on the quality of the end product. Backslopping, that is, inoculation of the raw material with a small quantity of a previously performed successful fermentation, was used to optimise spontaneous fermentation. This is still the basis for the home-scale production of several fermented food products, including, for example, yoghurt, kefir, traditional beer and boza. In this case, the best-adapted strain dominates. The dominant strains can be seen as a starter culture that shortens the fermentation process and reduces the risk of fermentation failure (Leroy & De Vuyst, 2004). Backslopping is still used in developing countries and even in the industrialised countries for production of sauerkraut and sourdough (Holzapfel, 1997). The use of starter cultures in large-scale production of fermented foods has become important for industries in developed countries, as it resulted in improved control over the fermentation process and a consistent end product. However, some disadvantages do occur due to the fact that commercial starter cultures were not selected in a rational way but, rather, on the basis of phage resistance and rapid acidification of the raw materials (Leroy & De Vuyst, 2004). With regard to the functionality and desired properties of

the end product, these starters are not very flexible, and they are frequently difficult to be keep even by modern microbiological procedures. Furthermore, it is believed that commercial starter cultures adapted to the food matrix led to a loss in genetic material (Leroy & De Vuyst, 2004). This may have contributed to the limited biodiversity of commercial starter cultures. Moreover, this leads to a product that lacks the uniqueness and characteristics that made the original product popular (Caplice & Fitzgerald, 1999).

Wild-type LAB that originate from the environment, raw materials and process apparatus serve as natural starter cultures ("inoculation") for many of the traditionally fermented foods (Böcker, Stolz, & Hammes, 1995; Weerkamp, Klijn, Neeter, & Smit, 1996). Recent studies focused on the use of wild-type strains isolated from traditional products for use as starter cultures (Beukes, Bester, & Mostert, 2001; De Vuyst et al., 2002; Hébert, Raya, Tailliez, & De Giori, 2000) as a resource of improved technological, beneficial and functional properties. When considering LAB as a starter culture, the following factors have to be taken into account: (1) not all LAB strains have the same practical and technical importance in food fermentations; (2) the genera *Lactobacillus* (*Lb. fermentum*, *Lb. plantarum*, *Lb. reuteri, Lb. rhamnosus*), *Leuconostoc*, and, to a lesser extent, *Lactococcus*, *Enterococcus*, *Pediococcus* and *Weisella* spp. are usually present in traditional fermented foods; (3) not all strains of the same species are suitable as starter cultures and (4) various industrial scale lactic acid fermentation processes are well controlled despite the fact that they are spontaneous (Holzapfel, 2002). Some of these LAB may be classified as functional and beneficial starters, due to their contribution to food safety, sensory properties and other nutritional advantages.

LAB are known to produce antimicrobial substances (including bacteriocins, organic acids, diacetyl and low-molecular-weight substances), polymers, sugars, sweeteners, nutraceuticals, aromatic compounds and various enzymes (including proteolytic enzymes). This leads to greater flexibility and wider application of LAB as starter cultures. It also represents a way by which chemical additives can be replaced by natural compounds and thus provide the consumer with new, appealing food products (Leroy & De Vuyst, 2004). Bacteriocins produced by LAB may prevent food spoilage, for example, late blowing of cheese by clostridia (Thomas, Clarkson, & Delves-Broughton, 2002). Some probiotic strains may also be used as functional starters or co-cultures in fermented food (Chandan, 1999; Jahreis et al., 2002; Ross, Stanton, Hill, Fitzgerald, & Coffey, 2000).

However, when considering the use of probiotic strains as functional starters or co-cultures, it is important that they do not enhance acidification and thereby reduce the shelf-life of the product, and that they do not have adverse effects on the aroma or taste of the product (Heller, 2001). Uncertainty still exists as to whether multifunctional strains possessing all desirable metabolic features would result from modern isolation techniques and selection procedures. Therefore, recent studies focus on the improvement of selected strains by the application of recombinant DNA technology. By its application, certain beneficial features, such as health-promoting properties, accelerated acid production, wholesomeness and overproduction of specific enzymes or bacteriocins can be improved (Holzapfel, 2002). Gene disruption may be used to

eliminate undesirable properties such as antibiotic and mycotoxin production by food-grade moulds (Geisen & Holzapfel, 1996; Hammes & Vogel, 1990). A large array of these optimised cultures is available but is not used because of legal regulations (Holzapfel, 2002).

6.6 Cereal-based probiotic foods

The concept of probiotic foods and feeds has been developed to quite an extent since its introduction to clinical nutrition and food science during the 1980s (Fuller, 1989; Shortt, 1999). The majority of probiotic food products available today are milk-based, although a few attempts have been made to use cereals. Cereal grains have a high nutritive value and are distributed worldwide, making cereal a very suitable raw material for the development of various fermented functional foods (Angelov, Gotcheva, Kuncheva, & Hristozova, 2006). Togwa, a lactic acid–fermented maize and sorghum gruel, inhibits the growth of some enterotoxin-producing bacteria in children <5 years of age. This suggests that togwa may possess probiotic properties (Kingamkono, Sjögren, & Svanberg, 1998). Vogel et al. (1999) found that the LAB present in various LAB-fermented foods, such as sourdough, is similar or, in some cases, identical to species found in the gastrointestinal tract of humans and animals. *Lactobacillus plantarum* indigenous to a variety of cereal-based fermented food is also associated with the gastrointestinal tract of humans (Ahrné et al., 1998; Molin, 2001). Colonisation of the intestinal mucosa with strains of *Lb. plantarum* isolated from sourdough has also been reported (Johansson, Molin, Jeppsson, Nobaek, & Ahrné, 1993).

The early Mayans used pozol as a source of nutrients and in ceremonies promoting the growth and harvest of maize. They also used this fermented beverage as a medicine to control diarrhoea, to reduce fever and to cure intestinal infections (Phister, O'Sullivan, & McKay, 2004). Microbiological studies on pozol provided evidence in support of these popular beliefs. It has been stated earlier that LAB are well known for their health-promoting capabilities, and, since LAB are the dominant microbial species in pozol, it is not surprising that this refreshing beverage possesses health-promoting properties. Antimicrobial compounds produced by *Bacillus* sp. strain CS93 isolated from pozol were identified and exhibited activities against several Gram-positive and Gram-negative bacteria, yeasts and moulds (Phister et al., 2004). It was also shown that *Agrobacterium azotophilum*, present in pozol, possesses bacteriocidal, bacteriolytic, bacteriostatic and fungistatic activities against a wide range of pathogenic microorganisms (Ulloa & Herrera, 1972). All of these findings, along with future studies, will surely help in promoting the consumption of this interesting Mayan drink.

Barley and oats contain β-glucan (Angelov et al., 2006), a prebiotic that may contribute to the reduction of LDL-cholesterol levels by 20–30%, and in this way may also contribute to a decrease in the risk of cardiovascular diseases (Gallaher, 2000; Stark & Madar, 1994; Wrick, 1994). For a polysaccharide or oligosaccharide to be characterised as a prebiotic, it should withstand digestion in the upper part of the gastrointestinal tract, be hydrolysable, be soluble, and stimulate the growth

and activity of beneficial microbiota in the gut (Gibson & Roberfroid, 1995). The low glycemic index of oats and barley is quite beneficial to diabetes in the gastrointestinal tract after ingestion, as it alters the level of fat emulsification and reduces lipase activity (Angelov et al., 2006). Furthermore, β-glucan stimulates the growth of beneficial bacteria associated with the colons of animals and humans (Jaskari et al., 1998; Wood & Beer, 1998).

To increase the number of beneficial bacteria in the gut, large numbers of probiotic bacteria have to be consumed by means of capsules or by using food as vector. Incorporating suitable dietary polysaccharides or oligosaccharides to the capsules may be even more effective. The latter is referred to as the prebiotic concept. Arabinoxylan is another prebiotic compound commonly found in wheat and rye (Crittenden et al., 2002; Jaskari et al., 1998; Karppinen, 2003).

6.6.1 Beneficial properties of lactic acid bacteria isolated from boza

According to the definition of the World Health Organisation, probiotics are live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host (Fuller & Gibson, 1997). This includes reduction of gastrointestinal infections and inflammatory bowel disease, and modulation of the immune system (Fuller & Gibson, 1997). Probiotic strains should survive the harsh conditions of the gastrointestinal tract and adhere to intestinal epithelial cells. They form a defense against the colonisation of pathogenic microorganisms by competing for adherence to mucus and epithelial cells and, in certain cases, production of hydrogen peroxide and bacteriocins (Boris &and Barbes, 2000; Lepargneur &and Rousseau, 2002; Reid &and Burton, 2002; Velraeds, Van De Belt-Gritter, Van Der Mei, Reid, & Busscher, 1998). Changes in diet, stress, contraceptives, medication and microbial infection disturb the microbial balance, which often leads to a decrease in the number of viable LAB (Fuller & Gibson, 1997). The subsequent uncontrolled proliferation of pathogenic bacteria may lead to diarrhoea and other clinical disorders such as cancer, inflammatory bowel disease and ulcerative colitis (Fooks, Fuller, & Gibson, 1999). A variety of *Lb. plantarum* strains are presently marketed as probiotics (De Vries, Vaughan, Kleerebezem, & De Vos, 2006). The best-studied probiotic strains are from the species *Lb. acidophilus, Lb. fermentum, Lb. plantarum, Lb. brevis, Lactobacillus jensenii, Lb. casei, Lb. delbrueckii, Lactobacillus vaginalis* and *Lactobacillus salivarius*. A number of surface-anchored proteins have been described for *Lb. plantarum* (Kleerebezem et al., 2003), suggesting that the species has the potential to associate with many different surfaces and substrates, and to adapt to changing environmental conditions.

Criteria for the selection of probiotic strains have been formulated by the Food and Agriculture Organisation of the United Nations and the World Health Organization (FAO/WHO, 2001). Some of the most important criteria are gastric and bile acid resistance, adhesion to mucus and human epithelial cells, competition with pathogens for adhesion sites, growth inhibition of potentially pathogenic bacteria, bile salt hydrolase activity and, in the case of vaginal applications, resistance to

contraceptives (FAO/WHO, 2001). The concept of probiotic foods has been developed extensively since its introduction to clinical nutrition and food science during the 1980s (Fuller, 1989; Shortt, 1999). Most probiotic foods available today are milk based, although a few attempts have been made to use cereals. Cereal grains are very suitable raw materials for the development of various fermented functional foods (Angelov et al., 2006). Examples of beneficial cereal-based fermented products are togwa, a LAB-fermented maize and sorghum gruel (Kingamkono et al., 1998). Vogel et al. (1999) reported on LAB present in various fermented foods, such as sourdough, that are similar or, in some cases, identical to species found in the gastrointestinal tracts of humans and animals. *Lactobacillus plantarum* indigenous to a variety of cereal-based fermented foods is also associated with the gastrointestinal tract of humans (Ahrné et al., 1998; Molin, 2001). Colonisation of the intestinal mucosa with strains of *Lb. plantarum* isolated from sourdough has also been reported (Johansson et al., 1993).

A number of LAB with probiotic properties have been isolated from boza, a traditional beverage produced from the fermentation of cereals. *Lactobacillus plantarum* (strains ST194BZ, ST414BZ and ST664BZ), *Lb. pentosus* (strain ST712BZ), *Lb. rhamnosus* (strains ST461BZ and ST462BZ) and *Lb. paracasei* (strains ST242BZ and ST284BZ) have been described previously as bacteriocin producers with activities against several Gram-positive and Gram-negative test organisms (Todorov & Dicks, 2006). Strains ST194BZ, ST242BZ, ST284BZ, ST414BZ, ST461BZ, ST462BZ, ST664BZ and ST712BZ were shown to have potential probiotic properties (Todorov et al., 2008), as all of them survived low pH conditions (pH 3.0), grew well at pH 9.0 and were not affected by the presence of 0.3% (w/v) oxbile. Cytotoxicity levels of the bacteriocins, expressed as CC_{50}, ranged from 38 µg/ml for bacteriocin ST194BZ to 3776 µg/ml for bacteriocin ST284BZ. Bacteriocin ST284BZ, bacteriocin ST461BZ and bacteriocin ST462BZ were the least cytotoxic. Bacteriocin ST284BZ revealed high activity (EC_{50} = 735 µg/ml) against the HSV-1 virus that causes encephalitis and oro-facial and genital lesions. Growth of *Mycobacterium tuberculosis* was repressed by 69% after 5 days of incubation in the presence of the bacteriocin ST194BZ. Various levels of auto- (self-) aggregation between the probiotic bacteria and co-aggregation with *Li. innocua* LMG 13568 were observed. Adhesion of strains ST194BZ, ST284BZ, ST414BZ, ST461BZ, ST462BZ and ST664BZ to HT-29 cells ranged from 18% to 22%, which is similar to that reported for *Lb. rhamnosus* GG. Adherence of strains ST194BZ, ST242BZ and ST712BZ to Caco-2 cells ranged between 7.0% and 9.0%, similar to values reported for *Lb. rhamnosus* GG. High hydrophobicity readings were recorded for most of the probiotic strains. Strain ST712BZ revealed only 38% hydrophobicity, but 63% of the cells adhered to HT-29 cells, compared to 32% adherence recorded for *Lb. rhamnosus* GG. Growth of strains ST194BZ, ST242BZ, ST284BZ, ST414BZ, ST461BZ, ST462BZ, ST664BZ and ST712BZ were inhibited by only seven of 24 medicaments tested (Todorov et al., 2008).

Enterococcus mundtii ST4V isolated from soya beans produces a broad-spectrum bacteriocin active against Gram-positive and Gram-negative bacteria and has antiviral activity (Todorov et al., 2009). The aim of the study of Todorov et al. (2009)

was to determine the probiotic properties of strain *Ent. mundtii* ST4V, to evaluate its survival in boza, and to study the antimicrobial activity of the strain *in situ*. For this purpose, boza was prepared based on the traditional recipe (Todorov, 2010; Todorov et al., 2009). Cereals have been cooked and inoculated with *Ent. mundtii* ST4V (a potential probiotic and bacteriocin-producing strain). Control batches have been prepared with commercial boza (as deliver vector of starter cultures). All samples received also *Sacch. cerevisiae*. Fermentation was carried out at 37 °C for 3 h. The organoleptic properties of fermented products were evaluated by a qualified taste panel. No significant differences in rheological properties were observed, suggesting that *Ent. mundtii* ST4V had no negative effect on the sensory qualities of the final product. Microbial cell numbers remained relatively unchanged during 1 week of storage. Sensory analysis of the six boza preparations was reported in the study of Todorov et al. (2009). A slightly greater bitterness was recorded when boza was produced by the starter culture of commercial boza. Similar results were obtained for this sample with regard to a stronger yeasty flavour of the product when commercial boza was used as starter culture. These differences, albeit statistically significant, were so small that they would probably not be detected by the consumers. The acid taste of all samples was relatively low, with the lowest recorded for boza prepared with *Ent. mundtii* ST4V. No correlation was found between the different sensory attributes tested. Fermentation of the product contributes to the acidity of the product (Todorov et al., 2009). The preservative properties of bacteriocin ST4V were evaluated by contaminating boza with *Lb. sakei* DSM 20017. Changes in microbial populations were monitored by using classical microbiological methods, PCR with species-specific primers and denaturing gradient gel electrophoresis (DGGE). *Enterococcus mundtii* ST4V survived 7 days in boza that was stored at 4 °C, and produced bacteriocin ST4V at levels high enough to prevent the growth of the target strain *Lb. sakei* DSM 20017^T. No off-flavours or abnormal textural changes were recorded in boza during storage. Boza could thus be developed as a vector to deliver strain ST4V (Todorov et al., 2009).

Incorporation of probiotic strains in cereal-based fermented foods is feasible and holds great promise for the future. Cereals are high in nutrition and confer specific health benefits. In addition, cooked bran provides an excellent substrate for several strains of probiotic bacteria. A snack exhibiting the combined postulated beneficial effects of bran and probiotic bacteria may serve as an alternative to soy-based and milk-based yogurts (Salovaara, 1996; Salovaara & Simonson, 2003). In general, oats are a suitable substrate for fermentation with probiotic LAB after appropriate processing (Johansson et al., 1993; Marklinder & Lonner, 1992; Salovaara, 1996; Salovaara & Simonson, 2003).

Acknowledgements

The author would like to thank CAPES, CNPq and FAPESP (Brasil) for their financial support.

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Advanced methods for the identification, enumeration, and characterization of microorganisms in fermented foods

7

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7.1 The fermented food microbial ecosystem

The complexity of food microbial ecosystems and the importance of investigating and understanding the microbial dynamics in fermented foods are well-known concepts in the field of microbial research. Although lactic acid bacteria (LAB) and *Saccharomyces cerevisiae* are mainly leading food fermentation, other microbial groups such as coagulase-negative cocci, coryneform bacteria, and non-*Saccharomyces* yeasts are able to colonize food matrices and to interact eventually compete with fermenting microbiota. This creates a more complex picture, which needs to be deeply understood. Traditional microbiology based on the isolation and cultivation of microorganisms has shown strong limits in the understanding of ecosystem biodiversity. Thus, culture-independent approaches based on direct microbial nucleic acid analysis have been optimized, together with new molecular techniques to provide greater insight into food fermentation.

In the past few years, the advantages and disadvantages of culture-dependent and culture-independent approaches have been extensively discussed. Microbiologists have agreed that cultivation conditions in synthetic media could not picture correctly the community structure for the imposition of new selective conditions. Culture-dependent approaches have shown limitations in terms of recovery rate, and the set of obtained isolates may not always truly reflect the microbial composition of the sample analyzed (Temmerman, Huys, & Swings, 2004). Thus, the culture collection will not totally represent the community, and the actual microbial diversity will be misinterpreted. The principal reason is a lack of knowledge of the real conditions under which most bacteria are growing in their natural habitat (in this case food), and the difficulty of developing media for cultivation accurately resembling these conditions (Ercolini, 2004). Consequently, species occurring in low numbers are often outnumbered in vitro by more abundant microbial species, and some species may be unable to grow in vitro although they are in a viable and perhaps metabolically active state in the food matrix.

For these reasons, the trend is now toward culture-independent methods, because they are believed to overcome problems associated with selective cultivation and

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00007-4

isolation of bacteria from food samples. The study of complex matrices, such as cheese, by culture-independent methods implies that microorganisms are detected in the matrix by the direct analysis of the total DNA and RNA without any culturing step. These methods are based on protocols in which total DNA or RNA is directly extracted from the substrate. In this way, the final "picture" of the ecosystem microbiota will be more realistic and reliable.

The development of culture-independent approaches for microbial analysis has revolutionized microbial ecology, and its application to food microbiology is leading to major new insights into this complex microbial ecosystem. Thus, in the last years, the scientific literature has been enriched with many manuscripts on the characterization of fermented food of dairy origin (Alessandria et al., 2010; Dolci et al., 2008; Randazzo, Vaughan, & Caggia, 2006; Rantsiou, Urso, Dolci, Comi, & Cocolin, 2008), meat products (Albano, Henriques, Correia, Hogg, & Teixeira, 2008; Cocolin et al., 2009; Nguyen et al., 2013), and vegetable matrices (Randazzo, Ribbera, Pitino, Romeo, & Caggia, 2012; Tofalo, Perpetuini, Schirone, Suzzi, & Corsetti, 2013) by both culture-dependent and culture-independent approaches.

To improve the characterization of these products, new techniques have been optimized and culture-independent approaches have been highlighting new information on fermented foods thanks to next-generation sequencing, in particular pyrosequencing, which provides high definition in the analysis of a fermentation process. That is due to the possibility of analyzing thousands of sequences in a single experiment, including low-abundance operational taxonomic units (OTUs).

7.2 Culture-dependent methods

The evaluation of microbial diversity in fermented food is problematic. It is often difficult to cultivate viable microorganisms using known media, as part of them might not be cultivable in vitro. Some species are outcompeted by numerically more abundant microbial species. Moreover, Fleet (1999) highlighted that adverse conditions in food ecosystem such as nutrient depletion, heat treatments, pH variations, or low temperatures could induce microbial cells in a stressed state characterized by the inability to produce colonies on media even if they are still able to perform metabolic activity. They enter in a viable but noncultivable state, which cannot be detected by culture-dependent methods.

Nevertheless, detection, identification, and both phenotypic and genotypic characterization of strains isolated from food matrices are still of great importance, especially for the food industry. The selection of new starter cultures with desirable competitive ability and metabolic properties is of paramount importance and starts from the screening of hundreds of isolates obtained from food. This has led to the development of polymerase chain reaction (PCR)-based molecular methods for strain identification and characterization. Here, we will discuss the most widely used advanced techniques in food fermentation.

7.2.1 Identification and characterization

When unknown bacterial isolates have to be identified, a powerful tool with high discriminatory power is the 16S or 23S rRNA gene sequencing. Yeast isolated from food matrices are commonly identified by sequencing of the D1/D2 region of the 26S rRNA gene. For the identification of genus or species, the partial or complete sequence of these genes is compared with sequences from known microorganisms by the aid of online databases of previously sequenced DNA. For the differentiation of bacteria such as LAB, other target genes have been considered, and the protein-coding genes *rpoB*, *recA*, and *pheS* have proved useful for studies by various authors (Martin-Platero, Maqueda, Valdivia, Purswani, & Martinez-Bueno, 2009; Rantsiou, Comi, & Cocolin, 2004; Torriani, Felis, & Dellaglio, 2001; Van Hoorde et al., 2010).

When researchers are approaching the identification of food microbial communities isolated on selective media, species-specific PCR could represent a valid and rapid alternative. Different researchers have optimized protocols for the identification of fermented food recurrent species such as *Lactobacillus helveticus* (Fortina, Ricci, Mora, Parini, & Manachini, 2001), *Streptococcus thermophilus* (Lick, Keller, Bockelmann, & Heller, 1996), *Lactococcus lactis* (Corroler, Desmasures, & Guéguen, 1998), and *Lactobacillus delbrueckii* (Torriani, Zapparoli, & Dellaglio, 1999), which have been widely applied in the study of food microbial populations (Dolci et al., 2008; Fonseca, Ouoba, Franco, & Carballo, 2013; Fortina et al., 2003; Robert, Gabriel, & Fontagné-Faucher, 2009).

Moreover, internal transcribed sequences (ITS) between rRNA genes can produce amplicon profiles characteristic of each microbial species. Jensen, Webster, and Strauss (1993) and Arroyo-Lopez, Duran-Quintana, Ruiz-Barba, Querol, and Garrido-Fernandez (2006) designed primers and developed the method for the identification of bacteria and yeasts, respectively. This approach, in particular, if followed by amplicon enzymatic restriction (ITS-RFLP), can reach a reliable definition at the species level and, in a few cases, at the strain level (Alves, Goncalves, & Quintas, 2012; Jeyaram, Singh, Capece, & Romano, 2008; Tanasupawat, Kommanee, Yukphan, Nakagawa, & Yamada, 2011).

In the past few years, the concept of strain has become more and more significant in terms of applications in food microbiology. Different strains present different genotypic, physiological, and technological characteristics to be taken into consideration, for example, in the selection of industrial starter cultures. Strains could present diverse attitudes in colonizing food matrices and competing with the resident microbiota. Moreover, the fermentation capability or the production of metabolites interesting for the characteristics of the final products can vary significantly among different strains. These findings have led to the development of continuously novel methods for intraspecific typing of microbial populations. Methods based on enzymatic restriction such as restriction fragment length polymorphism (RFLP), or on PCR such as random amplified polymorphic DNA (RAPD)-PCR, repetitive element sequenced-based (Rep)–PCR, multilocus sequence typing (MLST), and, more recently, Sau-PCR are only some examples.

Among PCR-based methods, RAPD-PCR has been the most popular technique applied to food ecosystems. In recent years, it has been successfully applied to detect the presence, the succession, and the persistence of LAB starter cultures inoculated in sourdoughs, cheeses, and meats, as well as to determine the variations of microbial populations in naturally fermented products such as artisan cheeses, olives, meats, sourdoughs, and indigenous fermented foods (Charteris, Kelly, Morelli, & Collins, 1997; Dolci et al., 2008; Gardiner, Ross, Collins, Fitzgerald, & Stanton, 1998; Giraffa & Neviani, 2000; Fitzsimons, Cogan, Condon, & Beresford, 2001; Urso, Comi, & Cocolin, 2006). Despite the fact that RAPD-PCR lacks high reproducibility unless a careful standardization of the experimental methodology has been carried out, this technique is still widely used in food microbiology. Aponte et al. (2012) tracked a *Lactobacillus pentosus* starter by M13 RAPD-PCR in Spanish-style table green olive fermentations. Likewise, the potential of implanting the probiotics *Lactobacillus fermentum* HL57 and *Pediococcus acidilactici* SP979 during manufacture of Iberian dry-fermented sausages, and their effect on the sensory properties of these meat products, was investigated by Ruiz-Moyano et al. (2011). Recent applications regarded also the impact of ecological factors on the stability of microbial associations in sourdough fermentation (Vogelmann & Hertel, 2011) and the characterization of *Leuconostoc* isolates used as adjunct starters in Manchego cheese (Nieto-Arribas, Sesena, Poveda, Palop, & Cabezas, 2010).

Repetitive chromosomal elements randomly distributed in microbial genomes are the target of rep-PCR. In particular, $(GTG)_5$-PCR was proved to be useful for the differentiation of lactobacilli at the strain level (Gevers, Huys, & Swings, 2001) and it was applied to characterize LAB isolated from fresh sausages (Cocolin et al., 2004), the microbiota in a Serbian homemade semi-hard cheese (Terzic-Vidojevic et al., 2007), and *Lactobacillus sanfranciscensis* isolates from Italian sourdoughs (De Angelis et al., 2007). Recently, this technique has been used for the microbiota characterization of different exotic products (Adimpong, Nielsen, Sorensen, Derkx, & Jespersen, 2012; Nguyen et al., 2013; Owusu-Kwarteng et al., 2012; Parkouda et al., 2010).

Enzymatic restriction of microbial genomes allows the detection of polymorphism in specific regions and so the differentiation of diverse strains. Based on this assumption, a few methods have been optimized in the past, such as RFLP and amplified fragment length polymorphism (AFLP) and have found application for the characterization of the microbiota detected from different fermented products (Arroyo-Lopez et al., 2012; Kubo et al., 2011; Kunene, Geornaras, von Holy, & Hastings, 2000; McLeod, Nyquist, Snipen, Naterstad, & Axelsson, 2008; Mendonca, Gouvea, Hungaro, Sodre, & Querol-Simon, 2013). More recently, Corich, Mattiazzi, Soldati, Carraro, and Giacomini (2005) developed a method based on the digestion of genomic DNA with the restriction endonuclease *Sau 3AI* and subsequent amplification with primers whose core sequence is based on the *Sau 3AI* recognition site. The molecular characterization of *Staphylococcus xylosus* isolated from naturally fermented Italian sausages (Iacumin, Comi, Cantoni, & Cocolin, 2006) and of *Lactococcus garvieae* strains isolated in northern Italy from dairy products and fishes (Foschino et al., 2008) have been carried out by comparing the results obtained by Sau-PCR with those from other typing techniques, and have confirmed the robustness of this method.

High reproducibility and discrimination can be reached by pulsed field gel electrophoresis (PFGE), a robust and easy-to-interpret technique that is based on the use of rare cutting restriction endonucleases on the whole chromosome structure. Because it requires expensive equipment and laborious protocols, its diffusion has been relatively limited despite the fact that it is considered the gold standard among typing methods. Recently, researchers have applied PFGE in the study of the biodiversity of strains belonging to microbial groups or genera recurring in fermented foods, such as staphylococci isolated from naturally fermented dry sausages (Leroy, Giammarinaro, Chacornac, Lebert, & Talon, 2010), fermented meat products (Marty et al., 2012), and *Lactobacillus* strains obtained from dairy products (Xu et al., 2012).

An approach that provides unambiguous results that are directly comparable between laboratories is the MLST, which is based on the high degree of variability of at least seven housekeeping or protein-coding genes for a given species (Maiden et al., 1998). The comparison of the DNA sequence of this specific subset of conserved genes allows distinguishing among subspecies or strains. At present, De las Rivas, Marcobal, and Munoz (2006) applied MLST to determine the genetic relationship among *Lactobacillus plantarum* strains and Tanganurat, Quinquis, Leelawatcharamas, and Bolotin (2009) to characterize *Lb. plantarum* strains isolated from fermented fruits and vegetables. Finally, Rademarker et al. (2008) and Taibi, Dabour, Lamoureux, Roy, and LaPointe (2010) used the technique to characterize *Lact. lactis* strains.

7.3 Culture-independent methods: diversity in microbial communities

In the past few years, approaches for studying microorganisms in food have undoubtedly changed.

Given the importance of fermented food in human life, much effort has been expended toward describing the microbial populations acting in the different food fermentations. A deeper understanding of microbial ecology can lead to an improvement in the quality and safety of products. The result has been the development of new techniques that were added to traditional microbial methods based on the cultivation of microorganisms and the resolution of the culture media bias distorting the real "picture" of microbial communities.

Culture-independent methods are based on the direct analysis of microbial nucleic acids of food samples. DNA analysis can be used to investigate the presence of microorganisms without distinguishing between viable and dead cells, owing to the stability of this molecule. In this sense, an approach based on DNA analysis describes the "history" of a sample, showing all of the species that succeeded. On the contrary, RNA is a better indicator of microbial vitality and activity, as it is degraded rapidly upon cell death (Cocolin, Alessandria, Dolci, Gorra, & Rantsiou, 2013; Santarelli, Gatti, Bernini, Zapparoli, & Neviani, 2008).

In recent years, molecular biology has encompassed a range of DNA- and RNA-based technologies, which are revolutionizing the way that microorganisms are studied

Table 7.1 Culture-independent methods as microbial community profiling tools: main advantages and disadvantages

Methods	Advantages	Disadvantages
(RT)-PCR-DGGE and (RT)-PCR-TGGE	Give a reliable broad-spectrum view of the dominant microbial communities present in a sample Clearly represent the evolution and dynamics of microbial populations in fermentation and spoilage processes Low-cost equipment	Unable to determine the relative abundance of the dominant species Time consuming, unsuitable for large-scale analysis Complex interpretation due to heterogeneous multi-copy genes and co-migrating bands
SSCP-PCR	No need for gradient gels Performed using an automated sequencer	Dependent on the availability of a reliable database
T-RFLP	High-throughput, sensitive, and automatable process Pseudo-quantitative method	Expensive equipment
LH-PCR	Gives a broad view of the dynamics of the whole community of microbial cells	Low sensitivity Biases inherent to the PCR process
(RT)-qPCR	Quantification of microbial targets analyzed Enhanced precision and specificity	Unable to study simultaneously large numbers of different targets
FISH	Possibility of localizing and observing target cells within their native environment	Unable to study simultaneously large numbers of different targets
Pyrosequencing	High sensitivity Exhaustive profiling of microbial communities due to the sequencing of thousands to billions of raw DNA fragment reads Enables phylogeny-based diversity	Need to develop new bioinformatic algorithms to manage large amounts of data

(Table 7.1). The analysis of both microbial genome and transcriptome represents a rich molecular toolbox for the study of microbial community in food matrices.

The diversity and dynamics of microbial populations in fermented food have been profiled by means of DNA-based experiments by different researchers (Alegrìa et al., 2009; Belén Flòrez & Mayo, 2006; Bonetta, Bonetta, Carraro, Rantsiou, & Cocolin, 2008; Casalta, Sorba, Aigle, & Ogier, 2009; Coppola, Blaiotta, Ercolini, & Moschetti, 2001; Dolci et al., 2008; Ercolini, Hill, & Dodd, 2003; Fonseca et al., 2013; Fontana, Vignolo, & Cocconcelli, 2005; Gala et al., 2008; Meroth, Hammes, & Hertel, 2003; Meroth, Walter, Hertel, Brandt, & Hammes, 2003; Randazzo, Heilig, Restuccia,

Giudici, & Caggia, 2005; Randazzo et al., 2012; Randazzo et al., 2006). Microbial RNA analysis has also been performed, but to a lesser extent, to obtain a picture of the species that are metabolically active at a particular sampling instant (Alessandria et al., 2010; Dolci, Alessandria, Rantsiou, Bertolino, & Cocolin, 2010; Masoud et al., 2011; Randazzo, Torriani, Akkermans, De Vos, & Vaughan, 2002; Rantsiou et al., 2008).

A main issue in culture-independent methods relates to the extraction of nucleic acids, because technical issues may arise: DNA may not be recovered from all genotypes, which could remain undetected due to low species abundance, insufficient homogenization of the matrix, or inadequate cell lysis that prevents the release of nucleic acids. Moreover, even if DNA yield is high, macromolecule inhibitors that have not been eliminated may lower PCR sensitivity, and PCR amplification may be inaccurate or inhibited (Jany & Barbier, 2008). For these reasons, protocols have to be accurately adapted to extract nucleic acids from all different types of microorganisms and matrices.

The microbial ecology of a fermented product and the dynamics of the different populations developing during the process can be well described by the direct analysis of the microbial DNA present in a food matrix. Over the years, different techniques have been optimized and applied to the study of fermented food; they can be divided mainly into methods based on the study of microbial DNA previously amplified by PCR, and methods based on the detection of microbial DNA directly in the samples, which allows the localization and distribution of microbial populations in the matrix.

7.3.1 PCR-based methods

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) have received great attention in the past 20 years for profiling microbial communities. Based on the separation of short amplicons of DNA along a chemical or temperature gradient, these methods allow the detection of the most dominant microorganisms in a given sample. The possibility of identifying microbial components in complex ecosystems is an important issue in both cases, for understanding whether a microbial transformation is proceeding correctly, for investigating spoilage processes, or, for examining the dynamics of the microbiota in a given fermented product. This interest has resulted in an impressive number of papers using DGGE and TGGE published in the last years (Cocolin et al., 2013; Ercolini, 2004). The diversity and dynamics of microbial populations in cheese and during cheese manufacturing have been profiled by means of DNA-based experiments by different authors (Alegrìa et al., 2009; Belén Flòrez & Mayo, 2006; Bonetta et al., 2008; Casalta et al., 2009; Coppola et al., 2001; Dolci et al., 2008; Ercolini et al., 2003; Gala et al., 2008; Randazzo et al., 2006). Likewise, the fermentation processes of different sausages (Cocolin, Manzano, Aggio, Cantoni, & Comi, 2001; Cocolin, Manzano, Cantoni, & Comi, 2001; Cocolin et al., 2004; Cocolin, Urso, Rantsiou, Cantoni, & Comi, 2006; Fontana et al., 2005; Rantsiou et al., 2005) and sourdoughs (Meroth, Hammes, et al., 2003; Meroth, Walter, et al., 2003; Randazzo et al., 2005) have been studied using this approach.

Coppola et al. (2001) first used DGGE to investigate a dairy microbial environment, specifically mozzarella cheese, and observed that the greatest diversity resulted

from the use of raw milk and the absence of starter cultures. Similar studies, which again used a DGGE approach but which focused on cheese produced from sheep's or goat's milk, have also revealed the presence of a more diverse biota in artisanal cheeses (Bonetta et al., 2008; Randazzo et al., 2006).

Cocolin et al. (2009) studied the bacterial biodiversity during the maturation process of three traditional sausages produced in northern Italy by using culture-dependent and culture-independent methods; these investigators showed how the environmental and processing conditions are able to select specific microbiota responsible for the main transformations during the fermentation and ripening of the sausages. This technique has also been applied to investigate complex ecosystems such as cheese surfaces. A first picture of dominant microorganisms colonizing Fontina protected denomination of origin(PDO) rinds was made possible by DGGE, and again the maturing environment seemed to influence the dynamics of microbial groups (Dolci et al., 2009).

The main issues in DGGE and TGGE use relate to the detection of the dominant species without the possibility to reliably determining their relative abundance. These are time-consuming methods unsuitable for large-scale analysis in which each representative band has to be sequenced from each single gel. Moreover, the presence, in the bacterial genome, of rRNA genes in heterogeneous multi-copies and of co-migrating bands further complicates the interpretation of the results.

The single-strand conformation polymorphism–PCR (SSCP-PCR) method has been widely applied for the study of dairy products (Callon, Delbes, Duthoit, & Montel, 2006; Delbes, Ali-Mandjee, & Montel, 2007; Feurer, Irlinger, Spinnler, Glaser, & Vallaeys, 2004; Saubusse, Millet, Delbes, Callon, & Montel, 2007). It is based on the differences in electrophoretic mobility, in non-denaturing gels, of single-stranded DNA folds into tertiary structures according to their nucleotide sequences and their physicochemical environment (e.g., temperature and ion strength). SSCP-PCR is potentially easier to carry out than PCR-D/TGGE, as there is no need for gradient gels and it can be performed using an automated sequencer either acrylamide gel or capillary based. At the same time, the robustness of this method is dependent on the availability of a reliable database for the reading and identification of the different picks.

Mounier, Monnet, Jacques, Antoinette, and Irlinger (2009) applied SSCP-PCR in the study of the surface of Livarot cheese by both culture-dependent and culture-independent approaches. A total of 40 yeasts and 40 bacteria from the cheese surface were collected, de-replicated using SSCP analysis, and identified using rRNA gene sequencing for the culture-dependent approach. The culture-independent approach involved cloning and sequencing of the 16S rRNA gene and SSCP analysis from total DNA extracted from the cheese. The main disadvantage of this technique lies in the difficulty of appending new data to an existing database: in fact, samples presenting unknown profiles cannot be directly sequenced because they are labeled.

Unlike DGGE and SSCP, terminal-restriction fragment length polymorphism (T-RFLP) has received much less attention by food microbiologist, probably because of the need for more expensive equipment. T-RFLP is a rapid and sensitive molecular method that targets ribosomal RNA (rRNA) genes among all individuals of a given community, and detects variations in fragment length using fluorescent dye-labeled primers (Marsh, 2005). Distinct fragments are detected using a capillary sequencer,

and identified peaks are then considered as OTUs. Unlike DGGE, T-RFLP is a high-throughput and automatable process that can compare much larger sample sets rapidly and efficiently. It can be considered a pseudo-quantitative method that calculates relative species abundance based on sensitive capillary electrophoresis separation and fluorescence detection. Rademaker, Hoolwerf, Wagendorp, and te Giffel (2006) used T-RFLP analysis for the characterization of bacterial population during yoghurt fermentation, detecting bacterial population structure and dynamics between short time spans. Eight samples taken at different times, within 5.5 h of yoghurt fermentation, revealed shifting relative signal intensities in TRF peaks between streptococci and lactobacilli, indicating a semi-quantitative relation of the T-RFLP signal with the actual numbers of bacteria. Recently, Arteau, Labrie, and Roy (2010) applied T-RFLP for profiling fungal communities in Camembert cheese. Isolating and identifying molds within a fungal community is challenging; fungi grow slowly and have high contamination potential. T-RFLP allowed the detection of ripening starters used in Camembert-type cheese making, namely *Penicillium camemberti*, *Geotrichum candidum*, and *Kluyveromyces lactis*, as well as other common dairy species found in the milk microbiota or environment, such as *Cladosporium*, *Debaryomyces*, *Mucor*, *Pichia*, *Saccharomyces*, and *Yarrowia*.

Like T-RFLP, length heterogeneity (LH)–PCR has not been widely carried out in the study of microbial ecology of fermented products. Whereas T-RFLP identifies PCR fragment length variations based on restriction site variability, LH-PCR distinguishes different organisms based on natural variations in the length of sequences of the 16S rRNA gene. However its low sensitivity and biases inherent to the PCR process (Lazzi, Rossetti, Zago, Neviani, & Giraffa, 2004) limited the applications in food microbiology. LH-PCR was applied, for the first time by Gatti et al. (2008) to study a fermented product. Microbial succession during Parmigiano-Reggiano cheese making was monitored at different stages of cheese production and ripening. Because of its low sensitivity, LH-PCR is not meant to provide a quantitative analysis. However, it has been a helpful method for following the dynamics of whole and lysed bacterial cells.

Even though it cannot be considered a community profiling techniques, quantitative PCR (qPCR) allows reliable detection of taxonomically defined group of microbial populations targeted. Although quantitative methods have many benefits, which, in addition to the ability to quantify, include enhanced precision and specificity, there are some disadvantages to take into consideration. qPCR cannot effectively quantify very large numbers of different targets in a single sample simultaneously, and therefore selection of target genes and the development of specific primers and probes is essential (Juste, Thomma, & Lievens, 2008). Recently, Carraro et al. (2011) used a combined approach of culture-dependent and culture-independent methods to study the microbiota of Montasio cheese, and qPCR was applied to follow specific microbial species linked to cheese production and ripening. Likewise, the combination of the results obtained from microbial counts, species, and genus-specific PCR as well as real-time qPCR allowed the identification for the dominant bacterial species as well as study of the variation in the community composition, over the ripening period, of a traditional dry-fermented Spanish sausage (Fonseca et al., 2013). Thus, the application

of qPCR in the study of a complex microbial ecosystem should be focused on the accurate and sensitive detection of individual microbial species or groups.

7.3.2 In situ methods

Fluorescence in situ hybridization (FISH) is a non-PCR technique based on fluorescence-labeled oligonucleotide probes targeting specific DNA sequences. Because only a few probes may be used simultaneously, FISH cannot be considered a high-throughput method for microbial profiling. The interest in this technique is based on the possibility of observing target cells within their native environment, and it is often associated with PCR-based methods for a more complete overview of the product analyzed. Cocolin et al. (2007) applied both PCR-DGGE and FISH techniques to fresh meats and fermented meat products and to fresh and ripened cheeses. Mounier et al. (2009) studied the microbiota of a specific product, Livarot cheese; FISH probes targeting the dominant yeasts present in the cheese, namely, *Candida catenulata*, *Candida intermedia*, *Geotrichum* spp., and *Yarrowia lipolytica*, were designed, and allowed the detection of these yeasts directly in cheese.

Fluorescently labeled oligonucleotide probes were developed to detect *Lact. lactis*, *Lb. plantarum*, and *Leuconostoc mesenteroides* in Stilton cheese by Ercolini et al. (2003). A combination of these probes allowed the assessment of the spatial distribution of the different microbial species in the dairy matrix, with implications of significance in understanding the ecology of Stilton matrix.

New light has been shed on Parmigiano Reggiano natural whey starter microbial composition by combining FISH and LH-PCR methods (Bottari, Santarelli, Neviani, & Gatti, 2009). Revealing different images of the same community, LH-PCR and FISH have given a more accurate view of the not-well-known Parmigiano Reggiano whey starter ecosystem, highlighting the importance of a polyphasic approach.

7.4 Culture-independent methods: metabolic activity in microbial communities

Because the half-life of DNA may vary to a great extent in dead bacterial cells and is highly dependent on environmental conditions (Cenciarini-Borde, Courtois, & La Scola, 2009; Keer & Birch, 2003), the use of retrotranscribed RNA as a template is suggested by many authors to overcome this critical issue. DNA from lysed cells can persist for a long time in the environment, whereas RNA is a better indicator of viable microorganisms, as it is degraded rapidly upon cell death (Santarelli et al., 2008). Thus, because the numbers of intact ribosomes approximately reflects the rates of protein synthesis, rRNA can be used as a marker of the general metabolic activity. The study of microbial metabolic activity in the fermentation of food products is fundamental in terms of understanding which microbiota are active in the transformation of the raw material, as well as the timing and the roles of the different populations during the maturing of the product.

Although it should be considered that rRNA molecules are characterized by a much higher level of protection than messenger RNA (mRNA), which has an average half-life that can be measured in minutes, the use of rRNA molecules as the target allows one to avoid practical problems of extracting detectable levels of intact mRNA and distinguishing mRNA from the corresponding homologous DNA sequences.

7.4.1 Microbial vitality population profiling

As reported in Section 7.3.1, PCR-DGGE has been widely used in food microbiology, and the diversity and dynamics of microbial populations during the manufacturing and ripening of different products have been profiled by means of DNA-based experiments. Reverse transcription (RT)-PCR-DGGE has also been performed, but to a lesser extent, to obtain a picture of the species that are metabolically active at particular sampling instants.

Diez et al. (2008) used RT-PCR-DGGE to evaluate whether high hydrostatic pressure treatment (HPP) could improve the shelf life of the blood sausage Morcilla de Burgos. The spoilage was caused by the metabolism of active populations, and, for this reason, the study of microbiota at the RNA level was fundamental. Other researchers carried out RT-PCR-DGGE to microbiologically characterize traditional products with PDO such as Castelmagno (Dolci et al., 2010) and feta cheese (Rantsiou et al., 2008), and with registered denomination of origin (RDO) and Ragusano cheese (Randazzo et al., 2002). The knowledge of the microbial populations that are metabolically active during the subsequent steps of the manufacturing and ripening of a fermented product highlights the contribution of each microbial group in the different technological processes. To understand the role of microbial species during the fermentation process as well as maturation can also be useful for the definition of new starter cultures at the industrial level.

Recently, Dolci et al. (2013) investigated Fontina PDO cheese surface microbiota complexity at both DNA and RNA levels. A detailed picture of the microbiota was observed when rRNA was targeted, and bacterial species that were not highlighted by means of the DNA analysis were detected. Thus, these authors suggest that RT-PCR-DGGE should be considered a better tool to profile microbial populations, and that the rRNA molecule is a worthwhile target for a better and more complete understanding of the microbial communities in food ecosystems.

Given the short half-life times of mRNA molecules, some studies have proposed using mRNA as a suitable viability marker and, in combination with quantitative reverse transcription PCR (RT-qPCR), to quantify yeasts and pathogenic bacteria (Gonzalez-Escalona et al., 2009) or to quantify bacteria during Emmental cheese manufacturing (Falentin et al., 2010). However, the susceptibility of RNA to degradation during sample extraction and the RNA copy number variability resulting from differential gene expression represent some limitations of this method. One promising recent strategy to detect and quantify only viable cells includes the use of impermeable nucleic acid binding dyes prior to DNA extraction and qPCR. The propidium monoazide (PMA)–qPCR procedure has been applied for the simultaneous

quantification of viable probiotic strains in fermented milk, and was also optimized with success in Cheddar cheese to monitor the viability of a mixture of three probiotic species (Desfossés-Foucault et al., 2012).

7.5 Recent insights: pyrosequencing

A new tool in the study of fermented food is represented by pyrosequencing, which allows one to sequence thousands to billions of raw DNA fragment reads in a single run, leading to a massive amount of information and to a more exhaustive profiling of microbial communities. Compared to Sanger sequencing, the need for electrophoresis gel, the low number of samples to be analyzed in parallel and the lack of process automatization are overcome. In pyrosequencing, short fragments are amplified using universal PCR primers targeting known marker genes, mainly prokaryotic 16S rRNA and fungal ITS genes. As the base incorporation in the growing DNA strand is detected using high-resolution optics, pyrosequencing is much more sensitive to low-abundance OTUs compared to DGGE or T-RFLP, and thus low-frequency community members can be detected more consistently by pyrosequencing. Moreover, pyrosequencing enables phylogeny-based diversity comparisons; thus microbial communities can be studied from the point of view of both biodiversity and phylogenic similarity. A critical step for reliable results is the choice of the reference database, which should have reasonable coverage of species of all taxa of interest.

Because of the advantages reported earlier, pyrosequencing has already been applied for the study of microbial successions in various fermented foods. Dairy products have been objects of study by numerous authors (Alegria, Szczesny, Mayo, Bardowski, & Kowalczyka, 2012; Ercolini, De Filippis, La Storia, & Iacono, 2012; Masoud et al., 2011; Quigley et al., 2012). By using this approach, the presence of several genera that were not previously associated with cheeses were revealed, confirming pyrosequencing as an efficient method for deeper investigation of microbial communities compared to the other methods. A more global view of the community structure including metabolically active populations can be offered by pyrosequencing. Finally, this technique has been applied for studying the bacterial diversity of traditional fermented foods such as pearl millet slurries (Humblot & Guyot, 2009), kimchi (Park et al., 2012), and narezuski (Kiyohara et al., 2012). Pyrosequencing has opened new and deeper insights in the diversity of and microbial interactions in food fermentation; however, a major disadvantage is the fact that quantitative data can only be obtained in conjunction with additional analytical techniques. Profiling data should be complemented by qPCR to quantify total microbial load and to track the most abundant or significant taxa detected by profiling tools. Actually, the foremost issue is computational analysis to process thousands to billions of reads. Thus, there is a need to develop a new bioinformatic algorithm to manage a large amount of data and to allow a deeper analysis and biological interpretation. Other limitations include the need for large amounts of DNA, relatively short reads, complexity of analysis, and the cost.

7.6 Conclusions

Currently, advanced molecular methods represent an invaluable tool in the study of food ecosystems and the strains responsible of fermentation processes. At the same time, especially in industrial or applied microbiology, phenotypic tests, which were used widely in the past for microbial identification, are still being considered for the characterization of strain metabolic properties, growth performance, resistance to industrial processes, and shelf life. However, due to their poor reproducibility and low discriminatory power, phenotypic methods have been almost abandoned for identification purposes. Their low taxonomic resolution often leads to differentiation only at the genus level, and they require a labor-intensive approach. On the contrary, genotypic techniques provide a more robust classification and identification, and their costs, over the years, have been decreasing.

Despite the already common use of molecular techniques, some traditional approaches, such as the use of microscopy, should not be completely forgotten for reason of the microbial characteristics that they are able to highlight. Microscopic analysis of an isolate is an immediate way to examine the dimensions, morphology, and aggregation state of a bacterium and, eventually, its belonging to Gram-positive or Gram-negative group or its ability to produce endospores. Microscopic observation allows one to detect asexual or sexual reproduction phases of yeasts or molds, leading quickly to a first indication of their belonging to a genus or species. Likewise, details of carbohydrate fermentation patterns remains a point of crucial importance for the knowledge and identification of isolates, and these approaches should still be taken into account. For these reasons, microbiologists should consider combining traditional and molecular methods for a more complete view of the different microbial aspects involved in fermentation processes.

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Systems biology and metabolic engineering of lactic acid bacteria for improved fermented foods

8

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8.1 Introduction

Lactic acid bacteria (LAB) form a phylogenetically homogeneous group of low G+C content Gram-positive bacteria (Makarova et al., 2006) that are able to produce a great variety of food products by the fermentative conversion of agro raw materials. Although there is a large variety in species, the industrial fermentations involve only a limited set of LAB, including *Lactococcus lactis*, *Streptococcus thermophilus*, and a limited number of *Lactobacillus* spp. The success of the fermentations initiated by these industrial LAB in many cases relies not only on the rapid or controlled conversion of sugars into lactic acid but also on the generation of flavor, texture, and health benefits that add the value of the final product (de Vos, 1996). Hence there is considerable interest in the metabolic engineering of these industrial LAB to improve existing and expand new metabolic conversions. Several factors have led to a renewed interest in doing so. First, their metabolism has been well described and characterized. Second controlled fermentations can be achieved by defined growth conditions and media. Finally, the genomes of these LAB have been well characterized, and a variety of functional genomics tools are available (Makarova et al., 2006; de Vos, 1996, 1999, 2011).

The vast majority of industrial LAB are homofermentative bacteria, and glycolysis is the major pathway associated with the rapid conversion of sugars. Growth and energy are obtained through substrate-level phosphorylation with the conversion of glucose to production of lactic acid. In some cases, mixed acid fermentation is observed when acetate, formate, and ethanol are formed. Finally, acetaldehyde, diacetyl, and other flavor compounds can be formed mostly from precursors other than sugars, such as citrate or amino acids. Moreover, the success of the industrial fermentations by LAB can also be attributed to the production of exopolysaccharides (EPS) and vitamins. Following the characterization of the metabolic pathways and the involved enzymatic activities, pathway engineering approaches have been applied to industrial LAB to steer the production of desired compounds from inexpensive substrates such as lactose or glucose (de Vos & Hugenholtz, 2004). Initial successes were realized before the genomics era by the high or almost complete conversion by *Lactococcus lactis* of glucose into diacetyl or alanine, respectively (Hols et al., 1999; Hugenholtz et al., 2000). These compounds are of significant interest, as diacetyl is an important flavor used in, among other things, butter

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00008-6

production and the confectionary industry, and alanine is not only an amino acid but also has applications as a food sweetener. Following these hallmarks, a series of engineering approaches have been described, mainly in *Lactococcus lactis*, *S. thermophilus,* and *Lactobacillus casei* and *Lactobacillus plantarum* (see Table 8.1 for an overview). In this chapter, we will provide an overview of these metabolic engineering approaches in industrial LABs. In particular, we summarize how molecular biology allowed the transformation of LAB into cell factories able to produce a large diversity of compounds. Subsequently, the combination of systems biology with metabolic engineering will be presented. Finally, we will discuss the future developments and the challenges in the metabolic engineering of LABs for improved food fermentations.

8.2 Metabolic engineering in industrial lactic acid bacteria (LAB)

Industrial LAB have been used for more than a decade as powerhouses for metabolic engineering. In the absence of complete genomic information, most attention has initially been focused on the well-known conversion of sugars (mainly glucose or lactose) into lactic acid. The glycolysis and the pyruvate branch represent the major highway in LAB metabolism. Rerouting of this central metabolism has been used toward the food-grade production of chemicals (lactic acid, ethanol, butanol), flavors and fragrances compounds (diacetyl, acetaldehyde, 2,3-butanediol), and food ingredients (alanine, polyols, EPS), and production of nutraceuticals, such as vitamins (Figure 8.1).

The industrial applicability of LAB has boosted the development of molecular biology tools that can be applied to LAB (de Vos, 2011). An important instrument has been the development of inducible gene expression systems (de Vos, 1999). These are of particular use in metabolic engineering, as the precise effect of increasing a specific metabolic conversion can be studied. The Nisin Controlled Expression (NICE) system that involves an extracellular food-grade inducer (the 34-residue peptide nisin or the supernatant of a nisin-producing *Lactococcus lactis*) has been of crucial importance in the controlled expression of single genes or gene cassettes (De Ruyter, Kuipers, & De Vos, 1996). A great number of proteins have been produced in LAB using the NICE and other systems, and many of these have been extensively reviewed (Kuipers, De Ruyter, Kleerebezem, & De Vos, 1997; Mierau & Kleerebezem, 2005; de Vos & Hugenholtz, 2004). As detailed in Table 8.1, a large diversity of compounds are now produced by metabolically engineered LAB strains. The central carbon metabolism and the pyruvate branch represent the major targets for metabolic engineering, as crucial precursors are formed in these conversions (Figure 8.1).

8.2.1 Engineering fermentation products and flavor compounds

The production of a variety of chemicals has been engineered by metabolic pathway engineering. Lactic acid production is dependent on lactate dehydrogenase (LDH) activities present in LAB. The lactic acid stereoisomerism is, in general, controlled by the enzymatic activity of LDH-L or LDH-D, although, in some cases, lactate

Table 8.1 Overview of relevant metabolic engineering approaches resulting in the design of new strains with improved properties (for further explanation, see text)

Product	Strain	Genes modified	References
Fermentation product and flavor compound			
Lactic acid	*Lactobacillus helveticus*	*↓ldhD or (↓ldhD and ↓ldhL)*	Bhowmik and Steele (1994) and Kyla-Nikkila et al. (2000)
	Lactobacillus johnsonii	*↓ldhD*	Lapierre et al. (1999)
	Lactobacillus plantarum	*↓ldhL*	Ferain et al. (1994)
Ethanol production	*Lactococcus lactis*	*↑pdc, ↓ldh, ↓ldhB, ↓ldhX, ↑adhB, ↓pta, ↓adhE*	Solem et al. (2013)
	Lactobacillus plantarum	*↑Spdc, ↓ldhL, ↓ldhD*	Liu et al. (2006)
Butanol	*Lactobacillus brevis*	*↑crt, ↑bcd, ↑etfB, ↑etfA, ↑hbd and ↑thl*	Berezina et al. (2010)
Acetaldehyde	*Streptococcus thermophilus*	*↑glyA, ↓glyA*	Chaves et al. (2002)
	Lactococcus lactis	*↑pdc, ↑ldh*	Bongers et al. (2005)
Diacetyl	*Lactococcus lactis*	*↑nox, ↓aldB*	Hugenholtz et al. (2000)
Diacetyl and acetoin	*Lactobacillus casei*	*↑ilvBN, ↓ldh, ↓pdc*	Nadal et al. (2009)
Nutraceutical, sweeteners, and vitamins			
Alanine	*Lactococcus lactis*	*↑alaD, ↓ldh, ↓alr*	Hols et al. (1999)
Mannitol and 2,3-butanediol	*Lactococcus lactis*	*↓ldh, ↓ldhB, ↓ldhX, ↑mtlD, ↑mtlP, mtlF, ↑als, ↑butA, ↓ adhE*	Gaspar et al. (2011)
Sorbitol	*Lactobacillus casei*	*↑gutF, ↓ldhL*	Nissen et al. (2005)
	Lactobacillus plantarum	*↑srlD1, ↑srlD2, ↓ldhD, ↓ldhL*	Ladero et al. (2007)
Xylitol	*Lactococcus lactis*	*↑XYL1, ↑xylT*	Nyyssölä et al. (2005)
Folate	*Lactococcus lactis*	*↑folkE*	Sybesma et al. (2003)
	Lactobacillus gasseri	*↑folA, ↑folB, ↑folKE, ↑folP, ↑ylgG, ↑folC*	Wegkamp et al. (2004)
	Lactobacillus reuteri	*Folate biosynthesis gene cluster*	Santos et al. (2008)
EPS production	*Lactococcus lactis*	*↑galU*	Boels et al. (2001)
	Streptococcus thermophilus	*↑galU, ↓pgmA*	Levander et al. (2002)
Histidine decarboxylation pathway	*Lactococcus lactis*	*↑hdcA, ↑hdcP, ↑hdcB*	Trip et al. (2012)

Source: Adapted from Gaspar et al. (2013).

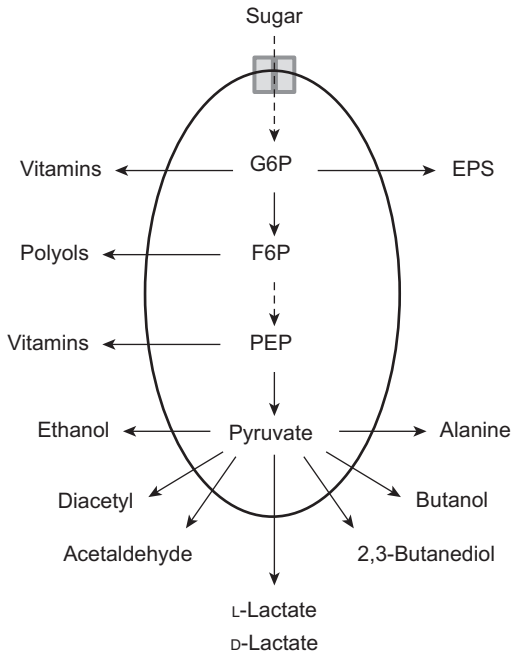

Figure 8.1 Main compounds produced by lactic acid bacteria in relation to the central metabolism.
Adapted from Gaspar et al. (2013).

racemases have been detected. Hence, LAB that contain only one of these LDH enzymes may produce the pure enantiomer. *Lactobacillus helveticus* has been the first LAB to be used and combined with *ldhD* inactivation, a derivative developed that was able to produce pure L-lactic acid (Bhowmik & Steele, 1994). With a similar approach, pure enantiomers of lactic acid have also been obtained by inactivation of the specific LDH in other LAB (Ferain, Garmyn, Bernard, Hols, & Delcour, 1994; Kyla-Nikkila, Hujanen, Leisola, & Palva, 2000). This also was combined with production of diacetyl and acetoin, as described for *Lactobacillus johnsonii* (Lapierre, Germond, Ott, Delley, & Mollet, 1999).

Increased ethanol production has been realized in *Lactobacillus plantarum* by the introduction of the heterologous pyruvate decarboxylase from *Sarcina ventriculi* combined with a double crossover knockout of the LDH gene (Liu, Nichols, Dien, & Cotta, 2006). Thereafter, high-level ethanol fermentation has been achieved in *Lactococcus lactis* via introduction of the pyruvate decarboxylase and the alcohol dehydrogenase from *Zymomonas mobilis* in a mutant of *Lactococcus lactis* MG1363 in combination with five genes inactivated (Solem, Dehli, & Jensen, 2013). Moreover, a butanol-producing *Lactobacillus brevis* has been generated by expressing a large set of genes from the clostridial *bcs*-operon (containing the *crt*, *bcd*, *etfB*, *etfA*, and *hbd* genes) in combination with a thiolase gene, opening avenues to use LAB not only for food but also biofuel applications (Berezina et al., 2010). However, the rerouting was

not very efficient, and issues with stability and solvent tolerance should be further addressed.

Rerouting metabolic highways such as glycolysis and the pyruvate branch can be done while affecting the redox state of the LAB (Figure 8.2). For instance, diacetyl has been produced in significant amounts by *Lactococcus lactis* by overexpressing the *nox* gene for a water-forming NADH oxidase in a mutant strain, where it has been placed under the control of *nisA* promoter of the NICE system and combined with an inactivated α-acetolactate decarboxylase (Hugenholtz et al., 2000). Under nongrowing conditions, 16% of glucose had been directly converted to diacetyl and 57% to acetolactate, the direct precursor, which is converted to diacetyl by a chemical oxidative conversion in the presence of oxygen. More recently, *Lactobacillus casei* has been used to produce diacetyl and acetoin in fed-batch growth condition with whey permeate in growth medium (Nadal, Rico, Perez-Martinez, Yebra, & Monedero, 2009). Despite the relatively low yield that was obtained, it appears relevant to produce those compounds from whey permeate, as it can contribute to valorizing this waste product of cheese making (de Vos et al., 1998).

Acetaldehyde, an end-product in glucose catabolism, presents the property to be an important flavor compound. Increase in acetaldehyde production has been first achieved in *S. thermophilus* while characterizing the enzymatic activity of the serine

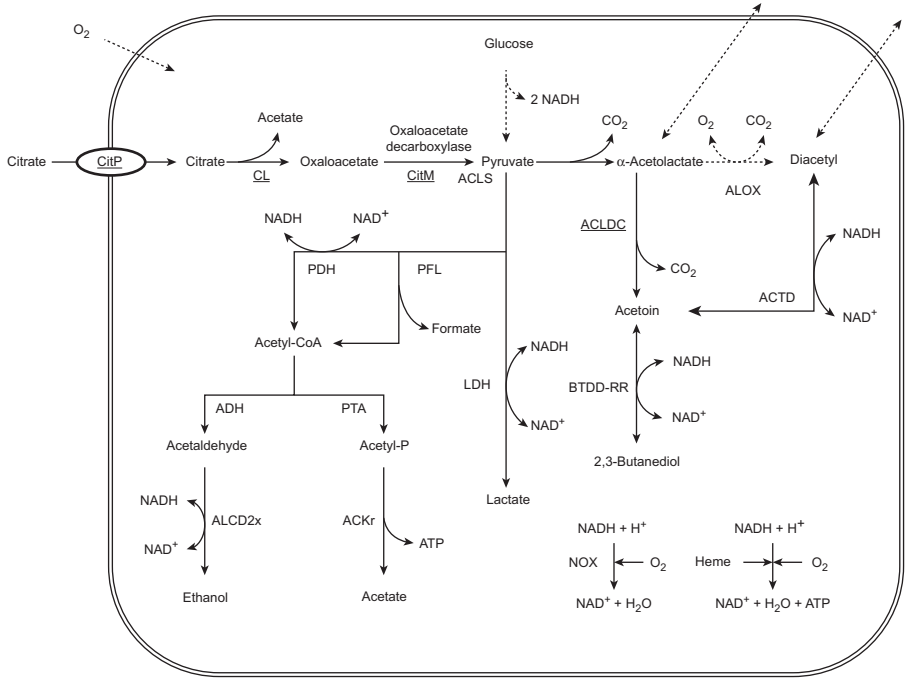

Figure 8.2 Pyruvate branch with diacetyl production and citrate metabolism. Underline indicates reactions that have to be modified to increase diacetyl production (for further explanation, see text).

hydroxymethyl transferase (*glyA*) for its threonine aldolase activity (Chaves et al., 2002). The authors succeeded in increasing by 82% the amount of acetaldehyde produced by replacing the wild-type gene by a construct under the strong *Lactococcus lactis* P_{LacA} promoter (Van Rooijen, Gasson, & De Vos, 1992), therefore illustrating that high production levels of a key enzyme allow an increase in the production of a target compound. Nonetheless, the highest amounts of acetaldehyde have been obtained with *Lactococcus lactis*, in which the central carbon metabolism has been modified by replacement of the native pyruvate decarboxylase (*pdc*) with an over-expressed PDC from *Z. mobilis* and its native NADH oxidase (*nox*) (Bongers, Hoefnagel, & Kleerebezem, 2005).

8.2.2 Overproducing nutraceuticals, sweeteners, and vitamins

For the dairy and other food industries, it is of interest to produce food sweeteners, vitamins, and other nutraceuticals. For that purpose, Hols et al. developed a homoalanine-producing *Lactococcus lactis* able to convert all glucose consumed into alanine (Hols et al., 1999). Introduction of the alanine dehydrogenase gene from *Bacillus sphaericus* under NICE control in a strain deficient for lactate dehydrogenase allowed complete rerouting of the carbon flux. Pure enantiomeric production of L-alanine was obtained by inactivation of alanine racemase. In the case of L-mannitol, a sweet-tasting polyol, Neves et al. reported its production after carbon source depletion in *Lactococcus lactis* (Neves et al., 2000; Neves, Ramos, Shearman, Gasson, & Santos, 2002). It was found that mannitol 1-phosphatase was a key enzyme in the production of mannitol (Wisselink et al., 2005). Subsequently, a strain with numerous gene inactivations was developed and was capable of achieving mannitol and 2,3-butanediol synthesis at the maximum theoretical yield (Gaspar, Neves, Gasson, Shearman, & Santos, 2011). This example illustrates that it is possible to realize metabolic engineering success with sufficient investments in resources. As food sweeteners have gained importance as nutraceuticals, a direct synthesis from central carbon metabolism has great advantages. Sorbitol is another sugar alcohol with high value in the food industry, due to its low-calorie properties as a sweetener and its ability to be used as a texturing agent. Various *Lactobacillus* spp. have been engineered to produce sorbitol, and two different approaches can be distinguished. Nissen et al. engineered a *Lactobacillus casei* with a similar approach to Chaves et al. for acetaldehyde production (Nissen, Pérez-Martínez, & Yebra, 2005). The D-sorbitol-6-phosphate dehydrogenase of *Lactobacillus casei* was integrated in a lactose operon to suppress catabolite repression in the native host. To improve production yield, the LDH gene was inactivated, and this resulted into a conversion rate of glucose to sorbitol of 4.3%. In a complementary approach, Ladero et al. reached high polyol production in *Lactobacillus plantarum* with insertion of the sorbitol-6-phosphate dehydrogenase under the P_{ldhL} control (Ladero et al., 2007). With their approach, resting cells have been able to convert up to 65% of glucose to sorbitol with only a small concomitant amount of mannitol production, whereas in pH-controlled growing conditions up to 25% of mannitol has been converted into sorbitol. Finally, xylitol synthesis was obtained by introduction of an heterologous

xylose transport system and a D-xylose reductase in *Lactococcus lactis,* and this succeeded in achieving a nearly complete reduction of xylose to xylitol in fed-batch condition (Nyyssölä, Pihlajaniemi, Palva, Von Weymarn, & Leisola, 2005).

Nutraceutical and other functional foods have received growing interest from consumers and the food industries, as these foods offer health benefits. Apart from amino acids, sweeteners, and polyols, vitamin production by LAB has received considerable attention from different research groups. An increased level of folate (also known as vitamin B11) has been realized in *Lactococcus lactis* by optimization of its biosynthesis pathway. Sybesma et al. increase by 10-fold the folate production in *Lactococcus lactis* by overexpressing *folKE* with NICE system (Sybesma et al., 2003). Subsequently, the same group demonstrated the high versatility of the NICE system while introducing it in *Lactobacillus gasseri*, which converted this former folate-consumer into a folate-producer (Wegkamp, Starrenburg, De Vos, Hugenholtz, & Sybesma, 2004). The complex metabolic pathway for vitamin B12 synthesis has been characterized in *Lactobacillus reuteri* and used to increase its production via medium optimization (Santos et al., 2009). Moreover, by expressing the *Lactobacillus plantarum* folate genes, a *Lactobacillus reuteri* strain capable of producing vitamin B12 and folic acid has been generated (Santos, Wegkamp, De Vos, Smid, & Hugenholtz, 2008). Similarly, a *Lactococcus lactis* strain simultaneously producing riboflavin (vitamin B2) and folate has been engineered (Sybesma, Burgess, Starrenburg, Van Sinderen, & Hugenholtz, 2004). This area of multivitamin production has significant potential, as has been recently reviewed (Leblanc et al., 2013).

Not only are LAB beneficial in food fermentations for their ability to produce flavor compounds, but they actively participate in the formation of texture of the final product, which represents a key factor in consumer choices. Specifically, yoghurt as well as the Nordic longvill and villii products are fermented dairy produce that are highly dependent on the production of EPS. In addition, EPS also has been considered as a neutraceutical, as it may have some health benefits (Van Kranenburg, Boels, Kleerebezem, & De Vos, 1999). *Lactococcus lactis* and *S. thermophilus* represent the natural targets of metabolic engineering for increased EPS production. A key enzyme for the production of EPS and its precursors is UDP-glucose pyrophosphorylase (GalU). Characterization of the *galU* gene in *Lactococcus lactis* and its induction by the NICE expression system demonstrated that precursor availability permits increase in EPS production (Boels, Ramos, Kleerebezem, & De Vos, 2001). A similar approach has been successful in *S. thermophilus,* but the highest yield of EPS production has been obtained with an inactivated phosphoglucomutase (*pgm*) in a Gal^+ strain (Levander, Svensson, & Radstrom, 2002). Although significant progress has been made in this area, the absence of high EPS-producing strains illustrates that challenges remain in further understanding of these complex pathways. In many engineering projects, the resistance of the production host to fermentation stress is a key factor. An original engineering solution has recently been described for *Lactococcus lactis,* in which the acid stress could be significantly increased by the introduction of the *S. thermophilus* histidine decarboxylation system (Trip, Mulder, & Lolkema, 2012).

8.3 Systems biology and metabolic engineering in LAB

System biology emerged over the past decades to become a major research field, and includes a cycle of analyzing the biological system, predicting its behavior by modeling, and testing this prediction with experiments. This results in an improved model that again can be tested and improved. When studying an organism or a set of organisms as a whole at the system level, two approaches can broadly be distinguished: a top-down approach, in which the system is considered like a "black box" for which no information is available, and a bottom-up approach, in which the genomic information is used (dos Santos, Muller, & Vos, 2010). It goes without saying that the bottom-up approach is the most accurate and knowledge-driven of the two. As the genomes of industrial LAB are all known, this bottom-up approach is possible, and hence we will focus on it here. Moreover, the system biology cycle via the bottom-up approach benefits from the functional genomics and other omics technologies that are now being applied to all industrial LAB. For metabolic engineering, a key part is the description of the specific interactions that control the fluxes through a metabolic pathway. The application of the omics technologies may result in the development of high-quality metabolic models that constitute the backbone of omics data integration. Here, we will present the different models that have been developed for industrial LAB, and modeling tools that can be applied.

8.3.1 Kinetic models and genome-scale metabolic models

Kinetic models and genome-scale models represent the two major type of models developed for LAB physiology (Table 8.2). The first attempts focused on the glycolysis of *Lactococcus lactis* and aimed to characterize the specific shift in its fermentation profile. The pioneering studies of Hoefnagel et al. constitute a major step in glycolytic flux modeling (Hoefnagel, Van Der Burgt, Martens, Hugenholtz, & Snoep, 2002). In particular, this first model described the increase of acetolactate synthesis via metabolic engineering and appeared highly valuable due to later experimental validation (Hoefnagel, Starrenburg, et al., 2002). This high-quality model has served as a scaffold for further developments regarding the influence of regulation (Voit, Neves, & Santos, 2006) and the influence of extracellular pH on the glycolytic flux (Andersen et al., 2009). Moreover, the *Lactococcus lactis* kinetic model has demonstrated its use as well as its value in comparative studies of medically relevant LAB (Levering et al., 2012). Costa et al. extended the glycolysis model to integrate 2,3-butanediol and mannitol biosynthetic pathways. Coupled with in vivo NMR to parameterize and test the model in different conditions, the authors succeeded in identifying several strategies, some already experimentally tested, and new hypotheses that would enhance mannitol and 2,3-butanediol production by identifying targets such as phosphofructokinase and mannitol 1-phosphatase, respectively (Costa, Hartmann, Gaspar, Neves, & Vinga, 2014).

Kinetic models capture the dynamics of the specific pathways and, in particular, interactions at the enzyme-substrate-product level that are translated into intracellular fluxes and transport dynamics. Thus a series of pathway modifications can be implemented, ranging from enzyme inactivation or enzyme adjunction to modification of

Table 8.2 In silico models of lactic acid bacteria and their application to bacterial physiology

	Species	Description	References
Kinetic models	*Lactococcus lactis* IL1403	First model of glycolysis in LAB	Hoefnagel, Starrenburg, et al. (2002) and Hoefnagel, Van Der Burgt, et al. (2002)
	Lactococcus lactis IL1403	Glycolysis model for primary metabolism regulation	Voit et al. (2006)
	Lactococcus lactis IL1403	Glycolysis model for pH acidification through fermentation	Andersen et al. (2009)
	Lactococcus lactis MG1363 *Streptococcus pyogenes* M49	Two glycolysis models revealing role of extracellular phosphate	Levering et al. (2012)
	Lactococcus lactis MG1363	Glycolysis model with mannitol and 2,3-Butanediol pathway	Costa et al. (2014)
Genome-scale metabolic models	*Lactococcus lactis* IL1403	First LAB genome-scale model; metabolic engineering of central metabolism for diacetyl production	Oliveira et al. (2005)
	Lactococcus lactis IL1403	Model used to increase recombinant protein expression	Oddone, Mills, and Block. (2009)
	Lactococcus lactis MG1363	Model coupling carbon and nitrogen metabolism that is used to predict flavor formation	Flahaut et al. (2013)
	Lactobacillus plantarum WCFS1	Model of growth on complex media. Zero-growth experiment detailed with model use.	Goffin et al. (2010) and Teusink et al. (2006)
	Streptococcus thermophilus LMG18311	Model used to illustrate incomplete metabolic pathways	Pastink et al. (2009)
	Lactobacillus reuteri JCM1112	Model used to study effects of glucose–glycerol co-fermentation and its impact on amino acid metabolism	Santos et al. (2009)
	Lactobacillus reuteri ATCC 55730 *Lactobacillus reuteri* ATCC PTA 6475	Models used to identify mechanistic determinants behind probiotic function and GI-tract persistence	Saulnier et al. (2011)

Source: Adapted from Branco Dos Santos et al. (2013).

substrate concentration. Kinetic models are in fact detailed models incorporating specific pathways, and hence have the disadvantage that they lack accurate enzymatic parameters. However, stoichiometric models contain this information, and, thanks to the increase in omics data, such models have gained significant interest. A genome-scale metabolic network (GEMM) contains, in a mathematical form (stoichiometric matrix) the genomic information from an organism coupled with the predicted reactions from the proteome, and hence the metabolite pool. With the developments of genome annotation pipelines complemented by manual curation efforts, a series of precise and intensive networks have now been reconstructed for a large variety of organisms (Durot, Bourguignon, & Schachter, 2009; Feist, Herrgard, Thiele, Reed, & Palsson, 2009). Thanks to the stoichiometric matrix obtained, linear algebra tools can be used. Among them, Flux Balance Analysis (FBA) is one of the major algorithm used (Orth, Thiele, & Palsson, 2010). FBA determines the flux distribution within the solution space of a metabolic network according to a defined objective function, for example, maximization of growth rate (Price, Reed, & Palsson, 2004). In parallel, specific algorithms have been developed for metabolic engineering studies. A tool for automatic prediction of gene deletions aimed at a yield increase has been described (Alper, Jin, Moxley, & Stephanopoulos, 2005) and successfully used for increasing lycopene production by *Escherichia coli* (Alper et al., 2005; Alper, Miyaoku, & Stephanopoulos, 2005). Another modeling approach on targeted gene deletion has been the development of OptKnock (Anthony, Priti, & Costas, 2003). OptKnock combines search for best gene deletion with the production of a targeted compound and has been successfully applied on lactic acid production by *E. coli* (Fong et al., 2005). Recently, ReacKnock has been developed as a new algorithm for gene inactivation strategy where optimization is computed through a single-level programing (Xu, Zheng, Sun, & Ma, 2013). Large-scale identification of genetic design strategies (GLDS) is another successful algorithm used for metabolic engineering strategies (Lun et al., 2009), and has been developed to overcome the limitations resulting from studies in large metabolic networks. Finally, whereas OptKnock and GDLS look at pathway reduction, OptStrain is looking at reactions to be added to the network to produce novel compounds (Pharkya, Burgard, & Maranas, 2004). In the following we will present a few successful examples of metabolic engineering strategies in LAB with specific attention for the application potential of genomic models for food fermentation.

Lactococcus lactis strain IL1403 is one of the models for dairy lactococci and has been the first LAB to have its GEMM reconstructed (Oliveira, Nielsen, & Forster, 2005). The network consists of 358 genes, 621 reactions, and 509 metabolites. Thanks to the identification of the amino acid auxotrophies, a minimal growth medium has been designed that sustained in silico growth. Furthermore, the impact of the presence of oxygen on the growth of the *Lactococcus lactis* strain was determined. As the maximum growth was always lower in the presence of oxygen, the limitation in NADH oxidase activity was identified. Pyruvate formate lyase is known to be inhibited by oxygen, leading to a flux through the pyruvate dehydrogenase complex to acetyl-CoA synthesis. Consequently, the capacities in NADH recycling limited the predicted maximum growth rate. Finally, Oliveira et al. used a constraint on the PFL flux to be able to represent the proper fermentation profile with increased production of lactic acid

and increased growth rate. This model represents an excellent framework to implement future large omics data (Lahtvee et al., 2011).

Lactobacillus plantarum WCFS1 is a model organism for lactobacilli and LAB. Teusink et al. reconstructed its first network (712 genes, 650 reactions, and 546 metabolites) and, through constraint-based modeling techniques, detailed the discrepancies between observed and predicted fermentation profiles (Teusink et al., 2006). In system biology, a well-curated GEMM represents an important basis to further integrate omics data. Goffin et al. used a retentostat to characterize the *Lactobacillus plantarum* phenotype in nongrowing conditions (Goffin et al., 2010). Thanks to metabolomics and transcriptomics data, the *Lactobacillus plantarum* model was of crucial help in determining how this paradigm for LAB had been able to sustain an active physiological state while still metabolizing glucose and some amino acids. In particular, the amino acid production in relation to its metabolism was detailed; thanks to the interaction of the different amino acid pathways to regenerate α-ketoglutarate, a key component of the amino acid metabolism. Thus an intensive combination of omics technologies with a genome-scale metabolic model has been successful in describing how *Lactobacillus plantarum* may benefit from a new metabolic network and sustain an active metabolism without stress response.

Flavor compounds are key metabolites produced through dairy fermentations. To understand how flavor formation is related to host metabolism, we developed a new genome-scale model of *Lactococcus lactis* MG1363 (Flahaut et al., 2013). Both *Lactococcus lactis* MG1363 and IL1403 are important plasmid-free model strains that have been used for panoply of fundamental and applied studies (de Vos, 2011). However, they differ in their taxonomic position, as strain IL1403 belong to *Lactococcus lactis* subsp. *lactis,* whereas MG1363 is a *Lactococcus lactis* subsp. *cremoris* strain to which most industrially used dairy lactococci belong. We reconstructed the MG1363 metabolic network (518 genes, 754 reactions, and 650 metabolites) with a special focus on flavor-forming pathways (59 reactions involved) based on its genome and the information available from the literature (Flahaut et al., 2013; Liu, Nauta, Francke, & Siezen, 2008). Our model succeeded in illustrating the interplay between amino acid catabolism and growth of the organism. In particular, production of flavor compounds relates to NADH oxidation. Thus, even low fluxes such as those involved in amino acid catabolism and formation of flavor compounds may represent targets for future engineering by releasing pressure on the central metabolism (Flahaut et al., 2013).

For LAB engineering, GEMM constitute a viable approach, as it produces a framework with all known metabolic reactions. In *S. thermophilus*, GEMM was applied to reconstruct the metabolic network, and this highlighted the large set of amino acids that can be derived from only three amino acids that have to be supplemented to the growth medium (Pastink et al., 2009). Furthermore, this *S. thermophilus* model emphasized the peculiar influence of the redox balance on its physiology. Since ethanol biosynthesis is not functional because of its pseudogenes, *S. thermophilus* has to recycle all NADH from glycolysis, resulting in homolactic fermentation. Furthermore, the pentose phosphate pathway in *S. thermophilus* is incomplete (directly affecting NADPH generation); by taking into consideration all of these constraints, the model opened a

new hypothesis on the interactions between central carbon metabolism and amino acid metabolism via redox constraints (Pastink et al., 2009).

Finally, three strains of *Lactobacillus reuteri* have been used for GEMM reconstruction. *Lactobacillus reuteri* JCM112 had been used for medium optimization to enhance vitamin B12 biosynthesis by insertion of the biosynthetic pathway from *Lactobacillus plantarum* WCFS1 (Santos et al., 2008). Furthermore, two isolates derived from breast milk have been used for metabolic model reconstructions (Saulnier et al., 2011). *Lactobacillus reuteri* strain 55730 has been identified as an important probiotic strain, and hence efforts were made to identify its functional properties via the integration of omics data, in particular, the amino acid biosynthesis capacity and vitamin production were detailed thanks to the model and proposed as part of traits enhancing probiotic effects. Such study represents an important step toward a metagenome-scale model and its application in industrial fermentations (Branco Dos Santos, De Vos, & Teusink, 2013).

8.3.2 *Metabolic engineering strategies: increasing diacetyl production in* Lactococcus lactis

Diacetyl is the main flavor compound responsible for the butter taste of various fermented dairy products. For instance, increasing the diacetyl level during butter production is of high value, in particular with the NIZO process (Charteris, 2007). Diacetyl is formed by oxidative decarboxylation of α-acetolactate; the α-acetolactate is produced by the α-acetolactate synthase condensing two molecules of pyruvate (Figure 8.1). To improve diacetyl production, various strategies have focused on increasing α-acetolactate to allow further abiotic formation of diacetyl. The biovariant diacetylactis of *Lactococcus lactis* has the ability to metabolize citrate to produce diacetyl. Citrate-consuming LAB tends to produce high-amounts of diacetyl, as citrate can be converted to pyruvate by citrate lyase and oxaloacetate decarboxylase, without the need to use any redox cofactor (Hugenholtz et al., 2000), whereas pyruvate produced through the glycolysis is under strong redox pressure. Citrate is a minor component in milk; thus, metabolic engineering strategies have been focused on diacetyl and α-acetolactate production from glucose and lactose. Hugenholtz et al. engineered the first lactococcal strain able to produce high amounts from glucose (Hugenholtz et al., 2000). The authors reported an important success with NICE-controlled overexpression of the NADH oxidase gene (*nox*) in a α-acetolactate decarboxylase mutant (ΔACLDC). A two-step fermentation was initially used to grow the cells in unaerated cultures with induced *nox* gene overexpression at the mid-exponential phase; subsequently, the cells were harvested and resuspended in phosphate buffer in the presence of glucose. This strategy was needed, as the researchers observed inhibition of NOX activity by the presence of diacetyl in the medium.

Hoefnagel et al. used their model (see Section 8.3.1) to predict which genes needed to be targeted at the pyruvate branch (Hoefnagel, Starrenburg, et al., 2002). The authors used metabolic control analysis (Kacser & Burns, 1995) to estimate which enzyme influenced most of this metabolic pathway. They predicted that *ldh* gene inactivation coupled with *nox* gene overexpression would be the most successful approach to increase the flux toward α-acetolactate branch. The genome-scale model

of *Lactococcus lactis* IL1403 has also been used to design and to test strategies to enhance in silico diacetyl production (via α-acetolactate increase synthesis) to be able to engineer new strains producing high levels of diacetyl (Oliveira et al., 2005). First, literature results were reproduced with inhibition of LDH, alcohol dehydrogenase (ADH), and acetolactate decarboxylase (ACLDC). Then the researchers defined a succession of simulations to characterize which gene inactivation would lead to an increase in α-acetolactate synthesis using FBA and MOMA as described previously (Segre, Vitkup, & Church, 2002). Phosphotransacetylase, 3-phosphglycerate dehydrogenase, formyltetrahydrofolate synthetase, and glucose-6-phosphate 1-dehydrogenase were identified as the relevant targets to enhance α-acetolactate production under growing conditions. It was predicted that those inactivations would lead to an increased yield toward α-acetolactate formation, by increasing by-product availability and diminishing flux toward biomass formation (Oliveira et al., 2005). However, no experimental data have been provided, so further support for this strategy is lacking.

The *Lactococcus lactis* MG1363 GEMM model that we recently published represents a useful framework for further hypothesis testing (Flahaut et al., 2013). Our approach has been based on the influence of the redox balance via cofactor availability. As observed by Hugenholtz et al. (2000), overexpressing the *nox* gene in *Lactococcus lactis* ΔACLDC strain released the need to restore the NAD^+ pool. Thus, we looked at alternative ways to interact with the intracellular redox balance. It has been observed that *Lactococcus lactis* is able to have a functional electron transport chain (ETC) when heme is added to the medium (Brooijmans, Poolman, Schuurman-Wolters, De Vos, & Hugenholtz, 2007; Duwat et al., 2001). The observed phenotypes included increased production of biomass and an improved long-term stability (Duwat et al., 2001; Gaudu et al., 2002; Rezaiki et al., 2004). An active ETC oxidizes NADH generated through glycolysis that otherwise should have been recycled thanks to LDH (Lechardeur et al., 2011). Several in vivo and in silico studies investigated which strategy could be used to refine the *Lactococcus lactis* metabolic network. Hence, we illustrate here how our earlier described model (Flahaut et al., 2013) can be used to test new hypotheses in silico and engineer the best growth conditions to obtain high production of α-acetolactate. We decided to test three different conditions: (1) the *Lactococcus lactis* wild-type strain MG1363, growing under aerobic conditions; (2) *Lactococcus lactis* ΔACLDC overexpressing *nox* gene; and (3) *Lactococcus lactis* wild-type strain MG1363 grown in the presence of heme, i.e., with an active ETC. FBA is traditionally used to compute flux distribution and flux rate for the objective function. A strong limitation in FBA is due to the multi solution that can be computed; therefore we focused on Flux Variability Analysis (FVA), which estimates the flux span of all network reactions in relation with the objective function (Mahadevan & Schilling, 2003). FVA allows the investigation of alternative optima solutions and appeared to be highly accurate to test metabolic engineering strategies (Xu et al., 2013). FVA applied to *Lactococcus lactis* wild-type strain MG1363 grown in the presence of glucose with unlimited oxygen flux predicts (Table 8.3) that 7% of glucose is converted in α-acetolactate (0.47 $mmol_{acls}/g_{DW}$). However, FVA predicts that the *Lactococcus lactis* ΔACLDC mutant that overexpresses the *nox* gene converts a very high amount of glucose to α-acetolactate (83% with 5.8 $mmol_{acls}/g_{DW}$). Thus, this latter *Lactococcus lactis* mutant is predicted to be

Table 8.3 Flux variability analysis (FVA) of maximum growth rate (1/h), α-acetolactate production rate (mmol/g_{DW}/h), and resulting yield predicted from the genome-scale model of *Lactococcus lactis* MG1363

	Wild-type *Lactococcus lactis*	*Nox* over-expressing *Lactococcus lactis* ΔACLDC	Wild-type *Lactococcus lactis* grown with Heme
Predicted maximum growth rate (1/h)	0.55	0.58	0.58
FVA maximum production rate ($mmol_{acls}/g_{DW}$/h)	0.85	9.99	6.80
Y_{acls} ($mmol_{acls}/g_{DW}$)	0.47	5.8	3.9

Three in silico conditions have been compared with glucose as the carbon source (10 mmol/g_{DW}/h): Wild-type *Lactococcus lactis* grown in aerobic conditions; *Lactococcus lactis* with acetolactate decarboxylase inactivation and overexpressing *nox* gene; wild-type *Lactococcus lactis* grown in aerobic conditions in the presence of heme. FVA provides insight into the flux span for each reaction in the metabolic network and therefore provides knowledge on the stoichiometric rates that can be observed though a reaction (Mahadevan & Schilling, 2003). Calculations were performed with the COBRA toolbox in Matlab with α-acetolactate set on a demand reaction, i.e., objective function (Schellenberger et al., 2011).

able to produce 10 times more α-acetolactate than the wild-type strain. Remarkably, these flux rates are in the same range that observed by Hugenholtz et al. (2000), with a little excess that appears to be related to amino acid catabolism. Thus, the phenotype observed by Hugenholtz et al. (2000) is reproduced at the same magnitude by our model and therefore validates it. At last, the simulation of the influence of the active ETC in *Lactococcus lactis* resulted in a predicted maximum growth rate in the same range as the wild-type and its mutant. The predicted α-acetolactate production rate has been predicted to be more than eight times that of the wild-type strain and more than two-thirds of the mutant rate (56% α-acetolactate formed and 3.9 $mmol_{acls}/g_{DW}$). So, after the validation of the predicted mutant's phenotype by in vivo experimental data, the model predicts a significant positive effect of an active ETC on α-acetolactate production. Nonetheless, the latter results need to be experimentally confirmed, as it is solely based on the model prediction. Despite of these caveats, the model-based approach appears to be of high value, as it shows that a non-GMO strain can be optimized for increasing the production of a target compound simply by engineering growth conditions Moreover, it illustrates that in silico predictions help to improve designing in vivo experiments. In conclusion, the modeling approach described earlier can be considered as an excellent example of a bottom-up approach.

8.4 Conclusions

Recent decades have shown a series of successes in the metabolic engineering in industrial and other LAB that have led to the enhanced production of a great

diversity of metabolites. Nowadays, a large set of experimental toolboxes and modeling approaches are available that appear to be decisive in developing strains with the targeted phenotype. Genome-based modeling approaches have the advantage of being cost-effective and hypothesis generating. Moreover, some tests can be performed in silico before the experimental approach is taken. Together with high-throughput engineering and synthetic biology approaches, this will appear to be a "game changer" in metabolic engineering. However, one has to realize that each model has its limitations, although models are constantly improved to increase their accuracy in the systems biology cycle. Moreover, genetic engineering is a powerful tool but cannot easily target all possible options in a living organism. For instance, cofactor imbalance is a strong bottleneck when aiming at rerouting bacterial metabolism. This and other limitations can be partly solved by adaptive evolution, in which selective pressure such as growth rate on a specific substrate is used to optimize an organism. This has shown to be a useful tool to complement systems approaches, and elegant examples of *Lactococcus lactis* have recently been described (Bachmann et al., 2013).

8.5 Sources of further information and advice

Details on *E. coli* GEMM, the most advanced and most commonly used model by the scientific community, will be found in Mccloskey, Palsson, and Feist (2013). A well-described method to reconstruct genome-scale metabolic model has been proposed by Thiele and Palsson (2010). Furthermore GEMM benefit from the high versatility algorithm present in the COBRA Toolbox and which is maintained by the scientific community (Schellenberger et al., 2011). To get more details on LAB physiology, we advise the reader to consult the latest reviews (Branco Dos Santos et al., 2013; Costa et al., 2014; Gaspar, Carvalho, Vinga, Santos, & Neves, 2013; Teusink, Bachmann, & Molenaar, 2011; de Vos, 2011).

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Designing wine yeast for the future

9

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9.1 Introduction

The past three decades have seen a global wine glut. So far, well-intended but wasteful and expensive market intervention has failed to drag the wine industry out of a chronic annual oversupply of roughly 15%. Can yeast research succeed where these approaches have failed by providing a means of improving wine quality, thereby making wine more appealing to consumers? To molecular biologists the "wine yeast" *Saccharomyces cerevisiae* is as intriguing as it is tractable. A simple unicellular eukaryote, it is an ideal model organism, enabling scientists to shed new light on some of the biggest scientific challenges such as the biology of cancer and ageing. It is amenable to almost any modification that modern biology can throw at a cell, making it an ideal host for genetic manipulation, whether by the application of traditional breeding or modern genetic techniques. Without the "right" yeast flourishing in a suitable nutritional environment, grape juice would be transformed into something closer to vinegar than wine. Therefore, to the winemaker, *S. cerevisiae* yeast is integral to crafting well-balanced, flavoursome wines from simple, sugar-rich grape juice. Thus any improvements that we can make to wine yeast fermentation performance or the sensory properties it imparts to wine will benefit winemakers and consumers. With this in mind, the application of frontier technologies, particularly the burgeoning "omics"-based fields of systems and synthetic biology, have much to offer in their pursuit of novel yeast strains to produce high quality wine. This chapter discusses the nexus between yeast research and winemaking. It also addresses how winemakers and scientists face up to the challenges of consumer perceptions and opinions regarding the intervention of science and technology; the greater this intervention, the stronger the criticism that wine is no longer "natural". How can wine researchers respond to the growing number of wine commentators and consumers who feel that scientific endeavours favour wine quantity over quality, and technical sophistication, fermentation reliability and product consistency over artisanal variation? Can the wine industry afford to ignore the opportunities offered by modern science and technology, and allow the "paralysing hand of playing it safe" to strangle genuine innovation in winemaking? Will the wine industry wilt away or will it embrace new offerings from science, challenging the perceptions of wine commentators and consumers? Can we, as yeast researchers, deliver on the expectations that come with shifts in public perception? This chapter seeks

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00009-8

to present yeast research in a new light and a new context, and it raises important questions about the direction of yeast research, its contribution to science and the future of winemaking.

9.2 Accidental beginnings and ancient wisdom

The wine yeast, *S. cerevisiae*, is so closely associated with humans it is rarely found in environs removed from human habitation (Martini, 1993). In fact, its evolutionary success can probably be explained by its relationship with humans, particularly in the production of alcoholic beverages, an activity that has been with us for at least 7000 years (McGovern et al., 2004; Verstrepen, Chambers, & Pretorius, 2006). Because of us, *S. cerevisiae* enjoys phenomenal reproductive success with, for example, an estimated 600,000 tons of baker's yeast being produced every year (Pretorius, Toit, & Van Rensburg, 2003).

But how did this close relationship get started? It is likely that the first alcoholic fermentations were happy accidents: harvested grapes were not eaten quickly enough and began to rot, *Saccharomyces* yeasts moved in and took advantage of the free sugary meal and the first wines were made (Chambers & Pretorius, 2010; Pretorius, Curtin, & Chambers, 2012). These early wines presumably tasted good and had an interesting, pleasing, psychotropic effect. One can only assume that early farmers learned from this experience and repeated the accidents of previous "vintages". Winemaking was born and wine yeast had a secure future in the hands of its human guardians.

9.3 Turning hindsight into foresight

If we step back for a moment, to grasp how far and how fast we have come, the relationship between scientific discovery, technological advancement and progress in wine production reveals common themes dominating the past, present and future of wine (Borneman, Schmidt, & Pretorius, 2013; Chambers & Pretorius, 2010; Pretorius, 2000).

In his "Race for Space" speech in 1962, President John F. Kennedy famously condensed 50,000 years of recorded human history into a time span of half a century. If we apply the same approach to the history of wine, we encounter an outstanding record of research, development and sustained innovation (Figure 9.1). Breathtaking in speed, it is a story of resilience, persistence and the unwavering pursuit of opportunity.

First of all, we must acknowledge that we know very little about the *first 40 years* of this "time capsule". What we do know is that anatomically modern humans, clothed in leaves and furry animal skins, emerged from their caves to construct other kinds of shelter about *10 years ago*. These hunter-gatherers established agriculture and the first great civilisation in Mesopotamia around the Tigris-Euphrates River system.

Seven years ago, between what is now known as the Black and Caspian Seas, humans started to gather berries from wild vines in the forest. They used the seeds to cultivate vines in their villages to provide a convenient source of food. The accidental fermentation of a mixture of water and perishable grape berries in sunlight produced

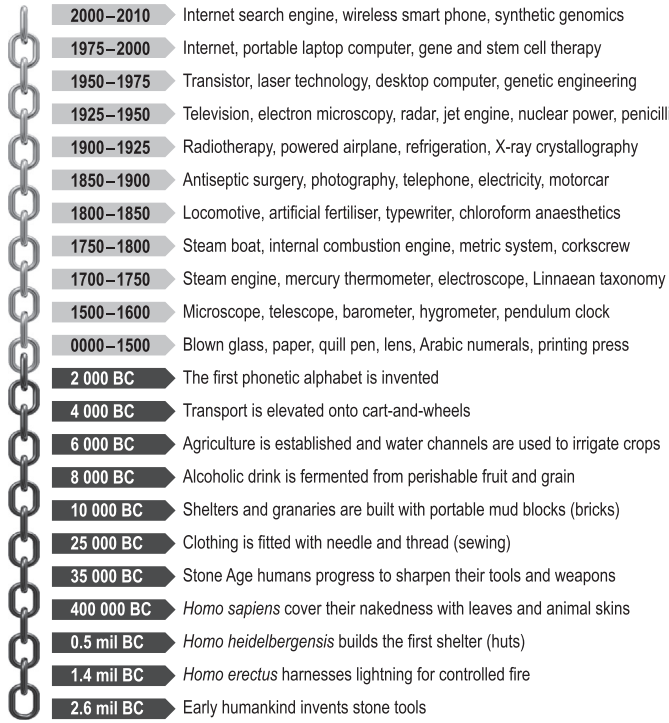

Figure 9.1 Selected milestones that mark the path of humankind's cultural and technological progress and the way we live today. The authors acknowledge that there is still fiery debate about the exact age of the earth and many aspects of the early parts of recorded human history. Therefore, the early steps of the timeline depicted in this diagram are not meant to express a definitive outcome of conflicting viewpoints; rather, they are included as part of the "Kennedy" metaphorical time capsule used in Section 9.3.

their first storable drink – a pleasurable drink preserved by alcohol. As we know from the discovery of Stone Age wine jugs, the intentional fermentation of grapes began soon after.

Just *five years ago*, humans learnt to write, to propel a boat with paddles and use a cart with wheels. One outcome was the spread of domesticated vines. Eventually those vines spread from the Near East to Europe and to as far afield as India and China (Figure 9.2).

When Christianity began just *two years ago* – on our condensed timeline – the connection between religion and wine was already strong. The first "miraculous sign" to be described in the scriptures involved turning water into "choice wine" for the guests at a wedding in Cana. But wine was not only embedded in religious ceremonies: it had become an integral part of many cultures, used as a source of nutrition and as a "social lubricant" for celebrations.

A year ago, Babylonian, Greek and Roman mathematicians, astronomers and philosophers, such as Pythagoras, Herodotus, Plato, Aristotle, Ptolemy, Copernicus and

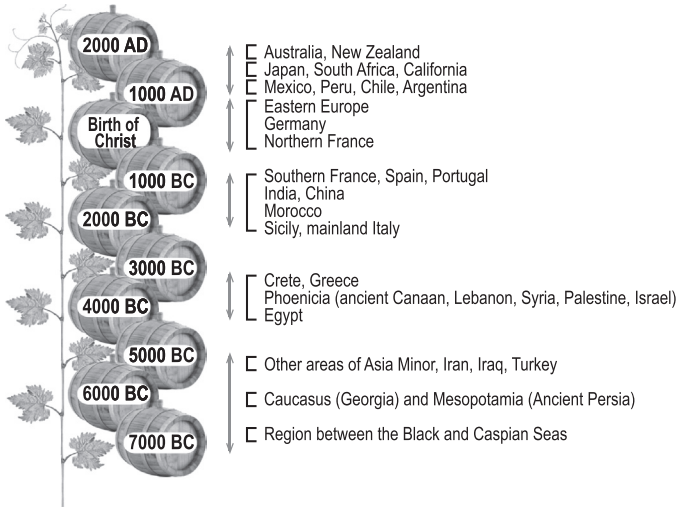

Figure 9.2 A generalised scheme of the spread of *Vitis vinifera* noble varieties of grapevine and winemaking from their centre of origin in Asia Minor to other parts of the world. Adapted from Chambers and Pretorius (2010).

Galileo, became convinced that the earth was a sphere within a larger sun-centred universe. They started to map the world.

Such maps enabled seafarers from Spain, Portugal, Holland and England to set sail for undiscovered lands. Thanks to the preservative power of alcohol, the only storable drink on the long and dangerous expeditions of Da Gama, Dias, Columbus, Van Riebeeck, Drake, Cook and others was wine – "sunlight, held together by water", as described by Galileo.

It comes, therefore, as no surprise that these explorers planted *Vitis vinifera* vines wherever they set foot ashore, marking the humble beginnings of wine production in the Americas, Japan, southern Africa and Australasia during the *second half of last year*.

This year, the Dutch merchant, Antonie van Leeuwenhoek, used a primitive light microscope to observe and describe the first yeast cells – the same cells that the French biochemist, Louis Pasteur, isolated as pure cultures *a few weeks later* (Figure 9.3). Pasteur also identified them as the living agents responsible for the conversion of grape sugar into wine alcohol and carbon dioxide. Fascinated by what he observed under the microscope and in the fermentation vessel, Pasteur stated that "a bottle of wine contains more philosophy than all the books in the world". Inspired by these discoveries, the German enzymologist, Eduard Buchner, was quick to find that this fermentation process was catalysed by enzymes contained in the fermenting yeast cells.

Meanwhile, the German oenologist, Hermann Müller-Thurgau, introduced the concept of inoculating wine ferments with specially selected pure yeast cultures from a species now known as *S. cerevisiae*. Müller-Thurgau and Robert Koch also demonstrated that bacteria – the malolactic bacterium now known as *Oenococcus oeni* – and not yeast, were responsible for the reduction of malic acid in wine. This process is

Figure 9.3 Selected milestones that mark the path of research in microbiology and yeast biology that impacted wine science and winemaking directly or indirectly. Adapted from Chambers and Pretorius (2010).

now known as malolactic fermentation, where malic acid (with its sharp taste of green apples) is converted into the softer-tasting lactic acid in wine.

Less than *six months ago*, the world's growing wine industry benefited immensely from the arrival of new forms of transport, such as steam-powered locomotives, bicycles, fuel-driven automobiles, tractors and airplanes. It also benefited from new forms of communication in the form of printing, telephones and telegraphs. Electricity and artificial light literally cast new light on scientific discoveries, and there were leaps forward in automation, introducing wineries to glass bottle-making machines, harvesters and cooling systems.

There was disease control with broad-spectrum fungicides and pesticides. There were new and stronger materials with trellising, stainless-steel pumps, pipes and

fermenters. There was new packaging with novel bottle-closures and casks. Science had come into its own.

Last month – on our timeline – Jim Watson and Francis Crick revealed the double-helix structure of DNA (Figure 9.4). This led to the unravelling of the universal genetic code of all living organisms.

Last week, shortly after we developed computers and landed the first man on the moon, we learnt how to clone DNA and engineer the genetic blueprint of any living cell. Only *yesterday*, a worldwide Internet – supported by search engines – allowed us to explore terabytes of information at the touch of a button on our newly developed laptop computers, wireless hand-held devices and smart phones. Thanks to these devices, irrigation of vineyards and the operation of large, complex wineries can now be controlled remotely by sophisticated computer networks, "artificial intelligence" and robotics.

This morning, we decoded the entire genetic blueprints of a few viruses, microbes, plants, animals and humans. We also decoded the genomes of the Pinot Noir grapevine variety, wine yeast strains and malolactic bacteria.

In the *past few minutes*, we achieved a breakthrough in reprogramming the natural "software" of life. For the first time, in a "hack-a-cadaver" approach, Craig Venter's

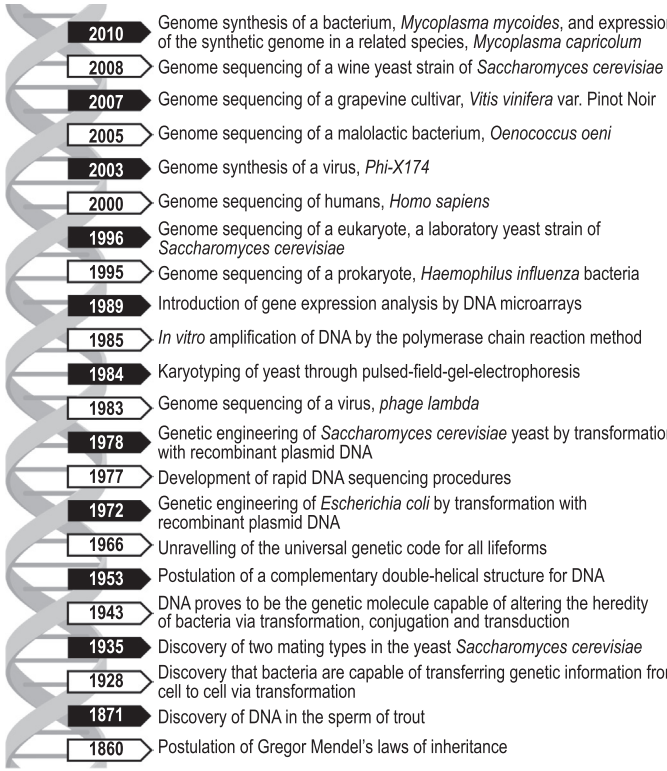

Figure 9.4 Selected milestones that mark the path of research in genetics and molecular biology that impacted wine science and winemaking directly or indirectly.
Adapted from Chambers and Pretorius (2010).

team successfully transplanted an artificially constructed genome of *Mycoplasma mycoides* – all 1.1 million base pairs of its DNA – into a closely related bacterial cell, *Mycoplasma capricolum*. This marked a world-first: a "synthetic" genome, created by computer, giving life to another living being with no ancestor. The potential for future advances in synthetic genomics seems endless.

Equipped with this new information, wine scientists are confident that by *this afternoon* we will be able to develop disease- and pest-resistant grapevine clones, low-alcohol and flavour-enhancing yeasts, and malolactic bacteria imparting fruity aromas to consumer-enticing wines.

And before *midnight tonight*, researchers believe that, backed by human genomic data, wine market developers will have deeper insights into taste preferences of specific consumer groups. This will allow scientists to design tailor-made yeast strains for specific wine styles, enabling winemakers to better align the chemical composition of their wines with the taste preferences of their target markets, anywhere in the world.

Now, back to real time…

9.4 The ancient art of winemaking meets frontier yeast science

A look at the evolution of wine production over the past seven millennia (Borneman, Schmidt, et al., 2013) and the global industry's value chain (Figure 9.5) makes it obvious that the scientific basis of the various steps of the winemaking process has

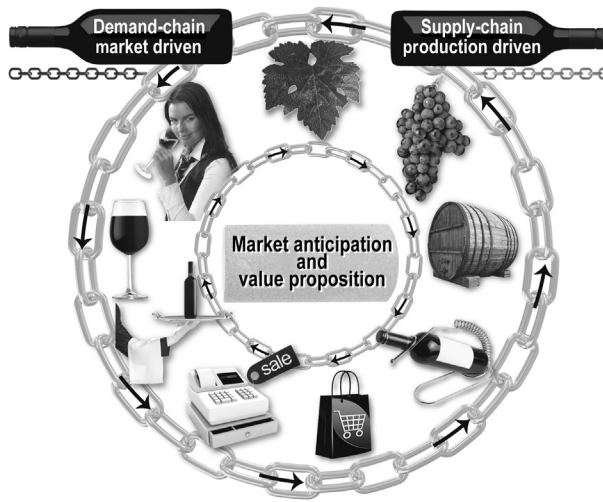

Figure 9.5 A schematic outline of the wine industry's value chain. In a globalised economy in which quality is defined as sustainable customer and consumer satisfaction, it is important to most producers to anticipate market trends and changing consumer preferences. A paradigm shift from a supply-chain approach to a demand-driven one has occurred over the past decade or so.

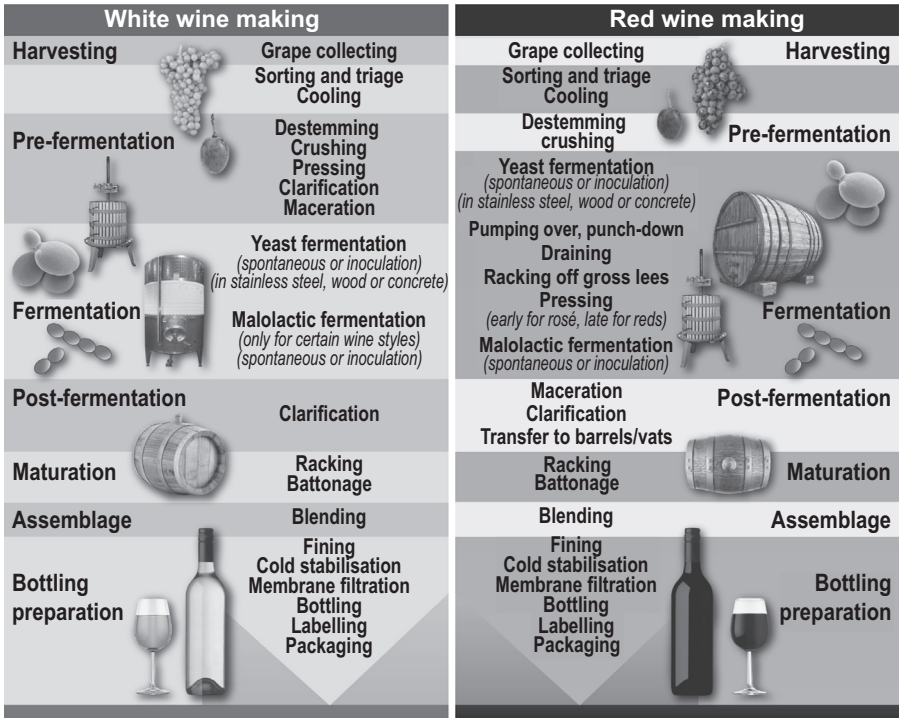

Figure 9.6 A schematic outline of the main steps in wine production. Some steps and the sequence thereof differ between the production of red and white wine.
Adapted from Pretorius (2000).

gradually become clearer, and many practices once thought impossible have now become routine (Figure 9.6).

It has probably been known since the earliest of times that wine is susceptible to spoilage. Winemaking is not a matter of chance or magic – left entirely to nature the result is variable, unreliable and can be undrinkable. Indeed, the completely natural result of fermenting grapes is vinegar (Lewin, 2010). The discovery that microbes are largely responsible for this has led, in more recent times, to a debate on whether wine should be "natural" or should we protect it from undesirable microorganisms by pasteurisation or the addition of sulfur dioxide. And this debate has broadened in the past decade to include questions on whether wine should be the fermentation product of its natural microbiota or of a controlled inoculated wine yeast (Pretorius et al., 2012).

Some winemakers and commentators believe that the ambient yeast population in the vineyard and winery constitute part of the characteristics of a natural wine (Lewin, 2010). They believe that the unique contributions of diverse yeast species – including non-*Saccharomyces* species – confer a complexity upon wine not seen in inoculated ferments. This might be true (Varela et al., 2009), but it comes with the risk of spoilage. There is also an increased risk that the fermentation will become "stuck" (Martini, 1993) – i.e. the ferment will stop and be difficult to restart. In addition,

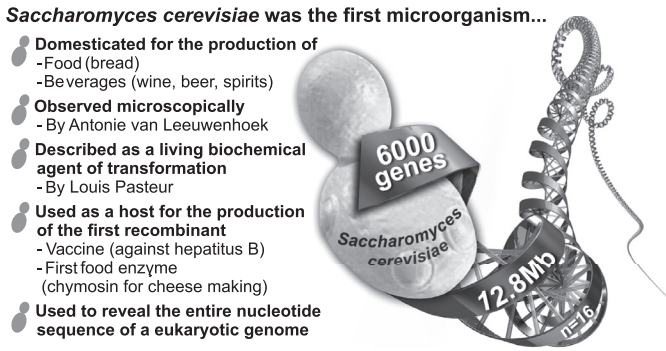

Figure 9.7 Since the beginning of recorded civilisation, *S. cerevisiae* has been instrumental in planting important milestones on the road of the development of modern humankind. In many instances, this versatile trailblazer was the first microbe with which breakthroughs were achieved.

spontaneous – "natural", "wild" or "feral" – ferments also tend to take longer to complete. Nevertheless, in recent times, some of these non-*Saccharomyces* yeasts have come into commercial use in winemaking. For example, *Torulaspora delbrueckii*, which was initially included in a commercially packaged mixture of yeast strains (Vinoflora® Melody.nsac and Vinoflora® Harmony.nsac) with *S. cerevisiae* and *Kluyveromyces thermotolerans* in 2003, and later on as a stand-alone seed culture by commercial yeast manufacturers such as Chr. Hansen, Lallemand and Laffort (Swiegers, Saerens, & Pretorius, 2014, chap. 1).

The debate has intensified and continues to set the backdrop for wine yeast research, but over the past two decades, active yeast strain development programs have been launched the world over to generate *S. cerevisiae* strains that can improve wine quality when used as inocula at the start of fermentation. This is a very fertile research field where advances in wine yeast strain development and fundamental yeast science have leveraged from one another.

This new era in wine yeast research, embracing cross-disciplinary expertise, is worthy of review. It started following the revelation that genes are made of DNA; the stage was set for an explosive growth of knowledge, driven by a convergence of genetics, biochemistry, cell biology, microbiology and computing. And work on yeast was often at the forefront of developments.

There were compelling reasons for molecular biologists from all fields to look on this simple single-celled fungus as the ideal guinea pig for fundamental and applied research (Figure 9.7). Our close relationship with *S. cerevisiae* in food and beverage production over millennia tells us that it is safe to work with; for example, it is designated Generally Recognised as Safe (GRAS) by the Food and Drug Administration in the USA (Verstrepen et al., 2006). In addition, it is a simple eukaryotic cell (Figure 9.8), which is inexpensive and easy to grow, and can be stored for long periods in suspended animation. But perhaps its best asset is an accessible genetic system that can be followed through asexual and sexual cycles (Figure 9.9). The three basic cell types – a, α and a/α cells – can undergo mitosis and reproduce through an asexual budding process

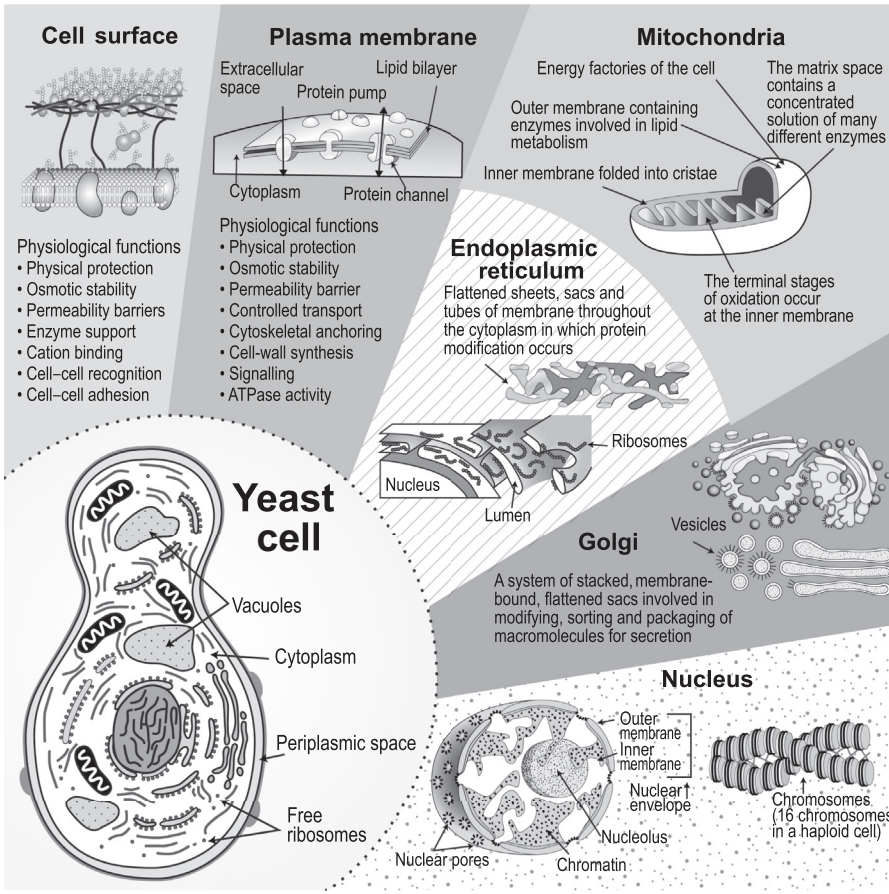

Figure 9.8 A schematic representation of the subcellular compartmentalisation of a wine yeast cell. The cell envelope, comprising a cell wall, periplasm and plasma membrane, surrounds and encases the yeast cytoplasm. The structural organisation of the intracellular milieu, containing organelles such as the nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria and vacuoles, is maintained by a cytoskeleton. Several of these organelles derive from an extended intramembranous system and are not completely independent of each other. Adapted from Pretorius (2000).

(Figure 9.10). The a and α haploid cells are also able to undergo mating, a sexual process that culminates in nuclear fusion and creation of a/α diploid cells, which can be induced to undergo meiosis to produce asci carrying four haploid spores.

Since the mid-1970s, when recombinant DNA technologies revolutionised the way research in biological sciences is conducted, *S. cerevisiae* has been one of the most important model organisms in molecular biology and emerging omics-based fields (Figure 9.11). For example, a haploid laboratory strain (S288c) was the first eukaryote to have its genome sequenced, a feat achieved through a collaborative international effort involving more than 600 scientists under the able leadership of

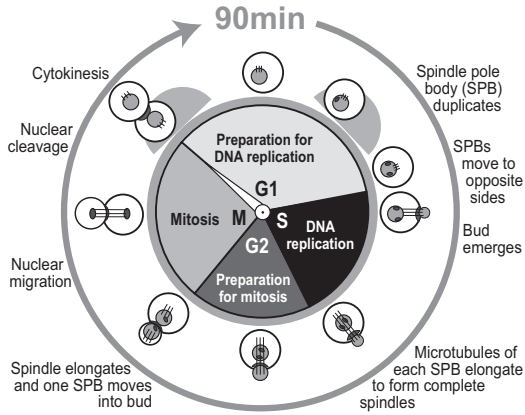

Figure 9.9 A schematic representation of the cell cycle of a budding wine yeast cell. Haploid and diploid cells reproduce asexually by multilateral budding during which each cell gives rise to a daughter cell made of entirely new cell surface material. Buds may arise at any point on the mother cell surface, but never again at the same site. Under optimal nutritional and cultural conditions *Saccharomyces cerevisiae* doubles its mass every 90 min. The cell division cycle consists of four phases: G1, S, G2 and M.
Adapted from Pretorius (2000).

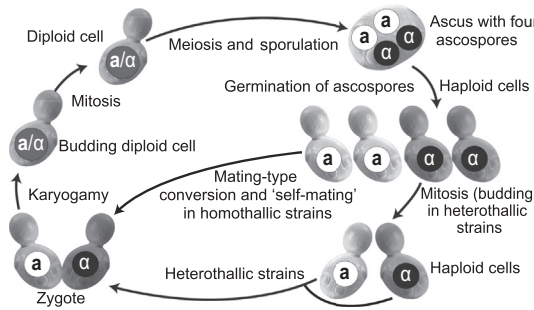

Figure 9.10 A schematic representation of the life-cycle of heterothallic and homothallic wine yeast strains. Haploid cells of *Saccharomyces cerevisiae* exist in two mating types, designated *MATa* and *MATα*. Cells of the *MATα* mating type produce a peptide of 13 amino acids, the *α* factor; while the a mating type cells produce a peptide of 12 amino acids, the a factor. When in close proximity, the *α* factor arrests the growth of *MATa* cells, permitting the cells to mate. The mating process results in cell and nuclear fusion. The *MATa/MATα* diploid cell formed by mating can neither produce nor respond to mating pheromones and will under satisfactory nutritional and cultural conditions grow and divide, maintaining the diploid state. Upon nutritional starvation, the *MATa/MATα* diploid cell undergoes meiosis, generating four haploid ascospores (two *MATa* and two *MATα* ascospores) encapsulated within a sac, the ascus. When released from the ascus, the ascospores germinate to commence new rounds of haploid existence. Strains that can be maintained stably for many generations as haploids are termed heterothallic. Strains in which sex reversals, cell fusion and diploid formation occur are termed homothallic.
Adapted from Pretorius (2000).

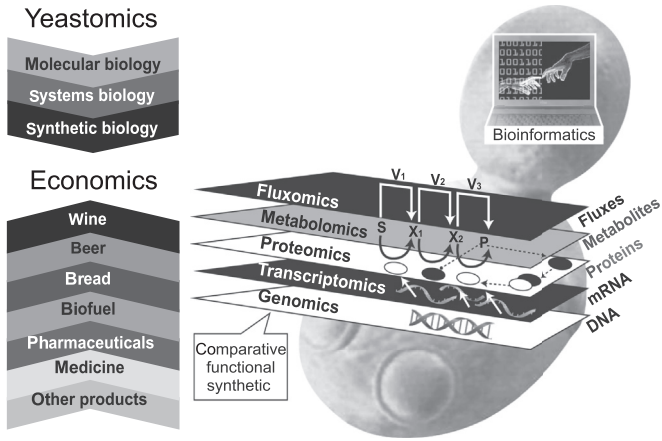

Figure 9.11 A diagrammatic representation of the main bioplatforms underpinning genomic, transcriptomic, proteomic and metabolomic datasets that drive the "yeastomics-to-economics" research approach. Bioinformatics integrates these omics platforms by converting massive amounts of "big data" into meaningful findings. In yeast strain development programmes, classical single-gene or reductionist strategies are being replaced by these whole-cell methodologies. These pan-cell approaches form the basis of systems and synthetic biology.

André Goffeau and Stephen Oliver (Goffeau et al., 1996; Oliver, 1996). This paved the way for the first chip-based gene array experiments (Schena, Shalon, Davis, & Brown, 1995).

Saccharomyces cerevisiae was also the first organism to be used to build a systematic collection of bar-coded gene deletion mutants enabling high-throughput functional genomics experiments (Winzeler et al., 1999). But the most important resource available to the yeast scientific community is the *Saccharomyces Genome Database* (SGD; www.yeastgenome.org), which provides, free of charge, access or links to the most comprehensive datasets (e.g. genomic, transcriptomic, proteomic and metabolomic data) available to a molecular biologist. All of this has been achieved by international collaborations on a grand scale.

What does all of this mean for wine research? The above international efforts have put the *winemaker's bug* centre stage in thousands of laboratories worldwide. And our knowledge is no longer limited to the S288c laboratory version of *S. cerevisiae* whose genome sequence was announced in 1996. It took another decade before genome sequences for additional strains of *S. cerevisiae* became publically available. The first wine yeast genome sequence (AWRI1631) was published in 2008 (Borneman, Forgan, Pretorius, & Chambers, 2008), and genomic data for >80 strains of *S. cerevisiae* are now available (see Borneman, Pretorius, & Chambers, 2013; Borneman, Schmidt, et al., 2013, and references therein). In total, the genome sequences of six commercial wine yeasts (AWRI796, EC1118, QA23, VIN7, VIN13 and VL3) and a dozen or so strains that were isolated from wine, grapes or vineyards have now been analysed. Five of the wine strains (AWRI796, QA23, VIN7, VIN13 and VL3) were sequenced in their industrially used forms (Borneman et al., 2012).

Genomic data for two strains, one derived from a vineyard isolate (RM11-1a) and the other a clinical isolate (YJM789), provided the first indication of *S. cerevisiae* inter-strain variation, with the analysis of the genome sequence of YJM789 uncovering >60,000 single nucleotide polymorphisms (SNPs) and several novel, strain-specific genes compared to the original reference genome sequence of the S288c laboratory strain (Wei et al., 2007). Subsequent analyses of the genome sequence data of *S. cerevisiae* wine yeasts have shown that wine strains form a tight geographic clade alongside environmental yeast isolates (Borneman et al., 2011; Borneman, Pretorius, et al., 2013; Borneman, Schmidt, et al., 2013; Dunn, Richter, Kvitek, Pugh, & Sherlock, 2012; Liti et al., 2009). In general, based on SNP variation, most commercial wine starter strains form a phylogenetically related group that is broadly related to strains from European environments (Figure 9.12). This is interesting because some isolates originated outside of Europe (e.g. RM11-1a isolated from an American vineyard and DBVPG1106 isolated from Australian grapes). The exportation of European wine yeast to other wine-producing regions of the world, either on grapes or through the use of commercial starter cultures at nearby wineries, might have led to interbreeding between these commercial strains and native strains (Borneman, Schmidt, et al., 2013). It is worth noting that, despite their common geographic origins and fermentation roles in alcoholic beverage production, wine strains are genetically distinct from the two ale strains, Foster's O and Foster's B (Borneman et al., 2011).

Comparing the genomic data derived from the first wine yeast genome sequence (AWRI1631), a laboratory strain (S288c) and a clinical isolate (YJM789), we uncovered a 0.6% difference in nucleotide sequence but, perhaps more importantly, there was 100 kb additional genome sequence – enough to carry at least 27 genes (Borneman et al., 2008). Open reading frames (ORFs) in the additional sequences do not resemble anything found in other species of *Saccharomyces*, but appear to be similar to genes found in distant fungal relatives. Blast searches indicated that some of the wine yeast-specific genes have similarities to genes encoding cell-wall proteins, perhaps contributing to the greater robustness of wine yeast compared to laboratory strains. Others may encode proteins associated with amino acid uptake, which is significant in the context of wine sensory attributes; amino acid metabolism is central to the production of many sensorially important volatile aroma compounds (Borneman et al., 2012).

When we compared the genome sequences of the commercial wine strains with those of the FosterO and FostersB ale strains, the RM11-1a vineyard isolate, the YJM789 clinical isolate and JAY291 biofuel strain, we found that the industrial yeasts displayed significant genotypic heterogeneity both between strains, but also between alleles present within strains (i.e. heterozygosity). This variation manifested as single-nucleotide polymorphisms (SNPs), small insertions and deletions, and as novel, strain and allele-specific ORFs. None had been found previously in the *S. cerevisiae* genome and may provide the basis for novel phenotypic characteristics.

More detailed analyses of the genomic datasets that became available recently confirmed the existence of strain-specific ORFs; heterozygosity (single nucleotide variants ranging from ~1000 to ~1900 heterozygous loci per strain); and large-scale structural variations (e.g. whole-chromosome aneuploidies and interspecific hybrids).

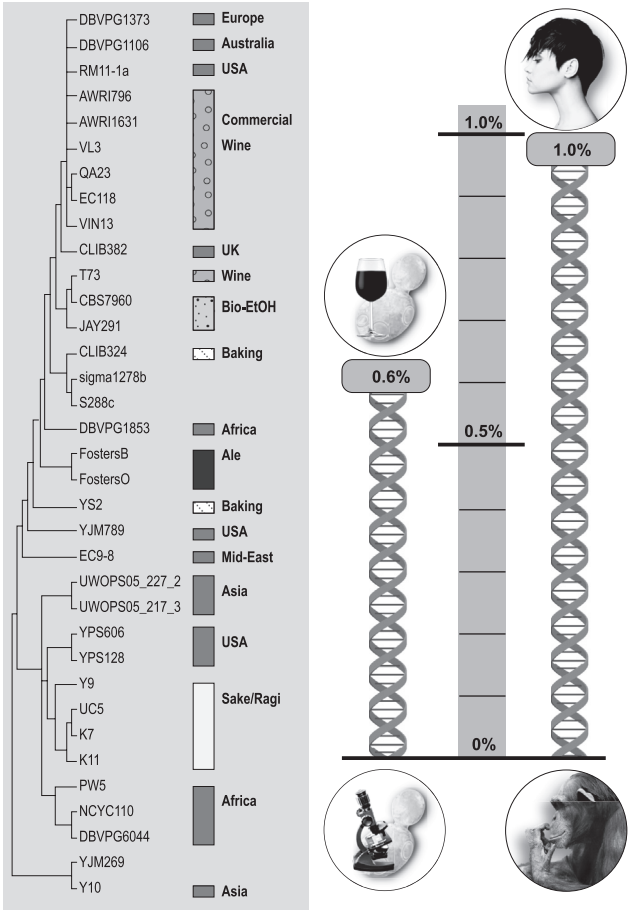

Figure 9.12 A cladogram depicting the genomic relationship among *Saccharomyces cerevisiae* strains originating from different geographical and industrial environments. Interestingly, there is a 0.6% difference in the nucleotide sequence of the S288c laboratory strain and that of the wine strain (AWRI1631) whose genome was first sequenced in 2008. By comparison, the difference between the nucleotide sequences of a human and a chimpanzee is 1%.
Adapted from Borneman, Pretorious, et al. (2013).

For example, the genome sequence of the thiol-releasing wine yeast, VIN7, revealed an allotriploid hybrid genome with *S. cerevisiae* and *Saccharomyces kudriavzevii* origins (Borneman et al., 2012). Another finding was that the genomes of three closely related wine strains – EC1118, QA23 and VL3 – contain at least two telomeric insertions (~65 kb on the right arm of chromosome XV and ~40 kb on the left arm of chromosome VI) relative to the S288c laboratory reference strain (Borneman et al., 2011; Novo et al., 2009). The insertion on chromosome XV contains at least three genes that might be of significance to these strains' oenological characteristics. The first one is a homolog of the *Saccharomyces pastorianus FSY1* gene, which encodes

a high-affinity fructose/H^+ symporter (Galeote et al., 2010). The presence of *FSY1* in strains facilitates active transport of fructose – a sugar that represents ~50% of the monosaccharide content of grape must – and provides a fermentative advantage over strains lacking this gene and which are limited to facilitated diffusion for the uptake of fructose (Galeote et al., 2010). The other two genes from this region on chromosome XV comprise a pair of tandemly duplicated oligopeptide transporters, which could potentially enable strains to utilise a broader range of small peptides as a nitrogen source (Damon et al., 2011; Novo et al., 2009). This could potentially enhance the fermentation performance of strains in nitrogen-deficient grape musts.

Analysis of the genome sequence of EC1118 revealed a cluster of five genes believed to have been horizontally transferred between *S. cerevisiae* and another yeast species, *Zygosaccharomyces* (Novo et al., 2009), possibly via the formation of a circular intermediate through an undetermined process that appears to be independent of classical recombinatorial or transposon-based duplication and insertion (Borneman et al., 2011; Galeote et al., 2011). This group of ORFs are present in several commercial wine strains but their location, copy-number and exact order of the five genes within the cluster is both strain-dependent and insertion-site specific. At this stage, it is unclear whether this cluster of five genes is important for wine-specific characteristics.

Overall, this work suggests that, despite the scrutiny that has been directed at the yeast genome, there remains a significant reservoir of ORFs and novel modes of genetic transmission that may have significant phenotypic impact in this important model and industrial species. As more and more genomic datasets are becoming publicly available, our ability to conduct finer and more definitive comparative genomic analyses will grow, which in turn, will help us better understand what makes wine yeasts "tick" and why there is such variation in *S. cerevisiae* "winemaking phenotypes" (Borneman et al., 2012). The availability of the means to swiftly characterise the scope of genetic diversity and to precisely define the genetic sources of specific yeast metabolites (Figure 9.13) and other phenotypes will facilitate the systematic assembly of multiple traits into tailored wine strains of *S. cerevisiae* (Borneman, Schmidt, et al., 2013).

9.5 Engineering yeast to make better wine

Wine research and wine yeast strain development are certainly well placed to benefit from the privileged place that *S. cerevisiae* occupies in the life sciences. Figure 9.14 summarises several targets of strain development programmes. These targets are normally, but not exclusively, focussed on the natural metabolites that various strains of *S. cerevisiae* produce under different fermentation conditions (Figure 9.15). The following includes some examples that demonstrate this.

9.5.1 Getting control of alcohol levels in wine

Without question the greatest challenge faced by the wine industry is rapidly mounting concerns over alcohol consumption; excess consumption creates problems for society and human health. In addition, too much alcohol in wine can overwhelm flavour and

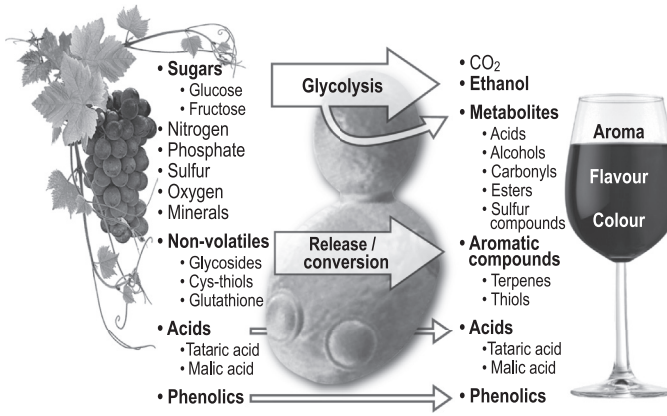

Figure 9.13 To the winemaker, yeast is integral to crafting wonderful, complex wines from simple, sugar-rich grape juice. Grape juice is converted into wine by the action of wine yeast. Some wine components are wholly generated by yeast as part of metabolism whilst others are essentially as created by the grapevine. The large number of compounds synthesised or modified by wine yeast have a major impact on wine quality and style. Commercial yeast strains possess different abilities to form and modulate compounds that impact on wine sensory properties. These compounds are produced as a result of yeast metabolic processes.

make the wine "hot" on the palate. The technical challenges associated with reducing the alcohol content of wine, however, are substantial (Kutyna, Varela, Henschke, Chambers, & Stanley, 2010).

Several genetic modification (GM)-based metabolic engineering strategies have been explored to generate wine yeasts that partially divert carbon metabolism away from ethanol production, with the aim of decreasing ethanol yields during vinification. Two glycerol-3-phosphate dehydrogenase isozymes, *GPD1* and *GPD2*, which divert carbon from glycolysis to glycerol production, have proven to be the best candidates to date (Figure 9.16).

Enhanced expression of either *GPD* paralog achieved the desired outcome with regard to ethanol yields (De Barros Lopes et al., 2000; Michnick, Roustan, Remize, Barre, & Dequin, 1997; Remize, Roustan, Sablayrolles, Barre, & Dequin, 1999); however, increased glycerol production was accompanied by undesirable increased concentrations of acetic acid. This was probably due to a perturbation in redox balance in the engineered strain, requiring the action of one or more of the five aldehyde dehydrogenase (Ald) isozymes; these enzymes help maintain redox balance by reducing coenzymes NAD^+ or $NADP^+$, when they oxidise acetaldehyde to acetic acid. The problem, however, was alleviated quite simply by knocking out *ALD6* (Cambon, Monteil, Remize, Camarasa, & Dequin, 2006; Eglinton et al., 2002).

Similar approaches (Kutyna et al., 2010) have targeted *S. cerevisiae* pyruvate decarboxylase isozymes, alcohol dehydrogenase isozymes and glycerol transporters, mostly leading to increased glycerol yields and accompanying reduced ethanol production. Alternative approaches have included expression of the *Aspergillus niger*

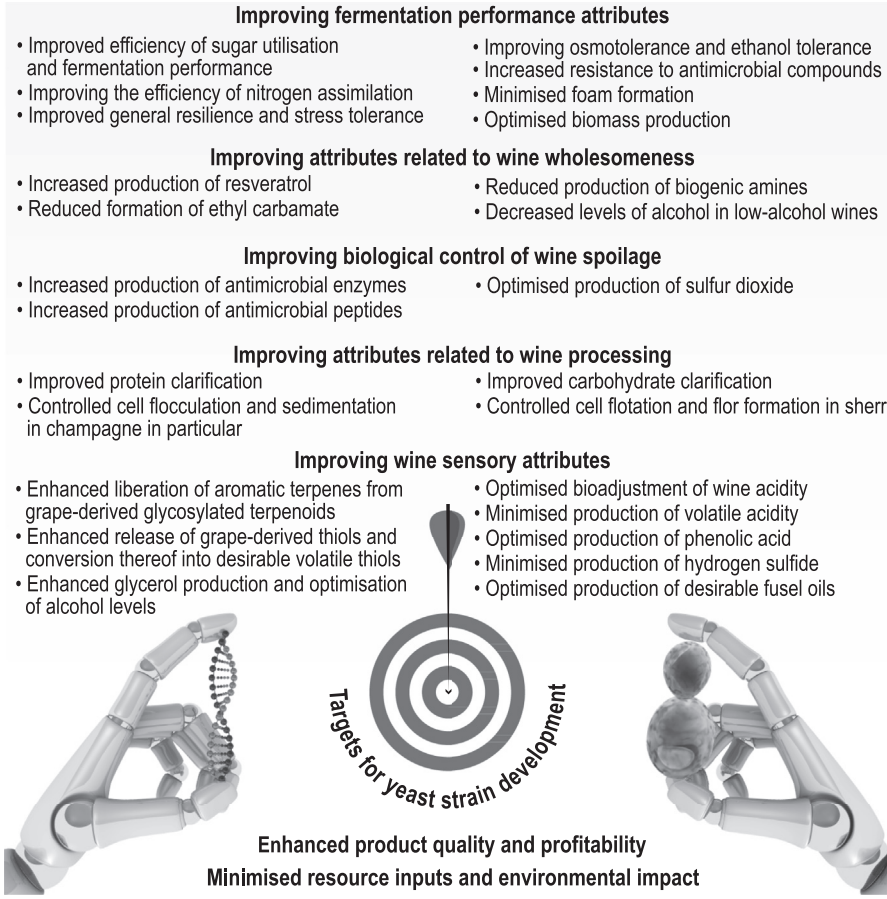

Figure 9.14 Potential targets for wine yeast strain development. The primary role of wine yeast is to conduct the alcoholic fermentation during which grape sugars (mainly glucose and fructose) are converted into ethanol, carbon dioxide and other minor, but important, metabolites. The main emphasis in strain development programmes is on the development of *Saccharomyces cerevisiae* strains with improved fermentation, processing and biopreservation abilities, and the capacity to enhance the wholesomeness and sensory quality of wine. It is hoped that such genetically improved yeast strains could enhance cost-effective production of wine with minimised resource inputs, improved quality and low environmental impact. Adapted from Pretorius (2000).

glucose oxidase encoding gene (*GOX*) in *S. cerevisiae*, which redirects glucose to gluconic acid, and extensive modification of *S. cerevisiae* hexose transporters, which forces the yeast to respire rather than ferment, regardless of the concentration of glucose and fructose it encounters. Arising from these research efforts are several promising candidate "low-ethanol" wine yeast strains awaiting widespread acceptance of their use in commercial winemaking. These strains could immediately enable production of wines that contain 12% alcohol instead of 15%, from optimally ripened grapes.

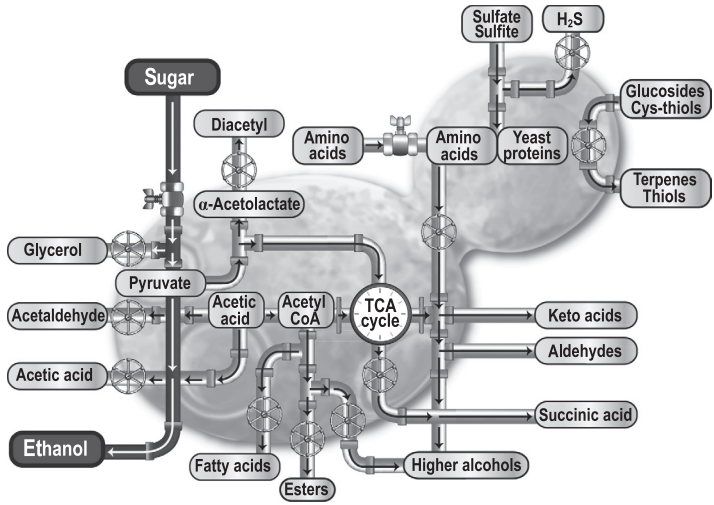

Figure 9.15 Commercial yeast strains possess different abilities to form and modulate compounds that affect wine sensory properties. These compounds are produced as a result of yeast metabolic processes.
Adapted from Pretorius et al. (2012).

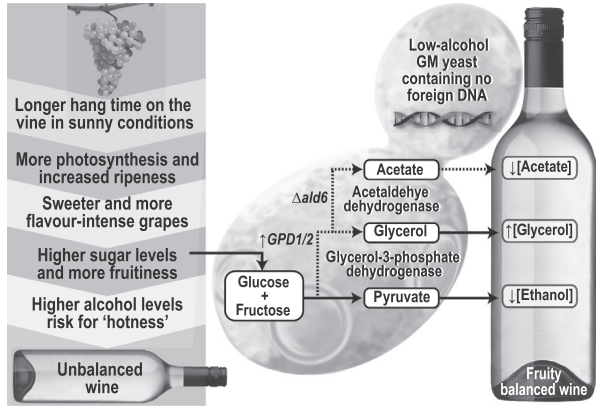

Figure 9.16 Reducing alcohol levels in wine: several GM-based strategies have been explored to generate wine yeasts that partially divert sugar metabolism away from ethanol production. Two glycerol-3-phosphate dehydrogenase isozymes, *GPD1* and *GPD2*, can be harnessed to divert carbon from glycolysis to glycerol production. However, increased glycerol production was accompanied by undesirable increased concentrations of acetic acid. This problem was alleviated by knocking out *ALD6*. Wild-type yeast convert most of the sugar they consume into ethanol and CO_2.
Adapted from Pretorius et al. (2012).

In a recent comprehensive study, we have generated and evaluated a large number of gene modifications that were predicted, or known, to impact on ethanol formation (Varela et al., 2012). Using the same yeast genetic background (AWRI1631), 41 modifications were assessed. These genetic modifications were chromosomally integrated

and included (1) gene deletions, where the ORF of the target gene was deleted; (2) promoter replacement, where the native promoter of the target gene was replaced with a strong constitutive yeast promoter; (3) gene cassette insertion, where a gene under the control of a strong constitutive yeast promoter was inserted in the chromosome and (4) discrete modifications, including nucleotide substitutions and deletions. From all modifications and strategies aimed at decreasing ethanol production evaluated in this study, those intended to increase glycerol formation were the most effective. For example, two of these modified strains, AWRI2531 and AWRI2532, carrying multiple, integrated overexpression cassettes of *GPD1,* were able to decrease the concentration of ethanol from 15.6% (v/v) to 13.2% (v/v) and from 15.6% (v/v) to 12% (v/v), respectively, in both Chardonnay and Cabernet Sauvignon juices (Varela et al., 2012). However, these strains still produced unacceptable levels of acetaldehyde and acetoin, indicating that further work remains to be done before a low-alcohol wine yeast might become commercially available.

9.5.2 Enhancing varietal wine flavour during fermentation

To casual wine drinkers it may seem fanciful, even pretentious, when a wine enthusiast states that Shiraz offers impressions of "black pepper"; Pinot Noir displays overtones of "earthy strawberries"; or that Sauvignon Blanc is characterised by traces of "asparagus" and "passionfruit" (Lewin, 2010). But these descriptors of wine flavours may become clearer to casual wine drinkers if they are informed that, for example, strawberry flavour is considered to be relatively complex among fruits, and contributed to by a large number of aroma compounds (Zabetakis & Holden, 1997); and that wine flavour is really the sum of complex interactions between more than a 1000 volatile compounds, many of which overlap with those found in strawberry, and some impact compounds that are, in fact, found in black pepper (e.g. rotundone; Wood et al., 2008) and passionfruit (e.g. polyfunctional thiols; Engel & Tressl, 1991). Therefore, perceiving the aromas of these aforementioned fruits, vegetables and spices in wine is not surprising. The relative amounts of each compound, and the resultant flavour profile, ultimately define differences among the vast array of wines and wine styles produced throughout the world (Cordente, Curtin, Varela, & Pretorius, 2012; Swiegers, Bartowsky, Henschke, & Pretorius, 2005).

Grape variety is the starting point for differentiation – many volatile compounds provide varietal distinction in addition to giving wine its basic structure. The concentrations of many volatile compounds are, however, dependent upon an almost infinite number of variations in production, whether in the vineyard or the winery. It is known, for example, that commercial yeast strains possess different abilities to form and modulate volatile compounds during alcoholic fermentation (Figure 9.15) that significantly affect the flavour and overall quality of wines (Swiegers et al., 2009). Therefore, while the proportion of wine volatiles modulated by yeast may be relatively low (Robinson, Boss, Heymann, Solomon, & Trengove, 2011), the choice of yeast strain controlling fermentation is an effective method for shaping wine aroma according to the preferences of consumers in target markets (King, Osidacz, Curtin, Bastian, & Francis, 2011).

A case in point is the incidence of powerful synergies between Sauvignon Blanc grapes and yeast strains in formation of the compounds responsible for "box-hedge" and

"tropical fruit" flavours – the polyfunctional thiols 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA). Odourless cysteine and glutathione conjugates of 3MH and 4MMP form in the grape berry, and during crushing, and can be found at higher concentrations in Sauvignon Blanc juice in comparison to other white varieties (Peña-Gallego, Hernández-Orte, Cacho, & Ferreira, 2012). Due to the potency of the free thiols, with perception thresholds in the ng/l range, only a fraction of the available conjugated precursors need to be released to impart strong "passionfruit", "grapefruit", "gooseberry" and "guava" flavours to wine. Yeast carbon-sulfur-lyase enzymes are responsible for the release of 4MMP and 3MH from their cysteine conjugates, while 3MHA is produced by yeast metabolism through the esterification of 3MH during fermentation (Figure 9.17).

By mining the *S. cerevisiae* genome for putative carbon-sulfur-lyase encoding genes, we identified four candidates (*BNA3*, *CYS3*, *GLO1* and *IRC7*) that when deleted decreased the ability of yeast to release 4MMP (Howell et al., 2005). Subsequent studies have narrowed this list to one gene, the β-lyase encoding *IRC7* (Thibon et al., 2008), and in fact, a particular allele of this gene (Roncoroni et al., 2011), as the main determinant of 4MMP formation during winemaking. This line of research has, therefore, provided a clear quantitative trait locus (QTL) for molecular breeding of wine yeast. 3MH release, on the other hand, is not monogenetically determined (Roncoroni et al., 2011; Thibon et al., 2008); therefore, optimisation of its release through non-GM approaches remains an empirical exercise.

Early research into precursors for the polyfunctional thiols utilised a column-immobilised *Escherichia coli* carbon-sulfur lyase enzyme, apo-tryptophanase, in a method designed to measure aromatic potential (Peyrot des Gachons,

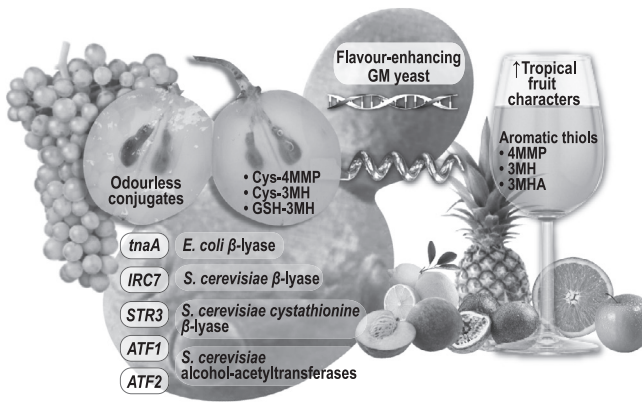

Figure 9.17 There are powerful synergies between Sauvignon Blanc grapes and yeast strains in formation of the compounds responsible for tropical fruit flavours: 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA). Odourless cysteine and glutathione conjugates are converted to aromatic thiols by carbon-sulfur-lyase enzymes. Alcohol acetyl transferase further modifies 3MH, converting it to the more potent 3MHA.
Adapted from Pretorius et al. (2012).

Tominaga, & Dubourdieu, 2000). We engineered a wine yeast, *VIN13*, to constitutively express the gene encoding this enzyme, *tna*A (Swiegers et al., 2007). Wine made from warm-climate Sauvignon Blanc grapes with this yeast exhibited intense tropical characters, while in model ferments the *VIN13-tnaA* strain released up to 20-fold more 3MH and 4MMP.

The same wine yeast, *VIN13*, engineered to overexpress *S. cerevisiae* alcohol-acetyltransferase encoding genes (*ATF1* and *ATF2*), was able to produce high concentrations of acetate esters (Lilly et al., 2006). In neutral-tasting grape varieties, such as Colombard, this results in lifted "banana" characters. Overexpression of *ATF1* in *VIN13* increased conversion of 3MH into its acetate ester, 3MHA, which, due to its lower perception threshold, is significantly more potent (Swiegers & Pretorius, 2007). Taken together, it is therefore possible to specifically manipulate 4MMP production by controlling the expression of an endogenous yeast gene, *IRC7,* to release high concentrations of both 3MH and 4MMP through the expression of *E. coli*'s *tnaA* gene in yeast, and increase the potency of "tropical fruit" aromas by boosting the conversion of 3MH into 3MHA by overexpression of *S. cerevisiae*'s own *ATF1* gene. While significantly less powerful than the *tnaA* gene product, modest enhancement of 3MH release is also possible through expression of the *S. cerevisiae* cystathionine β-lyase encoding gene, *STR3*, thereby paving the way for a range of "self-cloned" thiol-modulating wine yeast (Holt et al., 2011).

Polyfunctional thiols are not the only sulfur-containing aroma compounds that contribute to wine style. One need not be a wine expert to know that "reductive" aromas with descriptors such as "rotten egg", "burnt rubber", and "sewage" are not going to appeal to wine consumers. While there are several chemical and biological mechanisms that contribute to "reductive" aromas in wine,"rotten egg" gas, also known as hydrogen sulfide (H_2S), is largely a by-product of yeast metabolism. Under certain fermentation conditions, most wine strains of *S. cerevisiae* produce H_2S while incorporating inorganic sulfur into the amino acids methionine and cysteine – a process known as the sulfate reduction sequence (SRS) pathway.

Several GM strategies in the laboratory have been successful in limiting H_2S production by *S. cerevisiae*, and these are generally based on overexpression or inactivation of one or more genes involved in the SRS pathway (Omura, Shibano, Fukui, & Nakatani, 1995; Tezuka, Mori, Okumura, Kitabatake, & Tsumura, 1992). One of the targets has been sulfite reductase, which comprises two α- and two β-subunits ($\alpha_2\beta_2$) encoded by yeast's *MET10* and *MET5* genes, respectively. This knowledge informed a classical, non-GM mutagenesis approach to develop three "low-H_2S" strains, derived from the widely used commercial wine yeast *Maurivin PDM* (Figure 9.18) (Cordente, Heinrich, Pretorius, & Swiegers, 2009). These strains, commercialised under the names *Maurivin Advantage*, *Platinum* and *Distinction*, provide winemakers with new strategies to manage "reductive" aromas, especially in grape musts low in assimilable nitrogen.

In a follow-up study, we used a similar classical mutagenesis approach to isolate cerulenin-resistant mutants from the Maurivin PDM strain that produce significantly lower amounts of acetic acid during fermentation (Cordente, Cordero-Bueso, Pretorius, & Curtin, 2013). Acetic acid, a by-product formed during yeast alcoholic fermentation,

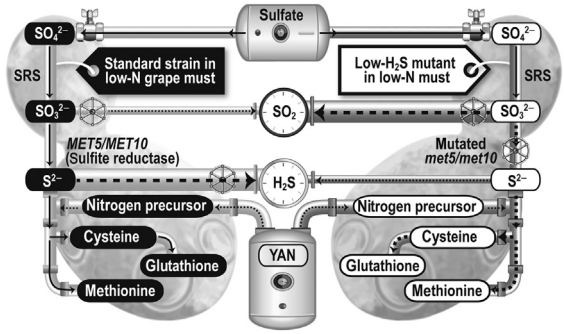

Figure 9.18 Building on knowledge from work utilising genetic modification (GM) strategies, a classical, non-GM mutagenesis approach was used to develop three "low-H_2S" strains. These strains have impaired sulfite reductase activity due to mutations in their *MET10* and *MET5* genes.
Adapted from Pretorius et al. (2012).

is the main component of volatile acidity (VA). When present in high concentrations in wine, acetic acid imparts an undesirable vinegary character that results in a significant reduction in quality and sales. We examined the relationship between mutations in *YAP1* and the low-VA phenotype of the cerulenin-resistant strains (Cordente et al., 2013). *YAP1* encodes a transcription factor that is essential for the normal response of cell to oxidative stress. We found that PDM strains carrying mutations in *YAP1* showed a consistent low-VA production phenotype after small-scale fermentation of different white and red grape musts. When integrated into the genome of a haploid wine strain, these mutated *YAP1* alleles partially reproduced the low-VA production phenotype of the diploid cerulenin-resistant strains, suggesting that *YAP1* might play a role in (regulating) acetic acid production during fermentation. In another study, it was found that a deletion mutant of the Fermentation Stress Response gene *AAF1* resulted in a significant decrease in acetic acid levels in Chardonnay wine without an increase in acetaldehyde (Luo, Walkey, Madilao, Measday, & Van Vuuren, in press). Both these studies offer prospects for the development of low-VA wine yeast starter.

9.5.3 Making the first modest moves with GM yeast strains

Australia, New Zealand and many European countries have effectively banned the use of genetically modified organisms (GMOs) in commercial wine production. A multitude of interconnected agronomic, business, regulatory, cultural and social factors that have led to these bans, but consumer sentiment is clearly one of the main drivers (Pretorius, 2000; Pretorius & Høj, 2005). Whilst it is unlikely that the situation will change in the near future, the first modest move to release a commercialised GM yeast to market was made in 2005; a transgenic wine yeast, ML01, was given the green light from regulatory authorities in the USA, Canada and Moldova.

ML01 carries genes that enable it to perform malolactic fermentation (MLF), in which grape-derived malic acid is de-acidified (decarboxylated) to lactic acid. MLF

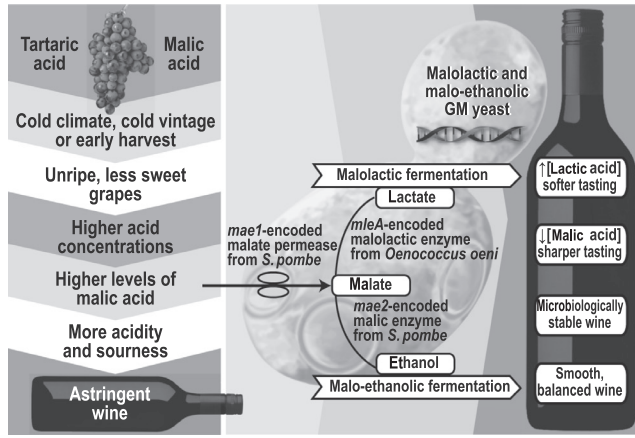

Figure 9.19 There are two options to genetically engineer extraneous malate utilisation in order to deacidify wine. One approach utilises the *Schizosaccharomyces pombe* malate transporter gene (*mae1*) and the *O. oeni* malolactic enzyme gene (*mleA*), enabling yeast to perform malolactic fermentation in parallel with alcoholic fermentation. Alternatively, *Saccharomyces cerevisiae* can be modified by the introduction of *mae1* and the *S. pombe* malic enzyme gene (*mae2*), thereby enabling the conversion of malate into ethanol.
Adapted from Pretorius et al. (2012).

is performed by lactic acid bacteria, particularly *O. oeni*, following alcoholic fermentation. However, *O. oeni* is rather fastidious, being inhibited by a range of conditions typical of fermented grape juice (e.g. low pH, high alcohol content and poor nutrient availability) and can become "stuck" or sluggish (Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985). In addition, some lactic acid bacteria produce biogenic amines that impose health risks. Clearly, a wine yeast that performs MLF should be of great interest to both winemakers and consumers.

ML01 carries the *Schizosaccharomyces pombe* malate transporter gene (*mae1*) and the *O. oeni* malolactic enzyme gene (*mleA*); both are chromosomally integrated and regulated by the *S. cerevisiae PGK1* promoter and terminator (Husnik et al., 2006). This enables the ML01 to perform MLF in parallel with alcoholic fermentation (Figure 9.19). In fermentation trials it was shown that 5 g/l of malic acid was decarboxylated to lactic acid within 5 days, without negative impacts on the sensory aspects on wine. Further analyses of the phenotype, genotype, transcriptome and proteome revealed that ML01 is substantially equivalent to its parental industrial wine yeast.

An alternative GM approach to lowering malic acid levels in wine has been to engineer a wine yeast that is able to conduct malo-ethanolic fermentation. In this case, malate is decarboxylated to pyruvate, which is then converted to ethanol. *S. cerevisiae* requires two heterologous genes for this, a malate transporter gene (*mae1*) and a malic enzyme gene (*mae2*), both of which come from *S. pombe*. Whilst this strategy appears to be successful, there has not been a commercially available version of a malo-ethanolic wine yeast released to market (Volschenk, Viljoen-Bloom, Van Staden, Husnik, & Van Vuuren, 2004).

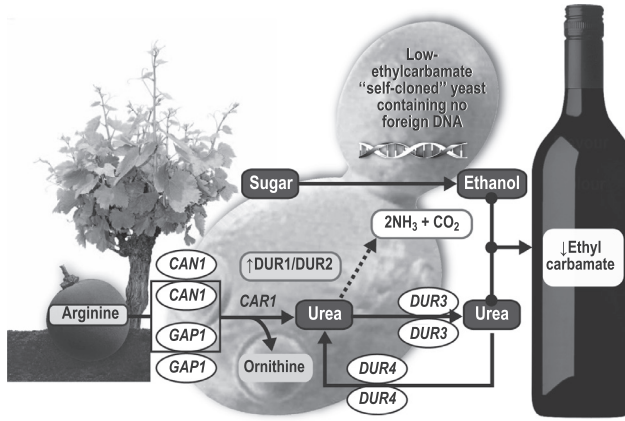

Figure 9.20 A wine yeast has been genetically engineered to reduce ethyl carbamate production during fermentation. Through increased expression of *DUR1/DUR2*, this yeast breaks down urea to ammonia and CO_2 before it is able to react with ethanol.
Adapted from Pretorius et al. (2012).

A second commercially available GM wine yeast, ECMo01, received clearance from the American and Canadian regulatory bodies in 2006. ECMo01 was engineered to reduce the risk of ethyl carbamate production during fermentation. Ethyl carbamate, a potential carcinogen, is the product of urea reacting with ethanol, but is typically produced at such low levels (if at all) in winemaking that it is generally not a concern. Nonetheless, in some fortified wines and in some wine-producing regions, it can make an appearance.

ECMo01 has an extra copy of the *S. cerevisiae DUR1,2* gene under the control of the yeast *PGK1* regulatory sequences (Coulon et al., 2006). *DUR1,2* encodes urea amidolyase, which converts urea into ammonia and carbon dioxide, thereby removing substrate for ethyl carbamate production (Figure 9.20). The ammonia that is produced is consumed as a preferred nitrogen source by yeast. ECMo01 has been shown to reduce ethyl carbamate in Chardonnay wine by almost 90%, and analyses of ECMo01's phenotype and transcriptome also revealed that the ECMo01 yeast is substantially equivalent to its parental strain.

Interestingly, this yeast is *cis* (or "self") cloned; it carries no foreign DNA, and therefore is not transgenic. Nevertheless, because it was generated using techniques that involved manipulation of DNA in vitro, the regulations of many countries require it to be classed as a GMO.

Because wine yeasts are classified as "processing aids" by American and Canadian regulators, wines made with GM yeasts are not required to be labelled as such. While no winemakers from these two countries have admitted to using ML01 or ECMo01, it is common knowledge that these GM yeasts have been used, albeit on a very limited scale; for understandable reasons, in the current anti-GMO climate, users prefer to keep this confidential.

For wine yeast researchers and many vintners it is frustrating that we cannot take full advantage of the many beneficial outcomes arising from the application of GM

technologies in the food and beverage sector. It is to be hoped that, in the near future, consumers will see through the misrepresentations and scaremongering of anti-GM lobby groups and be more accepting of what GM science has to offer. To hasten this, scientists who bioengineer yeasts and other microbes for industrial applications must be prepared to communicate their views to the wider community and ensure that the debate is not so one-sided. After all, there is no intrinsic fear of the technologies; indeed the pharmaceutical industry has been very successful in developing GM therapeutics, which the vast majority of us have welcomed because of their efficacy and safety.

9.6 Future trends

Looking over the horizon, as the various omics fields progress, it should be possible to build systems-based mathematical models of metabolism that will facilitate the in silico design of new wine yeast strains (Nielsen & Keasling, 2011). In parallel with this, we see the emergence of synthetic biology where, yet again, *S. cerevisiae* is a key player (Figure 9.21). It should not be too long before there are synthetically customised *S. cerevisiae* genomic components (e.g. regulatory elements to control expression

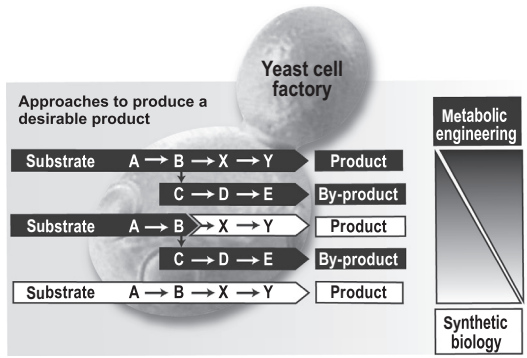

Figure 9.21 The overlap between *metabolic engineering* and *synthetic biology* by the use of three different approaches to produce a desirable product. This overlap was aptly described by Nielsen and Keasling (2011). The first approach is a classical approach where a naturally producing yeast is selected as the "cell factory" for production of the desirable product. Typically the flux toward the product is naturally low, but through the use of traditional, non-GM strain improvement or the use of directed genetic modifications – *metabolic engineering* – it is possible to increase the flux toward the product. In the second approach, the yeast cell does not naturally produce the product of interest. Through inclusion of a synthetically designed pathway into the yeast cell, the yeast can produce the product, often in small amounts initially. However, through pathway optimisation the flux through this synthetic pathway can be increased to ensure a high flux toward the product. This approach applies concepts from both *metabolic engineering* and *synthetic biology*. In the third approach, a complete synthetic yeast cell could potentially be constructed such that it is dedicated to produce a desirable product. Adapted from Nielsen and Keasling (2011).

of targeted genes; cassettes carrying genes encoding metabolic pathways to shape wine relevant traits, etc.) available "off the shelf" for designing, building and refining metabolic processes in wine yeast. But the key question remains: are consumers ready for this brave and exciting new world?

To answer this question, let us remind ourselves that truly great wines are born from great marriages between grape variety, climate, soil and landscape, on the one hand, and technology, innovation and craftsmanship on the other. Thus, the art in the science of winemaking lies in the choices made regarding which technological tools and innovations are selected and how they are applied to craft the infinite diversity of wine styles. Put differently, if we gave the same tools, i.e. paint, brushes and canvasses, to different artists – Da Vinci, Monet and Picasso – and all were asked to paint the same thing, they will invariably come up with very different masterpieces.

There is a fear that technological innovation – including the tailoring of wine yeast strains – could result in wine homogeneity and uniformity. Such fear is unfounded. The reality is that technology creates diversity by offering more options to vintners to respond to market needs and consumer preferences. These are options that the wine industry desperately needs as it faces so many challenges: an endemic oversupply of wine globally; prohibitionist-like propaganda campaigns from some anti-alcohol lobbyists; outbreaks of new diseases and pests in vineyards; climate change and environmental concerns.

The story of yeast research raises some important questions, therefore. It leads us to question public perception of the terms "natural" and "unnatural"; "technological" and "traditional". Where is the dividing line between natural and unnatural in the context of yeast and wine research, and indeed, science as a whole?

The story of yeast also highlights the importance of cross-disciplinary research. We may be wine scientists, microbiologists, molecular geneticists or researchers engaged in the new omics technologies – genomics, transcriptomics, proteomics, or metabolomics – but we all share a common goal. We all seek greater understanding of yeast as a simple, model organism: a microorganism that has the potential to shed new light on disease as well as processes such as fermentation. Wine science has made a significant contribution to understanding in this area of inquiry.

Finally, the story of yeast research raises questions about the future of omics technologies and their perception by society. Does yeast research bring into question, perhaps, the way that GMOs and synthetic genomes are perceived? We imagine that anyone reading this article holds his or her own views on this important and highly controversial subject. And this is the story of the journey of the winemaker's bug – 7000 years and counting…

Acknowledgments

This chapter is an updated, expanded and re-worked version of an article published in *Bioengineered Bugs* (2012) 3:147–156. Information is reproduced here with kind permission of the Editor, Dr Roy Sleator, Managing Editor, Dr Andrew Thompson, and the publisher, Landes Bioscience. Research at the *Australian Wine Research Institute* (AWRI) is financially supported by

Australia's grapegrowers and winemakers through their investment body the *Grape and Wine Research and Development Corporation*, with matching funds from the Australian Government.

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Modern approaches for isolation, selection, and improvement of bacterial strains for fermentation applications

10

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10.1 Introduction

The use of fermentation to preserve and improve the properties of food has a long history. For example, milk has been preserved by fermentation for at least seven millennia (Dunne et al., 2012). Initially, fermentation was a spontaneous process, probably with mixed results, and it was quickly learned that inoculation of the material to be fermented with a suitable inoculum would increase the likelihood of success. Traditionally, this was done by using part of a previous fermentation as an inoculum, but as microbiological knowledge increased, inoculation with specifically prepared fermentation starter cultures developed (Høier et al., 2010). This in turn gave a better control of the fermentation process and allowed for the development of new products with novel properties. This is well illustrated in the dairy industry, where a diversity of bacterial species is used to manufacture a large variety of fermented dairy products (Table 10.1).

Today, commercially produced starter cultures are extensively used in the food fermentation industry. Commercial starter cultures are produced by fermentation in large scale with volumes in excess of 40,000l. A single production run can yield several tons of harvested cells, which can be used to inoculate tens of thousands of tons of raw material. The resulting economy of scale means the cost of the starter culture is insignificant compared to the cost of the raw material to be fermented. However, the value added by the use of high-quality commercial starter cultures continues to increase. High demands are placed on commercial starter cultures, and the production procedures used are approaching pharmaceutical standards (Høier et al., 2010). The composition of the production medium is carefully designed to produce a high yield while avoiding the use of allergenic ingredients and ingredients that could conflict with religious beliefs of potential consumers of the final fermented food product. This allows the starter cultures to bear both Halal and Kosher labels as well as satisfying the needs of vegetarians that their food sources are free of animal-derived products.

Commercial starter cultures are available in both frozen and freeze-dried formats and can be used to directly inoculate the material to be fermented. The use of these types of cultures, referred to as direct vat set (DVS) cultures, has many advantages over the

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00010-4

Table 10.1 Species used in the production of various dairy products

Dairy product	Species included in starter cultures
Hard cheese (e.g., Cheddar, Feta) & Cottage cheese	*Lactococcus lactis* subsp. *lactis* *Lactococcus lactis* subsp. *cremoris* *Streptococcus thermophilus*
Cheese with small holes (e.g., Gouda, Edam) & Buttermilk	*Lactococcus lactis* subsp. *lactis* *Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* *Leuconostoc mesenteroides* subsp. *cremoris* *Leuconostoc pseudomesenteroides*
Cheese with large holes (e.g., Emmentaler, Jarlsberg)	*Streptococcus thermophilus* *Propionibacterium freudenreichii* *Lactobacillus helveticus* *Lactobacillus delbrueckii* subsp. *lactis*
Pizza cheese (e.g., Mozzarella)	*Streptococcus thermophilus*
Italian hard cheese (e.g., Grana, Parmesan)	*Streptococcus thermophilus* *Lactobacillus helveticus* *Lactobacillus delbrueckii* subsp. *bulgaricus* *Lactobacillus paracasei*
Blue mold cheese (e.g., Danish Blue, Roquefort)	Same as for cheeses with small holes with the addition of *Penicillium roquefortii*
White mold cheese (e.g., Camembert, Brie)	Same as for cheeses with small holes with the addition of *Penicillium candidum*
Red smear cheese (e.g., Esrum, Munster)	Same as for cheeses with small holes with a surface smear of *Brevibacterium linens*
Yogurt	*Streptococcus thermophilus* *Lactobacillus delbrueckii* subsp. *bulgaricus*
Probiotic dairy products	Same as for yogurt or buttermilk with the addition of *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus acidophilus*

use of traditional bulk starters produced on-site (Høier et al., 2010). Commercial starter cultures are put through an extensive quality control process ensuring consistent quality, performance, and the absence of undesirable microorganisms. Since they are produced away from the food to be fermented, the risk of accumulation of bacteriophage in the fermentation factory is reduced. Direct vat inoculation gives flexibility in fermented food production, as the amounts of the standardized inoculation material used can be adjusted to both the quantity and quality of the raw material to be fermented. Since the cultures are provided in formats with a long shelf life, a suitable starter culture can always be on hand. In addition, by blending specific strains, starter cultures can be customized to allow the production of fermented foods with unique, predetermined properties.

Consumer preferences are constantly changing and vary considerably around the globe. In addition, there is a considerable interest in new food experiences, which may be satisfied by fermentation of novel raw materials or by industrial production of foods traditionally produced by small-scale spontaneous fermentations. Thus, there is a need for continuous development of new starter cultures providing improved product performance. There are two potential sources of new microorganisms for inclusion in starter cultures: they can either be found in nature or they can be obtained by improvement of strains already in use by means of modern microbiological methods. As will be described in subsequent sections, both approaches are used and both have been optimized to give high success rates in developing new starter cultures. Examples provided will include:

- the use of laboratory automation to screen an extensive strain collection as well as to isolate mutants with specific properties,
- the use of positive selection methods to isolate mutants that would be difficult to obtain by other methods,
- the use of genome sequence analysis to understand the properties of the mutants obtained, and
- methods to change the genetic content of a strain without the use of controversial recombinant DNA technology.

Even though these examples are from the dairy industry, the principles involved are applicable to any industry using microbes for the production of fermented food or feed or for the bioconversion of biomass to valuable biochemical compounds.

10.2 Screening of strain collections

Bacterial diversity is estimated to cover more than four million different taxa based on 16S RNA gene differences (Curtis, Sloan, & Scannell, 2002) and reflects adaptation to a wide variety of environments, including such diverse habitats as water, air, soil, the human body, food, etc. Each species is comprised of a multitude of individual strains with unique properties. Due to this large diversity, screening of the total microbial population for any desired phenotype or property is unrealistic, and so screening is normally limited to a collection of bacteria obtained from certain relevant habitats. Bioremediation applications could start with a collection of bacteria isolated from soil; dairy applications, a collection derived from environments were milk is present, and so on. In addition, the ability to isolate and identify individual bacterial strains with specific desirable properties requires not only microbiological expertise but also highly automated processes.

A standardized work flow for strain acquisition, strain purification, primary characterization, and where necessary, classical strain improvement was initiated in our laboratories in 2008 and designated the strain supply chain (SSC). The introduction of these four standardized systematic work processes has resulted in a faster and more resource-efficient development of new strains, both wild-type strains and classically improved strains, for new product development. It is presented here to serve as an inspiration for other groups seeking to systematize their strain-screening activities (Figure 10.1).

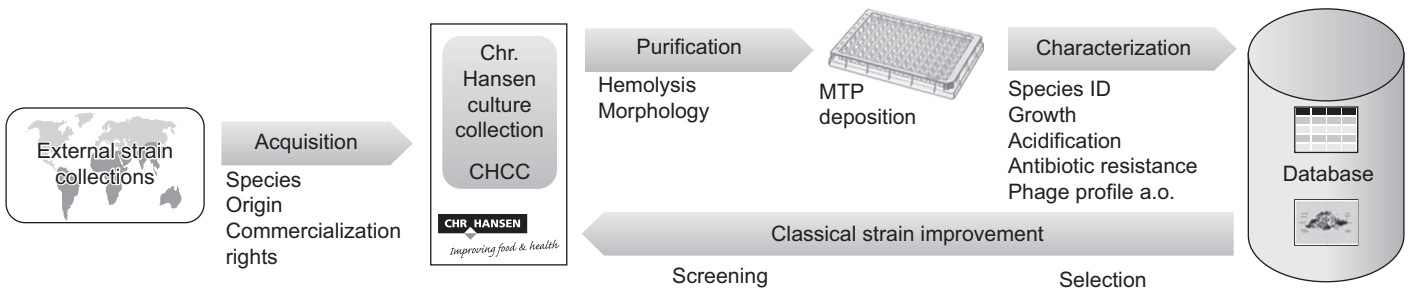

Figure 10.1 Schematic representation of the strain supply chain. The individual processes are described in detail in Sections 10.2 and 10.3. MTP, microtiter plate.

10.2.1 Laboratory automation

A key element of our strain-screening activities is automation. Two invaluable pieces of laboratory equipment are a colony picker and a liquid-handling robot. A typical colony picker has a robotic arm with multiple self-sterilizing pins (typically 96 pins) that samples cells from colonies on agar plates and deposits them in microtiter plates. The agar plates can be either traditional Petri dishes or large 20 cm by 20 cm plates with a capacity of 3000 colonies per plate. Microtiter plates are usually with 96 wells, but 384-well plates can also be used. Colonies on the agar surface are digitally recognized using a combination of a built-in camera and imaging software. This allows the picking of colonies with user-specified physical characteristics and ensures that only well-isolated colonies are picked. More than 2500 colonies can be picked per hour, a feat that cannot easily be matched by manual picking. In addition, the machine does not make mistakes nor suffer from boredom and fatigue, which is certainly a concern with manual colony picking.

Liquid-handling robots with a modular design are available allowing customization to give optimal performance and flexibility. For normal microbiological work, a liquid-handling robot needs facilities for pipetting liquids, typically provided by a row of 8 or 12 individually controlled pipetting channels, and for moving, heating, cooling, shaking, incubating, filtering, and centrifuging microtiter plates containing samples. Pipetting from tubes, plates, or any other container, that will fit on the deck of the liquid-handling unit, is possible; volumes between 1 and 1000 µl can be accurately aspirated or dispensed. In addition, built-in measuring capabilities, for example by inclusion of a spectrophotometer or a flow cytometer, allow automated measurement and data collection. Of course, the whole system is computer controlled and the software allows advanced programing, which gives a high degree of freedom for customizing experiments, but typically requires dedicated specialist personnel due to the complexity of the system. The generated data can be directly imported into relevant software for analysis. In our current setup, liquid handling is performed using a *Microlab Star* from Hamilton (Bonaduz, Switzerland) and optical density is measured using built-in spectrophotometers (BMG Labtech, Ortenberg, Germany) and a flow cytometer (HTFC, Intellicyt Corp., Albuquerque, USA). Equipment from other manufacturers can also be integrated.

10.2.2 Strain acquisition, purification, and identification

New strains can be obtained by isolation from natural sources or directly from other researchers who have isolated them. When acquiring strains from external sources, it is important to obtain documentation that ensures the rights to use, commercially develop, and produce the strains for relevant products. It is also important to obtain and retain as much information as possible concerning the exact source of the strains. This should include the type of sample that the strains were isolated from, geographic location, date of isolation, growth conditions and media, as well as the name and affiliation of the provider of the sample.

Upon receipt, samples are deposited in the Chr. Hansen Culture Collection (CHCC; described in Section 10.2.6) and stored at −80 °C, typically following addition of

glycerol to 20% v/v and rapid freezing. In order to avoid working on impure isolates, all strains entering the SSC are purified prior to genotypic and phenotypic characterization. This is done by streaking to single colonies on an appropriate growth medium, e.g., MRS agar (de Man, Rogosa, & Sharpe, 1960) for *Lactobacillus* isolates or M17 agar (Terzaghi & Sandine, 1975) for *Lactococcus* isolates, and selection of well-isolated single colonies. This process is repeated three times, after which the strains are observed by light microscopy to determine cell morphology and to confirm that the population appears homogenous. An aliquot of the purified isolate is plated on blood agar to test for the ability to produce hemolysins; hemolytic isolates are usually discarded as these have limited use in the food industry. The remaining isolates are re-deposited as pure cultures in the CHCC and also deposited in 96-well microtiter plates. Two master plates and 10 replica plates are made and stored in a −80 °C freezer, ready for fast retrieval and use in the primary characterization of the strains.

10.2.3 Strain characterization

Primary characterization of each strain is performed to get initial knowledge about the strains and to determine their suitability for further product development. The strains are characterized for both desirable and undesirable traits, e.g., growth rate in various fermentation media as an example of a positive trait, or atypical resistance to antibiotics as an example of an undesirable trait. The primary characterization of strains in the SSC consists of several different phenotypic tests, typically performed in 96-well microtiter plates, in volumes of 100–200 µl. In order to allow fast throughput and high accuracy, several processes are automated, as described in Section 10.2.1. A full primary characterization of a collection of strains in microtiter plate can be completed in 2 weeks.

The taxonomic identity of each strain is determined following DNA extraction and partial 16S rRNA gene sequencing. This typically establishes genus, species, and, in some cases, subspecies identity. For some closely related species or subspecies, additional testing is required to allow unambiguous discrimination. For example, sequencing of the gene *rpoA*, encoding the RNA polymerase alpha subunit, can be used to distinguish between *Lactobacillus helveticus* and *Lactobacillus gallinarum* (Naser et al., 2007). In other instances, phenotypic differentiation is used to discriminate species. *Lactobacillus delbrueckii* subsp. *bulgaricus* can be differentiated from *Lactobacillus delbrueckii* susbsp. *lactis* by differences in the carbohydrates they are able to metabolize (Tanigawa & Watanabe, 2011). This can suitably be determined using the API 50 CH carbohydrate fermentation kit (BioMérieux, Inc., Marcy l'Etoile, France) or as part of the determination of the growth characteristics of the strains as described below. Determination of the taxonomic identity of a strain is important, as it only makes sense to work with strains that can be accepted for the desired purpose. An extensive list of species that have a technologic benefit when used in foods has been established (Bourdichon et al., 2012).

The growth characteristics of the strains are determined by cultivation in a variety of proprietary fermentation media as well as traditional laboratory media with different carbon sources. For *Lactobacillus* strains, we typically use MRS broth base (without glucose), supplemented with 1.8% (w/v) glucose, lactose, fructose, galactose, sucrose,

maltose, trehalose, xylose, or water (negative control). Growth measurements can be either end-point, typically after 18 h incubation, or continuous measurements at 1–2 h intervals for 18 h, allowing measurement of both total optical density increase and determination of maximum growth rate. Most strains are examined during growth at several different temperatures (30, 37, 40, and 43 °C), allowing assessment of optimal growth temperature as well. Growth experiments are performed in microtiter plates and automated optical density readings are done using the built-in spectrophotometer in the liquid-handling robot.

Strains for use in dairy applications are examined for their ability to acidify milk at various temperatures in microtiter plates. The change in the pH of the milk is followed by the inclusion of pH indicators (bromocresol purple and bromocresol green) in the milk and scanning on a flatbed scanner typically at 6-min intervals. The data are exported to a suitable spreadsheet such as Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) for ease of calculation. Acidification curves are obtained, and descriptors like total pH drop, maximum acidification rate, and specific time intervals such as time to acidify from pH 6.0 to pH 5.5 are easily derived in the spreadsheet. The formation of volatile organic compounds in the acidified milk is measured by head space gas chromatography, allowing quantitative assessment of the production of relevant metabolites such as acetaldehyde, acetoin, and 3-methyl-butanal. Tolerance to NaCl is also tested in milk by following the acidification in the presence and absence of 4% (w/v) NaCl.

One major concern in the dairy industry is the devastating effect of bacteriophage attack on acidification (Høier et al., 2010). Consequently, all *Streptococcus thermophilus* and *Lactococcus* strains are tested for bacteriophage tolerance during growth in milk in the presence of a specific bacteriophage pool. Each bacteriophage pool consists of multiple unique bacteriophage specific for the respective genera. The acidification curves from pure milk inoculations are compared to bacteriophage-inoculated milk acidifications, to determine if the strains are tolerant or sensitive to the tested bacteriophages. If a strain is sensitive to one or more of the tested bacteriophages, milk acidification will be perturbed, whereas a normal acidification indicates tolerance to the tested bacteriophages.

All strains are tested for resistance to the antibiotics tetracycline and erythromycin as part of the primary characterization. Resistance to these antibiotics is frequently observed in natural isolates from a variety of food and feed sources (Domig et al., 2008). Since antibiotic resistant microbes are undesirable in the food chain, resistant strains will not normally be selected for further product development work. Additional extensive antibiotic-resistance testing is done at a later stage to rule out the presence of resistance to other antibiotics with relevance to medical and veterinary practice.

The levels of several intra- and extracellular enzyme activities are also determined. Whole-cell proteolytic activity is determined by measuring fluorescence development after exposure to fluorescein isothiocyanate (FITC) – tagged casein (Twining, 1984). This proteolytic activity is important for acidification of milk and flavor development (Høier et al., 2010). Intracellular enzyme activities are measured following cell lysis by sonication using a custom-made 96-pin sonication head (Misonix Inc.,

Farmingdale, USA) combined with glass beads. Supernatants from lysed cells are examined for enzyme activities believed to be of importance for flavor development in cheese. These include a variety of aminopeptidase and aminotransferase activities that are involved in the conversion of the peptides and amino acids derived from the breakdown of casein to a variety of flavor compounds.

The availability of an easily retrievable, microtiter plate-based strain-screening collection also opens the possibility to test large collections for more specific parameters like bioconversion of certain compounds or production of specific metabolites in a very short time. Combining the data from such specific screens with the data generated in the primary characterization provides a good starting point for targeted product development activities.

10.2.4 *Characterization of an extensive* Lactococcus lactis *collection*

More than 800 *Lactococcus lactis* strains were systematically characterized in a series of five experiments using the automated methods described above. Three experiments involved cells which were pre-grown in M17 medium (Terzaghi & Sandine, 1975) followed by incubation in milk at 30, 37, or 40 °C (designated M17_30, M17_37, and M17_40, respectively). In one experiment, cells were pre-grown in a proprietary fermentation medium followed by incubation in milk at 30 °C (designated BD53_30); while in the fifth experiment, cells were pre-grown in the same proprietary fermentation medium, transferred to milk at 30 °C, and after 2 h stressed with NaCl at a final concentration of 4% (w/v) (designated BD53S_30). The results were analyzed using multivariate statistics (SIMCA P+, Umetrics, Umeå, Sweden) and are presented in Figure 10.2. Two experimental descriptors from each experiment were used for the plot: maximum pH difference observed, ΔpH (DpH), and maximum acidification rate, ΔpH/hour, determined over a 3 h period (Vmax3hr) and eight clusters of strains were identified (Figure 10.2). Clusters 1 and 3 consist mainly of *Lactococcus lactis* subsp. *cremoris*, whereas cluster 8 consists mainly of *Lactococcus lactis* subsp. *lactis.*

The strains represented by the eight clusters observed in Figure 10.2 have unique acidification patterns (Figure 10.3). The five columns of this figure represent the five different experiments described above. It is apparent that clusters 1 and 3, containing mainly *L. lactis* subsp. *cremoris*, are very sensitive to stress like 4% NaCl or high temperature (40 °C), whereas the *L. lactis* subsp. *lactis* strains in cluster 8 are relatively stress tolerant. These clusters also correlate with other phenotypic characteristics such as the effect of cultivation in the presence of arginine on optical density. Strains in cluster 8 are stimulated by the presence of arginine in the growth medium while those in clusters 1 and 3 are not. The presence of an active intracellular arginine deiminase pathway results in conversion of arginine to ornithine and generation of ATP and release of NH_4^+ ions (Larsen, Buist, Kuipers, & Kok, 2004). This provides additional energy to the cell as well as neutralization of some of the lactic acid produced by fermentation of lactose. Growth stimulation by arginine has been used to differentiate *L. lactis* subsp. *lactis* from *L. lactis* subsp. *cremoris* (Mundt, 1986). Our analysis fully supports this differentiation.

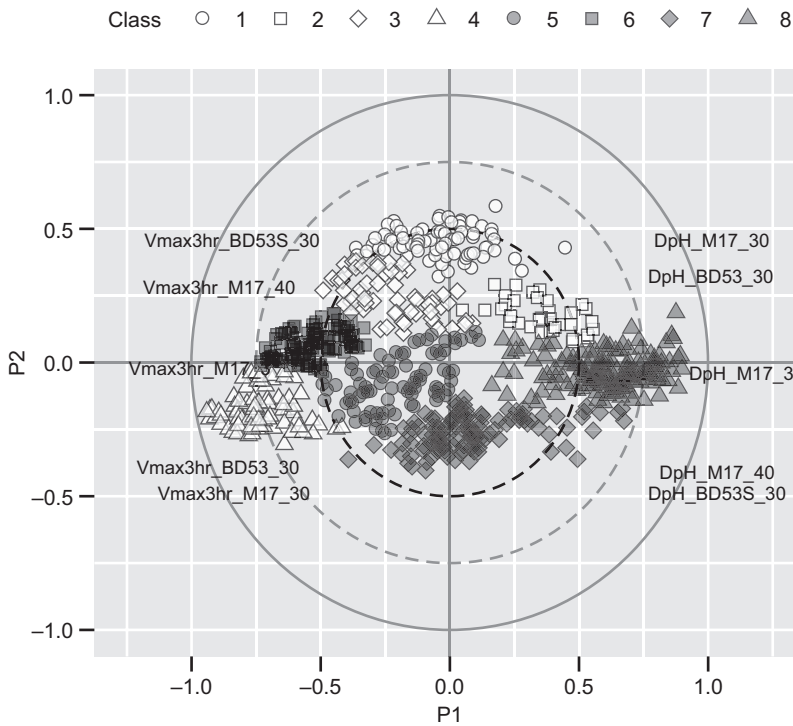

Figure 10.2 Principal component plot of more than 800 *Lactococcus* strains. Each strain is represented by one of eight different symbols, corresponding to the cluster they belong to as determined by analysis of their acidification properties in the five experiments illustrated in Figure 10.3. Pre-growth was in either traditional laboratory medium (M17 broth, Terzaghi & Sandine, 1975) or proprietary fermentation broth (BD53) at 30 °C, followed by incubation in milk at 30 °C (M17_30 and BD53_30), 37 °C (M17_37), or 40 °C (M17_40). BD53S_30 denotes pre-growing the strains in BD53 followed by incubating in milk at 30 °C for 2 h after which salt stress is induced with NaCl at a final concentration of 4% (w/v). Two experimental descriptors are used: maximum pH difference observed, ΔpH (denoted DpH); and maximum acidification rate, ΔpH/h, determined over a 3-h window (denoted Vmax3hr).

This comprehensive collection of well-characterized *Lactococcus* strains facilitates fast development of new cultures in the event a strain in a commercial culture is attacked by bacteriophage. Strains that strongly resemble the attacked strain, but with a different bacteriophage sensitivity, can simply be selected based on their previously determined phenotypic characteristics and tested for suitability for use in specific applications, thus eliminating the need for new, time-consuming, strain-screening experiments.

10.2.5 *Characterization of an extensive* Lactobacillus *collection*

New starter cultures, specifically for the production of low-fat yogurts, were developed based on the phenotypic characterization of more than 250 *Lactobacillus* isolates.

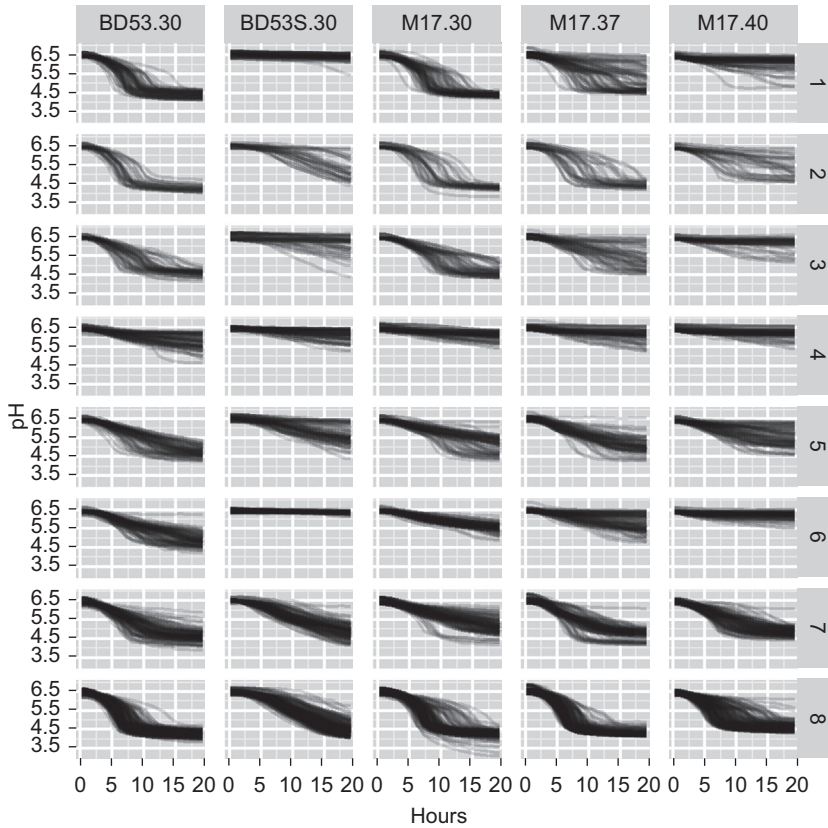

Figure 10.3 Milk acidification curves for the eight clusters determined by the principal component analysis. Each row represents one class, numbered 1 through 8 (see Figure 10.2) and each column one experimental setup: BD53.30 denotes pre-growth in a proprietary fermentation medium at 30 °C followed by growth in milk at 30 °C; BD53S.30 denotes pre-growth under the same conditions followed by growth in milk at 30 °C for 2 h with subsequent salt stress with NaCl at a final concentration of 4% (w/v); M17.30, M17.37, and M17.40 denote pre-growth in M17 broth (Terzaghi & Sandine, 1975) at 30 °C followed by growth in milk at 30, 37, and 40 °C, respectively.

Strains were examined for their ability to produce acetoin and diacetyl in milk; especially diacetyl is associated with creaminess in fermented milk products (Burdock, 2004). In addition, strains were tested for the ability to produce acetaldehyde (a characteristic flavor note in yogurt), the absence of atypical antibiotic resistance, and the ability to grow at elevated temperatures. Measurement of volatile organic compounds after milk fermentation in microtiter plates gave quantitative values for both acetaldehyde and acetoin production (Figure 10.4). Several strains, in particular from the species *Lactobacillus rhamnosus* and *Lactobacillus paracasei*, had increased acetoin levels, and after testing in small-scale yogurt production, one *L. rhamnosus* strain was selected to be part of the new low-fat yogurt cultures, where it contributes to the flavor profile of the final product (Jiminez et al., 2012).

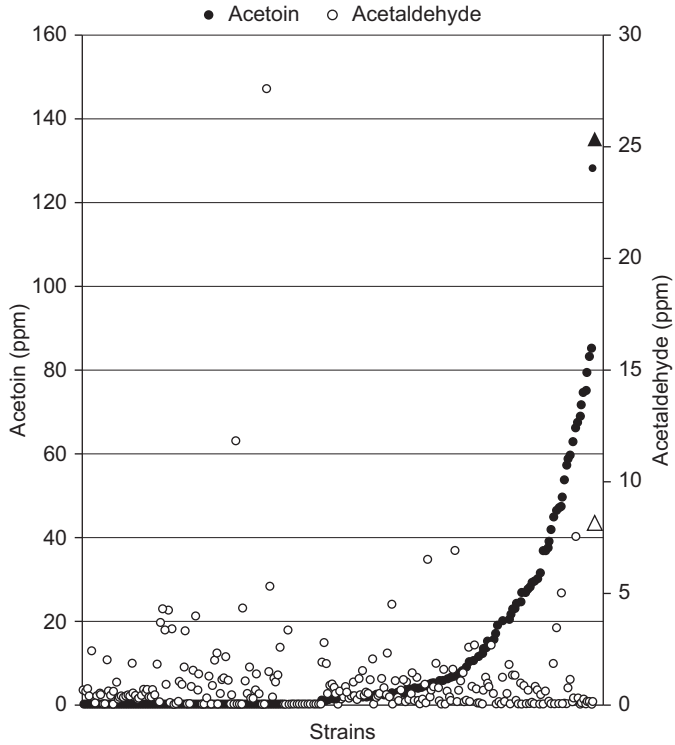

Figure 10.4 Production of acetaldehyde (open circles) and acetoin (filled circles) during overnight growth in milk. The left vertical axis represents the acetoin concentration (ppm); the right vertical axis the acetaldehyde concentration (ppm); and the horizontal axis the individual strains. The *L. rhamnosus* strain with the highest acetoin concentration was selected for use in low-fat yogurt and is represented by triangles.

10.2.6 The Chr. Hansen culture collection

Chr. Hansen A/S has been collecting microorganisms for many decades and today is in possession of an extensive collection of bacteria, yeast, and molds that can be used in the development of industrial fermentation processes. This collection contains more than 20,000 deposits with the majority of these being pure single strains of bacteria. As can be seen in Figure 10.5, there is a strong bias toward bacteria that can be used in the dairy industry. The main species of the indicated genera are those listed in Table 10.1. The genera not traditionally used in food, *Bacillus* and *Enterococcus*, are primarily for use in animal feed applications.

10.3 Classical strain improvement

It is not always possible to obtain a strain with the exact combination of properties required for a specific use by screening of natural isolates. Thus, it is often necessary

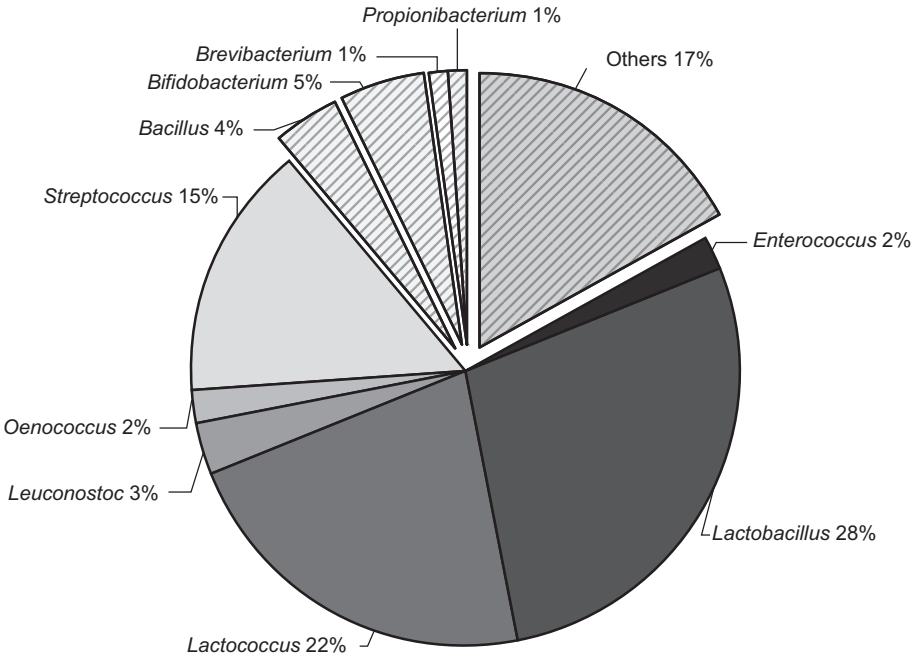

Figure 10.5 Taxonomic distribution of strains deposited in the Chr Hansen culture collection. The six solid sectors depict bacteria belonging to the genera normally considered to be lactic acid bacteria. The cross-hatched sectors depict bacteria belonging to other genera.

to improve an existing, well-characterized strain. Since the use of recombinant DNA technology in the food industry is highly controversial (see Section 10.4), this is usually done by classical strain improvement (CSI), occasionally involving the use of chemical or physical mutagens to generate genetic variation in a population of cells.

Mutagenic treatments that are commonly used include irradiation with ultraviolet light and exposure to chemical mutagens such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, ethyl-methane sulfonate, ethidium bromide, or nitrous acid. From a practical viewpoint, ultraviolet light mutagenesis is preferred as it is easy to control and does not result in toxic waste, which must be safely disposed of. It is also a relatively mild mutagenesis, so the risk of undesirable second site mutations is lower than with chemical mutagenesis.

These treatments are relatively nonspecific and generate mutations at random in the genome. Cells with the desired properties are subsequently obtained from this population by screening of a large number of isolates or selection of variants with the desired property.

10.3.1 Screening

The tools developed for screening of natural isolates (Section 10.2.3) can also be used for screening of the survivors of a mutagenesis procedure for strains with desired properties. This will be illustrated with a few examples from our own laboratories.

An important factor in the shelf life of yogurt is referred to as post-acidification, implying the continued production of lactic acid by the starter culture during storage at refrigerator temperature. The development of a lower pH affects the organoleptic properties of the product and reduces the viability of bacteria contained in the yogurt. This latter concern is especially relevant for products containing probiotics, which are by definition required to be viable at the time of consumption in order to exert their beneficial effect (FAO/WHO, 2001). One way to control post-acidification is to control the lactic acid production by the starter cultures. We have successfully used random mutagenesis and screening to isolate low post-acidifying mutants of *L. delbrueckii* subsp. *bulgaricus*. Several thousand survivors of a chemical mutagenesis treatment were screened in microtiter plates for their ability to acidify and post-acidify milk (see Section 10.2.3). The mutants that showed the best acidification properties were subsequently screened in 200-ml fermentations to determine their ability to form a desirable texture in yogurt. Based on these two screening parameters, it was possible to isolate strains that not only give a reduced post-acidification but also have improved texturing properties relative to their mother strains (Jensen & Johansen, 2007).

It is well known that all members of *Bifidobacterium animalis* subsp. *lactis* are resistant to low levels of tetracycline due to the presence of a *tet*(W) gene on the chromosome (Gueimonde et al., 2010). While this is not considered to have any food safety consequences (Wassenaar & Alter, 2012), it could still be desirable to obtain mutants in which this property is eliminated. Cells of *B. animalis* subsp. *lactis* were subjected to a combination of chemical mutagenesis with ethidium bromide and physical mutagenesis with ultraviolet light (Stroeman, 2009). Following mutagenesis, the cells were subjected to ampicillin enrichment, a common method to enrich cells that are unable to grow under specific conditions (Miller, 1972). Since tetracycline-sensitive strains do not grow in the presence of tetracycline, this procedure could be used to enrich for tetracycline-sensitive mutants. Screening of several thousand survivors using automated methods resulted in the isolation of several mutants of *B. animalis* subsp. *lactis* with an inactivated *tet*(W) gene, and subsequent testing revealed one mutant in which the mutation had an extremely low reversion rate (Stroeman, 2009). Genome sequencing (see Section 10.3.4) revealed that this strain has a frameshift mutation in the *tet*(W) gene rendering it sensitive to tetracycline. According to a recent opinion, use of such a strain as a probiotic could take place without additional safety or efficacy testing (Sanders et al., 2014).

10.3.2 Positive selection schemes

The examples in Section 10.3.1 are based on screening of a large number of survivors of a mutagenesis protocol for specific properties. In order to have a reasonable chance of obtaining mutants with the desired properties, an efficient mutagenesis is required. The unfortunate consequence of this is that any given survivor will have multiple mutations. Many of these are irrelevant to the desired phenotype, but some of them might be detrimental to the industrial fermentation the strain was sought for. Also, even though the screening process is automated, it consumes significant amounts of time and resources. Thus, methods of obtaining mutants without mutagenesis and

screening are desirable. Several examples from our own laboratories of positive selection of strains with useful properties are described below.

One area where direct selection has traditionally been successfully used is in the development of strains with enhanced resistance to bacteriophage attack. In this procedure, a bacteriophage-sensitive parent strain is targeted by one or a small number of relevant bacteriophages in a challenge assay and new bacteriophage-insensitive mutants (BIMs) are selected because they survive this challenge and form colonies on an agar medium. This is especially relevant in dairy applications, where bacteriophage attack is still one of the main causes of production failure. New bacteriophages are constantly being isolated, and variants of existing strains that are resistant to these are constantly being sought. It is important that the survivors of a bacteriophage challenge are thoroughly characterized because it is not uncommon for the BIMs to have subtle but important changes in performance (Høier et al., 2010).

Even though galactose is one of the two monosaccharides present in lactose, its presence in fermented dairy products is not always desirable. For example, cheese with a high level of galactose tends to turn brown upon heating due to the Maillard reaction (Fox & McSweeney, 1998). The majority of *S. thermophilus* strains are unable to ferment galactose, and although the genes responsible for galactose fermentation are present in *S. thermophilus,* they are generally not sufficiently active (Hols et al., 2005). By directly selecting for strains that are able to grow on agar plates where galactose is the sole carbon source, Janzen and Christiansen (2012) were able to isolate galactose-fermenting mutants. Characterization of these mutants revealed that they had a mutation in the promoter region of the *galK* gene that resulted in an increased gene expression and production of galactokinase-enabling fermentation of galactose (Figure 10.6). These galactose-fermenting mutants also showed an enhanced production of exopolysaccharides and generated a higher viscosity in fermented milk than the parental strain. This property was retained when the strains were used for the production of yogurt together with *L. delbrueckii* subsp. *bulgarcius.* Since only a very limited number of genetic changes can lead to an increased expression of galactokinase, the mutants described in this section would have been extremely unlikely to have been found by the screening methods described in Section 10.3.1. Hence, a positive selection is the only realistic way of obtaining these mutants.

The bacterial cell wall is the first line of defense against environmental stress in gram-positive bacteria. Thus, selection schemes that change the cell wall structure can potentially make cells more resistant to environmental stress. D-cycloserine is an antibiotic that inhibits alanine racemase and D-alanine D-alanine ligase, both involved in cell wall biosynthesis. Mutants resistant to this antibiotic have been isolated in a number of gram-positive species and shown to have differences in surface properties (Clark & Young, 1977; Reitz, Slade, & Neuhaus, 1967). We have used D-cycloserine as a selective agent to directly select mutants in a variety of strains and found that the mutants obtained have properties useful in industrial fermentation processes.

Lactobacillus plantarum is used in the wine industry, where it performs malolactic fermentation following the alcoholic fermentation by the wine yeast (Lerm, Engelbrecht, & du Toit, 2011). This fermentation is important for the organoleptic properties of the wine because the relatively harsh malic acid is converted to the

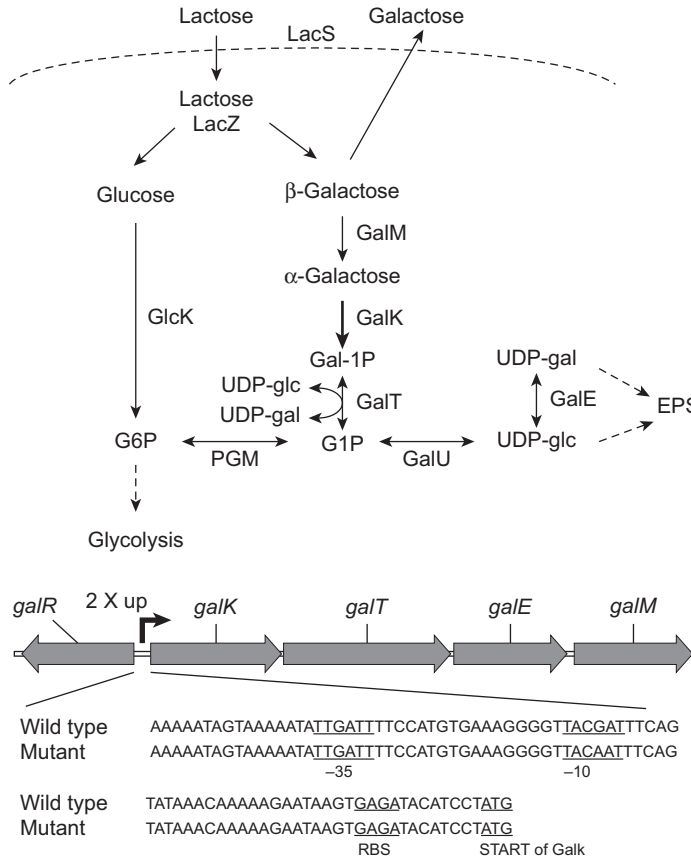

Figure 10.6 Galactose and lactose metabolism in *Streptococcus thermophilus,* inspired by Levander, Svensson, and Rådström (2002). Abbreviations: LacS, lactose transporter; LacZ, β-galactosidase; GlcK, glucose kinase; GalM, mutarotase; GalK, galactokinase; PGM, α-phosphoglucomutase; GalT, galactose-1-phosphate udridyltransferase; GalE, UDP glucose 4-epimerase; GalU, UDP glucose pyrophosphorylase; Gal-1P, galactose-1-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; UDP-glc, UDP-glucose; and UDP-gal, UDP-galactose. The promoter −35 and −10 region and ribosomal binding site (RBS) are underlined; the mutation in the promoter region of the mutant strain is in the −10 region. The two-fold enhanced expression of *galK* is indicated by a thickened arrow in the pathway and an arrow in the promoter region of the operon.

milder lactic acid. Due to the warm growing seasons in recent years, the sugar content of grapes has increased, resulting in wines with alcohol contents of more than 14%. This is a substantial challenge for the malolactic fermenting microorganisms, often resulting in insufficient conversion of malic acid to lactic acid. Mutants of *L. plantarum* that were resistant to D-cycloserine were selected on agar plates. Screening of these revealed that many of them had increased tolerance to ethanol (Sørensen, Kibenich, & Johansen, 2012).

The bacterial surface is also involved in the formation of texture in fermented dairy products such as yogurt. Consumers today prefer products that have a mild flavor and a high level of texture. Mutants of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* resistant to D-cycloserine were selected and tested for their ability to grow in milk. It was observed that several of the mutants produce more texture and less whey than the parent strain and combining mutants of each species produced yogurt with properties satisfying the current consumer preference (Kibenich, Sørensen, & Johansen, 2012).

10.3.3 Traditional bacterial genetics

The previous sections describe the use of screening and selection techniques to obtain strains with useful new properties following the creation of genetic variability in the population. An alternative approach is to use the pre-existing variability in known strains and to combine the desired properties from different strains into a single new isolate. This requires the transfer of genetic material from one strain to another. In traditional bacterial genetics, this can be accomplished by three different naturally occurring processes: transformation, conjugation, and transduction.

Transformation is a process whereby a bacterial cell takes up DNA from the environment and incorporates some or all of the genetic information present in the DNA into its genome. The DNA that is taken up can be chromosomal or plasmid DNA and can be retained by the cell following homologous recombination or by establishment of the plasmid in the cell. The ability to take up exogenous DNA, referred to as natural competence for transformation, is controlled by the physiological status of the cell and differs significantly between bacterial species and even differs between strains of a given species (Gardan, Besset, Guillot, Gitton, & Monnet, 2009). The best-known examples of species showing natural competence are *Bacillus subtilis* and *Streptococcus pneumoniae*. Among the lactic acid bacteria, only *Leuconostoc carnosum* and *S. thermophilus* have been shown to be naturally competent (Blomqvist, Steinmoen, & Håvarstein, 2006; Helmark, Hansen, Jelle, Sørensen, & Jensen, 2004). *Lactococcus* and a few species of *Lactobacillus* have many of the required competence genes but natural competence has not yet been demonstrated (Johnsborg, Eldholm, & Havarstein, 2007). The competence of *S. thermophilus* is highly dependent on the environment and cells are competent only when grown in a peptide-free defined medium (Gardan et al., 2009), suggesting that establishing competence in other species might be a matter of finding the optimal growth conditions.

In bacterial conjugation, genetic material is transferred between bacterial cells either by direct cell-to-cell contact or through bridge-like connections called pili (de la Cruz & Davies, 2000). Conjugation is widespread in prokaryotes and is routinely used in research for constructing new strains. The conjugal transfer of naturally occurring plasmids between strains of lactic acid bacteria has been described, and *Lactococcus* strains improved by conjugation have been on the market for many years (Høier et al., 2010).

Transduction is the process whereby a bacteriophage serves as an intermediate for the transfer of genetic material from one bacterium to another, and unlike conjugation does not require direct cell-to-cell contact. Two major types of transduction are known and referred to as generalized transduction and specialized transduction. Generalized transduction involves the transfer of random pieces of bacterial DNA from

one cell to another, whereas specialized transduction involves the transfer of specific DNA regions between cells. Transfer of plasmid DNA from one *Lactococcus* strain to another by transduction has been described (McKay, Cords, & Baldwin, 1973). More recently, transfer of plasmid DNA from *S. thermophilus* to *L. lactis* was documented (Ammann, Neve, Geis, & Heller, 2008). To the best of our knowledge, lactic acid bacteria strains improved by transduction have not been introduced to the market.

The preceding paragraphs have described traditional methods of introducing genetic material into a strain. In some situations, it is actually desirable to eliminate genetic material from a strain. This material can include antibiotic-resistance genes or the ability to produce undesirable metabolites like biogenic amines. Antibiotic-resistance genes often reside on mobile genetic elements such as plasmids or transposons. This causes problems because transmissible antibiotic-resistance genes are considered to be a hazard to public safety and unacceptable in the food supply (European Food Safety Authority, 2009). Fortunately, most transmissible genes are inherently unstable, probably as a consequence of the very structures that make them mobile. Plasmid curing demonstrated that technologically important properties of *L. lactis* were plasmid encoded (Gasson, 1983). Plasmid curing has subsequently been used in several instances to eliminate a plasmid containing antibiotic-resistance genes from different *Lactobacillus* strains (Danielsen, 2002; Rosander, Connolly, & Roos, 2008). Curing of chromosomal transposon-based antibiotic-resistance genes can be accomplished by treatments such as heat shock that increase the transcription of the transposase genes located in the IS elements flanking the antibiotic-resistance gene (Strøman, Müller, & Sørensen, 2003). Similar methods were unable to eliminate the *tet*(W) gene from the chromosome of *B. animalis* subsp. *lactis* (Stroeman, 2009), consistent with the lack of IS elements flanking this gene and its documented lack of transmissibility (Bennedsen, Stuer-Lauridsen, Danielsen, & Johansen, 2011; Gueimonde et al., 2010). As described in Section 10.3.1, this was instead accomplished by mutagenesis and screening. Similar approaches would be required to eliminate the ability to produce biogenic amines, as this property is not normally plasmid or transposon based.

10.3.4 Genomics

The determination and analysis of the complete sequence of the genetic material in an organism is commonly referred to as genomics. Due to a significant reduction in both the time required and the cost of genome sequencing, this has become a powerful method of strain characterization (Danielsen & Johansen, 2009). Currently there are more than 2900 bacterial genome sequences, including more than 100 genome sequences of lactic acid bacteria, available in public databases. In addition, many companies have unpublished genome sequences for their most important industrial strains.

Knowledge of the complete genome sequence can be very useful for developing strains for industrial fermentations and allows a rapid approximation of the metabolic potential of the strain. Methods exist for ruling out the presence of unwanted genes, such as antibiotic-resistance genes and virulence factors, in a strain of interest through genome sequence analysis (Bennedsen et al., 2011). Comparison of genome sequences can also be a very effective way of differentiating closely related strains and can provide clues to differences in the properties of the specific strains (Garrigues, Johansen,

& Crittenden, 2013). In addition, possession of the complete genome sequence of an organism facilitates the development of several powerful analytical methods (Garrigues, Stuer-Lauridsen, & Johansen, 2005). Microarrays can be designed based on a genome sequence and used for comparative genome hybridization and analysis of gene expression under a variety of conditions. Proteomics is facilitated by the prediction of the amino acid sequence of the various proteins found in the cell, allowing accurate identification of specific proteins in a sample. Finally, possession of a complete genome sequence can be used to rationalize strain improvement activities. The selection of *S. thermophilus* mutants able to grow on galactose (Section 10.3.2) would be quite futile if the galactokinase gene were absent.

One particularly promising use of genome sequence analysis is in the identification of the alterations introduced into a strain using the classical strain improvement methods described in Section 10.3. For example, we have determined that a citrate nonfermenting *Oenococcus oeni* strain useful for malolactic fermentation in white wine has a nonsense mutation in the citrate transporter gene, that a mutant of *L. lactis* that no longer produces lactic acid but that scavenges oxygen has a mutation in the lactate dehydrogenase gene, and that a tetracycline-sensitive variant of *B. animalis* subsp. *lactis* has a frameshift mutation in the *tet*(W) gene ubiquitous in this subspecies. Most importantly, analysis of the genome sequence allows the identification of unintended mutations in a strain that could have an impact on the industrial performance.

10.4 Future trends

Recombinant DNA technology can be used to genetically improve bacterial strains for use in industrial processes including food applications. A working definition of "food-grade" genetically modified organisms has been elaborated (Johansen, 1999) and a large variety of food-grade genetic modifications can be made. Specific genes can be partially or totally eliminated from a strain or replaced with different alleles from other strains of the same genus. Likewise, new properties can be introduced into a strain by the transfer of a gene absent in the specific strain but present in other members of the genus. The presence of recombinant DNA can be used to isolate mutants with interesting properties (Curic, Stuer-Lauridsen, Renault, & Nilsson, 1999). Expression of genes can be increased, either by cloning onto a food-grade plasmid (Sørensen, Larsen, Kibenich, Junge, & Johansen, 2000), or by changing the gene regulatory sequences. Examples of microorganisms fitting the definition of "food-grade" that can be used to improve flavor development in cheese have been published (Guldfeldt et al., 2001; Joutsjoki et al., 2002) but, as described below, have not been used commercially. Genome sequence analysis (see Section 10.3.4) can be used to confirm the totality of the genetic changes introduced into a genetically modified organism and confirm that the strain indeed conforms to the working definition of "food-grade".

In spite of the speed and precision with which genetically modified organisms can be produced, these are not commercially used in industrial fermentation processes. One notable exception is in the production of specific enzymes that are subsequently separated from the production organism. The reasons for this lack of use are many-fold and

include regulatory hurdles, labeling requirements, and a lack of consumer acceptance for foods made using recombinant DNA technology in many parts of the world. Clearly, future development of strains for fermentation applications will be based on screening of natural isolates and classical strain improvement as described in Sections 10.2 and 10.3.

10.5 Sources of further information and advice

The patent literature is an often neglected source of scientific information. Due to the economic value of novel bacterial strains, many companies and universities seek patent protection of these specific strains and the methods used to improve key properties. A quick search using the Google Patent search engine (Google Inc., Mountain View, USA) reveals more than 20,000 hits when the search terms "lactic acid bacteria" and "strain improvement" or "strain screening" are used. Clearly, the United States Patent and Trademark Office and European Patent Office files searched contain a vast amount of data, much of which is not mined by routine scientific literature searches. Several of the examples described in this chapter have not been previously mentioned in the scientific literature but are referred to in the indicated patent applications. To assure an in-depth review of strain screening and strain improvement methodologies and techniques it is therefore highly recommended to include patent literature as a source of information.

Several available textbooks review strain screening and selection methodologies. For example, Crook and Alper (2013) describe several strategies for strain improvement and strategies to improve phenotypic parameters, while the methodology behind the isolation of bacteriophage-resistant mutants is described by Høier et al. (2010). Finally, it can be mentioned that the web sites of companies providing equipment for automation of laboratory procedures is a rich source of information on what types of equipment are available as well as inspiration for what this equipment can be used for.

Acknowledgments

The authors are grateful to Jannik Vindeløv for data and assistance with the preparation of some of the figures. We also thank Thomas Janzen, Elke Brockmann, and Hentie Swiegers for helpful discussions.

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Advances in starter culture technology: focus on drying processes

11

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11.1 Introduction

In the production of fermented food, starter cultures are used to prevent fermentation failure and to ensure high-product quality. Starter cultures are cultures with well-defined properties that ensure a fast, safe, and defined fermentation and lead to fermented food products with high and constant product quality. The use of defined starter cultures is state-of-the-art in the dairy industry, and replaces traditional procedures in the production of meat and bakery products and other fermented commodities. Starter cultures are mostly produced by specialized companies that distribute the cultures worldwide, and are being used increasingly in concentrated forms for direct inoculation into the food matrix (direct-to-vat-set cultures, DVS; Hansen, 2002).

Gentle and energy-saving preservation processes ensuring high vitality and metabolic activity are decisive for effective production and distribution of starter cultures. Therefore, the preservation technology has to reach four targets:

1. **Viability.** The number of viable cells must be maximal after preservation, as each loss in viable cell numbers means a higher number of expensive and energy-intensive fermentation batches.
2. **Vitality.** The metabolic activity must be high. As a large group of starter cultures are producing organic acids (particularly lactic acid), the rate of acidification must be high and the lag phase must be short to ensure a safe fermentation and to prevent spoilage and phage infections.
3. **Storage stability.** The third important quality attribute is the storage stability. As the cultures are distributed worldwide, they must withstand adverse conditions during transport. Furthermore, they must retain their viability and vitality during storage, preferably at ambient temperatures (nonrefrigerated).
4. **Handling.** Depending on the preservation technology, the handling of the cell concentration must be easy and, in case of a powder, the rehydration must be fast. The powder should not be prone to caking and should be easy to allot.

Freezing and freeze drying of starter cultures are regarded as gentle processes in the preparation of starter cultures and are therefore state-of-the-art, although cellular damage can result from the freezing process (de Valdez & de Giori, 1993). For frozen cultures, the viability and vitality is generally high (Santivarangkna, Higl, & Foerst, 2008), but transport is energy-intensive and expensive as it has to be carried

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00011-6

out at subzero temperatures on dry ice. The storage temperature for frozen cultures must be below −40 °C. Freeze-dried cultures are much lighter than frozen cultures, as about 90% of the water has been removed during drying; therefore, transport of significantly lower bulk is much more simple and cheaper than for frozen cultures. Freeze-dried cultures should be kept at −18 °C during storage for high-storage stability; however, during transport the cold chain can be interrupted for a short time. As freezing and freeze drying are energy-intensive preservation technologies, alternative technologies are pursued. Different drying techniques are investigated with regard to their suitability to preserve bacterial cultures (Melin, Hakansson, & Schnurer, 2007; Peighambardoust, Goshan Tafti, & Hesari, 2011; Santivarangkna, Kulozik, & Foerst, 2007). Examples of these techniques include convective air drying methods such as spray drying and fluidized bed drying, contact drying methods such as vacuum belt drying, and radiative drying methods such as microwave drying.

11.2 Protective agents

In order to minimize damage during drying, protective agents are added to the cell suspension before drying. Protective agents can be simple substances such as mono-, di-, oligo-, or polysaccharides, or more complex substances such as skim milk powder. Several mechanisms for the protective effect are proposed: (1) preferential hydration, (2) water replacement, and (3) glass transition (Santivarangkna, Higl, & Foerst, 2008).

1. The protection due to preferential hydration is effective at water content higher than ~0.3 g H_2O/g dry weight. However, a number of cell components, especially the lipid membrane, are damaged at lower water content.
2. In normal membranes, phospholipids are hydrated, and the removal of water to less than 0.25 g H_2O/g dry weight may cause leakage of membrane components due to packing defects from phase separation between liquid-like and solid-like domains. It has been proposed that protectants can stabilize the cell membrane by replacing water and interacting with phospholipids during drying.
3. The glass matrix of a protectant can improve storage stability of dried cells by raising the T_g of starter cultures. The high viscosity of glasses below or around their T_g is generally believed to retard molecular mobility and hence slow down the deteriorative reactions.

It is not yet possible to predict the most suitable compound for protection, and therefore time-consuming optimization procedures are required. Furthermore, the protective effect of one specific compound is dependent on the drying method and on the previous processing steps, especially the fermentation step. There are strong interrelationships between the fermentation step and the drying step with regard to the effects of added protectants; i.e., the same protectant may be more or less effective depending on the previous fermentation step (Carvalho et al., 2003, 2004). These characteristics make process development a very complicated and time-consuming task. It is extremely important to understand the protective effects on living cells on a molecular level.

11.3 Starter culture fermentation process

For efficient stabilization of starter cultures, a holistic approach has to be pursued and the interaction between the single processes in the production process of starter cultures must be taken into account (Bauer, Schneider, Behr, Kulozik, & Foerst, 2012; Carvalho et al., 2004). The single steps of the production process are depicted in Figure 11.1. In Figure 11.2, a fishbone diagram is shown that lists the most decisive factors influencing product quality in the different process steps.

The most decisive process steps are the fermentation process and the drying process. The factors during the fermentation process that influence vitality and viability after drying are the fermentation medium (especially the carbon source), the temperature and pH, the neutralization agent, the harvesting time, and the cell concentration before drying (Meng, Stanton, Fitzgerald, Daly, & Ross, 2008; Palmfeldt, Radstrom, & Hahn-Hagerdal, 2003). Furthermore, the impact of drying conditions is not independent from fermentation conditions; i.e., the optimum drying conditions will be

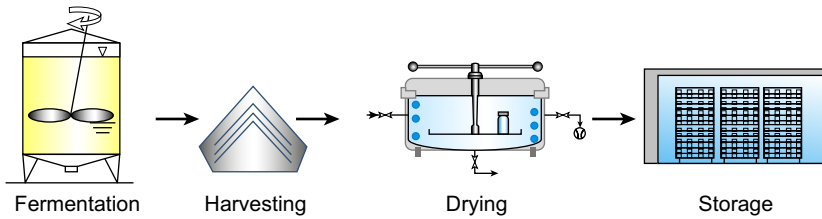

Figure 11.1 Schematic drawing of process steps in the production chain of concentrated and dried starter cultures.

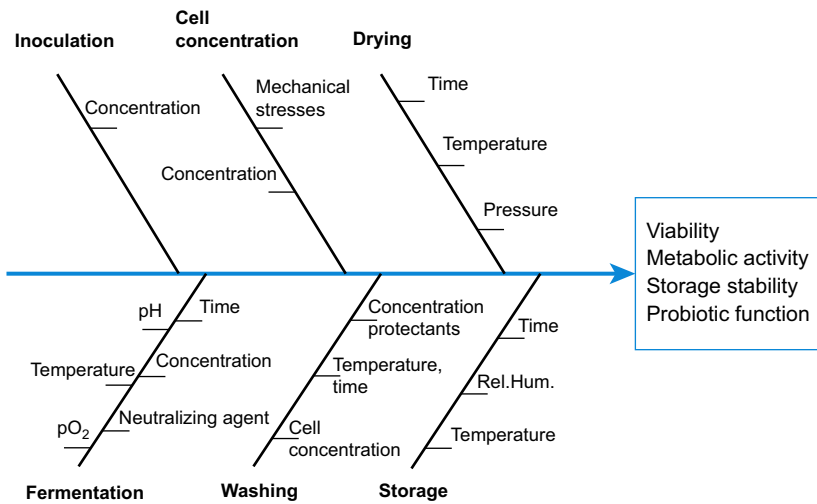

Figure 11.2 Fishbone diagram of the factors influencing the survival of microbial cells after drying.

determined by the fermentation conditions, especially the pH during fermentation (Bauer et al., 2012).

Growing the pure culture of cells is the first step for the preparation of starter cultures. The culture media and conditions can influence viability of the starters during preservation. Lactic starter cultures grow generally better in the rich media than in milk, but the media are too expensive for the industrial-scale preparation of bulk starters. Skim milk and whey-based media are more common and economical for the industries. Growth-promoting nutrients are often added and some of them can improve stability of starter cultures. The acidification activity of *Streptococcus thermophilus* was improved after frozen storage at −20 °C for 8 weeks when adding oleic acid in whey-based medium (Beal, Fonseca, & Corrieu, 2001). Moreover, different sugars in growth media may affect the production of some important metabolites, and some metabolites may be responsible for the distinct stability of dried starter cultures (Carvalho et al., 2003). For example, *Weisella cibaria*, *Lactobacillus plantarum*, and *Pediococcus pentosaceus* (Di Cagno et al., 2006) produced EPS when sucrose was used as a carbon source. Lactose, fructose, and galactose increased the amounts of EPS produced by *Lactobacillus helveticus* (Torino, Hebert, & Mozzi, 2005), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Carvalho et al., 2004), and *Lactobacillus casei* (Mozzi, Rollan, & Giori, 2001), respectively. Mannitol, which is able to stabilize cells at low water activity and prevent cells from oxidative damage, is produced in high concentrations by *Leuconostoc* (pseudo) *mesenteroides* (Wisselink, Weusthuis, Eggink, Hugenholtz, & Grobben, 2002). The industrial-scale cultivation of lactic starter cultures is free-cell batch fermentation. The fermentation pH is maintained at optimal ranges to obtain high biomass yield. Neutralizing agents used are NH_4OH, Na_2CO_3, KOH, NaOH, and gaseous NH_3. Among them, calcium hydroxide was shown to improve viability of *Lactobacillus bulgaricus* after freezing and freeze drying (Wright & Klaenhammer, 1983), while growth of lactic starter cultures is less inhibited by calcium lactate than ammonium lactate or sodium lactate (Stadhouders, Janson, & Hup, 1969). Nonetheless, it was reported that cells of *L. delbrueckii* subsp. *bulgaricus* grown under uncontrolled pH (end fermentation pH was ~4) show higher stability after spray drying and heating (Silva et al., 2005).

Recently, a change from the traditional anaerobic fermentation to an aerobic respiration process has been introduced for *Lactococcus* spp. The lactic acid bacteria exhibit a respiratory lifestyle in the presence of oxygen and heme. Consequences of the respiration are the higher biomass and lower amount of lactic acid produced. However, the aeration and heme did not increase the biomass yield of *Streptococcus thermophiles*, *L. delbrueckii subsp. bulgaricus*, or *L. helveticus* (Pedersen, Iversen, Sorensen, & Johansen, 2005).

In addition to batch fermentation, membrane bioreactor (Taniguchi, Kotani, & Kobayashi, 1987; Taniguchi et al., 1987b) and immobilized cell systems (Doleyres, Fliss, & Lacroix, 2004) were also investigated. Cells are retained in a bioreactor with continuous feeding of fresh medium by the membrane filtration and the entrapment of cells in biopolymer matrices, respectively. The continuous fermentation processes give higher yields and productivity, but a disadvantage is that the process is more susceptible to contamination and cell characteristics can be lost over time (Lacroix & Yidirim, 2007). So far, continuous fermentation is still mainly for experimental purposes.

Following the cultivation, cells must be harvested and concentrated before the production of frozen and dried concentrated lactic starter cultures. Harvest of cells at the stationary phase yields a higher amount of biomass and increases the tolerance of cells to stresses. In comparison to log-phase cells, higher cryotolerance were reported in stationary-phase cells of *Lactococcus lactis* subsp. *lactis* (Kim, Ren, & Dunn, 1999; Wouters, Rombouts, Vos, de, & Abee, 1999), *L. bulgaricus* and *S. thermophilus* (Fonseca, Beal, & Corrieu, 2001), and *L. lactis* subsp. *diacetylactis* (Aerts, Lauwers, & Heinen, 1985; Lee, 2004). Similarly, higher viability after spray drying was reported for stationary-phase cells of *Lactobacillus rhamnosus* (Corcoran, Ross, Fitzgerald, & Stanton, 2004) and *L. delbrueckii* subsp. *bulgaricus* (Teixeira, Castro, & Kirby, 1995).

Centrifugation is conventionally employed to harvest and concentrate cells. A high viscosity medium needs stronger centrifugal conditions to harvest cells. Moreover, in some cultures it is difficult to obtain a complete recovery of cells by centrifugation. In a study with four *Bifidobacterium longum* strains, *B. longum* S9 and *B. longum* III produced firm cell pellets, whereas *B. longum* ATCC 15,707 and *B. longum* II produced soft cell pellets. The soft pellet is an indication of difficulty in recovering cells by centrifugation (Reilly & Gilliland, 1999).

Alternatively, cross-flow membrane filtration can be used to concentrate cells. An advantage of cross-flow filtration is a possibility to obtain the complete recovery of cells and the reduced costs (van Reis & Zydney, 2001). Disadvantages of membrane filtration are the longer separation time and the formation of filter cake. The filtration may also affect cell physiology. Following the filtration, *L. bulgaricus* CFL1 had increased unsaturated to saturated and cyclic to saturated fatty acid ratios, resulting in improved cryotolerance of cells to freezing and frozen storage (Streit, Athes, Bchir, Corrieu, & Beal, 2011).

11.4 Freeze drying for the production of dried starter cultures

The second very important factor in the production chain is the preservation process, i.e., the method of dehydration for the production of dried starter cultures. Freeze drying is state-of-the-art and is considered to be the most sensitive drying method; however, depending on the strain to be dried, the freezing damage can be very high and dominate the effect of lyophilization on survival. This is especially true for freeze-sensitive cultures such as *L. delbrueckii* subsp. *bulgaricus* (de Valdez & de Giori, 1993; Bauer et al., 2012; To & Etzel, 1997b).

Freeze drying, also called lyophilization, is a process where the concentrated cell suspension (with added protectants such as sugars and antioxidants) is first frozen under atmospheric pressure, and then the water is sublimed at reduced pressure (sublimation), and at the end the residual water is desorbed at very low pressure (desorption) (Oetjen & Haseley, 2004). The different steps of the lyophilization process are shown in Figure 11.3. The freezing step is mostly carried out by immersion in liquid nitrogen, e.g., in form of liquid droplets or filled in cans. In this case the freezing rate is very high. The optimum freezing rate is dependent on the strain (Santivarangkna, Kulozik, & Foerst, 2008). After freezing, the frozen cell concentrate is placed on heatable shelves in the freeze dryer where the pressure is decreased to a point below the

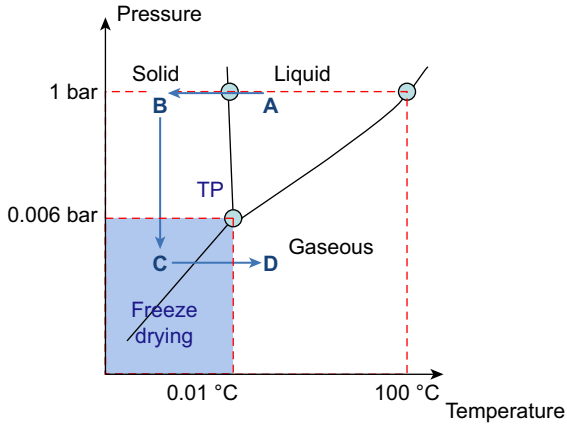

Figure 11.3 Lyophilization process shown in the phase diagram of water (A to D).

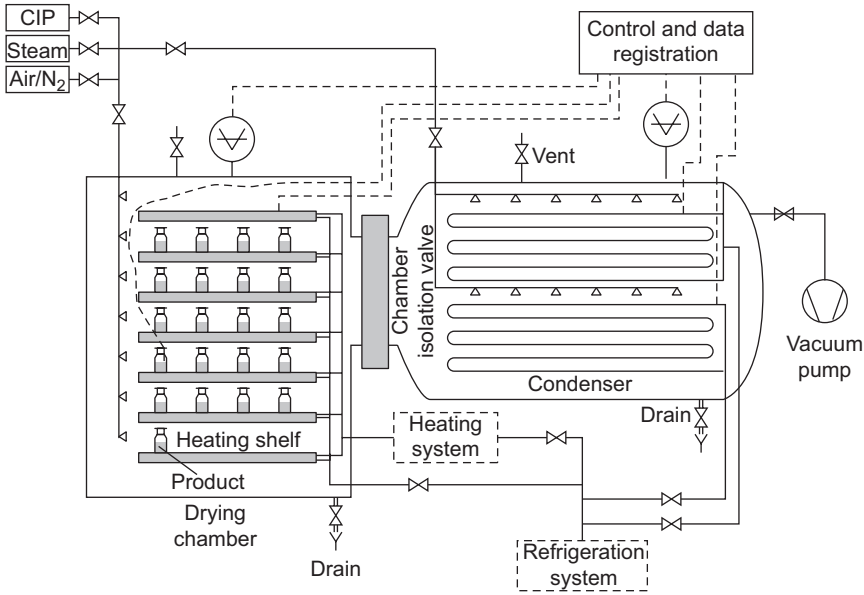

Figure 11.4 Schematic drawing of a freeze drier.
Modified according to Pupp and Hartmann (1991); Oetjen and Haseley (2004).

triple point of water ($p < 6.1$ mbar) to facilitate sublimation. The pressure determines the product temperature during sublimation according to the sublimation curve (see Figure 11.1). A pressure of 1 mbar refers to a sublimation temperature of −20 °C. In order to keep the sublimation process up, heat has to be supplied during sublimation (sublimation enthalpy at 1 mbar $r = 2837$ kJ/kg). This is done by heating the shelves up to a temperature higher than 0 °C. The developing vapor during drying is removed by a condenser, which is usually cooled down to −50 to −60 °C (see Figure 11.4). The

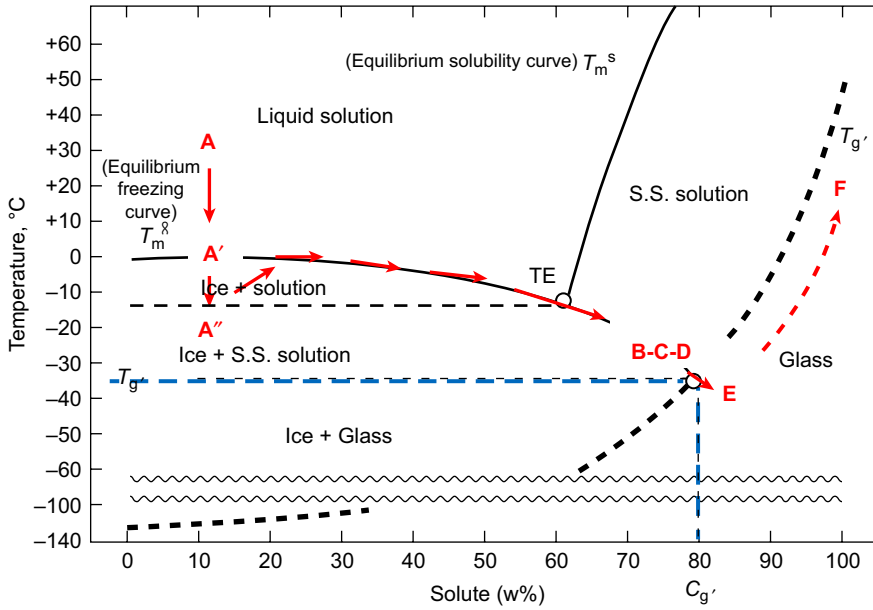

Figure 11.5 State diagram for an aqueous sugar solution and typical freeze-drying curve ABCDEF.

Modified according to Roos (1995), copyright 1995, with permission from Elsevier.

driving force for the process is therefore the temperature difference between sublimation temperature and temperature of the heated shelf (shelf temperature).

During sublimation, the frozen water is removed from the sample. Depending on the freezing temperature and the temperature $T_{g'}$ that refers to maximum freeze concentration, part of the water may not be frozen. Toward the end of the sublimation process, the temperature increases because of the decreasing sublimation rate. This behavior marks the beginning of the third drying phase, the desorption phase. In this phase, the shelf temperature is further increased and the pressure further decreased to accelerate the removal of the last portion of water. The temperature–concentration curve of a typical freeze-drying process is depicted in Figure 11.5. A–A″ depicts the cooling including undercooling. Due to freeze concentration of the remaining solution, freezing-point depression, and supersaturation of the solute, the main drying takes place at a temperature below the eutectic point of the solute, close to the temperature of maximum freeze concentration. After the sublimation is completed (B–D), the temperature is raised and further water is removed (E, F). The final water content should be below 4% (wet basis) in order to ensure sufficient stability during storage (Gardiner et al., 2000). The impact of water content on storage stability is addressed in Section 11.5.

The advantage of freeze drying is that the drying method is suitable to maintain the quality of sensitive products, though it is a highly time-consuming and expensive drying technique because of the very low pressures and temperatures. There is no shrinkage during drying, as only the water is removed from the frozen sample and the

solid substances build a solid and porous framework after drying (Oetjen & Haseley, 2004). The porous structure is influenced by the freezing process, and a fast cooling with a high undercooling leads to small ice crystals and a large inner surface (Franks, 1998). Due to the high porosity, the freeze-dried cell suspension has a high specific surface area. This influences the sorption behavior as well as rehydration and storage characteristics (Haque & Roos, 2005). Freeze-dried samples are easy to rehydrate and therefore exhibit good instant properties.

As only frozen water is removed during sublimation, the temperature during main drying (sublimation steps C and D in Figure 11.4) has to be low enough that the maximum possible fraction of water is frozen. This temperature is referred to as $T_{g'}$ and can be derived from the state diagram of the cell suspension. It has been suggested that the formation of a maximally freeze-concentrated matrix containing the protectant with entrapped microbial cells is essential for high viability after freeze drying (Pehkonen, Roos, Miao, Ross, & Stanton, 2008). Mostly, the protectants are glass-forming carbohydrates. It has been shown that the state diagram of the cell carbohydrate mixture is dominated by the carbohydrate. A glass transition of the cells could not be shown so far (Fonseca, Passot, Lieben, & Marin, 2004). The choice of suitable protectants is therefore important with regard to $T_{g'}$ and the required product temperature during sublimation (Aschenbrenner, Kulozik, & Foerst, 2012; Santivarangkna, Higl & Foerst, 2008).

Recently, the collapse temperature was proposed as critical temperature during sublimation; i.e., as the temperature that should not be exceeded to prevent the solid structure from flowing and therefore from a structural collapse (Fonseca, Passot, Cunin, & Marin, 2004). It is proposed that the collapse temperature is a more realistic measure for structural stability as it is determined by visual observation under realistic conditions (Fonseca et al., 2004). In contrast, glass transition temperature $T_{g'}$ is determined by differential scanning calorimetry at ambient pressure. Furthermore, it has been shown that the presence of the cells in the matrix increases the collapse temperature, but not the glass transition temperature. Therefore the cells serve as structural stabilizer. This finding was also repeated with inert glass spheres as structural stabilizer (Aschenbrenner, 2014). This finding is of high practical importance as a structural collapse can be avoided by a sufficiently high cell concentration and the main drying can be conducted at higher temperatures. An increase in 1 °C during main drying already leads to a reduction in drying time of 13% and also to energy reduction, as drying can be carried out at higher pressure and higher condenser temperatures (Barresi, Ghio, Fissore, & Pisano, 2009; Tang & Pikal, 2004).

More recently, it was even hypothesized that a structural collapse would not lead to a deleterious product quality as—unlike for pharmaceutical applications—optical appearance of the product is not a quality criterion for starter cultures (see Section 11.1). First investigations even show that the storage stability of collapsed products may be higher, probably due to the lower porosity and also drying time may be shorter (Schersch et al., 2010, 2012). This aspect including all the consequences on product quality has to be investigated further.

In general, freeze drying leads to high survival rates, high metabolic activity, and good rehydratability and therefore to a high product quality (Kiviharju, Leisola, & Eerikainen, 2005; Oetjen & Haseley, 2004). However, investment costs and energy

Table 11.1 Investment and energy costs of different drying technologies relative to the freeze-drying process

Drying technology	Rel. investment costs at same throughput (%)	Spec. energy costs (kWh/kg water)
Freeze drying	100	2.0
Vacuum drying	65.2	1.3
Spray drying	52.2	1.6
Air drying	43.5	1.9

Modified from Regier et al. (2004).

cost per kg removed water are higher than for other drying processes (see Table 11.1). Furthermore, some strains exist that exhibit a very low survival after freeze drying (e.g., *L. delbrueckii* subsp. *bulgaricus*, de Valdez & de Giori, 1993).

Regarding the recent developments in process and formulation optimization together with the actual work on collapse drying (Aschenbrenner, 2014; Barresi et al., 2009), it becomes obvious that the potential of optimization the freeze-drying process is very large, especially with regard to acceleration of the process and energy saving. Nevertheless, there is a need for alternative drying technologies that are faster and more energy-efficient and may be beneficial for some strains. Furthermore, alternative drying processes may lead to enhanced long-term stability, which is one of the most important quality criteria for starter cultures.

11.5 Spray drying for the production of dried starter cultures

As it has not been clearly shown for starter cultures that the glassy state is an essential prerequisite for high cell vitality, and because of the high costs of the freeze-drying process, and also the long drying times, alternative drying techniques for starter cultures are becoming more and more important. The alternative drying processes that are mostly investigated for the drying of starter cultures are spray drying and vacuum drying. The greater focus in literature lies on spray drying, as it is a continuous process with high throughput, common in the food industry, particularly in the dairy industry. Furthermore, spray drying is considered to be a sensitive drying technique as the maximum product temperature always stays below the air outlet temperature and the residence time is short (Fu & Chen, 2011; Peighambardoust et al., 2011; Perdana, Fox, Schutyser, & Boom, 2011, 2012).

For spray drying, the cell suspension is pumped as liquid concentrate to the dryer and separated in small droplets by means of a pressure nozzle (one or two fluid nozzles) or a rotary disc. The mode of particle formation in the drier also influences the viability of the bacteria after drying (Ghandi, Powell, Chen, & Adhikari, 2013). A schematic spray drier is shown in Figure 11.6.

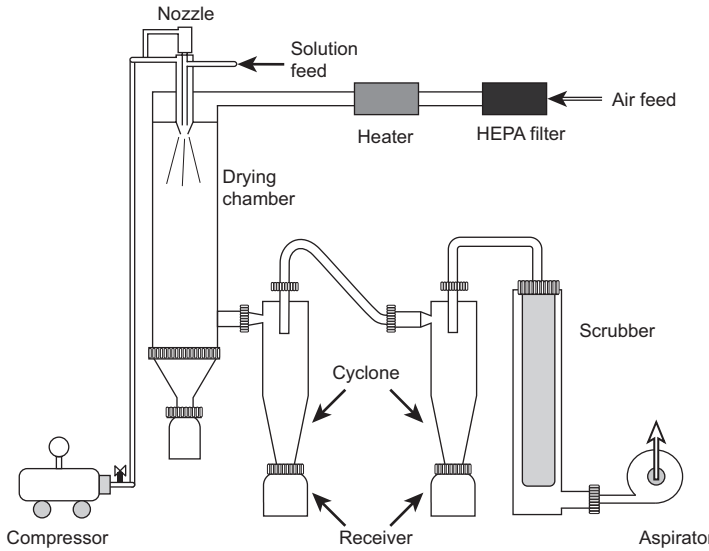

Figure 11.6 Schematic drawing of a co-current spray drier. HEPA, high-efficiency particulate air. Modified according to Devakate et al. (2009), copyright 2009, with permission from Elsevier.

The droplets are then mixed with a large amount of hot and dry air (inlet temperatures between ~150 and 200 °C) (Peighambardoust et al., 2011), and the water at the surface is immediately evaporating and the surface temperature cools down to wet bulb temperature due to evaporation during the constant rate period of drying. The wet bulb temperature is considerably lower than the inlet temperature. For example, inlet air with a temperature of 200 °C and a water content of $x = 0.01$ g water/g dry air has a wet bulb temperature lower than 50 °C. The wet bulb temperature is influenced by the condition of the inflowing air (water content and temperature) and can be derived from the Mollier-h-x diagram for wet air.

In the falling-rate period of drying, the particle temperature rises above the wet bulb temperature as the rate of evaporation is decreasing due to falling water content. The temperature of the particle at the end of drying depends on the outlet air temperature. The outlet air temperature is the decisive factor for survival after drying, and a low outlet air temperature correlates with high survival. However, the final water content of the particles is also coupled to the outlet air temperature, and a low outlet temperature leads to high residual water content. This, however, means low storage stability (Ananta, Volkert, & Knorr, 2005; Desmond, Ross, O'Callaghan, Fitzgerald, & Stanton, 2002; Kim & Bhowmik, 1990; To & Etzel, 1997a). The outlet air temperature depends on the inlet air temperature, airflow rate, product feed rate, medium composition, and atomized droplet size (Santivarangkna, Kulozik, & Foerst, 2007, 2008). As the outlet temperature is dependent on many factors and is not an independent variable, it is difficult to control.

The residence time distribution of the particles depends on the design of the drier, the airflow pattern, and the particle size distribution. For starter culture drying it is

important to achieve a short residence time and narrow residence time distribution in the drier (Ghandi et al., 2013). For lysine blockage in infant formula, it has been shown in our own group that it is significantly influenced by the residence time distribution in the drier. This behavior is also applicable to the spray drying of microorganisms, as the exposition time at a lethal temperature determines the survival rate after drying (Fu & Chen, 2011). Typical survival rates for different strains after spray drying are listed in by Santivarangkna et al. (2007). For optimum conditions, i.e., a short residence time and narrow residence time distribution, and depending on the strain, survival rates can be close to 100%.

Several attempts have been made to overcome the disadvantages of the spray drying process, i.e., the high inactivation in the falling rate period due to high temperatures and the long residence time when lower air temperatures are applied. Semyonov, Ramon, and Shimoni (2011) developed an ultrasonic vacuum spray drier combined with fluidized bed drier to produce highly viable probiotics under reduced oxidative and thermal stresses. Chávez and Ledeboer (2007) combined spray drying with vacuum drying. The spray drying was carried out mainly in the constant rate period where the temperature is low, and the vacuum drying was carried out in the second drying stage to prevent thermal and oxidative stresses. Stadhouders et al. (1969) also used vacuum drying as a postdrying step after spray drying.

11.6 Vacuum drying for the production of dried starter cultures

As mentioned in the previous section, vacuum drying is used as a postdrying step, because of the gentle drying temperatures due to reduced pressure and reduced boiling point of water (Santivarangkna et al., 2007). Vacuum drying can also be used as sole drying method, and is currently explored by several research groups as a potential alternative to freeze drying due to the gentle drying (Bauer et al., 2012; Bauer, Kulozik, & Foerst, 2013; Higl, Santivarangkna, & Foerst, 2008; Tymczyszyn, Del Rosario, Gomez-Zavaglia, & Disalvo, 2007; Tymczyszyn et al., 2008). The vacuum drying process is mostly a conductive drying process where the heat is transferred via conduction, but can also be combined with microwaves in the microwave vacuum drying process. For a batch conductive vacuum drying process, the sample is placed in suitable containers on a heatable shelf. The equipment is similar to that of freeze drying (Figure 11.4), but the setup is more simple as no condenser is needed and the requirements for the vacuum pump are lower as the operating pressure is higher than for freeze drying. The working area for vacuum drying is schematically depicted in Figure 11.3. For the continuous vacuum drying process, the sample is spread on a moving belt. For the microwave vacuum drying process, the energy is supplied via a magnetron that applies microwave irradiation with a frequency of either 2450 or 915 MHz (McLoughlin, McMinn, & Magee, 2003). According to the phase diagram of water, the boiling point of water is reduced at low pressure, leading to the possibility to carry out the drying

process at low temperatures. Furthermore, the oxygen partial pressure is reduced and therefore oxidative reactions are minimized.

If the vacuum drying process is driven close to the triple point of water ($p_{tr} = 6.1$ mbar, $T_{tr} = 273.16$ K), then the process is referred to as controlled low-temperature vacuum drying. The working range for the low-temperature vacuum drying process is close to the operating conditions of freeze drying, but the sample is not frozen (Higl, 2008). The product temperature during the constant rate period can be derived from the process conditions and the phase diagram of water (Figure 11.3). In the falling rate period, the product temperatures rise until reaching the shelf temperature at the end of drying. In comparison to spray drying, typical drying times are longer and are on the order of several hours.

Depending on the process parameters, the damages during vacuum drying may be reduced to only dehydration damages, leading to a high viability of dried microorganisms. King and Su (1993) have shown that the survival rate of *Lactobacillus acidophilus* after low temperature vacuum drying is comparable to that of freeze drying. Similar results were found by Higl (2008) and Bauer et al. (2012). It is shown for *Lactobacillus paracasei* subsp. *paracasei* that the survival rate at optimum conditions is comparable to freeze and vacuum drying and that the optimum working ranges for both drying techniques are around the triple point of water (for freeze drying below and vacuum drying above the triple point). Bauer et al. (2012) demonstrated that the optimum process conditions for vacuum drying depend on the previous fermentation step. This characteristic makes the optimization procedure to produce highly viable starter cultures a very complex task. An interrelation between fermentation conditions and the effect of protectants during vacuum drying was also found by Tymczyszyn, Gomez-Zavaglia, and Disalvo (2007).

The survival rate after vacuum drying can be increased by the use of an appropriate drying medium. As the most suitable protectants are dependent on the drying process, and the protective mechanisms are not yet fully understood, the most appropriate protectants must be found in elaborative experiments. For vacuum drying, the following protectants were shown to be effective: sorbitol (Foerst, Reitmaier, & Kulozik, 2006; Santivarangkna, Kulozik, & Foerst, 2006), trehalose and sucrose (Tymczyszyn et al., 2007), and skim milk powder (King, Lin, & Liu, 1998).

Due to the fact that vacuum drying is mainly carried out as conductive drying method, one limiting factor is the heat transfer in the product. Another limiting factor that makes the vacuum drying process a lengthy process is the mass transfer of vapor out of the sample. Due to the missing freezing step, the sample is not frozen before drying, and therefore the liquid sample shrinks during drying. This characteristic leads to a very compact structure of the vacuum-dried sample. The diffusion limitation leads to a long falling rate period, making the vacuum drying process lengthy. As the inactivation during drying is a time-dependent process, very long drying times should be avoided (Li, Lin, Chen, Chen, & Pearce, 2006). Even after long drying times, the residual water contents after vacuum drying are higher than for freeze drying (Bauer et al., 2012; Higl et al., 2008).

For optimization of the vacuum drying process it is important to know the concentration and temperature-dependent inactivation kinetics and to link them to the drying

kinetics (Foerst & Kulozik, 2012; Li et al., 2006). The inactivation kinetics depend on the inactivation mechanisms. It was shown that in a low-temperature regime, inactivation mechanisms are different from a high-temperature regime (Aljarallah & Adams, 2007; Tymczyszyn et al., 2008). The concentration dependence of the inactivation rate is different at low temperatures compared to higher temperatures above 50 °C, and it was shown that a medium water activity exists where microbial inactivation is highest. This leads to a drying process with dynamically adapted process parameters where the most detrimental water activity range is passed either very fast or at reduced temperatures and/or pressures. In order to tackle this, the impact of process parameters on drying rate and product temperature must be known. Due to heat and mass transfer limitations, the process-control possibilities are limited. Furthermore, control possibilities to run the process are required.

In order to speed up the vacuum drying process and to get a less compact structure and a larger inner surface similar to freeze drying, the vacuum drying process can be combined with radiative heat transfer to the microwave vacuum drying process. With microwaves the energy is brought volumetrically into the sample and high energy densities may be possible leading to a volume expansion (Zhang, Zhang, Shan, & Fang, 2007). Another advantage is the selective heating of water in the sample as long as the residual moisture content is high. No temperature gradient is therefore needed for heating up the sample and therefore potential overheating is minimized. The drying rate during microwave drying can be well controlled via the chamber pressure and the absorbed energy in the sample. The absorbed energy depends on the dielectric properties of the sample components. In order to avoid the formation of hot spots it is important to bring in the radiation energy evenly. This is mainly done by rotating the sample by a turntable. With microwaves the vacuum drying process can be accelerated up to five times (Ahmad, Yaghmaee, & Durance, 2012). The survival rates after microwave vacuum drying are comparable to those after vacuum drying if optimal process parameters are chosen (Ahmad et al., 2012; Cardona, Driscoll, Paterson, Srzednicki, & Kim, 2002; Kim et al., 1997). It has been shown that the power input is the most decisive factor for survival, as power input is directly correlated with product temperature (Ahmad et al., 2012).

As the microwave drying process can be controlled by the absorbed energy in the sample and the energy input is more uniform than for contact drying (lower temperature gradients within the sample), the dynamic adaptation of process parameters seems to be possible. This process is subject of actual research of our group.

11.7 Product characteristics and storage stability

The dried cell concentrate is typically produced by specialized suppliers and distributed worldwide. As the producers of fermented foods want to build up some stock, the cultures should be stable for at least one year. As the activity of the cultures after drying depends on the previous fermentation step (see Section 11.3), and the storage stability depends on the drying process as we will show in this section, the starter culture preparation process must be looked at in a holistic approach. This

means that it does not make sense to optimize survival and vitality in each process step separately. Furthermore, a lower yield in one process step could still lead to a higher overall performance of the process. This highlights the importance of studying the interrelationships between the processing steps; i.e., fermentation, drying, and storage processes. In many cases the inactivation during storage is described by a first-order reaction, i.e., the decline in cell number depends on the cell concentration (Ananta et al., 2005; Foerst, Kulozik, Schmitt, Bauer, & Santivarangkna, 2012; Higl et al., 2007):

$$\frac{N}{N_0} = e^{-k \cdot t} \tag{11.1}$$

Here, N is the number of living cells at time t, N_0 is the initial number of cells, k is the inactivation constant, and t is the time. The inactivation constant k is in general a function of moisture and temperature (Foerst & Kulozik, 2012; Fu & Chen, 2011). Some authors observed a deviation of first-order kinetics and found a stronger decline at the beginning of storage (Teixeira, Castro, Malcata, & Kirby, 1995). This behavior could be due to nonequilibrium conditions at the beginning of storage. Then the stronger decline at the beginning of storage would coincide with the time to reach equilibrium. It is important to apply the reaction kinetics to conditions in equilibrium, i.e., if the water activity after drying differs from the relative humidity of the storage atmosphere, the sample moisture equalizes first, leading to a deviation of first-order kinetics.

In general, the storage stability of dried cultures for a given strain depends on the moisture content, the storage temperature, the presence of protectants, the presence of oxygen, and the physical state of the sample.

With regard to moisture, the stability increases with decreasing moisture content down to a certain moisture level. Below this level the stability becomes less. The maximum stability is found for a moisture level of 3–4% (wet basis) (Pehkonen et al., 2008; Scott, 1958; Zayed & Roos, 2004). The impact of moisture on stability is temperature dependent. At higher storage temperatures, the moisture has a higher impact on stability (Aschenbrenner et al., 2012; Higl et al., 2007).

The glassy state of the drying matrix is assumed to be a trigger for stability as it limits bimolecular reactions (Schmitz-Schug, Gianfrancesco, Kulozik, & Foerst, 2013). If the inactivation constant is plotted against $T–T_g$, the distance of the storage temperature to the glass transition temperature, the inactivation constant should sharply increase above the glass transition temperature. One plot is shown in Figure 11.7 for freeze-dried *L. paracasei* ssp. *paracasei* (F19). As water acts as a plasticizer, the glass transition temperature of the drying matrix is a function of moisture and decreases with increasing moisture. Therefore, the distance ($T–T_g$) is a function of both moisture and temperature. It is shown that absolute stability is only given for values of about ($T–T_g$) = −50 °C, as all molecular motions are frozen at that temperature (Schoug, Olsson, Carlfors, Schnurer, & Hakansson, 2006). However, Aschenbrenner et al. (2012) have shown that this value also depends on the protectant.

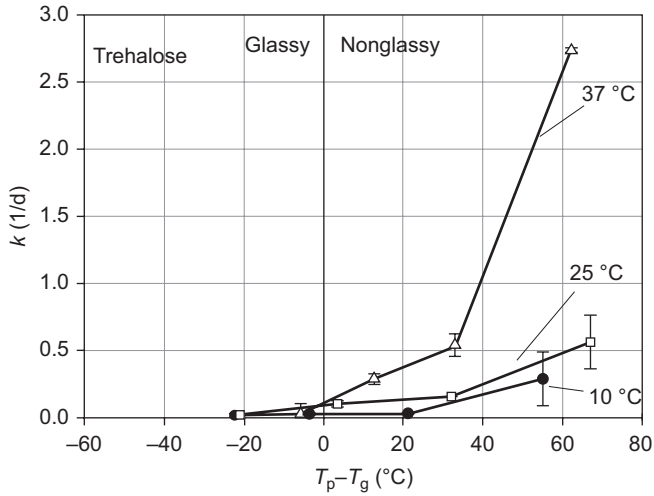

Figure 11.7 Inactivation constant during storage of *L. paracaei* ssp. *paracasei* as a function of (T–T_g) with trehalose as drying matrix. Right-side of the solid line, the matrix is in the rubbery state; left-side of the solid line, the matrix is in the glassy state.
Reprinted from Aschenbrenner et al. (2012), copyright 2012, with permission from Elsevier.

With regard to temperature, storage temperature has a large impact on stability. The temperature dependence of the inactivation constant is described by the Arrhenius function:

$$k = k_0 \cdot e^{\left(-\frac{E_a}{R \cdot T}\right)} \tag{11.2}$$

where k is the inactivation constant, k_0 the inactivation constant at infinite temperature, E_a the activation energy, R the universal gas constant, and T the temperature. Therefore, the storage stability exponentially decreases with increasing temperature. This makes storage at ambient temperatures much more difficult than at deep temperatures. It has also been shown that in the region above the glass transition temperature of the matrix (rubber region), the temperature dependence of the inactivation constant is even stronger than with Arrhenius behavior and can be described by the WLF kinetic with adapted WLF constants (Aschenbrenner et al., 2012; Higl et al., 2007).

The drying technology has a strong impact on microstructure of the dried sample and therefore on the sorption characteristics (Delgic, Pekmez, & Belibagli, 2012; Haque & Roos, 2005). It has been shown that the drying technology has a large impact on storage stability. This can be either due to a sublethal damage of the cells already during drying or to a different microstructure. For instance, a higher surface of a porous material could lead to more oxidation damages.

The storage stability of freeze-dried starter cultures may be lower than for products dried with other technologies, as the specific surface is large (Haque & Roos, 2005). This may lead to a higher rate of detrimental reactions such as oxidation during storage (Higl et al., 2007; Kurtmann, Carlsen, Risbo, & Skibsted, 2009). The storage

Table 11.2 Product viability after drying, storage stability, and handling for the different drying technologies

Description	Liquid culture (as reference)	Freeze dried	Spray dried	Vacuum dried
Initial viability	High	Intermediate	Low	Intermediate
Storage stability, room temperature	Low	High	Intermediate	High
Storage stability, refrigerated	Intermediate	High	High	High
Handling	High	High	Intermediate	Intermediate

Modified from Melin et al. (2007).

has to occur at low humidity in closed packages and low temperature. Commercially available freeze-dried starters for direct inoculation (DVS cultures) are stable for at least one year when stored below −18 °C.

The storage stability after spray drying is lower, especially at higher storage temperatures (Desmond et al., 2002). For vacuum-dried cultures the storage stability is superior to freeze-dried and spray-dried cultures, and even for adverse storage conditions in combination with suitable protectants the cultures are very stable. Foerst et al. (2012) have shown for *L. paracasei* ssp. *paracasei* that, when dried with sorbitol, almost no loss occurred at storage temperature of 20 °C even at high humidity. A good stability could even be achieved at a high temperature of 37 °C combined with low moisture ($a_w = 0.07$). An overview of the product characteristics with regard to handling and storage stability for the different drying technologies is given in Table 11.2.

11.8 Conclusion

Dried starter cultures are stable at nonfreezing temperatures and easier to handle than frozen cultures, which in contrast have higher viability and vitality/activity. The production of dried starter culture with high viability and vitality is a holistic process, where several factors in each step, starting from starter culture inoculation to storage of the dried cultures, can affect their viability and activity. Among drying processes, freeze drying is gentle and therefore results in a dried product with high survival, high metabolic activity, and good rehydratability. A major drawback of freeze drying is its high cost, which can be reduced by the process optimization, especially with regard to acceleration of the process and energy saving. Alternatively, drying processes with much lower costs have been explored and tested with starter cultures. These promising processes are, for example, spray drying and vacuum drying. The key advantage of spray drying is a continuous process with high productivity. Given the productivity, the vacuum drying can be accelerated up to five times with microwaves, whose power input is the most decisive factor for survival of the dried cultures. The storage stability of dried cultures depends on the moisture content, storage temperature, protectants,

the presence of oxygen, and the physical state of the sample, as well as the drying process used. Vacuum-dried starter cultures, with appropriate protectants, are more stable than spray- and freeze- dried cultures, especially at high storage temperature and relative humidity.

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Controlling the formation of biogenic amines in fermented foods

12

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12.1 Introduction

Fermentation is an ancient technique used for the preservation of a variety of perishable foods over an extended period of time. Fermented foods are the basis of many diets in the world, often handed down over the centuries, due to their nutritional value, practical storage, and palatability.

Nonetheless, toxic compounds, including biogenic amines (BAs) and ethyl carbamate, which are a potential risk to human health, may be synthesized by microorganisms present in the food. Ethyl carbamate is a genotoxic and carcinogenic molecule that may be present mainly in fermented beverages but also in bread, fermented milk products, and soy sauce. Wine is mostly at risk, since during the malolactic fermentation (MLF) different ethyl carbamate precursors such as citrulline, and to a lesser extent carbamoyl phosphate, are produced by yeasts or bacteria from proteins (Weber & Sharypov, 2009).

Apart from wine, increasing attention has been paid over recent years to the occurrence of BAs in fermented foods (Silla Santos, 1996; Spano et al., 2010). Therefore, in this chapter the current knowledge and perspectives concerning the origin, detection, and control of BA production in food will be described.

BAs are basic organic molecules of low molecular weight, generally resulting from the microbial decarboxylation of certain amino acids. The ability to form BAs is strain-specific yet quite widespread among species of lactic acid bacteria (LAB) and Gram-negative bacteria (Halász, Baráth, Simon-Sarkadi, & Holzapfel, 1994), and is based on the presence on amino acid decarboxylase enzymes. Thus, both the key microorganisms for the food fermentations and contaminating spoilage bacteria can contribute to BA formation in foods. Generally, histamine, tyramine, tryptamine, β-phenylethylamine, and the polyamines putrescine, cadaverine, spermine, and spermidine represent the most important BAs in foods (ten Brink, Damink, Joosten, & Huis in't Veld, 1990; Shalaby, 1996).

Under normal physiological conditions BAs are degraded in the gut lumen by the mono- and di-aminoxidase enzymes. However, higher levels than the normal intake of BAs from the diet, or pathological alterations of the detoxification system, can provoke several disorders in humans. In addition, substances such as alcohol or some drugs can inhibit the amino oxidase activities, and therefore reduce the critical

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00012-8

threshold for BA poisoning (Spano et al., 2010). The most important toxicological effects from BAs, known as histaminosis and "cheese reaction", are caused by high ingestion of histamine and tyramine, respectively. Since histamine plays a biological role as a neurotransmitter and vasodilator, an increase of its concentration in the bloodstream can result in a number of clinical manifestations affecting the hemodynamic (hypotension) and neurological functions (headache, palpitations, tingling, burning, itching); the gastrointestinal tract (nausea, vomiting, diarrhoea) and the skin (rash, urticaria, oedema and localized inflammation) (Spano et al., 2010). Histamine has been reported in dry fermented sausages and cheeses, where it is produced by the metabolism of *Lactobacillus* spp. (for instance, *Lactobacillus buchneri*, *Lactobacillus curvatus*), *Streptococcus thermophilus* and *Staphylococcus xylosus* (Cruz Martín, Fernández, Linares, & Álvarez, 2005; Gardini et al., 2012; Linares et al., 2012; Suzzi & Gardini, 2003). In fermented beverages, histamine – mainly produced by *Oenococcus oeni*, *Pediococcus parvulus*, *Pediococcus damnosus,* and some *Lactobacillus* spp. (Garai, Dueñas, Irastoza, & Moreno-Arribas, 2007; Ladero, Coton, et al., 2011; Lonvaud-Funel, 2001) – represents a potential risk due to the concomitant presence of alcohol. In addition, strains belonging to the same species may also be able to synthesize putrescine (Arena & Manca de Nadra, 2001; Beneduce et al., 2010). Polyamines, mainly putrescine and cadaverine, are generally considered less hazardous BAs, since at high levels they produce disagreeable "off flavors", which prevents the use of the contaminated food for consumption. Nonetheless, their presence may enhance the effect of histamine. Furthermore, polyamines are involved in cell proliferation and therefore associated with cancer events (Gerner & Meyskens, 2004). Moreover, polyamines can act as possible mutagenic precursors by formation of carcinogenic nitrosamine, when exposed to nitrite. For this reason, nitrate and nitrite usually added during sausage production are considered basically undesirable (Silla Santos, 1996).

Tyramine, together with tryptamine and β-phenylethylamine, are classified as vasoactive amines, as at high levels they can provoke a dangerous hypertensive crisis. The consumption of some antidepressant drugs strongly inhibits monoaminoxidase activity, thereby increasing the risk of tyramine ingested with meals. In addition, high tyramine levels are especially reported in artisanal produced cheeses. For example, very high concentrations of tyramine are found in blue cheeses, up to a maximum of 2010 mg/kg in Egyptian blue cheese (Fernández, Linares, Rodríguez, & Álvarez, 2007; Novella-Rodríguez, Veciana-Nogués, Izquerdo-Pulido, & Vidal-Carou, 2003; Rabie, Elsaidy, el-Badawy, Siliha, & Malcata, 2011). Strains belonging to the species *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans* are the main tyramine producers in cheese (Burdychová & Komprda, 2007; Fernández, Linares, & Álvarez, 2004; Fernández et al., 2007), fermented sausages (Bover-Cid, Hugas, Izquierdo-Pulido, & Vidal-Carou, 2000) and unfrequently in wine (Capozzi et al., 2011). In dairy products and sausages, *L. curvatus*, *Lactobacillus brevis* (Bover-Cid et al., 2000), *Carnobacterium* spp. (Masson, Talon, & Montel, 1996), *Staphylococcus* spp. and *Micrococcus* spp. (Straub, Kicherer, Schilcher, & Hammes, 1995) are also responsible for the final concentration of tyramine. Furthermore, De las Rivas et al. (2008) reported that *Staphylococcus carnosus* and *L. curvatus* produce significant concentrations of β-phenylethylamine in Spanish dry-cured chorizo sausage. In fermented

beverages, *L. brevis*, *Lactobacillus hilgardii*, *Lactobacillus plantarum* and *Leuconostoc* species have been reported as tyramine producers (Coton, Romano, et al., 2010).

Nowadays, the food industry has to ensure an adequate response to the requirement for food safety. In this work we report a global overview of the metabolic pathways, physiological factors, and monitoring of BAs, with the aim of identifying strategies for their reduction. Overall, we are confident that an in-depth understanding of the mechanisms that control the production of BAs is the most important step in eliminating their presence in food.

12.2 Molecular determinants of biogenic amine formation

The BA biosynthetic pathways are generally composed of a transport protein embedded in the cytoplasmic membrane, which is responsible for both the uptake of the precursor and the secretion of the BA; and a metabolic enzyme, which converts an amino acid into the corresponding amine by a decarboxylation reaction (Figure 12.1(a)). Exceptions are the putrescine biosynthetic pathways from agmatine or arginine, in which several enzymes are required for production of this BA (Figure 12.1(b)).

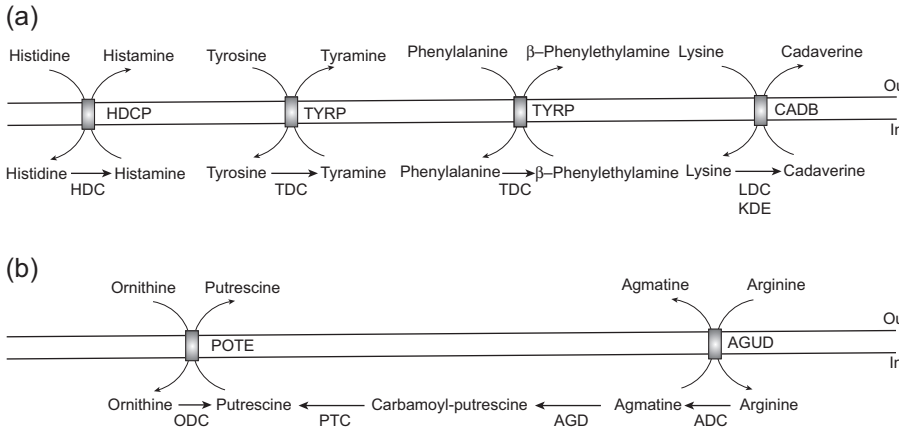

Figure 12.1 Metabolic and transport biosynthetic pathways of biogenic amines. The pathways for histamine, tyramine, β-phenylethylamine and cadaverine (a) as well as for putrescine and agmantine (b) are shown. The transporters, attached to the cytoplasmic membrane, for histidine/histamine (HDCP), tyrosine/tyramine and phenylalanine/β-phenylethylamine (TYRP), lysine/cadaverine (CADB), ornithine/putrescine (POTE) and arginine/agmatine (AGUD) exchanges are indicated. The reactions catalysed by histidine (HDC), tyrosine, phenylalanine (TDC), lysine (CDC), arginine (ADC) and ornithine (ODC) decarboxylases are shown, as well as those catalysed by agmatine deiminase (AGD), arginine deiminase (AD) and putrescine carbamoyl transferase (PTC) (Linares et al., 2011; Suzzi & Gardini, 2003).

Currently, genes encoding the proteins involved in the different BA-producing biosynthetic pathways have been identified, and most of them are carried by LAB (Linares, Martín, Ladero, Álvarez, & Fernández, 2011). The knowledge that these genes are located in genomic islands (Figure 12.2) in the chromosomes of BA-producer strains or in unstable plasmids, and that the formation of BAs is strain-specific and not species-specific, supports the hypothesis that horizontal gene transfer has occurred between different organisms producing BAs (Landete, De las Rivas, Marcobal, & Muñoz, 2008; Linares et al., 2011; Spano et al., 2010).

The most important BAs, both qualitatively and quantitatively, in foods and beverages are histamine, tyramine, putrescine, cadaverine, tryptamine and β-phenylalanine. Therefore, the molecular bases of their production will be discussed.

Figure 12.2 Genetic determinants of biogenic amines biosynthesis. The gene clusters involved in transport and synthesis of histamine, tyramine, cadaverine and putrescine are indicated (Landete, Pardo, & Ferrer, 2008; Marcobal et al., 2012).

The synthesis of histamine is by decarboxylation of histidine (Figure 12.1(a)) catalysed by histidine decarboxylase (HDC). This BA is the most abundant BA found in food and beverages, such as fish and wine. HDC has been identified in both Gram-negative and Gram-positive bacteria. These enzymes have been classified into two distinct classes: a pyruvoyl-phosphate-dependent HDC apparently restricted to Gram-positive bacteria, and a pyridoxal-phosphate-dependent HDC present in Gram-negative bacteria (Van Poelje & Snell, 1990). The coding gene of the pyruvoyl-dependent HDC (*hdcA*) has been identified in a number of LAB (Figure 12.2), including *Oenococcus oeni* (Coton, Rollan, & Lonvaud-Funel, 1998), *Lactobacillus* 30A (Vanderslice, Copeland, & Robertus, 1986), *L. hilgardii* (Lucas, Wolken, Claisse, Lolkema, & Lonvaud-Funel, 2005), *L. buchneri* (Cruz Martín et al., 2005), *Tetragenococcus halophilus* (Satomi, Furushita, Oikawa, & Yano, 2011), *L. reuteri* and *S. thermophilus* (Calles-Enriquez et al., 2010); in other Gram-positive bacteria such as *Clostridium perfringens* and *Staphylococcus capitis* (De las Rivas, Rodríguez, Carrascosa, & Muñoz, 2008) and in strains of the genus *Micrococcus* (Romantsev & Prozorovskii, 1984). The HDC of *Lactobacillus* 30a has been extensively studied and is the prototype of this class of enzymes. This HDC is active at low pH and is synthesized as an inactive pro-enzyme called the π chain (Gallagher, Rozwarski, Ernst, & Hackert, 1993). Other HDCs from *L. buchneri*, *C. perfringens* (Van Poelje & Snell, 1990) and *Micrococcus* spp. (Rashkovetskii & Prozorovskii, 1983) have also been studied. Downstream of *hdcA* is located *hdcB* (Figure 12.2), and Trip, Mulder, Rattray, and Lolkema (2011) have shown that its product HdcB is involved in the conversion of the HdcA pro-enzyme to the active decarboxylase. Upstream of *hdcA* is located *hdcP* (Figure 12.2), which encodes a histidine/histamine antiporter not yet identified in some histamine-producing LAB (Linares et al., 2011). In lactobacilli, a fourth gene is located downstream of *hdcB*, called *hisRS* (Figure 12.2), which encodes a protein similar to histidyl t-RNA synthetase (Landete, De las Rivas, et al., 2008). In the case of *S. thermophilus*, the gene order is *hdcAPB*, similar to that in *S. capitis*, *Staphylococcus epidermidis* and *C. perfringens*, except that in these clusters the *hdcB* gene is absent (Calles-Enriquez et al., 2010, Figure 12.2).

In most of the histamine producer strains, the histamine cluster is located in the bacterial chromosome, while in *L. hilgardii*, *O. oeni* and *T. muriaticus* the cluster is located in an 80-kb unstable plasmid (Lucas, Claisse, & Lonvaud-Funel, 2008; Lucas et al., 2005). In Gram-negative bacteria, the gene encoding the pyridoxal-phosphate dependent HDC (*hdcA*) has been identified in *Morganella morganii*, *Raoultella planticola*, *Enterobacter aerogenes* and *Photobacterium phosphoreum*. However, in these bacteria the gene encoding the transporter involved in amino acid/amine interchange has not been detected (Landete, De las Rivas, et al., 2008).

The aromatic BA, tyramine and β-phenylethylamine are produced, respectively, by decarboxylation of tyrosine and phenylalanine by the enzyme tyrosine decarboxylase (TDC) (Landete, De las Rivas, Marcobal, & Muñoz, 2007; Pessione et al., 2009). TDC was purified from the strain *L. brevis* IOEB 9809 isolated from wine (Moreno-Arribas & Lonvaud-Funel, 2001; Russo et al., 2012). Lucas and Lonvaud-Funel (2002) described that in *L. brevis* IOEB 9809, the tyramine biosynthetic pathway is encoded by a cluster of four genes (Figure 12.2). All strains carrying the TDC cluster produce both tyramine and β-phenylethylamine, but the levels of the latter are four to

five-fold lower than the former (Marcobal, De Las Rivas, Landete, Tabera, & Muñoz, 2012). The first gene (*tyrS*) of the cluster has strong similarities to tyrosyl-tRNA synthetase genes; the second gene (*tdc*) corresponds to tyrosine decarboxylase; the third gene (*tyrP*) encodes a tyrosine/tyramine exchanger (Wolken, Lucas, Lonvaud-Funel, & Lolkema, 2006) and the last gene (*nhaC*) is related to the Na^+/H^+ antiporter genes. The existence of the same gene organization has been described in other tyramine-producing LAB: *E. faecalis* (Connil et al., 2002), *E. durans* (Linares et al., 2012; Linares, Fernández, Martín, & Álvarez, 2009), *E. faecium* (Capozzi et al., 2011), *Lactobacillus fermentum* and *S. epidermidis* (Torriani et al., 2008).

Proteins with high similarity to TDC have been reported in Gram-negative bacteria, but the production of tyramine itself has not been reported (Marcobal et al., 2012).

Although bacterial decarboxylation of aromatic amino acids, such as tyrosine and histidine, has been widely investigated in different strains, the study of tryptophan decarboxylation to yield tryptamine, and its genetic characterization, has been much less studied. The formation of tryptamine has been detected in strains belonging to the genera *Acinetobacter*, *Micrococcus* and *Staphylococcus*, with the highest decarboxylase activity being present in the genus *Micrococcus* (Nakazawa, Kumagai, & Yamada, 1977) and in some strains of *Lactobacillus* (Gonzalez de Llano, Cuesta, & Rodriguez, 1998). Nakazawa, Kumagai, and Yamada (1981) were able to crystallize the tryptophan decarboxylase from the crude extracts of *Micrococcus percitreus*. Studies performed with this enzyme showed that it is pyridoxal-phosphate dependent and that the decarboxylation of tryptophan is noncompetitively inhibited by the presence of other BAs such as tyrosine and phenylalanine (Nakazawa, 1987).

Cadaverine is a BA with two amine groups (a diamine), that can be found in wine (Romano et al., 2012), cheese (Fiechter, Sivec, & Mayer, 2013), cider (Coton, Romano, et al., 2010), sausage (Cachaldora, Fonseca, Franco, & Carballo, 2013) and fishery products (Hu, Huang, Li, & Yang, 2012). The cadaverine-producing strains usually belong to the genera *Enterobacteriaceae*, *Bacillus*, *Staphylococcus* and *Lactobacillus* (Maifreni et al., 2013; Papavergou, Savvaidis, & Ambrosiadis, 2012; Suzzi & Gardini, 2003). Cadaverine is synthesized from lysine by lysine decarboxylase (LDC). LDC activity has been mostly associated with Gram-negative bacteria such as *Escherichia coli* (Haneburger et al., 2012), *Salmonella enterica* serovar Typhimurium (Georgnaras, Dykes, & Von Holy, 1995) and *Vibrio vulnificus* (Kang, Kim, Kim, & Lee, 2007), and until now only in one Gram-positive bacterium, *Lactobacillus saerimneri* 30a. The cadaverine biosynthetic locus has been extensively studied in *E. coli* and it is composed of three genes (Figure 12.2): *cadA*, encoding the lysine decarboxylase (LDC); preceded by *cadB*, which encodes a transporter protein; and by a third gene, *cadC*, encoding a regulatory protein (Meng & Bennett, 1992). The LDC protein has not been detected in LAB. In these bacteria, with the exception of *L. saerimneri* 30a, lysine could possibly be decarboxylated by ornithine decarboxylase (ODC) but with low affinity. Recently, Romano, Trip, Lolkema, and Lucas (2013) have characterized the enzyme responsible for lysine decarboxylation in *L. saerimneri* 30a. This enzyme differs from LDC from Gram-negative bacteria. For this reason, the authors have denominated KDE to the lysine decarboxylase protein of *L. saerimneri* 30a. The KDE coding gene (Figure 12.2) is adjacent

to a transporter gene (*aat*) that encodes both a lysine/cadaverine as well an ornitine/putrescine exchanger protein (Romano et al., 2013).

Putrescine (1,4-diaminobutane) is the most abundant BA in alcoholic beverages, such as wines and cider. These BAs are considered as indicators of freshness (or lack of it) in fish and meat products, because they are produced during food storage. Putrescine can be synthesized from ornithine, via ODC, or from agmatine, via the reactions of an agmatine deiminase and a putrescine transcarbamylase (Figure 12.1(b)). The first pathway is abundantly found in Gram-negative bacteria but rarely in Gram-positive bacteria (*O. oeni*, *S. epidermidis* and some strains of genus *Lactobacillus*), whereas the latter has been described in LAB implicated in fermentation of dairy products and in others Gram-positive bacteria such as strains of the genus *Enterococcus* (Linares et al., 2011). The ornithine decarboxylase cluster (Figure 12.2) consists of an *odc* gene, which encodes the ODC, and a second gene, called *potE*, which encodes an ornithine/putrescine antiporter (Coton, Mulder, et al., 2010; Marcobal, De las Rivas, Moreno-Arribas, & Muñoz, 2006). The order of the genes in the cluster varies in different bacteria (Figure 12.2). The agmatine deiminase pathway is composed of five genes (Figure 12.2): *aguR*, which encodes a transcriptional regulator; and the genes encoding a putrescine transcarbamylase (*aguB*), an agmatine/putrescine antiporter (*aguD*), an agmatine deiminase (*aguA*) and a carbamate kinase (*aguC*). In the case of *Lactobacillus*, *Pediococcus* and *Listeria*, the *aguR* gene is located at the end of the cluster, and this group has a second putative agmatine deiminase gene (*aguA2*), located between *aguA1* and *aguR* genes (Ladero, Rattray, et al., 2011). In the absence of ornithine, putrescine can be synthesized by decarboxylation of arginine (Figure 12.1(b)). However, arginine decarboxylase activity has been only reported in *L. hilgardii* X1B (Arena & Manca de Nadra, 2001).

12.3 Environmental factors involved in the production of biogenic amines

Summaries of some enological and technological factors involved in BA production in wine and different fermented food, so far identified, are presented respectively in Tables 12.1 and 12.2.

Often the prime factor for the potential formation of BAs in fermented foods is the raw materials used, which may contain both the substrates and the microorganisms necessary for BA production. For instance, the quality of wine is strictly linked to the grape and therefore to all the related viticultural aspects. Consequently, factors such as agronomical practices, soil composition, climatic conditions, vintage, degree of maturation, and integrity and health of the grape berry can all contribute to the total levels of BAs in the wine (reviewed by Smit, du Toit, & du Toit, 2008; Ancín-Azpilicueta, González-Marco, & Jiménez-Moreno, 2008). In particular, in recent years several studies have determined a correlation between grape variety and/or geographical origin with the occurrence of some BAs (Del Prete et al., 2009; Glória, Watson, Simon-Sarkadi, & Daeschel, 1998; Herbert, Cabrita, Ratola, Laureano, & Alves, 2005; Landete et al.,

Table 12.1 Some enological factors affecting the formation of biogenic amines in wine

Enological factor	Change in the formation of biogenic amines	References
Maceration practices	Higher levels of precursor amino acids and biogenic amines (BAs) found in the absence of skin contact, extended maceration, and to a lesser extent in conventional maceration. Cold maceration before fermentation resulted in the lowest concentrations of BAs in wine.	Smit, du Toit, Stander, and du Toit (2013)
Malolactic fermentation (MLF) (spontaneous; co-inoculum; inoculum after alcoholic fermentation) and yeast lees	Co-inoculation reduces BA production over time compared to spontaneous MLF and conventional inoculation. The presence of yeast lees during aging led to higher final concentrations of BAs in wines.	Smit and du Toit (2013)
Ganimede vinification method	The concentration of BAs, in particular histamine and tyramine, was higher in the wine made by the Ganimede method than that elaborated by the traditional maceration method.	Ancín-Azpilicueta, González-Marco, and Jiménez-Moreno (2010)
Organic wine/nonorganic wine; press wine addition to the free-run wine and yeast mannoproteins	Organic wine showed higher levels of BA than nonorganic wine. Addition of press wine to the free-run wine, and the treatment with yeast mannoproteins, both increase the BA levels.	García-Marino, Trigueros, and Escribano-Bailón (2010)
Grape cultivars	Grape varieties are related to the presence of certain BAs in wines, and climatic conditions also affect their accumulation in grapes.	Del Prete, Costantini, Cecchini, Morassut, and García-Moruno (2009)
Grape varieties; MLF (spontaneous or inoculum) and aging in oak barrels	More amines were produced in wine of the Tempranillo variety than in Cabernet Sauvignon. Commercial MLF starters minimized the levels of BAs. The aging time had a significant influence on the evolution of the volatile compounds.	Hernández-Orte et al. (2008)
Viticulture region; grape variety; antifungal treatment of grapes; fermentation activators; malolactic starters and storage on lees	Viticulture region and grape variety affect type and levels of BAs. Wines inoculated with MLF starters present lower amounts of BAs. The wine storage on lees contributes to an increase of BA content.	Marques, Leitão, and San Romão (2008)

Table 12.1 Continued

Enological factor	Change in the formation of biogenic amines	References
Vintage; pectolytic enzymes usage; aging with lees; maceration practices and inoculation with commercial starter bacteria	Vintage can clearly influence the BA contents in wines. Aging of wine on lees and longer grape skin maceration strongly increased BA concentration. Addition of pectolytic enzymes did not favor the accumulation of any BA. The inoculation with commercial malolactic starters minimizes the BA levels.	Martín-Álvarez, Marcobal, Polo, and Moreno-Arribas (2006)
Grape varieties; type of vinification; wine pH, malolactic fermentation and storage	Important differences in putrescine and histamine concentrations among regions, varieties of grape, and type of wine. MLF and short storage periods in bottle increase the histamine concentration, whereas longer periods of storage led to its decrease.	Landete, Ferrer, Polo, and Pardo (2005)

Note: MLF, malolactic fermentation; BA, biogenic amines.

2005). However, despite the foregoing, the key factor affecting BA formation is the microbial population in the fermentation process. Microorganisms can occur naturally in the food substrate or can be introduced accidentally by contamination or deliberately by adding starter cultures. The ability for BA production among LAB and other bacteria during food processing has been extensively reported (Coton, Romano, et al., 2010; Silla Santos, 1996). Therefore, there is a high risk of the presence of indigenous decarboxylase-positive bacteria in the food matrix. Indeed, it is clear that foods prepared using not-selected wild microbes are usually related to a higher content of BAs. This is particularly evident in organic production or in traditional regional fermented foods not produced on industrial scale like artisanal cheeses, ciders and wines (Beneduce et al., 2010; Garai, Dueñas, Irastorza, Martín-Álvarez, & Moreno-Arribas, 2006; Linares et al., 2011). Moreover, high BA levels were found in oriental and African specialties like soya bean food products (Guan et al., 2013; Shukla, Park, Kim, & Kim, 2010; Yongmei et al., 2009) and traditional alcoholic beverages (Lasekan & Lasekan, 2000; Yeğin & Üren, 2008) not fermented with starter cultures.

Although fermentation is a historical method to preserve various foodstuffs, decrease of the starting microbial load of the matrix is a necessary preliminary step, prior to the inoculation of selected cultures, in order to reduce the risk of pathogen-, spoilage- or bacteria-producing toxic compounds such as BAs. Thus, a pasteurization treatment is required during cheese and sauerkraut manufacturing, while the use of sulphites is a worldwide enological practice. These methods, many times associated with a reduction in final BA content, prevent the formation of these compounds by reducing the

Table 12.2 Some technological factors affecting the formation of biogenic amines in different fermented foods

Foodstuff	Starter culture	Technological factor	Ripening/ storage conditions	Change in the formation of biogenic amines	References
Sauerkraut	*Lactobacillus plantarum, Leuconostoc mesenteroides*	NaCl (0.5–1.5%)	4 °C, 3 months	BA content was lower at 0.5% NaCl than at 1.5%, and increased with the time of storage. *L. mesenteroides* produced a lower content of BAs than *L. plantarum.*	Peñas, Frías, Sidro, and Vidal-Valverde (2010)
Fresh fermented sausage	*L. plantarum* plus *Bifidobacterium lactis* *L. plantarum* plus *Bifidobacterium bifidum* *L. plantarum* plus *B. lactis* and *B. bifidum*	Influence of starter culture inoculation	20–22 °C, 72 h 4°C, 14 days	Sausages produced by fermentation with starters had a lower amount of BAs than those obtained by natural fermentation. A great reduction in BA content was achieved when *Bifidobacterium* sp. was inoculated.	Mokhtar, Mostafa, Taha, and Eldeep (2012)
Sucuk fermented sausage	*Lactobacillus sakei, Pediococcus pentosaceus, Staphylococcus carnosus, Staphylococcus xylosus*	Nisin and nitrite	20 °C, 13 days	The interactive effect of nisin and nitrite significantly reduced putrescine production.	Kurt and Zorba (2010)

Spanish dry fermented sausage	Spontaneous	Sausages diameter: *fuet* (2.5 cm) and *longaniza* (4.5 cm); relative humidity (RH)	20–23 °C, 3 days 12–14 °C, 20 days or 12–13 °C, 23 days	BA accumulation was faster and higher at higher temperature and RH. *Longaniza* had higher BA contents compared with *fuet*.	Latorre-Moratalla, Bover-Cid, and Vidal-Carou (2010)
Chorizo dry sausage	*L. sakei* K29, *Pediococcus* P22, *Pediococcus* P208	Sugar (glucose, lactose, sucrose)	23 °C, 48 h 13 °C, 18 days	*L. sakei* K29 and sugar concentrations of 0.5% or 1% markedly decreased the formation of putrescine and tyramine.	González-Fernández, Santos, Jaime, and Rovira (2003)
Smear-ripened cheese		Storage temperature, shape, cheese age, and season	5 °C, 66 days or 20 °C, 66 days	Tyramine, cadaverine, and putrescine content in cheeses increased during storage. Tyramine content was twice as much in disc-shaped cheeses as compared to bars. Storage temperature and season affected the variability in BA content by 46% and 13%, respectively.	Komprda, Rejchrtová, Sládková, Zemánek, and Vymlátilová (2012)
Dutch-type cheese	*Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris*	Elevated storage temperature	10 °C, 56 days or 16 °C, 56 days	The elevated ripening temperature caused greater production of BAs such as tyramine (500 mg/kg), putrescine, and cadaverine.	Pachlová, Bunka, Flasarová, Válková, and Bunková (2012)
Dutch-type cheese	*L. lactis* subsp. *lactis* *L. lactis* subsp. *cremoris*	Ripening and storage conditions	10 °C, 98 days or 10 °C, 38 days 5 °C, 60 days	The highest content of tyramine, putrescine, and cadaverine was produced in cheeses stored in a ripening cellar at 10 °C during the whole observation period.	Bunková et al. (2010)

Continued

Table 12.2 Continued

Foodstuff	Starter culture	Technological factor	Ripening/ storage conditions	Change in the formation of biogenic amines	References
Cheeses		Time of ripening pasteurization		BA concentrations increased with cheese ripening, and were higher in cheeses made from raw milk than in those made from pasteurized milk. Tyramine was the most commonly recorded and abundant BA.	Fernández et al. (2007)
Goat cheese		Raw milk stored or not for 48 h at 4 °C		The storage of milk under refrigeration caused significant increases in the levels of putrescine, histamine and β-phenylethylamine (two-fold higher).	Novella-Rodríguez, Veciana-Nogués, Roig-Sagués, Trujillo-Mesa, and Vidal-Carou (2004)
Azeitão cheese		Temperatures of storage	4 °C, 2 weeks 25 °C, 2 weeks	Room temperature promoted a significant increase of tyramine and putrescine contents.	Pinho, Ferreira, Mendes, Oliveira, and Ferreira (2001)
Gouda cheese	*Lactobacillus delbrueckii, Lactobacillus buchneri, Lactobacillus brevis*	Addition of proteolytic enzymes	14 °C, 12 weeks	Conditions of accelerated ripening of cheese by amino acid decarboxylase-positive *Lactobacillus* sp. result in increase of histamine and tyramine concentrations.	Leuschner, Kurihara, and Hammes (1998)

microbial concentration in foods (Marcobal et al., 2006; Novella-Rodríguez et al., 2004). Nonetheless, although the use of selected microbiota is widespread in the food industry, some authors reported on starter cultures that carry the decarboxylase activities for phenylalanine, tyrosine and histidine (Ansorena et al., 2002; Linares et al., 2012). Also the addition of sodium chloride has been reported as a current method to reduce the undesired microbiota in sausages and dairy products and consequently their BA content (Gardini et al., 2001; Gardini et al., 2008; Roseiro, Santos, Sol, Silva, & Fernandes, 2006). In contrast to these results, Peñas et al. (2010) showed that in sauerkraut production, starter-induced fermentation of white cabbage with high salt concentration (1.5% NaCl) was linked to a larger total BA content. Similarly, Buňková, Buňka, Dráb, Kráčmar, and Kubáň (2012) and Buňková, Buňka, Pollaková, Podesvová, and Dráb (2011) found that *E. durans* and *Lactococcus lactis* were able to produce more tyramine in a growth medium containing 20 g/l than 10 g/l of NaCl. These results were interpreted as being due to a stimulatory effect of the higher osmotic pressure on the decarboxylase enzymes activity, due to the involvement of BA production in regulation of intracellular pH (Wolken et al., 2006).

During the fermentation process, LAB metabolism modifies the composition and the physico-chemical features of the foods, with acidification of the media being one of the most important changes. The lowering of pH impacts at different levels on BA formation: it hampers the growth of contaminant microorganisms and modulates the activity of decarboxylases and proteolytic enzymes (Linares et al., 2012).

In dry sausages fermented by selected cultures, it is usual to add sugars as substrates for lactic acid production by LAB in order to ensure the quick pH decrease. Thus, it would prevent the growth of contaminant bacteria. In fact, a correlation between sugar usage and a decrease of BA production has been reported in *chorizo* dry sausages (Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 2001; González-Fernández et al., 2003). Several authors reported that high pH values in wine are associated with the greatest production of BAs as a consequence of an easier total growth and of the greater bacterial diversity (Landete et al., 2005; Lonvaud-Funel, 2001). However, harsh environments should encourage BA production by LAB, which seems to be a mechanism to survive in the acidic environment or to supply alternative metabolic energy when bacterial cells are exposed to suboptimal substrate conditions (Spano et al., 2010). Transcriptional analysis performed on *L. brevis* IOEB 9809 showed that the genes responsible for tyramine and putrescine production can be induced at a low pH (Arena, Russo, Capozzi, Beneduce, & Spano, 2011). However, the optimum pH for decarboxylase activity was observed between 4.5 and 5.5, although the enzymes can remain active in cell-free extracts in a wider pH range (Moreno-Arribas & Lonvaud-Funel, 1999; Schelp, Worley, Monzingo, Ernst, & Robertus, 2001). Fernández et al. (2007) found the maximum production of tyramine at pH 5.0 by the dairy *E. durans* IPLA 655, while in wine TDC activity could be inhibited at a low pH (Leitão, Teixeira, Barreto Crespo, & San Romão, 2000). A relationship between pH and BA content has been reported by Miguélez-Arrizado, Bover-Cid, Latorre-Moratalla, and Vidal-Carou (2006), who found higher cadaverine and histamine contents in high acidic sausages.

The increase of acidity also encourages proteolysis during the ripening of cheeses and sausages, which has been described as a potential factor favoring the accumulation of BAs (Fernández et al., 2007; Vidal-Carou, Latorre-Moratalla, Veciana-Nogués, & Bover-Cid, 2007). Free amino acid availability extends the growth of strains with decarboxylase activity including spoilage lactobacilli (Gardini et al., 2001; Leuschner et al., 1998). Several studies reported that proteolysis can be increased by the combined effects of physico-chemical or technological conditions. Therefore, selection of starter cultures, temperature, addition of adequate amounts of proteinases, size or diameter of the food particles, and sodium chloride concentration, are features that can determine the final BA concentration in cheeses and dry fermented sausages (Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 1999; Fernández-García, Tomillo, & Núñez, 1999; Latorre-Moratalla et al., 2010; Roseiro, Gomes, Gonçalves, Cercas, & Santos, 2010; Tabanelli et al., 2012). Valsamaki et al. (2000) reported that the total BA content in mature Feta cheese (60 days) was 330 and 617 mg/kg after a 120-day storage, with tyramine and putrescine being the main representatives (around 70%). Buňková et al. (2010) observed the lowest BA concentrations in Edam cheeses moved into a cold storage device (5 °C) after a shorter storage in a ripening cellar (10 °C). Consistently, the increase of temperature by 6 °C (from 10 to 16 °C) can halve the ripening time in cellars, but caused greater production of BAs (almost 500 mg/kg) such as tyramine, putrescine and cadaverine (Pachlová et al., 2012).

Several studies have been carried out to determine the contribution of typical metabolites to BA synthesis. However, it is very difficult to define the importance of each of the interrelated factors occurring in a complex milieu like wine. Thus, some authors reported the positive influence of glucose and malic acid presence, respectively, on tyramine and putrescine production (Mangani, Geurrini, Granchi, & Vincenzini, 2005; Moreno-Arribas, Torlois, Joyex, Bertrand, & Lonvaud-Funel, 2000). In contrast, the occurrence of metabolic sources, like glucose, fructose or organic acids, were often related to low BA content, since they negatively affected the expression decarboxylase genes or their enzymatic activity (Arena, Fiocco, Manca de Nadra, Pardo, & Spano, 2007; Arena, Landete, Manca de Nadra, Pardo, & Ferrer, 2008; Landete, Pardo, et al., 2008). Interestingly, the occurrence of phenolic compounds in red wines seems to hinder putrescine synthesis (Alberto, Arena, & Manca de Nadra, 2007). Recently, Smit et al. (2013) detected higher levels of BAs in the absence of grape skin contact during the alcoholic fermentation, suggesting that the shortage of phenolic compounds improves the conditions for BA formation. Maceration is a critical enological practice, because the time of contact between grape skin and the must strongly determine its free amino acid content. Ancín-Azpilicueta et al. (2010) found that Ganimede, a new vinification method that utilizes carbon dioxide to achieve a more effective maceration of the skins, results in a higher concentration of histamine and tyramine in all the wines analysed. However, cold maceration appears to have a major protective effect against BA accumulation during malolactic fermentation (Smit, du Toit, Stander, & du Toit, 2013). Aging and storage of the wine also can affect the BA concentration probably due to a residual bacterial metabolism or to the activity of extracellular decarboxylase enzymes (Herbert et al., 2005; Jiménez Moreno & Ancín-Azpilicueta, 2004; Landete et al., 2005; Martín-Álvarez et al., 2006). Hernández-Orte et al. (2008) investigated

some factors occurring during the aging of wines fermented in oak barrels. Technological practices such as the addition of mannoproteins or of press wine to the free run wine would permit an increase in the content of nitrogen compounds, which could act as precursors for the synthesis of BAs (García-Marino et al., 2010). Similarly, the uncontrolled presence of lees from yeast autolysis can increase the amount of hydrolysed peptides and free amino acids (Alcaide-Hidalgo, Moreno-Arribas, Martín-Alvarez, & Polo, 2007; Marques et al., 2008; Martín-Álvarez et al., 2006). These results were recently confirmed by Smit and du Toit (2013), who emphasized that the separation of the fermentation lees after MLF is a good practice in order to minimize the risk of BA production.

12.4 Techniques for the detection of biogenic amine-producing bacteria

Several methods to detect BA-producing LAB have been described. The most reliable and direct procedure consists of growing LAB in an appropriated medium containing the precursor amino acids and, after the required time of growth, the BA concentration present in the culture supernatants is determined by HPLC or other physico-chemical methods. However, these methods require expensive equipment and are time consuming (several days). Therefore, alternative strategies that are cheaper and less complicated have been proposed. They generally require the use of a differential growth medium containing a pH indicator, such as bromocresol purple, the precursor amino acids, and other basal components (Bover-Cid and Holzapfel, 1999; Maijala, 1993). The medium has a low buffering power and it is adjusted to a pH value, which is slightly lower than the value at which the pH indicator changes colour. Production of the more alkaline BA from the corresponding precursor amino acid induces a change of the medium colour to purple in response to the shift to a more alkaline pH. This method has been widely used for screening LAB samples from laboratory collections and from food analyses with the aim to detect BA producers. Nevertheless, a comparison of results obtained by this method and by HPLC showed that the former is not reliable, in the sense that it can give false-positive and false-negative results (Actis, Smoot, Barancin, & Findlay, 1999; Linares et al., 2011; Marcobal, De las Rivas, & Muñoz, 2006). In addition, both methods require the isolation of the strains and can take days before a positive result is obtained. Therefore, rapid molecular methods have been developed in order to detect and quantify the content of BA producers in dairy products as well as in wine to ensure quality and safety. Thus, PCR was proposed in 1995 as a technique for the detection of BA-producing strains (Le Jeune, Lonvaud-Funel, Ten Brink, Hofstra, & Van Der Vossen, 1995) by use of primers specific for the genes encoding the amino acid decarboxylases. Le Jeune et al. (1995) developed a PCR method to detect histamine-producing strains. The author designed primers specific for the *hdcA* gene based on the comparison of *hdcA* gene sequences from *Lactobacillus* 30A and *C. perfringens* and the amino acid sequences of HDC purified from *Lb. buchneri* and *Micrococcus*. This approach allowed PCR detection of

the *hdcA* genes of several LAB strains isolated from cheese and two *O. oeni* strains from wine. Similarly, Lucas and Lonvaud-Funel (2002), by use of primers specific for the TDC-coding gene of *L. brevis* IOEB 9809, detected other *L. brevis* strains carrying a similar gene. However, additional *tdcA* gene sequences were required for designing degenerate PCR primers specific for all LAB species present in fermented food and beverages (Fernández, Del Rio, Linares, Martín, & Álvarez, 2006; Nannelli et al., 2008). In addition, a multiplex PCR method was developed for the simultaneous detection of foodborne bacteria producing histamine, tyramine, putrescine and cadaverine (De las Rivas, Marcobal, Carrascosa, & Muñoz, 2006; Marcobal, De las Rivas, Moreno-Arribas, & Muñoz, 2005). Later, various real-time quantitative RT-PCR analyses have been developed for detection of BA-biosynthetic pathways expression in food matrices (Coton, Romano, et al., 2010; Ladero, Coton, et al., 2011; Nannelli et al., 2008). Finally, the most recently advance in this detection has been performed by Sciancalepore et al. (2013), which consists of a microdroplet-based multiplex PCR method, directly on a food matrix, for the simultaneous detection of bacterial genes involved in BA biosynthesis. The system was successfully applied for detection in wine of tyramine and putrescine synthesized by *L. brevis* IOEB 9809.

12.5 Techniques for the detection of biogenic amines

Detection of BAs in foods encompasses several key issues: (1) to link the BA content of a foodstuff to a foodborne event, (2) to establish the toxic threshold of a specific BA, (3) to ensure a high standard of safety and freshness of the products according to governmental regulations and (4) monitoring quality during the food production process. Therefore, although an accurate quantification of BAs in food is generally required, in some cases a fast detection of BAs is preferable.

Over the years, many analytical methods for BA detection have been developed, including qualitative or semiquantitative methods (i.e. enzymatic and thin layer chromatography (TLC)), and quantitative chromatographic techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE) and ion-exchange chromatography (IEC) (reviewed by Önal, 2007; Önal, Tekkeli, & Önal, 2013; Peña-Gallego, Hernández-Orte, Cacho, & Ferreira, 2012; Smit et al., 2008). Currently, HPLC-based approaches are considered the most reliable and sensitive methods for the detection of BAs in food.

Analytical quantification of BAs may be difficult due to the complexity of some food matrices and the low concentrations of BAs generally encountered in the majority of foodstuffs. In addition, the low volatility of these compounds and the lack of chromophores for most of the BAs, does not allow the rapid direct detection by ultraviolet and visible (UV and vis) spectrometric or fluorimetric (FL) methods. In general, in order to obtain an optimal analysis, extraction, clean-up, concentration, and derivatization procedures are required. Extraction methods usually based on liquid-liquid or solid-phase extraction with C18 or ion-exchange cartridges can be applied to improve selectivity and sensitivity (Giannotti et al., 2008; Peña-Gallego, Hernández-Orte, Cacho, & Ferreira, 2009). Alternative approaches, such as solid-phase microextraction

(Awan, Fleet, & Thomas, 2008), hollow-fibre liquid-phase microextraction (Saaid et al., 2009), and recently developed dispersive liquid–liquid microextraction (DLLME) (Almeida, Fernandes, & Cunha, 2012) and gas-diffusion microextraction (Valente, Santos, Gonçalves, Rodrigues, & Barros, 2012), have also been reported as alternative methods to detect BAs in food.

Chromatographic methods require a pre- or post-column derivatization of BAs and a subsequent ultraviolet or fluorescent detection of the corresponding derivative. Several labelling reagents such as *o*-phthaldialdehyde (OPA), dansyl chloride (dansyl-Cl), 4-chloro-3,5-dinitrobenzotrifluoride (CNBF), 1,2-naphthoquinone-4-sulfonate (NQS), 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), fluorenylmethylchloroformate, *N*-hydroxysuccinimide ester, diethyl ethoxymethylenemalonate (DEEMM) and dabsyl chloride (dabsyl-Cl) are reported for the analytical detection of BA in food samples (reviewed by Hernández-Cassou and Saurina, 2011; Önal et al., 2013; Smit et al., 2008). Dansyl-Cl is probably the most widely used reagent for pre-column derivatization, while OPA is mostly used for post-column derivatization (Alberto, Arena, & De Nadra, 2004). However, the influence of food matrix on the derivatization process should be evaluated. For instance, the wine sample composition indicates that pre-column labelling may undergo more severe matrix effects (Hernández-Cassou & Saurina, 2011).

In recent years, several workers reported the simultaneous detection of BAs and the corresponding amino acids, showing either a comprehensive picture of the food samples analysed or reducing times and costs of the analysis. For instance, an HPLC method with combined diode array and fluorescence detection was developed by Korös, Varga, and Molnar-Perl (2008) resulting in the identification and quantification of 21 amino acids and 9 amines from a single solution. Furthermore, the method was positively applied to the determination of the amino acid and BA contents of different cheese samples (Korös et al., 2008). Kelly, Blaise, and Larroque (2010) proposed a new rapid (less than 40 min) and economical procedure without sample cleanup for routine determination of 24 amino acids and BAs in grapes and wine. The HPLC-UV method after a pre-column derivatization with dansyl-Cl was successfully applied to the simultaneous determination of BAs and their precursor amino acids in different food samples, such as cheese, clams, salami and beer (Mazzucco et al., 2010). Bach, Le Quere, Vuchot, Grinbaum, and Barnavon (2012) proposed an optimized method for the simultaneous analysis of 8 BAs in wine, simplifying the derivatization of the sample and increasing the preservation of the column. Finally, Jia, Kang, Park, Lee, and Kwon (2011) proposed a novel liquid chromatography coupled with a quadruple time-of-flight mass spectrometry method allowing the simultaneous determination of 23 amino acids and 7 BAs in food samples. The matrix effect was minimal, suggesting the application of this method to the analysis of amino acids and BAs in beer, cheese, and sausage samples (Jia et al., 2011). The coupling of chromatographic separation to mass spectrometric detection is recommended when extremely high levels of sensitivity and precision of the analysis are required (García-Villar, Hernandez-Cassou, & Saurina, 2009). The contents of BAs in beer samples was recently quantified by gas chromatography mass spectrometry (GC–MS) using a novel DLLME. This method allowed a simultaneous extraction/derivatization and quantification of the 18 BAs

(Almeida et al., 2012). Similarly, GC–MS after fast chloroformate extraction/derivatization enabled the simultaneous quantification of 22 BAs in Port wines and grape juices (Cunha, Faria, & Fernandes, 2011).

Ultra-high-pressure liquid chromatography (UHPLC) is a new emerging chromatographic technique that operates at higher pressures, up to 15,000 psi, enhancing the efficiency of HPLC in terms of increased resolution, sensitivity and analysis speed. However, the expensive cost of laboratory equipment and the need for specialized operators make UHPLC a viable opportunity only for high-throughput applications. UHPLC analysis has been performed on fermented food samples to determine their BA content in wine, fish, cheese and dry fermented sausages (Latorre-Moratalla et al., 2009); cheese (Mayer, Fiechter, & Fischer, 2011) and *cheonggukjang*, a fermented Korean soya bean product (Lee, Eom, Yoo, Cho, & Shin, 2011). In addition, Fiechter et al. (2013) have validated a UHPLC method for the simultaneous determination of amino acids and BAs in ripened acid-curd cheeses. In a single run, 23 amino acids and 15 BAs were separated in a retention time of only 9 min, showing very sensitive limits of detection (0.05–0.29 mg 100/g) and quantization (0.16–0.97 mg 100/g). Similarly, a derivatization treatment with DEEMM followed by UHPLC allowed the simultaneous quantization of 22 amino acids, 7 Bas, and ammonium ions in cheese samples in 10 min (Redruello et al., 2013). Therefore, this approach will be valuable to monitor the content of BAs during the ripening of cheese and identify those foods potentially hazardous for their high levels of free amino acid precursors.

CE methods are quantitative methods becoming attractive due to their speed and high resolving power. These techniques allow the separation and identification of highly polar compounds that cannot be easily separated by traditional HPLC methods. The low sensitivity of CE can be overcome by coupling to mass spectrometric detection. Several CE-based quantifications have been optimized for the analysis of BA in wines and no treatment other than filtration of the sample were required (García-Villar, Saurina, & Hernández-Cassou, 2006; Gomez, Monasterio, Vargas, & Silva, 2012; Santos, Simonet, Rios, & Valcarcel, 2004; Simó, Moreno-Arribas, & Cifuentes, 2008). Kvasnička and Voldřich (2006) analysed six BAs in different fermented foods (salami, cheese, wine and beer), developing a CE method with conductometric sensitivity (μmol/l), speed (less than 15 min) and a detection procedure that does not require derivatization. Therefore, this method seems to be an interesting alternative to chromatographic techniques for routine analysis.

Among the semiquantitative methods, TLC is an analytically inexpensive but time-consuming technique that can be easily implemented for routine quality control in laboratories. TLC was satisfactory for the rapid screening of BA-producer LAB strains from food origin in synthetic media (Costantini, Cerosimo, Del Prete, & Garcia-Moruno, 2006; Coton, Romano, et al., 2010; García-Moruno, Carrascosa, & Muñoz, 2005). Costantini, Vaudano, Del Prete, Danei, and Garcia-Moruno (2009) were able to detect, by TLC analysis, contaminant BA-producer bacteria in commercial yeast starters usually used in winemaking. Recently, Romano et al. (2012) optimized a new method, based on amine dansylation and TLC/densitometry, which allows the direct determination in wine of histamine, tyramine, putrescine and cadaverine at concentrations between 1 and 20 mg/l. The same authors suggest a simpler,

semiquantitative version of the method, based on visual evaluation of spot intensity adequate for the needs of routine wine analysis.

Moreover, in recent years, enzymatic sensors reduce the time needed for analysis and may offer a rapid and reliable screening method for industrial food quality testing. Enzyme sensors able to detect the presence of BAs in dry fermented sausages have been developed, and they can constitute a useful tool for quality control in the meat industry (Hernández-Cázares, Aristoy, & Toldrá, 2012). An overview of the developments and issues in the construction of biosensors for the detection of most common BAs found in food has been recently reported (Kivirand & Rinken, 2011).

12.6 Future trends

Several analyses carried out on products purchased from farms or the retail market showed that the presence of high levels of BAs is a real problem in commercialized foods (Table 12.3). Schirone et al. (2013) found that the total BA content reached 5861 mg/kg in a commercial cheese produced in the Abruzzo region. Recently, a survey on 50 dry fermented sausages sold in Greek retail markets showed that the histamine content of 28% of the samples exceeded the limit of 100 mg/kg (Papavergou, Savvaidis, & Ambrosiadis, 2012). Analyzing 30 samples of Herby cheese, Andic, Genccelep, and Kose (2010) detected a maximum of 1125.5 and 1844.5 mg/kg for tyramine and cadaverine, respectively. Similar results were reported for the BA content of Asian products placed on the market (Byun & Mah, 2012; Guan et al., 2013; Kung, Lee, Chang, Wei, & Tsai, 2007). In addition, in vitro experiments revealed that *Enterococcus* and *Lactobacillus* strains isolated from artisanal cheese and wine are able to produce tyramine and putrescine in conditions that resemble the human digestive tract (Fernández de Palencia et al., 2011; Russo et al., 2012). Moreover, the production of tyramine by *E. durans* IPLA 655 resulted in higher adhesion to and immunomodulation of Caco-2 human epithelial cells (Fernández de Palencia et al., 2011). Production of tyramine and putrescine increased survival of *L. brevis* IOEB 9809 under digestive tract conditions (Russo et al., 2012). These results indicate that BA producers from food and beverage origins can survive in the intestinal environment and synthesize BAs in the colon.

Therefore, the need to minimize the presence of BAs and BA producers in fermented food is obvious.

Current knowledge indicates that control of the food microbiota is the best strategy to minimize BA production. Thus, the use of non-BA producing starters has been shown to diminish production of BAs in fermented food (Table 12.2) and wine (Halász, Baráth, & Holzapfel, 1999; Martín-Álvarez et al., 2006; Spano et al., 2010). However, this is not the optimal solution, since these starters cannot counteract BA production by contaminating bacteria. Thus, an alternative is the use of bacteria able to degrade BAs. Various studies have been performed to detect and test bacteria possessing amino oxidase activities, and LAB as well as strains belonging to the *Staphylococcus* and *Bacillus* genera seem to diminish BA production under laboratory conditions simulating food fermentations (reviewed by

Table 12.3 **Maximum levels (mg/kg) of biogenic amines detected in foodstuffs purchased from retail markets**

Food	Geograph. origin	No. of samples	HIM	TYM	PUT	CAD	SPD	SPM	TRY	PHE	Total BA	Detection method	Derivatization	References
Sufu	China	38	196.9	446.6	316.9	85.8	4.0	6.9	104.1	36.3	801.2	HPLC-DAD	Benzoyl chloride	Guan et al. (2013)
Sufu	China	10	36.0	48.0	47.3	n.i.	27.1	22.9	57.0	4.2	n.i.	HPLC-UV	CNBF	Tang et al. (2011)
Miso	Korea	22	24.4	66.7	14.09	1.3	28.3	2.8	9.7	11.7	n.i.	HPLC-UV–Vis	Dansyl-Cl	Byun and Mah (2012)
Boza	Turkey	21	4.0	82.8	9.8	17.7	n.i.	n.i.	13.8	4.5	101.1	HPLC-DAD	Dansyl-Cl	Cosansu (2009)
Boza	Turkey	10	7.9	65	5.6	1.5	3.5	4.0	3.3	5.0	69	HPLC-UV–Vis	Benzoyl chloride	Yeğin and Üren (2008)
Rice wine	China	39	22.4	37.6	58.9	29.9	n.i.	n.i.	n.i.	n.i.	260	HPLC-UV	Benzoyl chloride	Zhong et al. (2012)
Doenjang	Korea	23	279.5	661.6	429.2	323.6	880.4	972.9	280.8	870.5	n.i.	HPLC-UV–Vis	Dansyl-Cl	Shukla et al. (2010)
Natto	Korea	21	34.4	300.2	43.1	36.8	478.1	80.1	40.7	51.5	n.i.	HPLC-UV–Vis	Dansyl-Cl	Kim, Lee, Kim, Mah, Hwang, and Kim (2011)
Soy sauce	China	40	592	673	n.i.	550	486	145	n.i.	n.i.	1358	HPLC-DAD	Dansyl-Cl	Yongmei et al. (2009)
Sucuk	Turkey	30	136	676	364	199	10.7	16.4	82.3	20.3	n.i.	HPLC-UV	Dansyl-Cl	Gençcelep, Kaban, İrfan-Aksu, Öz, and Kaya (2008)
Fermented cabbage juices	Germany	5	83.7	73.0	366	59.4	96.9	n.d.	99.6	50.8	826	HPLC-(ESI-MS)	DNBZ-Cl	Kirschbaum, Rebscher, and Brückner (2000)

Soy sauces	South Korea	2	157	172	52.3	n.d.	25.0	n.d.	n.d.	68.5	482			Kirschbaum et al. (2000)
Miso	South Korea	2	389	215	93.1	n.d.	13.7	n.d.	n.d.	6.1	726			Kirschbaum et al. (2000)
Cider	Spain	14	16	7	34	34	n.i.	n.i.	n.i.	n.i.	67	RP-HPLC	Dansyl-Cl/ dabsyl-Cl	Ladero, Coton, et al. (2011)
	France	29	5	14	1	3	n.i.	n.i.	n.i.	n.i.	14			Ladero, Coton, et al. (2011)
Beer	Portugal	22	0.34	5.92	12.77	1.38	n.i.	n.i.	n.i.	0.15	n.i.	DLLME-GC–MS	Isobutyl chloroformate	Almeida et al. (2012)
Beer	China	18	4.62	7.15	8.23	n.i.	2.55	3.96	1.73	0.42	n.i.	HPLC-DAD	CNBF	Tang et al. (2009)
Wine	Italy	65	7.21	11.94	15.99	1.11	0.70	n.i.	n.i.	1.28	n.i.	HPLC-PDA	Dansyl-Cl	Martuscelli et al. (2013)
Wine	Spain	14	18.7	17.8	35.7	n.d.	n.i.	n.i.	16.2	n.i.	96.8	RP-HPLC-FL	OPA	Arrieta and Prats-Moya (2012)
Wine	South Korea	18	2.84	2.54	3.12	0.59	0.80	n.i.	0.42	1.17	8.88	UPLC/Q-TOFMS	Dansyl-Cl	Jia et al. (2011)
Wine	Chile	27	10.49	7.24	25.99	2.45	6.39	2.62	n.i.	2.41	39.84	HPLC-UV–VIS	Dansyl-Cl	Pineda et al. (2012)
Wine	Spain	8	15.9	9.3	45	0.458	n.i.	n.i.	n.i.	1.26	n.i.	HPLC-APCI-MS	NQS	Garcia-Villar et al. (2009)
Wine	Spain	4	25.2	20.0	83.2	7.0	n.i.	n.i.	n.i.	7.3	n.i.	CE–IT-MS	–	Simó et al. (2008)
Wine	Spain	100	8.22	3.20	13.00	0.68	1.10	0.19	0.98	4.02	n.i.	LC-ESI-ITMS	–	Millán et al. (2007)
Wine	China	20	10.45	19.1	19.0	13.0	3.82	0.75	n.d.	4.58	29.3	HPLC-FL	Dansyl-Cl	Zhijun, Yongning, Gong, Yunfeng, and Changhu (2007)

Continued

Table 12.3 Continued

Food	Geograph. origin	No. of samples	HIM	TYM	PUT	CAD	SPD	SPM	TRY	PHE	Total BA	Detection method	Derivatization	References
Sausages	Greece	50	514.5	509.9	505.3	689.9	10.23	36.74	49.8	25.2	2228.2	HPLC-FL	OPA	Papavergou et al. (2012)
Sausages	Greece	40	375.7	381.4	491.7	1014.1	19.5	60.1	60.5	56.40	n.i.	HPLC-FL	OPA	Papavergou (2011)
Sausages	China	42	101.34	771.52	449.98	1435.24	85.32	214.88	28.22	216.56	n.i.	HPLC-DAD	Dansyl-Cl	Lu et al. (2010)
Cheese	Austria	47	509.0	1597.0	840	1268	n.i.	n.i.	161	n.i.	3123	UHPLC	AQC	Fiechter et al. (2013)
Cheese	Spain	6	337.9	2519.98	105.78	774.51	n.i.	n.i.	72.10	67.86	n.i.	UHPLC	DEEMM	Redruello et al. (2013)
Cheese	Italy	12	761.4	1771.3	986.0	2127.6	n.i.	n.i.	n.i.	232.4	5860.6	HPLC-UV	Dansyl-Cl	Schirone et al. (2013)
Cheese	Italy	40	57.7	147.1	82.9	64.7	73.1	47.4	n.i.	136.6	n.i.	LC-ELSD	–	Spizzirri et al. (2013)
Cheese	Austria	58	1159.7	486.4	523.2	748.2	75.1	61.0	312.2	61.3	1938.2	UHPLC	AQC	Mayer et al. (2011)
Cheese	Italy	10	21.8	1171.3	394.1	276.1	143.9	n.i.	n.i.	127.1	2393.0	HPLC-UV	Dansyl-Cl	Schirone, Tofalo, Mazzone, Corsetti, and Suzzi (2011)

Note: HIM, histamine; TYM, tyramine; PUT, putrescine; CAD, cadaverine; SPD, spermidine; SPM, spermine; TRY, tryptamine; PHE, phenylalanine.
n.i., not investigated; n.d., not detected.
Detection methods: HPLC-DAD, HPLC with diode-array detection. HPLC-(ESI-MS), HPLC with electrospray ionization mass spectrometry. RP-HPLC, reversed-phase HPLC. UPLC/Q-TOFMS, UPLC with quadrupole-time of flight mass spectrometry. HPLC-APCI-MS, HPLC with atmospheric pressure chemical ionization mass spectrometry. CE–IT-MS, capillary electrophoresis–ion-trap mass spectrometry. LC–ESI-ITMS, liquid chromatography–electrospray ionization ion trap mass spectrometry. LC-ELSD, LC with evaporative light scattering detector. DNBZ-Cl, 3,5-dinitrobenzoyl chloride.

Chong, Abu Bakar, Russly, Jamilah, & Mahyudin, 2011). In addition, recent work has shown that lactobacilli and pediococci isolated from musts, wines and fermentation lees are able to metabolize histamine, tyramine and putrescine (Callejón, Sendra, Ferrer, & Pardo, 2012; García-Ruiz, González-Rompinelli, Bartolomé, & Moreno-Arribas, 2011). Moreover, wine *L. plantarum* strains able to degrade tyramine and putrescine have been detected, and showed to have potential as malolactic starters (Capozzi et al., 2012). However, the studies performed until now do not prove that these bacteria are effective under real manufacturing process conditions. Therefore, further testing is required before transferring this promising strategy to the industrial environment.

Another potential future strategy that has not been explored until now, is the use of non-BA-producers starters able to synthesize and secrete bacteriocins that could eliminate BA producers. Supporting this hypothesis, the use of nisin in combination with nitrite during elaboration of fermented sausages resulted in a significant decrease of putrescine production (Table 12.2; Kurt & Zorba, 2010). Moreover, two bacteriocin-producing enterococci and a nisin-producing *Lactococcus lactis* strain inhibited growth of a histamine-producing *L. buchneri* during cheese-making (Joosten & Nunez, 1996).

In addition, methods to avoid growth of BA producers during food processing can include irradiation, hydrostatic pressures, controlled atmosphere of packing and salting (Chong et al., 2011). However, health authority recommendations and the health perceptions of consumers increase the demand for low-salt foods, and this will probably diminish the use of salting.

As depicted in Tables 12.1 and 12.2 and stated above, several environmental or technological factors concur to affect BA formation in wine and fermented food, thus resulting in a deterioration of quality of foodstuffs. Understanding the mechanisms throughout the whole path from farm to fork is therefore the most important strategy to control the threat of BAs in fermented foods, and more research in this field is expected in the near future. Current knowledge reveals that, in particular, the time of ripening, and storage and drying environmental conditions, should be strictly controlled to reduce the total BA concentration in cheeses and dry fermented sausages. Moreover, for food and beverage raw materials, pH and storage temperature should be also controlled. Finally, the very sensitive methods recently developed to detect BA-producer strains and BA content in food will also help dairy and wine companies to evaluate ingredients and raw materials, and in early detection of BA production during fermentation processes, and in selecting new non-BA producing starters.

12.7 Legislation concerning biogenic amine content in food

Food safety should always be guaranteed to the consumer. Nonetheless, the establishment of community rules on the occurrence of BAs in fermented foods is still an open question. Although BAs are present in many different foods and beverages and their

concentrations vary widely between and within food types, a common regulation limiting the amounts of BAs in foods is still lacking (except for histamine in fish). Information regarding their presence in foods is also important for the food trade sector (in particular import and export), because recommended upper levels of BA content vary between countries.

It is reasonable to assume that BAs in food possess a potential risk hard to quantify. It is probable that cases of poisoning by BAs are underestimated, since generally they are not reported to the health authorities either because the symptoms can be often misdiagnosed or because in mild disorders medical reports are not required (Russo et al., 2010). Moreover, it is always difficult to establish a critical threshold of BAs in foods due to the intrinsic intra- and inter-individual variations in sensitivity or to the concomitant interference of inhibitors of the detoxification pathways such as antidepressants drugs or alcohol (Spano et al., 2010). Furthermore, monitoring and surveillance of the incidence food poisoning is not regulated in a uniform way across European Union (EU) countries, and a global overview on the BA intake during a meal should be evaluated.

To date, the only specific legislation for BAs in foods covers only histamine in fishery products (EFSA, 2005) and subsequent amendments (EC 1441/2007 and EC 365/2010). For these foodstuffs, the EU legislation considers a critical level of histamine to range between 200 and 100 mg/kg according to whether the products have undergone enzymic maturation treatment in brine or not (EC 2073/2005). A more stringent limit of 50 mg/kg is required by the FDA (Anonymous, 2001). However, it should be noted that in fish products, the formation of BAs is due to the decarboxylation of free histidine, abundant in the muscles of fishes, by Gram-negative bacteria, and it is not related to fermentation processes (Kim et al., 2003; Lehane & Olley, 2000).

Risks related to BAs were investigated by the European Food Safety Authority (EFSA), and a final report on a qualitative risk assessment of BA in fermented foods was issued by the Panel on Biological Hazards (EFSA, 2011). In response to EFSA's request to report data on BA occurrence in food, nine European countries submitted to EFSA a number of samples, with histamine being the most representative (10,123 samples), although tyramine was even indicated as the most relevant BA for food safety (EFSA, 2011). Data provided on food products were primarily of the fish and fish product category, followed by cheese and meat products. In addition, the study performed by EFSA Comprehensive European Food Consumption Database (EFSA, 2011) calculates the exposure for the BAs in all identified food categories in order to determine the food categories constituting a major risk for consumers and to quantify an overall exposure per day to BAs from the relevant food categories. The collected data led to a tentative figure for a cumulative daily intake, related to the food categories in question (EFSA, 2011).

Finally, even though this information on BAs was included only recently in a food composition database, information on their existence, distribution and concentration in fermented foods is crucial and useful for the food industry, health professionals and consumers.

12.8 Sources of further information and advice

Recently, findings of toxic levels of BAs in fermented food in member states of the European Union led the EFSA Panel on Biological Hazards (BIOHAZ), in collaboration with a selected working group of experts in the field, to produce a scientific opinion on risk-based control of BA formation in fermented foods.

Furthermore, BA formation in foodstuffs was examined in depth in the recent project BIAMFOOD (Controlling Biogenic Amines in Traditional Food Fermentations in Regional Europe), funded by the EU in the 7th Framework. This project was a continuation of the successful FP5 project "Fermentation of Food Products: Optimized Lactic Acid Bacteria Strains with Reduced Potential to Accumulate Biogenic Amines", coordinated by P. López, that ended in March 2006. BIAMFOOD involved the main research groups investigating alcoholic beverages (wine and cider) and cheese. The consortium coordinated by J. Lolkema (University of Groningen, Netherlands) also included the research groups of P. López (Centro de Investigaciones Biológicas, Spain), A. Lonvaud (Universite Victor Segalen - Bordeaux II, France), H. Alexandre (Université De Bourgogne, France), G. Spano (University of Foggia, Italy), M. Alvarez (Instituto de Productos Lácteos de Asturias, Spain) and E. Coton (ADRIA Normandie, France). However, since food safety is of global interest, there are worldwide studies focusing on the BA topic. For instance, research groups in Italy (Torriani, S.; Suzzi, G.; Gardini, F.), Spain (Bover-Cid, S.; Muñoz, R.; Pardo, I., Vidal-Carou), the Czech Republic (Komprda, T.), Turkey (Önal, A.), South Africa (du Toit, M.) and Argentina (Magni, C.; Manca de Nadra, M.C.) have long investigated BA formation in wine, fermented dry sausages and cheeses. In addition in recent years several authors from China and South Korea have reported on the detection and control of BAs in traditional Asian foods.

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Biopreservation effects in fermented foods

13

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13.1 Preservation and biopreservation

Microorganisms are naturally associated with the raw material used to produce foods, and if not reduced or removed they can spoil the food or be ingested with it and, in the case of pathogens, cause food-borne intoxication of the consumers.

That implies that a food needs to be preserved in order to be safe and maintain its nutritive and rheological characteristics till the moment of consumption. Food preservation relies on an array of techniques, and the use of chemical compounds represents one of the most frequently applied for many years. By definition, a preservative can be considered as a chemical substance useful to retard or arrest microbial growth in order to prevent food deterioration and/or safety loss (Surekha & Reddy, 2000).

Based on this definition, biopreservation is the use of compounds of "natural" origin, e.g., products deriving from the microbial metabolism to preserve foods. One of the oldest ways to naturally enrich a food with inhibitory substances against food spoilers and pathogens is represented by fermentation. Of course, besides releasing of compounds deriving from their metabolism, fermenting microorganisms can develop in food and exert a competitive effect against the resident microbiota. In this context, one can broadly define biopreservation as the use of safe microorganisms and/or their antimicrobial substances, naturally present or deliberately added, to extend the shelf-life and enhance the safety of foods. Lactic acid bacteria (LAB) represent the main actors in food biopreservation, as they are safe to consume (GRAS: "generally recognized as safe" status), as demonstrated by their long history of safe use in food, and are rapidly becoming the dominating microbiota during spontaneous or started fermentation and persisting during the storage of fermented foods.

LAB are a functional group of gram-positive, catalase-negative, non-spore-forming acid-tolerant bacteria, including different genera and more than 200 species, highly related from a phylogenetic point of view but showing ample metabolic differences (Axelsson, 2009, pp. 1–72). They generally show a fermentative metabolism leading to the release of lactic acid as the main metabolic end-product of carbohydrate fermentation. Among all the microorganisms, LAB can be considered as the best producers of natural preservatives in foods. Even if in the past the antimicrobial activity of LAB has been mainly attributed to the production of lactic and acetic acids, besides the consequent pH drop, more recently different kinds of bioactive molecules, such as diacetyl, hydrogen peroxide, reuterin, and

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00013-X

reutericyclin—as well as an array of different antifungal peptides, 3-hydroxy fatty acids, and bacteriocins—have been considered in the context of biopreservation (Stiles, 1996).

Among all the possible substances reported as antimicrobials on the basis of *in vitro* assays, the next sections will focus especially on those compounds for which studies exist about their possible efficacy *in situ*, as well as on metabolic pathways for production and possible mode of action.

13.2 Biopreservative effect of lactic and acetic acids

Even if, according to the "hurdle concept", it is difficult, and incorrect from a conceptual point of view, to separate the effect of each single factor acting against the growth of spoilage/pathogenic microorganisms in such a complex system as a food, the preservative effect in fermented milks, cheeses, or other foods prepared by raw materials rich in carbohydrates has been traditionally attributed to the presence of weak organic acids (mainly lactic acid and acetic acid) derived from the carbohydrate metabolism by LAB.

According to Hammes and Vogel (1995), LAB are classified in three different metabolic groups, as reported in Table 13.1. By considering the species of LAB naturally related to fermented foods, or deliberately added to them as starter cultures, all three of the metabolic categories are represented, with prevalence/combination of species as imposed by the intrinsic and extrinsic parameters acting during the process for the production of a specific food.

LAB belonging to the genera *Lactococcus*, *Enterococcus*, *Streptococcus*, and *Lactobacillus* are the main representatives responsible for the biopreservation effect in fermented milks and cheeses as based on their homofermentative metabolism of carbohydrate, releasing 2 mol of lactic acid for each mole of glucose consumed.

Table 13.1 Lactic acid bacteria metabolic groups and their characteristics (Hammes & Vogel, 1995)

Metabolic group	Main features
Obligately homofermentative	Exclusively ferment hexoses and produce lactic acid by the EMP (Embden–Meyerhof–Parnas) pathway
Obligately heterofermentative	Ferment both hexoses and pentoses by the 6-PG/PK (6-phosphogluconate/phosphoketolase pathway) and produce lactic acid, ethanol/acetic acid, and CO_2 (this latter only from hexoses) in the ratio 1:1:1
Facultatively heterofermentative	Ferment hexoses by the EMP pathway and pentoses (besides gluconate), by the 6-PG/PK pathway

Of course, LAB show different metabolic pathways to convert the lactose of milk to glucose subsequently fermented by the glycolysis (Figure 13.1).

The lactic acid accumulated during the production of fermented milks and cheeses, besides the related pH drop, represents the key component for the antimicrobial effect of dairy LAB against many spoilage and/or pathogenic bacteria. On the other hand, in some dairy products, and mainly in vegetable-based fermented foods or in intermediate food products such as sourdough, acetic acid released by facultatively or obligately heterofermentative LAB can account for an additive preservative effect related to fermentation.

13.2.1 Mode of action of weak organic acids on microbial cells

According to Jay, Loessner, and Golden (2005, pp. 1–701), while some foods protect themselves from microbial deterioration by an intrinsic acidity (e.g., malic, citric, or tartaric acids in fruits), in other cases both food acidity and low pH rely on microbial activity. In this case one can define this acidity as "biological." In each case, independently from its origin, the effect of the acidity on food preservation seems to be the same, but it is noteworthy that not all organic acids act in the same way and none of them has got a complete spectrum of activity against all the target microorganisms (Surekha & Reddy, 2000). Although the preservation of food by organic acids, included those produced by LAB fermentation, has been used for thousands of years in order to assure the safety and prolong the nutritional value of basic, perishable materials (e.g., milk or cereal mash), the possible mechanisms for microbial growth

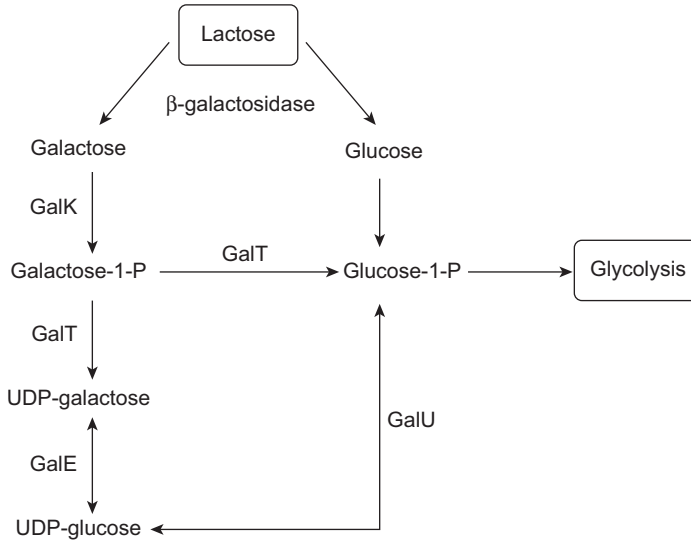

Figure 13.1 Lactose metabolism in lactic acid bacteria. GalK, galactokinase; GalT, galactose-1-phosphate uridyltransferase; GalE, UDP-galactose-4′-epimerase; GalU, glucose-1-phosphate uridyltransferase; UPD, uridine diphosphate.

inhibition have been addressed only in recent years. By considering the main aim of this chapter, only the organic acids released by the fermentative action from LAB will be considered here, namely lactic and acetic acids.

Early studies reported that the antimicrobial action of weak organic acids was essentially related to their capability to decrease the intracellular pH of the target microorganisms. This conclusion represents the basis for the so called "weak acid" theory (Stratford, 1999) (Figure 13.2): in low pH synthetic media or food systems, weak monocarboxylic organic acids, such as lactic and acetic acid, are only partially dissociated in solution, reaching a pH-dependent equilibrium, related to the pK_a of each organic acid (3.66 and 4.75 for lactic and acetic acid, respectively) between the undissociated, uncharged form and anions plus protons. The lower the pH, the higher the concentration of the undissociated form (Stratford, 1999). Since both lactic and acetic acids are lipophilic, their uncharged form is an essential requisite to enter the cell, bypassing the semipermeable cytoplasmic membrane by simple diffusion. This then rapidly diffuses into the cell and, at the near neutral pH of the cytoplasm, dissociates releasing protons and anions until an equilibrium between the external and internal concentrations of the uncharged form is reached. In this way cell growth can be inhibited if the accumulation of protons inside the cytoplasm exceeds the buffering capability of the cell or its capability to pump protons out by H^+-ATPase carriers.

Following the weak acid theory, the following causes can lead to growth inhibition or cell death: (1) lowering internal pH, which in its turn causes enzyme and nucleic acid denaturation; (2) impairment of the ΔH^+ gradient, which prevents active transport based on pmf (proton motive force); and (3) excessive consumption of ATP, which depletes the energy required for microbial growth (Hirshfield, Terzulli, & O'Byrne, 2003; Strafford, 1999).

Nevertheless, more recent investigations lightened the possible role of acid anions besides protons. Applying some basic chemistry principles and using a model system, Carpenter and Broadbent (2009) demonstrated the importance of correct experiment design in order to separate the general effects of the acidity from the specific effects of organic acids and their anions, in order to elucidate the mechanisms at the basis of the antimicrobial effect of organic acids. In particular, those authors concluded that

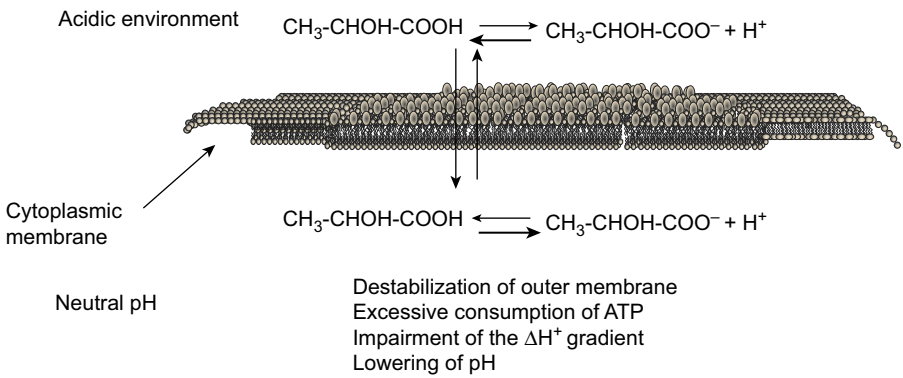

Figure 13.2 Action mode of weak organic acids according to Stratford (1999).

intracellular accumulation of anions is driven by two factors, external anion concentration and external acidity. Especially in foods with a pH around 5.0, a value characteristic of many fermented foods at least in some phases of their production, a high intracellular anion concentration can inhibit the microbial growth by (1) increasing of cellular turgor, until a level not compatible with regular cell metabolism is reached and possibly inducing an osmotic lysis; (2) inducing the expulsion of cellular anions, e.g., glutamate, which contributes to low cytoplasmic pH; and (3) influencing vital metabolic pathways by a feedback control, as with the inhibition of methionine biosynthesis by acetate accumulation in *Escherichia coli*, which induces an intracellular accumulation of the toxic intermediate homocysteine (Carpenter & Broadbent, 2009; Hirshfield et al., 2003).

Besides the mechanisms described above, some organic acids (especially sorbic but also lactic acid) can act by chelating ions from the medium as well as from bacterial cell walls, thus affecting both microbial function and structure (Stratford, 1999). Moreover, the lipophilic nature of acetic and lactic acids can also account for possible damage to the structure of the cytoplasmic membrane of target microorganisms, which interferes with its fluidity and regular functionality (Hirshfield et al., 2003; Strafford, 1999).

Overall, considering that many experiments leading to the above conclusions have been done in synthetic media and model systems, it should be considered that the real impact of the antimicrobial activity of weak organic acids in foods should be related to the possible interference of those compounds with all the components of the food matrix.

13.3 Biopreservative effect of phenyllactic acid

Many recent studies demonstrated that various LAB, especially *Lactobacillus plantarum* strains, can extend the shelf-life and improve the safety of many fermented foods and feed-stuffs by inhibiting mold growth. One of the most potent antifungal compounds has been identified as phenyllactic acid (PLA). Its production is strain-dependent (Vermeulen, Gänzle, & Vogel, 2006) and has been demonstrated *in situ* during the fermentation of sourdough (Lavermicocca et al., 2000; Lavermicocca, Valerio, & Visconti, 2003; Ryan, Dal Bello, Czerny, et al., 2009) and silage (Broberg, Jacobsson, Strom, & Schnurer, 2007) started with different, selected *Lb. plantarum* strains. In particular, Ryan, Dal Bello, Czerny, et al. (2009), by applying high-resolution gas chromatography-mass spectrometry analysis, demonstrated the production of 33.47 mg of PLA/kg of dough during the fermentation of a sourdough inoculated with the antifungal strain *Lb. plantarum* FST 1.7. In *in vitro* experiments, using a synthetic medium at pH 4.0, Lavermicocca et al. (2003) showed that less than 7.5 mg of PLA/ml was required to obtain 90% growth inhibition for all target mold strains evaluated in the study, and that fungicidal activity against 19 mold strains was shown by 10 mg/ml. Vermeulen, Gänzle, and Vogel (2006) showed that *Lb. plantarum* TMW 1.468 produced up to 1.1 mM PLA/kg during sourdough fermentation, while 45 mM PLA are required to inhibit mold growth in the presence of lactic and acetic acids (Lavermicocca et al., 2003). Taking together the above findings, it can be assumed that PLA represents a

component of a more complex mixture of compounds requested for an effective antifungal effect, including both lactic and acetic acids (Lavermicocca et al., 2003).

Moreover, various authors have pointed out that many other LAB such as *Lactobacillus alimentarius*, *Lactobacillus rhamnosus*, *Lactobacillus sanfranciscensis*, *Lactobacillus hilgardii*, *Leuconostoc citreum*, *Lactobacillus brevis*, *Lactobacillus acidophilus*, and *Leuconostoc mesenteroides* subsp. *mesenteroides* (Valerio, Lavermicocca, Pascale, & Visconti, 2004), as well as *Weissella* spp. (Ndagano, Lamoureux, Dortu, Vandermoten, & Thonart, 2011), can produce PLA in a synthetic medium. In particular, PLA has been proved to be inhibitory against many molds such as *Aspergillus niger*, *Aspergillus ochraceus*, *Penicillium roqueforti*, *Penicillium verrucosum*, and *Penicillium citrinum* contaminating bakery products, flour, cereals, and other foodstuffs (Valerio et al., 2004). LAB are not the only microorganisms releasing PLA (Mu, Yu, Zhu, Zhang, & Jiang, 2012); Dieleveux, Van Der Pyl, Chataud, and Gueguen (1998) were the first to demonstrate the production of d-3-phenyllactic acid by the yeast *Geotrichum candidum* and its effectiveness against some bacteria found in human and other foodstuffs, such as gram-positive *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Enterococcus faecalis* (Dieuleveux et al., 1998; Lavermicocca et al., 2000, 2003; Valerio et al., 2004). Other microorganisms inhibited by PLA are gram-negative *Providencia stuartii* and *Klebsiella oxytoca*, as well as *Salmonella* spp. (Rodriguez, Salgado, Cortes, & Dominguez, 2012) and *E. coli* O157:H7 (Ohhira et al., 2004).

The pathway accounting for PLA production involves phenylalanine catabolism. The first step relies on the transamination of the amino acid to phenylpyruvic acid by an aromatic aminotransferase, which catalyzes the transfer of the amino group from the amino acid to the α-keto acid acceptor α-ketoglutarate. The second step is the reduction of phenylpyruvic acid to PLA by a dehydrogenase (Figure 13.3), especially the lactate-dehydrogenases (LDH) (Mu et al., 2012). This metabolism has been particularly investigated by Vermeulen et al. (2006) who, by using a nitrogen-limited medium, addressed the influence of amino-acid/peptide supply on the phenylalanine metabolism by two key microorganisms involved in sourdough fermentation, namely *L. plantarum* and *L. sanfranciscensis*. In particular, the authors highlighted the low effectiveness of the amino acid transport system in lactobacilli, which resulted in just

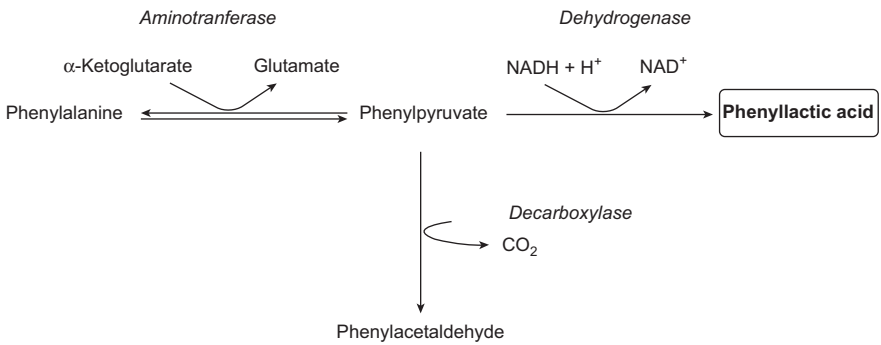

Figure 13.3 Metabolic pathway for phenyllactic acid production.
Adapted from Vermeulen et al. (2006).

1% conversion of the phenylalanine added to the medium in PLA, while a supply of phenylalanine-containing peptides increased the PLA yield two- to four-fold. The above findings seem not relevant in a real system, such as sourdough fermentation, due to the high level of peptides in dough, which lowers peptide transport in LAB (Vermeulen, Pavlovic, Ehrmann, Gänzle, & Vogel, 2005). Nevertheless, as the amino acid metabolism in LAB is limited by the availability of amino acceptors in the transamination reaction, the addition of α-ketoglutarate, the main ketoacid acceptor in some LAB, determines a surplus of up to 30% of PLA in the presence of *L. plantarum* TMW1.468, both in a synthetic nitrogen-limited medium and in sourdough (Vermeulen et al., 2006), indicating the possibility to direct the fermentation in order to improve the product shelf-life toward a correct strain selection and application besides environment modification. In the same context, it is interesting to observe that PLA production by LAB can be improved by adding to the medium its direct precursor, e.g., phenylpyruvic acid, or alternative electron acceptors, e.g., citrate, fructose, or glucose (Dellagnol et al., 2011; Li, Jiang, & Pan, 2007). Combining some of the above strategies, Vermeulen et al. (2006) reported that the addition of both α-ketoglutarate and citrate stimulated an overproduction of PLA by *L. sanfranciscensis* DSM20451^T, both in vitro and in situ, e.g., during sourdough fermentation.

13.3.1 Mode of action of PLA

As for other organic acids, the inhibitory activity of PLA is pH-related, depending on its pK_a (3.46) and could be explained by the weak acid theory (described earlier) (Lavermicocca et al., 2003). Nevertheless, Dieleveux et al. (1998), evaluating the antimicrobial effect of PLA against *L. monocytogenes* by using scanning electron microscopy, showed that D-3-phenyllactic acid acts particularly on the cell wall, first inducing the formation of cellular aggregates, followed by a lost of the cell wall rigidity, and finally leading to the complete cell lysis.

13.4 Biopreservative effect of diacetyl

Some species of the LAB group such as *Leuconostoc mesenteroides* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *dextranicum*, and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, are known for their capability to produce diacetyl (2,3-butanedione) from citrate, and this metabolism appears especially relevant in the field of dairy products (Figure 13.4). Actually, selected strains belonging to the above species are currently added as starter cultures to those products, e.g., butter, in which diacetyl imparts the distinctive and peculiar aroma. Nevertheless, in particular conditions where there is a pyruvate surplus in the medium (e.g., in the presence of an alternative source of pyruvate than the fermented carbohydrate, such as citrate in milk; or in the presence of an alternative electron acceptor available for NAD^+ regeneration) (Axelsson, 2009, pp. 1–72), even other LAB such as lactobacilli and pediococci can produce diacetyl by the scanted pyruvate (Figure 13.5). Thus, in addition to butter and dairy products, diacetyl can be present in other fermented foods and feeds, such as wine and ensilage (Jay, 1982).

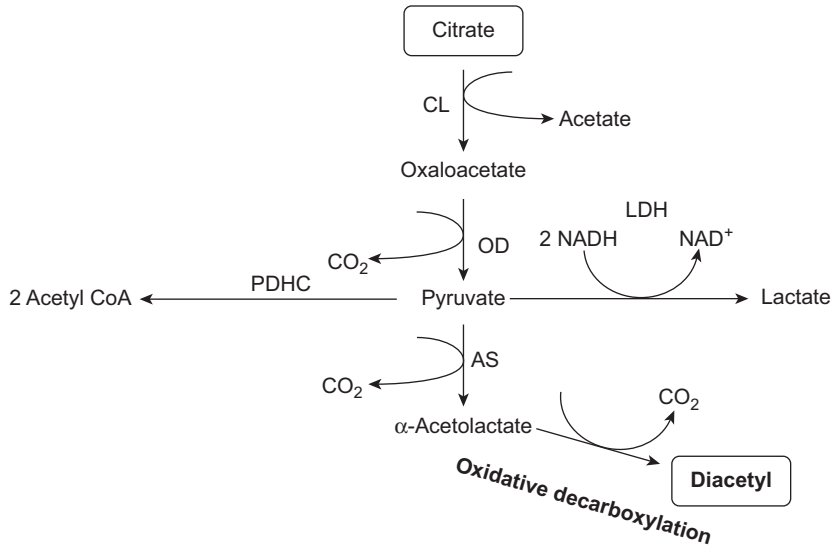

Figure 13.4 Diacetyl production from citrate. CL, citrate lyase; OD, oxaloacetate decarboxylase; LDH, lactate dehydrogenase; AS, α-acetolactate synthase; PDHC, pyruvate dehydrogenase complex.

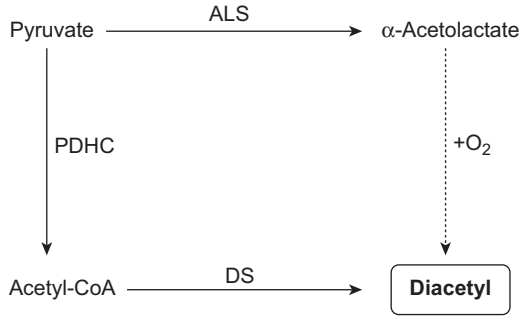

Figure 13.5 Diacetyl production from pyruvate. The chemical oxidative decarboxylation of α-acetolactate into diacetyl is shown by a dotted arrow. ALS, α-acetolactate synthase; PDHC, pyruvate dehydrogenase complex; DS, diacetyl synthase.
Adapted from Axelsson (2009).

Diacetyl has been reported to have an antimicrobial effect against food spoilage and food-borne pathogens, and has also attracted the attention of the scientific community for many years for its demonstrated absence of toxicity in humans and animals (Jay, 1982; Lanciotti, Patrignani, Bagnolini, Guerzoni, & Gardini, 2003; Newberne et al., 2000). It has been reported that diacetyl particularly inhibits growing cells of gram-negative bacteria and fungi at concentrations by 200 ppm, while gram-positive bacteria seems less affected and LAB in particular are not inhibited in the range

100–350 ppm at pH 5.0–7.0 (Jay, 1982). Lanciotti et al. (2003) showed that in a medium containing 300 ppm of diacetyl, *E. coli* was more sensitive than *S. aureus* or *L. monocytogenes*, and that the addition of NaCl to the liquid medium strengthened the antimicrobial efficacy against *E. coli*. Moreover, Kang and Fung (1999) reported the inhibition of *E. coli* O157:H7 and *Salmonella* Typhimurium by 50 ppm of diacetyl in a liquid medium in the presence of *Pediococcus acidilactici*.

In general, investigations aiming at evaluating the antimicrobial activity of diacetyl have been performed by using commercial preparation of such volatile compounds and not necessarily by exploiting the in situ activity of producer microorganisms or the compounds purified from cells. In any event, Jay (1982) concluded that the antibacterial and antifungal activities of diacetyl from seven different commercial preparations were shown to be essentially similar.

Moreover, most experiments have been performed in vitro by using synthetic media with added diacetyl, while only a few papers investigated the effect of diacetyl in fermented foods in situ. As reported by Escamilla, Valdes, Soriano, and Tomasini (2000), concentrations of diacetyl ranging from 0.5–20 to 44.0–66.5 mg/kg can be present in fermented foods such as dairy and bakery products, respectively. The above concentrations, taking into account all the possible interactions of the inhibitory compounds with the various components of a food matrix, could justify a possible antimicrobial role of diacetyl in situ. Kang and Fung (1999) demonstrated that, while 50 ppm diacetyl were sufficient in a synthetic medium (see above), 300 ppm were needed to inhibit *E. coli* O157:H7 and *Salmonella* Typhimurium in sausages fermented with the meat starter culture *P. acidilactici*, with growth and acidification capability remaining unaffected. The lower antimicrobial effectiveness in the meat can result from many factors such as the lower homogeneity of the matrix and/or the presence of fat that limit the contact between diacetyl and the target cells.

13.4.1 Mode of action of diacetyl

Helander, von Wright, and Matilla-Sandholm (1997) report that diacetyl can enter the cell of gram-negative bacteria throughout the porin protein, thus without altering the outer membrane permeability. The proposed mode for antimicrobial action relies on the reaction between diacetyl and the periplasmic proteins binding the arginine, thus interfering with the metabolism of such an amino acid. Lacking of similar binding proteins and the possibility to use a wider pattern of amino acids confer the gram-positive bacteria a greater resistance to this compound (Jay et al., 2005).

13.5 Biopreservative effect of cyclic dipeptides (2,5-diketopiperazines)

Cyclic dipeptides, also known as 2,5-diketopiperazines, are compounds well represented in nature as well as in foods and beverages (Gautschi et al., 1997; Hilton, Prasad, Vo, & Mouton, 1991). Their active role in foods has been related especially

to sensory aspects and, possibly, to antifungal activity (Magnusson, & Schnürer, 2001; Ryan, Dal Bello, Arendt, & Koehler, 2009), while an inhibitory action against pathogenic bacteria such as *S. aureus* has been suggested in the nonfood matrix (Li, Wang, Xu, Magarvey, & McCormick, 2011). Various bacteria, including LAB, have the potential to produce different cyclic dipeptides (Holden et al., 1999; Li et al., 2011; Niku-Paavola, Laitila, Mattila-Sandholm, & Haikara, 1999; Prasad, 1995). Strom, Sjogren, Broberg, and Schnurer (2002) isolated a strain of *Lb. plantarum*, labeled as MiLAB393, from grass silage and demonstrated the production, in liquid medium, of two cyclic dipedides, cyclo (L-Phe–L-Pro) and cyclo (L-Phe–trans-4 -OH-L-Pro), which showed inhibitory activity against many common food- and feed-borne molds, among which *Fusarium sporotrichioides* and *Aspergillus fumigatus* were the most sensitive. Later, as a result of screening of 1200 LAB strains isolated from various sources, not including fermented foods, Magnusson, Strom, Roos, Sjogren, and Schnurer (2003) attributed the capability to produce diketopiperazines also to other LAB such as *Lactobacillus coryniformis*, *Lactobacillus sakei*, and *Pediococcus pentosaceus* as well as to different strains of *Lb. plantarum*, pointing out a strong antimould activity against *Aspergillus nidulans* and *Penicillium commune*, in addition to the fungi already mentioned. The above results have been obtained *in vitro*, e.g., in a dual-culture agar plate assay, after growing LAB in a liquid medium; and so far an insufficient number of dedicated experiments have been devoted to verify the real production/effectiveness of cyclic dipeptides *in situ*, e.g., during the production of a fermented food. Consequently, one can just hypothesize that LAB can release a sufficient quantity of those compounds useful to protect a fermented food against fungal spoilage.

Ryan, Dal Bello, Arendt, & Koehler. (2009) addressed this topic by quantifying the level of *cis*-cyclo (L-Leu–L-Pro) and *cis*-cyclo (L-Phe–L-Pro) in acidified dough and bread, demonstrating that the production of 2,5 diketopiperazines depends on the acidification of the dough, in addition to the presence of divalent metal ions, without differences between chemical or biological acidification obtained by the fermentative activity of the strain *Lb. plantarum* FST 1.7. Thus, the role of LAB in the production of 2,5-diketopiperazine seems related essentially to the acidification effect and not to specific metabolic pathways. In particular, Ryan, Dal Bello, Arendt, & Koehler. (2009) pointed out that 2,5-diketopiperazines accumulate in dough following the progress in pH drop for three main reasons:

1. Activation of endogenous proteases and peptidases leading to the release of linear dipeptides.
2. Dipeptide cyclization toward the protonation of free carboxyl groups followed by the release of water and the formation of new intramolecular peptide bonds.
3. Activation of endogenous phytases, leading to the release of metal ions.

In general, in the presence of substrate, LAB, due to a broad spectrum of peptidases, could greatly contribute to the accumulation of 2,5-diketopiperazine in fermented foods, but at present, experimental evidence is still lacking.

Moreover, besides the above mechanism of production, some authors (Strom et al., 2002) hypothesized the direct synthesis of cyclic dipeptides by LAB, similarly to other bacteria. While this hypothesis has not been further addressed for LAB, recent research (Wyatt et al., 2010; Zimmermann & Fischbach, 2010) pointed out that

S. aureus possess a highly conserved non-ribosomal peptide synthetase gene cluster to synthesize cyclic dipeptides.

In general, based on the current literature, it seems that 2,5-diketopiperazines cannot be considered the main antifungal compounds in the food matrix, but they can possibly contribute to the shelf-life of some fermented foods acting in a synergistic way with other compounds (Ström et al., 2002). In fact, as reported by Ström et al. (2002) and Ryan, Dal Bello, and Arendt (2008), the MIC for both *cis*-cyclo (L-Leu–L-Pro) and *cis*-cyclo (L-Phe–L-Pro) is near 20 mg/g, that is, about 1000 times higher than the level that Ryan, Dal Bello, Arendt, & Koehler. (2009) detected in their experimental study on dough.

13.5.1 Mode of action of cyclic dipeptides

Only a few studies have been devoted to investigating the mode of action of cyclic dipeptides; Li et al. (2011) addressed this subject, by considering the inhibitory effect of a human vaginal isolated strain, *Lactobacillus reuteri* RC-14, against a pathogenic strain of *S. aureus,* isolated from the same source and responsible for the production of an exotoxin leading to menstruation-associated toxic shock syndrome (TSS). When cultivated in liquid medium, *Lb. reuteri* releases small signaling molecules, identified as the cyclic dipeptides cyclo (L-Tyr–L-Pro) and cyclo (L-Phe–L-Pro), which interfere with the staphylococcal quorum-sensing system *agr*, which functions as a key regulator of virulence genes, thus repressing the exotoxin synthesis without interfering with the pathogen survival. In that context it is interesting to underline that the availability of molecules targeting the bacterial virulence could decrease the selective pressure of antimicrobial compounds leading to the selection of drug-resistant strains (Li et al., 2011), a prominent problem in the field of bacteriocin application in food preservation (see below).

Li et al. (2011), based on the work of Waters and Bassler (2005), defined the antagonism of *Lb. reuteri* RC-14 against the quorum-sensing mechanism of *S. aureus* as "quorum quenching". A concept that deserves further attention in the field of food microbiology. Actually, *Lb. reuteri* represents a recognized probiotic strain that could be vehicled by many fermented foods but, in order to extend the above findings to food and feed systems, specifically designed studies are warranted.

13.6 Biopreservative effect of bacteriocins

Bacteriocins are low-molecular-mass peptides or proteins ribosomally synthesized by many bacteria, showing a bactericidal or bacteriostatic effect against other bacteria belonging to the same species (narrow spectrum) or to different genera (broad-spectrum) (Cotter, Hill, & Ross, 2005; Klaenhammer, 1988; Tagg, Dajani, & Wannamaker, 1976). Generally, LAB bacteriocins are active against gram-positives, and like the "producer" bacteria, are considered GRAS compounds, being ingested with foods naturally containing bacteriocinogenic strains since people consume fermented foods (Settanni & Corsetti, 2008). For that reason, bacteriocins from LAB have attracted the interest of the scientific community for many years, as they can be used to confer

a sort of innate, "biological" immunity to foodstuffs (Cotter et al., 2005). In this context, three different strategies can be considered to preserve a food by bacteriocins (Anonou, Maqueda, Martinez-Bueno, Galvez, & Valdivia, 2007):

1. Inoculation of bacteriocinogenic (e.g., bacteriocin-producer) selected LAB strain, able to release the active bacteriocin *in situ*, as co-culture, protective culture, or starter culture.
2. Addition to the food processing of a pre-fermented food containing bacteriocinogenic strains.
3. Addition to the food of a previously produced and purified bacteriocin.

Despite the renewed interest in the application of natural compounds in food preservation and extensive studies dealing with potential new bacteriocins, to date only two LAB bacteriocins, nisin and pediocin PA1, are commercially available as Nisaplin (Danisco, Copenhagen, Denmark), produced by *Lactococcus lactis*; and ALTA 2351 (Kerry Bioscience, Carrigaline, Co. Cork, Ireland) produced by *P. acidilactici*, respectively, and are currently applied as food additives (Mills et al., 2011; Settanni & Corsetti, 2008). As the use of LAB metabolites as additives falls out of the scope of this chapter and many reviews are dealing with this subject (Castellano, Belfiore, Fadda, & Vignolo, 2008; Deegan, Cotter, Hill, & Ross, 2006; De Vuyst & Leroy, 2007; Galvez, Abriouel, Benomar, & Lucas, 2010; Galvez, Abriouel, Lopez, & Ben Omar, 2007; Galvez, Lopez, Abriouel, Valdivia, & Omar, 2008; Settanni & Corsetti, 2008; Sobrino-Lopez & Martin-Belloso, 2008), the reader is directed to those papers for further details. Here, some examples based on investigations dealing with the possible applications of bacteriocinogenic strains as part of the biopreservative effect in some fermented foods are reported. In this context, it could be useful to remember that while antimicrobial activity constitutes the only requested characteristic for protective cultures or co-cultures (even indicated as adjunct cultures), it may represent a secondary effect for starter cultures selected on the basis of technological traits (Holzapfel, Geisen, & Schillinger, 1995).

Regarding the above point, Foegeding and Stanley (1991) investigated the inactivation of a mix of five strains of *L. monocytogenes* during a meat fermentation challenge started with a pediocin-producing (Bac^+) *P. acidilactici* strain. By using an isogenic pediocin-negative (Bac^-) derivative as negative control, the authors demonstrated that *L. monocytogenes* can survive the fermentation process when the pH was not lowered under 4.9, but in that case, pediocin released *in situ* contributed to inactivation of the pathogen during the drying process.

McAuliffe, Hill, and Ross (1999) addressed the application of bacteriocinogenic strains of *Lc. lactis* as protective culture to improve the safety of cottage cheese. In experimental cheeses, started with *Lc. lactis* DPC4268-producing lacticin 3147 and with 4 log CFU/g of *L. monocytogenes* Scott A added, the authors measured bacteriocin activity in the curd of 2560 AU/ml throughout the first week of storage at 4 °C and a consequent reduction of the *L. monocytogenes* target strain up to 99.9% within 5 days, while the initial numbers of the pathogen remained unchanged in the control cheese started with the non-producer strain *Lc. lactis* DPC 4275. As the authors were unable to recover any *Listeria* in cheese through pre-enrichment over a week of storage at different temperatures, they could confirm the bactericidal nature of the lacticin 3147.

More recently, Dal Bello et al. (2012) applied four strains of bacteriocinogenic *Lc. lactis* (one nisin Z producer, one nisin A producer, and two lacticin 481 producers), isolated from cheese and previously evaluated for desired technological characteristics, as starter cultures in cottage cheese production, with the aim to control the growth of *L. monocytogenes*. In particular, the strain *Lc. lactis* subsp. *cremoris* 40FEL3 (producer of nisin A) in combination with the high acidity reached during cheese manufacturing, was able to control and partially reduce the growth of the *L. monocytogenes* target strain inoculated in cheese at 3 log CFU/g. By considering that the *in situ* inhibitory effects were reduced in comparison to those observed *in vitro*, the authors considered the application of the above strain in cheese production as a possible additional measure to control *Listeria* spp. contamination.

In general, the lower efficacy of bacteriocins in food with respect to their inhibitory activity as evaluated with *in vitro* assays is a common problem in the field of bacteriocin application in real systems, and has been related to many factors, such as partitioning into polar or nonpolar food components and binding of the bacteriocins to food fat or protein particles and food additives (e.g., triglyceride oils), NaCl concentration, proteolytic degradation or inactivation by other inhibitors, changes in solubility and charge, changes in the cell envelope of the target bacteria, as well as the development of bacteriocin-resistant strains, a quite common problem in the field of bacteriocin application in food biopreservation (Aesen et al., 2003; Chollet, Sebti, Martial-Gros, & Degraeve, 2008; Degnan & Luchansky, 1992; Gänzle, Weber, & Hammes, 1999; Jung, Bodyfelt, & Daeschel, 1992; Leroy & De Vuyst, 1999; Murray & Richard, 1997; Nishie, Nagao, & Sonomoto, 2012). A more detailed list of the factors limiting the bacteriocin efficacy in foods is given in Figure 13.6. Thus, even if many examples can be found in literature dealing with the possibility of using in situ released bacteriocins as food biopreservatives, it is actually recognized that this approach is unlikely to efficiently protect a food against microbial contamination (Deegan et al., 2006) if other preservation methods (e.g., other chemical compounds and/or physical treatments) are not applied in conjunction (Galvez et al., 2007, 2008). On the other hand, in the presence of adjunctive hurdles useful to destabilize the outer membrane, LAB bacteriocins also become effective against gram-negative pathogens (Deegan et al., 2006; Stevens, Sheldon, Klapes, & Klaenhammer, 1991).

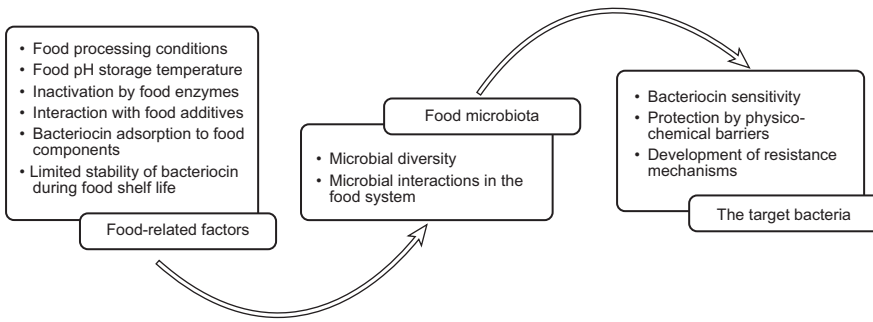

Figure 13.6 Main factors influencing bacteriocin activity in foods.
Adapted from Galvez et al. (2007).

Nevertheless, even if the effectiveness of a single bacteriocinogenic strain *in situ* against target pathogens seems still controversial, the release of bacteriocins *in situ* can generally account for the implantation of starter strains, thus contributing, even indirectly, to the preservative effect in fermented foods.

In this context, Ruiz-Barba, Cathcart, Warner, and Jiménez-Díaz (1994), by comparing the growth and persistence of the bacteriocin-producer strain *Lb. plantarum* LPCO10 with its non-bacteriocin-producing, bacteriocin$^-$ immune derivative *Lb. plantarum* 55-1, in natural Spanish-style green olive fermentations, showed that while the latter strain could not be isolated after 7 weeks, the strain LPCO10 dominated the contaminating microbiota and persisted, beside spontaneous lactobacilli, until 12 weeks of fermentation, assuring a higher concentration of lactic acid in brine. Recently, the same authors (Ruiz-Barba, Caballero-Guerrero, Maldonado-Barragan, & Jimenez-Diaz, 2010) used the bacteriocin producer *Lb. plantarum* NC8 in table olive fermentation in co-culture with two specific bacteriocin production-inducing strains, i.e., *Enterococcus faecium* 6T1a-20 and *P. pentosaceus* FBB63, pointing out the use of the above mixed starter accounting for enhancing the viability of the bacteriocin producer.

Settanni, Massitti, Van Sinderen, and Corsetti (2005) showed that the *in situ* release of lacticin 3147 by *Lc. lactis* subsp. *lactis* M30 contributed to the microbial stability of sourdough during a 20-day period of propagation. In particular, the presence of the bacteriocinogenic strain enables the persistence of the selected, insensitive strain *Lb. sanfranciscensis* CB1, useful to confer desired characteristics to the dough, by inhibiting the indicator strain *Lb. plantarum* 20, able to rapidly overcome the strain CB1 in control fermentation in the presence of a non-bacteriocin producing *Lc. lactis* strain. The last can be considered as an example of the application of a bacteriocinogenic strain as a co-culture to support the starter implantation; in that case, the bacteriocin producer does not need to contribute to the fermentation, and in the meantime, it does not interfere with the function of the starter, which of course needs to be insensitive to the bacteriocin (Galvez et al., 2007).

13.6.1 Mode of action of bacteriocins

Based on the classification of Cotter et al. (2005), LAB bacteriocins have been grouped into two classes, namely "lantibiotics" (containing lanthionine) and "non-lanthionine-containing bacteriocins" that are further divided into two and four groups, respectively (Nishie et al., 2012). In general, the mode of action is linked to the primary structure of the peptide; thus, a variety of modes of action have been reported, but it is also demonstrated that a single bacteriocin can act by more than one way to kill target bacteria (Hasper et al., 2006; Nishie et al., 2012; Widemann et al., 2001). In general, two main mechanisms of action, often related, can be shown for many LAB bacteriocins: (1) binding of the bacteriocin to the surface of the cell membrane of the target bacteria, mediated or not by a specific receptor, finally leading to the pore formation throughout the plasma membrane, and the consequent efflux of intracellular molecules; and/or (2) cell-wall biosynthesis inhibition (Nishie et al., 2012). By considering, as example of the first step of the bacterium–bacteriocin interaction, one of the most-studied LAB bacteriocins, nisin, besides other lantibiotics, recognizes

as a receptor the membrane-bound cell-wall precursor lipid II (Breukink & de Kruijff, 2006). Lacticin 3147, a two-peptide lantibiotic (LtnA1 and LtnA2), binds first the lipid II by the LtnA1 component. Pediocin PA-1/AcH, a class IIA bacteriocin, recognizes as receptor two components of the membrane-linked protein carrier mannose-phosphotranferase (man-PTS) (Nishie et al., 2012). In contrast, some class IIc cyclic bacteriocins such as enterocin AS-48, and some class IId bacteriocins such as Lacticin Q, form nonselective pores, thus acting without any specific receptor (Galvez, Maqueda, Martinez-Bueno, & Valdivia, 1991). In each case, independently from the mode of action, it is important to point out that the bacteriocin activity in food can be greatly reduced by interaction with food components (see Figure 13.6).

13.7 Biopreservative effect of other compounds

Reuterin (h-hydroxypropionaldehyde) is a broad-spectrum antimicrobial compound produced by *Lb. reuteri* during anaerobic growth on a mixture of glucose and glycerol or glyceraldehydes (Axelsson, Chung, Dobrogosz, & Lindgren, 1989). Reuterin-producing strains of *Lb. reuteri* show strong inhibitory effects against gram-positive bacteria, e.g., *B. cereus*, *S. aureus*, and *L. monocytogenes*, and gram-negative bacteria, e.g., *E. coli*, *Yersinia enterocolitica*, and *Pseudomonas fluorescens*, in synthetic media (El-Ziney et al., 1999). The mode of action of reuterin is not completely understood, but probably it inhibits ribonucleotide reductase and thioredoxin activities and thereby the synthesis of DNA (Vollenweider & Lacroix, 2004).

Reutericyclin is a highly hydrophobic, charged inhibitory compound produced by sourdough isolates of *Lb. reuteri* (Gänzle, Holtzel, Walter, Jung, & Hammes, 2000; Gänzle & Vogel, 2003). This compound is bacteriostatic or bactericidal against a broad range of gram-positive bacteria including spoilage microorganisms and pathogens such as *S. aureus*, *L. monocytogenes,* and *B. cereus*, based on its activity as a proton-ionophore. Regarding the mode of action, it seems that reutericyclin is active against the cytoplasmic membrane (Gänzle & Vogel, 2003). Probably it acts as proton ionophore; in fact, low pH and high salt concentrations increase the inhibitory activity of reutericyclin, whereas divalent cations inhibit its activity (Gänzle et al., 2000; Gänzle & Vogel, 2003). Gram-negative bacteria are not inhibited by reutericyclin due to the permeability barrier of their outer membrane (Gänzle, 2004). Therefore, food applications based on fermentations with reutericyclin-producing strains can be envisaged. Generally, the application of reutericyclin is possible through addition of the purified compound to food products, by fermentation with reutericyclin-producing strains, or through addition of metabolically inactive cells to foods in which the product composition allows for the desorption of reutericyclin associated with the cells. This compound is associated with the stability of some German sourdoughs (Messens & De Vuyst, 2002). In particular, Gänzle and Vogel (2003) showed that reutericyclin production provides a competitive advantage to the producer strains and contributed to the stable persistence of *Lb. reuteri* in an industrial sourdough fermentation over a period of 10 years inhibiting other LAB such as *Lb. sanfranciscensis*. Its application as biopreservative is due also to its ability to inhibit rope-forming *Bacillus subtilis*

strains in bread (Gänzle et al., 2000). Reutericyclin is produced in active concentrations during wheat sourdough fermentations (Gänzle & Vogel, 2003) and remains active after baking. Thus, reutericyclin-producing strains may be applied to extend the shelf-life of baked goods.

Other potential antimicrobials produced by LAB that can play a role in food biopreservation are fatty acids (FAs), CO_2, and hydrogen peroxide (H_2O_2), but no studies concerning their involvement in food biopreservation following the *in situ* release by fermenting microorganisms are available. Besides the synthesis of these inhibitory compounds, LAB may release, through proteolysis, antimicrobial peptides from food proteins with antifungal activity (Coda et al., 2011). Okkers, Dicks, Silvester, Joubert, and Odendaal (1999) described a peptide, called TV35b, from *Lactobacillus pentosus*. This compound induced formation of pseudohyphae and had fungistatic effects against *Candida albicans*. Two other antifungal proteinaceous compounds from LAB have been described: a proteinaceous compound from *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 showing inhibitory effect against a broad range of filamentous fungi, molds and to a lesser extent against yeasts (Magnusson & Schnurer, 2001); and a "bacteriocin-like substance" isolated from *Lactobacillus paracasei* subsp. *paracasei* M3, with antibacterial activity and fungistatic effects against strains of *C. albicans*, *C. pseudointermedia*, *C. blankii*, and *Saccharomyces cerevisiae* (Atanossova et al., 2003).

A number of FAs are known to have potent antimicrobial activities (Hou & Forman, 2000). The mechanism by which they exert their antimicrobial activity is still not clear. Generally, the antifungal activity of FAs is due to their detergent-like properties, which could alter the structure of cell membranes of the target microorganisms leading to an increase of membrane permeability and so to the release of intracellular electrolytes and proteins and to cell lysis (Petschow, Batema, & Ford, 1996; Sjögren, Magnusson, Broberg, Schnürer, & Kenne, 2003). LAB are able to produce short-chain fatty acids with antifungal activity. Four antifungal 3-hydroxy FAs (3-(R)-hydroxydecanoic acid, 3-hydroxy-5-*cis*-dodecenoic acid, 3-(R)-hydroxydodecanoic acid, and 3-(R)-hydroxytetradecanoic acid produced by *Lb. plantarum* MiLAB 14 have been characterized by Sjogren et al. (2003) and showed antimicrobial activity against yeasts and fungi. Yeasts were more sensitive than fungi, and *P. roqueforti* was the most sensitive (5–50 μg/ml), whereas *A. fumigatus* was the least sensitive (25–100 μg/ml).

CO_2 is produced by heterofermentative LAB. The antimicrobial activity of this compound is due to its ability to replace the existent molecular oxygen in food products, creating anaerobic conditions toxic to some aerobic food microorganisms through its action on cell membranes and its ability to reduce internal and external pH (Caplice & Fitzgerald, 1999). This activity is important in vegetable fermentations to prevent the growth of spoilage fungi (Bela, Zaiton, & Sadon, 2011). In fact, concentrations of CO_2 ranging from 20% to 50% are able to inhibit common fruit spoilage organisms such as *Botrytis*, *Rhizopus,* and *Penicillum* (Bela et al., 2011).

Regading H_2O_2, it is produced by most of the LAB when oxygen is available using such enzymes as the flavo-protein oxidoreductases, NADH peroxidase, NADH oxidase, and glycerophosphate oxidase (Ross, Morgan, & Hill, 2002). Hydrogen peroxide is well studied and the mode of action is well known. It is related to the strong

oxidizing effect on the bacterial cell, and to the destruction of basic molecular structures of cellular proteins (Bela et al., 2011). Some authors suggested the use of H_2O_2 for food biopreservation (Bela et al., 2011; Ponts, Pinson-Gadais, Verdal-Bonnin, Barreau, & Richard-Forget, 2006; Ross et al., 2002; Venturini, Blanco, & Oria, 2002). The potential of hydrogen peroxidase produced by LAB for food preservation may be limited by the oxidizing nature of the molecule, and free radicals produced may have profound effects on the sensory quality, causing rancidity of fats and oils and discoloration reactions.

13.8 Conclusions

Consumers are increasingly concerned about possible adverse health effects from the presence of chemical additives in foods. This perception, together with the growing demand for minimally processed foods with long shelf life, has led to an increased interest of the scientific community in the application of consumer-friendly biopreservatives, especially as part of compounds related to the metabolic activity of spontaneous or voluntarily added microorganisms in fermented foods. As reported in this chapter, fermentation by LAB plays a key role in food biopreservation, due to an array of compounds sometimes acting in a synergistic way in the complex food system, possibly against both spoilage and pathogenic microorganisms. Nevertheless, the mode of action of many antimicrobials remains unclear, and some new compounds are still poorly investigated for their effectiveness *in situ*. Further advancement in that context can warrant the development of new technologies for fermented food production and preservation, and contribute to their distribution through industrialized countries.

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Lactic acid bacteria as antifungal agents

14

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14.1 Introduction

In the environment, microorganisms coexist; and it is natural that by sharing their habitat they may influence one another. Fungi, especially molds, are no different and can have their growth and mycotoxin production influenced by their surroundings. Many factors could result in this biocontrol, including competitive growth (competition for space and nutrients in general), competition for nutrients required for mycotoxin production but not for growth, production of antimycotic and antimycotoxigenic metabolites by coexisting microorganisms, changes in pH of the substratum, or a combination of these factors (Bianchini & Bullerman, 2010).

The first report of biocontrol including molds and mycotoxin production was in the 1960s, when Ciegler, Lillehoj, Peterson, and Hall (1966) identified *Flavobacterium aurantiacum* as capable of irreversibly removing aflatoxin from solutions while evaluating over 1000 microorganisms for their ability to degrade aflatoxins. Further studies on this ability of *Flav. aurantiacum* indicated that pH and temperature influenced the toxin removal (Lillehoj, Ciegler, & Hall, 1967). Since then, many other studies have described the control exerted by molds, yeasts, and bacteria over the growth of certain molds. Reports have indicated that the growth of *Fusarium moniliforme* and *Aspergillus flavus* is affected by *Trichoderma harzianum* and *T. viride* (Calistru, McLean, & Berjak, 1997), where complementary action of antibiosis, nutrient competition, and production of cell wall-degrading enzymes results in the biocontrol. Other instances where molds are biocontrolled by yeasts can be exemplified by the influence of *Kluyveromyces* spp. on the growth of *Aspergillus* section Flavi (La Penna, Nesci, & Etcheverry, 2004).

Another potential source of organisms that could offer biocontrol over molds and/or produce natural antifungal agents is lactic acid bacteria (LAB). According to current taxonomy, the lactic acid bacteria group presently (in 2014) consists of six families and at least 38 genera (Vandamme, De Bruyne, & Pot, 2014), including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Vagococcus*, and *Weissella* (Hutkins, 2006). This group is commonly referred to as LAB and some of them are traditionally used as starter cultures for dairy, vegetable, cereal, and meat fermentations because of their contribution to flavor development and preservative potential (Buckenhüskes, 2006; Holzapfel, Geisen, & Schillinger, 1995; Lindgren & Dobrogosz, 2006; Olson, 2006; Urbach, 1995). It has been estimated that 25% of the

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00014-1

European diet and 60% of the diet in many developing countries consist of fermented foods (Stiles, 1996).

Lactic acid bacteria have a GRAS (Generally Recognized as Safe) status and research has shown that they may positively influence the gastrointestinal tract of humans and other mammals as probiotics (Sandine, 1996). Trias, Bañeras, Montesinos, and Badosa (2008) determined the occurrence of LAB in fruits and vegetables and found them in the range of 10^2–10^6 CFU/g in these products, with the highest concentration in ready-to-eat vegetables. Indigenous LAB are also commonly found in retail foods, which suggests that the public is already consuming viable LAB in many ready-to-eat products (Garver & Muriana, 1993). Additionally, they have been used to ferment foods for many years, which suggests the nontoxic nature of metabolites produced by these organisms (Garver & Muriana, 1993; Klaenhammer, 1998). It is believed that the metabolic activity of LAB that may contribute in a number of ways to the control of bacterial pathogens, and improvement of shelf life and sensory qualities (Holzapfel et al., 1995; Klaenhammer, 1998), might also have applications for preventing mold growth.

This chapter will describe some natural antifungal compounds produced by LAB, the factors that influence their production by this group of bacteria, and some practical applications of such antifungal compounds. In addition, the effect that these bacteria have on the production of mycotoxin by toxigenic fungi will be discussed, along with their potential as mycotoxin binders (by adsorption) in liquid systems.

14.2 Natural antifungal compounds produced by lactic acid bacteria

There have been a number of reports of mold inhibition by lactic acid bacteria (Gourama & Bullerman, 1995a, 1995b; Karunaratne, Wezenberg, & Bullerman, 1990; Lavermicocca et al., 2000; Plocková, Stiles, Chumchalová, & Halfarova, 2001; Schnürer & Magnusson, 2005; Stiles, 1999; Stiles, Penkar, Plocková, Chumchalová, & Bullerman, 2002; Stiles, Plocková, Toth, & Chumchalová, 1999; Trias et al., 2008); however, while the majority describes the inhibitory activity of LAB, few report on the characterization of compounds or mechanisms of action. Table 14.1 shows some of the publications that have demonstrated the antifungal activity of LAB, which varies from broad to very specific among and within species; while Figure 14.1 illustrates the antifungal activity of LAB on DeMan-Rogosa-Sharpe (MRS) agar.

Lactobacillus paracasei isolated from sourdough starter cultures has shown antifungal activity against several species from three mold genera: *Aspergillus*, *Penicillium*, and *Fusarium*, including both toxigenic and deteriorative fungi. Experiment results indicated that *L. paracasei* has a strong activity against all three *Fusarium* species tested, followed by *Penicillium* species to a lesser degree. All four *Aspergillus* species tested were the most difficult to inhibit (Hassan & Bullerman, 2008). The inhibition pattern described by Hassan and Bullerman (2008) was also

Table 14.1 Publications reporting the antifungal activity of lactic acid bacteria, their source of isolation, and reference to published scientific papers

LAB isolate	Source	Activity spectrum	Reference
Streptococcus lactis C10	Cheeses	*Aspergillus parasiticus*	Wiseman and Marth (1981)
Lactobacillus casei var. *rhamnosus*	NRRL culture collection	Broad spectrum	King, Fowler, and Vandenbergh (1986)
Lactobacillus reuteri	Human gastrointestinal tract	Broad spectrum	Talarico et al. (1988)
Lactobacillus casei subsp. *pseudoplantarum*	Silage inoculant	*Aspergillus flavus* subsp. *parasiticus*	Gourama and Bullerman (1997)
Lactococcus lactis subsp. *lactis*	Cheddar cheese and raw buffalo milk	*Aspergillus flavus* *Aspergillus parasiticus* *Fusarium* spp.	Roy et al. (1996)
Lactobacillus casei	Dairy products, vegetables and fruits	*Penicillium* spp.	Gourama (1997)
Lactobacillus sanfrancisco CB1	Sourdough	*Fusarium* spp. *Penicillium* spp. *Aspergillus* spp. *Monilia* spp.	Corsetti et al. (1998)
Lactobacillus plantarum	Sourdough	*Eurotium* spp. *Penicillium* spp. *Endomyces fibuliger* *Aspergillus* spp. *Fusarium graminearum*	Lavermicocca et al. (2000)
Lactobacillus plantarum MiLAB 393	Grass silage	*Fusarium sporotrichioides* *Aspergillus* spp. *Kluyveromyces marxianus* *Penicillium roqueforti*	Ström et al. (2002)
Lactobacillus plantarum VTT E-78076 and VTT E-79098	Beer and pickled cabbage	*Fusarium* spp.	Laitila et al. (2002)
Lactobacillus paracasei subsp. *paracasei*	Raw milk, cheese, yogurt, olives, and sourdough, as well as corn and grass silage	Yeast *Penicillium* spp.	Schwenninger, Ah, Niederer, Teuber, and Meile (2005)

Continued

Table 14.1 Continued

LAB isolate	Source	Activity spectrum	Reference
Weissella cibaria	Durum wheat semolina	*Aspergillus niger* *P. roqueforti* *E. fibuliger*	Valerio et al. (2009)
Lactobacillus paracasei subsp. *tolerans*	Sourdough bread	*Fusarium* spp. *Penicillium* spp. *Aspergillus* spp.	Hassan and Bullerman (2008)
Lactobacillus amylovorus	Cereal environment (patented as DSM strain)	*Aspergillus fumigatus* *Fusarium culmorum*	Ryan et al. (2011)

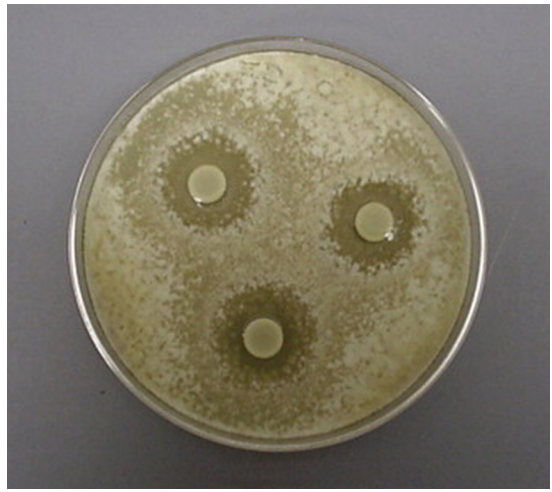

Figure 14.1 Halos (clear zones) of inhibition of growth of *Penicillium expansum* formed around colonies of lactic acid bacteria on MRS agar.
Reprinted from Hassan and Bullerman (2008) with permission from Elsevier.

observed by Gerez, Torino, Rollán, and Font de Valdez (2009) while studying antifungal activity of 95 homo- and heterofermentative strains of LAB. They evaluated the effect of those LAB on the conidial germination of *Aspergillus*, *Penicillium*, and *Fusarium* species. Their results indicated that 63 strains showed high inhibition of conidial germination (more than 70%) against *Fusarium graminearum*, moderate inhibition (40–70%) against *Penicillium* sp., and low inhibition (less than 40%) on the conidial germination of *Aspergillus niger.* However, when the antifungal activity was evaluated on the vegetative growth of these molds, slightly different results were observed by Gerez et al. (2009), with very few LAB strains being able to inhibit vegetative growth. From all 95 strains tested, only two (representing *Lactobacillus plantarum* and *Lactobacillus casei*) were able to inhibit *A. niger*; four

strains showed activity against *F. graminearum* (*L. plantarum*, *Lactobacillus reuteri*, and two strains of *Lactobacillus brevis*); and no action was observed on the vegetative growth of *Penicillium* sp.

Mauch, Dal Bello, Coffey, and Arendt (2010) studied in depth the effect of *L. brevis* on the conidial germination of *Fusarium culmorum.* They observed that germination of *F. culmorum* was completely delayed for either 6 or 48 h, depending upon the *L. brevis* strain. When observing the germination pattern of the mold spores, in the presence of cell-free supernatant (5% level) of inhibitory *L. brevis*, the macroconidia showed more than two germ tubes that mainly emerged from nonapical cells. These alterations from normal germination patterns (two germ tubes emerging from apical cells) were suggested as indication of stress (Mauch et al., 2010). Other authors have also described the emergence of germ tubes from internal compartments of the macroconidia when nonlethal concentrations of antifungal substances were present in the environment (Chitarra, Breeuwer, Rombouts, Abee, & Dijksterhuis, 2005; Harris, 2005).

Most of the studies that describe the antifungal activity of LAB do so either by using a cell-free supernatant from bacterial culture to inhibit mold growth or by culturing the two organisms consecutively (bacteria previously grown on an agar plate is overlaid with agar containing mold spores). However, a few studies have tried to demonstrate a potential competitive growth between these two groups of organisms by cultivating them at the same time. One report showed the antifungal activity of *Lactobacillus rhamnosus* isolated from a Czech tartar sauce, when bacteria and mold were cultivated together for 10 days at 30 °C. In this assay the ability of *L. rhamnosus* to inhibit mycelial growth of *Penicillium commune* and *A. niger* was attributed to an antagonistic behavior. Results from this experiment showed that reduction in the weight of dried mycelial mass was observed in *P. commune* mostly after 4 days of incubation, while *A. niger* was inhibited from the beginning of the experiment (Stiles, Carter, & Bullerman, 2002a).

Scientific research has also suggested that this inhibitory activity of LAB over molds may result from the production of metabolites such as organic acids (in particular, lactic, propionic, and acetic acids), carbon dioxide, ethanol, hydrogen peroxide, diacetyl, reuterin, and other small molecular weight metabolites; production of proteinaceous compounds or low-molecular weight peptides; competitive growth; decrease in the pH caused by acid production; or a combination of these factors (Bianchini & Bullerman, 2010).

While studying the inhibitory activity of LAB over *Penicillium digitatum* and *Geotrichum citri-aurantii*, Gerez, Carbajo, Rollán, Torres Leal, and Font de Valdez (2010) reported *L. plantarum*, *L. casei*, *Lactobacillus acidophilus*, *L. paracasei*, and *Pediococcus pentosaceus* as capable of inhibiting the target organisms. However, the rate of fungal inhibition was markedly different, depending upon the LAB and the fungal strain evaluated. In their studies, because the antifungal activity was lost after neutralization but not after either heating or treating the cell-free bacterial supernatant with proteinase K, it was suggested that the antifungal effect observed was acidic in nature. Evaluation of the supernatant showed that lactic and acetic acids were produced in a broad range, while phenyllactic acid (PLA) was produced at very low concentrations. Upon evaluation of the mode of action of these three acids, the authors indicated

that the effect of lactic acid on *P. digitatum* was greater than the effect caused by acetic acid or PLA. They also concluded that the inhibitory effect of lactic acid was linear and directly proportional to its concentration; while the inhibition by acetic acid or PLA was not correlated with its concentration, indicating other mechanisms of action (i.e., inhibition of enzyme activity) besides the undissociated active fraction of these organic acids (Gerez et al., 2010).

In a different study, Valerio et al. (2009) isolated LAB from wheat flour and used repetitive sequence-based polymerase chain reaction to establish the clonal relationship of 125 isolates. Based on their results, 17 different patterns were identified and one isolate representing each group was used for identification. Organisms were identified to species level; species included in those 17 different patterns were *Weissella* spp., *Leuconostoc* spp., *Lactococcus* spp., and *Lactobacillus* spp., with *Weissella* spp. being the most frequent ones. These researchers evaluated the antifungal activity of isolates representing those 17 patterns and correlated it with the production of organic acids, mostly acetic and lactic acids. To confirm the role of these acids, the inhibitory effect of pure lactic and acetic acids, at the concentrations found in one of the most active cell-free supernatants, was tested and the results indicated an inhibitory effect similar to the one given by the supernatant itself (Valerio et al., 2009).

Even though the role of organic acids on mold inhibition is very well established, knowing exactly which ones produced by LAB result in antifungal activity can sometimes be challenging. Research has shown that different species, and sometimes different strains of the same species, may produce different mixtures of acids that all result in antifungal activity. Corsetti, Gobbetti, Rossi, and Damiani (1998) have described *Lactobacillus sanfrancisco* (*Lactobacillus sanfranciscensis*) as producer of a mixture of acetic, formic, propionic, butyric, valeric, and caproic acids, where caproic acid played a key role in antifungal activity. Many LAB have been reported as producers of PLA and derivatives (i.e., 4-hydroxy-phenyllactic acids), including *L. plantarum, Lactobacillus coryniformis*, *Lactobacillus sakei*, and *P. pentosaceus* (Lavermicocca et al., 2000; Magnusson, Ström, Roos, Sjögren, & Schnürer, 2003; Ström, Sjögren, Broberg, & Schnürer, 2002). Some authors indicate that even though PLA has activity against yeasts and molds only at high concentrations (mg/ml), it still may contribute to the overall antifungal effect (Schnürer, 2007).

Sjögren, Magnusson, Broberg, Schnürer, and Kenne (2003) described the identification of four 3-hydroxy fatty acids (3-OH-FAs) from *L. plantarum*, which showed different degrees of inhibition against a series of molds and yeasts. The authors mentioned that although the minimum inhibitory concentration (MIC) for total growth inhibition for each of the 3-OH-FAs was 10–200 times higher than the amount of the fatty acids found in the supernatant, these compounds could still contribute to the antifungal activity of *L. plantarum* MiLAB 14 (Sjögren et al., 2003).

Other than organic acids and fatty acids, a few reports have described other active compounds produced by LAB; among them are cyclic dipeptides, proteinaceous compounds, and others. Reuterin (β-hydroxypropionaldehyde), first described as produced by *L. reuteri* (Talarico, Casas, Chung, & Dobrogosz, 1988), has antimicrobial activity against several different groups of microorganisms, including fungi. Its effect has been shown on the growth inhibition of *Candida*, *Torulopsis*, *Saccharomyces*, *Aspergillus*,

and *Fusarium* (Chung, Axelsson, Lindgren, & Dobrogosz, 1989). The production of reuterin can be achieved from glycerol by starving cells under anaerobic conditions and it has been reported as produced by *L. reuteri*, *Lactobacillus collinoides*, and *L. coryniformis* (Claisse & Lonvaud-Funel, 2000; Magnusson, 2003; Nakanishi et al., 2002).

The proteinaceous nature of some LAB active compounds, such as the ones produced by *Lactococcus lactis*, has been suggested based on evidence of lost activity after treatment with proteolytic enzymes (Batish, Grover, & Lal, 1989; Gourama, 1997; Roy, Batish, Grover, & Neelakantan, 1996). Roy et al. (1996) reported antifungal activity of *Lactococcus lactis* subsp. *lactis* that was lost after treatment with chymotrypsin, trypsin, and pronase E. Gourama and Bullerman (1995b, 1997) described the antifungal and antiaflatoxigenic activity of *L. casei* subsp. *pseudoplantarum*, isolated from a silage inoculant. The active compound was found to be a small peptide (<1 kDa) sensitive to trypsin and α-chymotrypsin. While investigating the antifungal activity of *L. coryniformis* subsp. *coryniforms*, Magnusson and Schnürer (2001) showed strong inhibitory effect against at least nine different molds, including but not limited to the genera *Aspergillus*, *Penicillium*, and *Fusarium.* The antifungal compound was most active at pH ranging from 3.0 to 4.5, stable under heat treatment, degraded by proteolytic enzymes, and had a molecular mass estimated around 3 kDa. Also, proteinaceous compounds with fungistatic activity against *Candida albicans* were purified by Okkers, Dicks, Silvester, Joubert, and Odendaal (1999) when investigating the antifungal activity of *Lactobacillus pentosus*.

Ström et al. (2002) described the production of two cyclic dipeptides (cyclo(L-Phe-L-Pro) and cyclo(L-Phe-*trans*-4-OH-L-Pro)) and 3-phenyllactic acid by *L. plantarum* MiLAB 393. The synergistic effect of the compounds was evaluated, and the results showed only a weak interaction of these metabolites in the inhibition of mold growth (Ström et al., 2002). Magnusson et al. (2003) also described the production of these cyclic dipeptides by *P. pentosaceus*, *L. sakei*, and *L. coryniformis*, suggesting that they might be common LAB metabolites (Schnürer & Magnusson, 2005). Dal Bello et al. (2007) isolated antimicrobial compounds from cell-free supernatant of *L. plantarum* FST 1.7 and identified two cyclic dipeptides (cyclo (L-Leu-L-Pro) and cyclo (L-Phe-L-Pro)), lactic acid, and phenyllactic acid as the major components responsible for the antifungal activity. Yang and Chang (2010) reported the production of a cyclo (Leu–Leu), a class of 2,5-diketopiperazines, when studying the antifungal activity of *L. plantarum* AF1. Furthermore, when *Lactobacillus amylovorus* was evaluated as an antifungal agent by Ryan et al. (2011), 17 bioactive compounds were identified in its cell-free supernatant with activity against *Aspergillus fumigatus*, from which four were cyclic dipeptides: cyclo (L-Met-L-Pro), cyclo (L-Pro-L-Pro), cyclo (L-Tyr-L-Pro), and cyclo (L-His-L-Pro).

Coda et al. (2011) studied the antifungal activity of *L. plantarum* against *Penicillium roqueforti* when the LAB was used for sourdough fermentation. In their experiments, after dough production the water/salt-soluble extract obtained from the sourdough lost its inhibitory activity against *P. roqueforti* after treatment trypsin. Upon further investigation, nine novel antifungal peptides were identified. When the sequences of the peptides were cross-referenced using the nr database of the National Center for Biotechnology Information they were identified as encrypted sequences of *Oryza sativa*

proteins. Others have also indicated that perhaps, besides the synthesis of inhibitory compounds, LAB may also release antimicrobial peptides from food proteins through proteolysis (Coda et al., 2008; Rizzello, Cassone, Coda, & Gobbetti, 2011).

Therefore, based on evidence from scientific studies, lactic acid bacteria produce various substances during fermentation, including acids (lactic, acetic, and PLA), fatty acids, proteinaceous compounds, cyclic dipeptides, reuterin, and other metabolites that may have antifungal activity. Some of these compounds are desirable not only for their effects on food flavor and texture, but also because of their inhibitory activity on undesirable microbial flora (Gilliland & Speck, 1977; Holzapfel et al., 1995; Klaenhammer, 1998; Schnürer & Magnusson, 2005). However, due to complex and commonly synergistic interaction between these different compounds, the mechanism of action for microbial inhibition by LAB is difficult to elucidate (Corsetti et al., 1998; Niku-Paavola, Laitila, Mattila-Sandholm, & Haikara, 1999).

14.3 Factors affecting production of antifungal compounds by lactic acid bacteria

Another area of knowledge that also needs more research is the effect of nutritional and abiotic factors on the production of active antifungal compounds by LAB. There have been numerous reports on the effect of extrinsic factors (nutritional and abiotic) on the production of bacteriocins, which are biologically active proteins produced by LAB, with activity against many Gram-positive bacteria, including related LAB, food-spoiling organisms, and pathogens (Cheigh et al., 2002; Guerra, Rua, & Pastrana, 2001; Li, Bai, Cai, & Ouyang, 2002; Mataragas, Drosinos, & Metaxopoulos, 2003; Todorov & Dicks, 2006). However, how these factors would affect the production of antifungal compounds has not yet been well described. Just a few reports mentioned how the incubation time, temperature, pH, and addition of nutrients to the culture medium could influence the production of antifungals by LAB.

Roy et al. (1996) studied the effect of incubation time and temperature on the production of antifungal compounds by *Lactococcus lactis* subsp. *lactis,* and they reported an optimum production at 30 °C after 48 h of incubation. Corsetti et al. (1998) also reported maximum production of organic acids, with antifungal activity, by *L. sanfrancisco* (*L. sanfranciscensis*) after 48 h of incubation at 30 °C, when the initial pH of the medium was 6.0.

Magnusson and Schnürer (2001) reported an increase in the antifungal activity of *L. coryniformis* strain Si3 in liquid culture when the medium was supplemented with either ethanol, formic acid, or acetic acid; while Magnusson (2003) showed that addition of glycerol to an overlay assay gave a dramatic increase in the inhibitory effect of *L. coryniformis* strain Si3 against several filamentous fungi and yeasts.

Improvement in the production of phenyllactic acid by *L. plantarum* by addition of phenylalanine and low amounts of tyrosine to the culture medium has been reported (Valerio, Lavermicocca, Pascale, & Visconti, 2004). This effect in *L. plantarum* was further studied by Vermeulen, Gänzle, and Vogel (2006) by the addition of several

different compounds to the culture medium that could influence the synthesis of PLA, such as peptides, phenylalanine, glutamate, and α-ketoglutarate. Results showed that production of PLA is an ongoing process during the stationary phase and it is enhanced more by the addition of peptides than single amino acids to the culture medium. Because the transamination step on the synthesis of PLA is another bottleneck, Vermeulen et al. (2006) reported an increase in PLA production when α-ketoglutarate was added to the medium. This upregulation of PLA production seems to be strain specific, since Vermeulen et al. (2006) also showed that *L. sanfranciscensis* only responded positively when a combination of citric acid and α-ketoglutarate was used.

Mu, Chen, Li, Zhang, and Jiang (2009) also studied the effect of medium composition on the production of PLA by lactic acid bacteria. Attempting to optimize a medium for the commercial production of PLA, the authors used a response surface methodology and the results showed that addition of phenylpyruvic acid (PPA) increased the yield of PLA. The authors suggested that because the transamination step is a bottleneck in the synthesis of PLA, the addition of PPA allowed for a shortcut that bypassed the transamination of phenylalanine to PPA, which is normally the first step when phenylalanine is used as the source for PLA.

14.4 Potential applications of lactic acid bacteria as antifungal compounds

Several studies have described the application of LAB as antifungal compounds in real or simulated food systems with results that are encouraging. Based on the results reported in the literature, it seems that the genus *Lactobacillus* is a promising one regarding potential application of LAB as antifungal agents. The potential use of *L. plantarum* to prevent growth of *Fusarium* species during malting of barley was evaluated by Laitila, Alakomi, Raaska, Mattila-Sandholm, and Haikara (2002). They reported an inhibitory effect of the indigenous *Fusarium* flora by the addition of *L. plantarum* to the steeping water. However, the effect was dependent on the initial level of contamination of the barley and also on the fungal species and strains present.

The potential for *L. plantarum* to prolong the shelf life of fresh vegetables was studied by Sathe, Nawani, Dhakephalkar, and Kapadnis (2007). To show the antifungal activity of this LAB, cucumbers were wounded, inoculated with *L. plantarum*, and challenged with several spoilage molds, such as *Aspergillus flavus*, *F. graminearum*, *Rhizopus stolonifer*, and *Botrytis cinerea.* The size of the lesions were evaluated after 9 days of incubation (20 °C and 85% relative humidity), and results indicated that *L. plantarum* was able to reduce up to 95% the diameter of the lesions caused by *R. stolonifer* and 88–93% the size of the ones caused by the other molds tested (Table 14.2).

Lavermicocca et al. (2000) also showed the potential application of *L. plantarum* as an antifungal agent in food systems. In their experiments, bread fermented with this bacterium along with *Saccharomyces cerevisiae* delayed *A. niger* growth for up to 7 days of storage at 20 °C, when compared to control bread prepared only with *Saccharomyces*, which was spoiled by the mold after 2 days of storage. Because LAB

Table 14.2 Effect of *L. plantarum* treatment on lesion diameters (mm) produced by spoilage fungi on wounded cucumber after 9 days of incubation at 20 °C

Spoilage fungi	Spoilage fungus only: lesion diameter (mm)	Spoilage fungus + *L. plantarum*: lesion diameter (mm)
Aspergillus flavus	22.6	1.5
Fusarium graminearum	14.8	1.8
Rhizopus stolonifer	28.6	1.2
Botrytis cinerea	28.4	2.4

Source: Reprinted from Sathe et al. (2007), copyright © 2007, John Wiley and Sons.

is such an integral part of sourdough cultures it is only natural that this trait as antifungal agents be explored by the bakery industry. And several scientific reports have supported the common knowledge that sourdough bread tends to be less susceptible to mold spoilage than breads made only with yeasts, when chemical preservatives (i.e., calcium propionate) are not used.

Dal Bello et al. (2007) evaluated the microbiological quality of sourdough bread produced using *L. plantarum* when compared to bread obtained using common sourdough starters, e.g., *L. sanfranciscensis*. Their results showed that breads produced with *L. plantarum* had the ability to retard the growth of both *F. culmorum* and *F. graminearum*, improving the shelf life of wheat bread. The use of *L. plantarum* was also evaluated in the production of gluten-free breads, and the results showed a product with increased quality and shelf life, when the breads were challenged with *F. culmorum* (Moore, Dal Bello, & Arendt, 2008). The use of a reduced amount of calcium propionate in combination with fermentation using *L. plantarum* was evaluated by Ryan, Dal Bello, and Arendt (2008) for the ability to inhibit growth of common bread spoilage fungi. Their results showed that the use of calcium propionate at 1000 ppm inhibited mold growth only when *L. plantarum* was used in combination to ferment the bread, and the increase in shelf life achieved was higher than that obtained using 3000 ppm of calcium propionate alone.

Other LAB have also been described as effective in controlling mold spoilage in breads when they are part of the sourdough starter culture. Ryan et al. (2011) described the effect of *L. amylovorus* DSM 19280 on improving the shelf life of bread and compared its efficacy with calcium propionate at 0.3%. The addition of the chemical preservative increased the shelf life of the breads by 1–7 days, depending upon which mold was used to challenge the food system; while *L. amylovorus* increased the shelf life by 1–9 days. A mixture of LAB was also tested on their ability to extend shelf life of sourdough bread when compared with bread fermented only with yeasts (Gerez et al., 2009). Results showed that the addition of LAB starter improved the leavening power of the dough, without affecting yeast growth, and extended the shelf life of the product by 3 days when compared to control (bread leavened by yeast, without calcium propionate). Researchers also showed that the LAB starter provided the same

shelf life as the one achieved with bread leavened with yeasts and to which 0.3% calcium propionate was added.

While studying the antifungal activity of *L. rhamnosus* on mold growth in commercial cottage cheese, Stiles, Carter, and Bullerman (2002b) reported that when live bacterial cells were added to cheese that was simultaneously inoculated with *P. commune* and *A. niger*, mold growth was reduced 40% and 70%, respectively, after 10 days of incubation.

The effect of LAB isolated from fresh fruits and vegetables was studied by Trias et al. (2008) on the inhibition of a series of phytopathogens, including *Penicillium expansum*, *Monilia laxa*, and *Botrytis cinerea.* Based on their results, during the in vitro assays, isolates showed different levels of antifungal activity against the different molds, with none of the LAB strains tested being able to prevent growth of *P. expansum.* However, when all the strains were tested using a food system—golden delicious apples—134 out of the 496 tested showed ability to reduce rot diameters caused by *P. expansum* by 10–50%. The best results were obtained with a strain of *Weissella cibaria.* The authors suggest that mechanisms such as competition for substrate and space and competitive exclusion of the pathogen from the entry sites in the fruit might explain the inhibitory effect of LAB on fungal infection in apples. They also highlighted the importance of ex vivo analysis, since the in vitro assays were not fully predictive of the antifungal activity of these LAB.

14.5 Lactic acid bacteria and mycotoxins

However, while some reports have indeed described the nature of the active LAB metabolites, the great majority of them have only described the inhibitory effect on mold growth. Taking the research one step further toward the effect of these active compounds on toxin production by the molds is critical. This would help in understanding the complex interactions between different metabolites that result in the antifungal effect and possible antimycotoxigenic activity of LAB.

Lactic acid bacteria have been reported to inhibit or stimulate the production of mycotoxins by toxigenic molds (Gourama & Bullerman, 1995b, 1997; Luchese & Harrigan, 1990); and once the toxin is present in the substratum, such as liquid media, they have also been described as able to bind and remove toxins (El-Nezami, Chrevatidis, Auriola, Salminen, & Mykkänen, 2002; El-Nezami, Polychronaki, Salminen, & Mykkänen, 2002; Fuchs et al., 2008; Haskard, El-Nezami, Kankaanpää, Salminen, & Ahokas, 2001; Peltonen, El-Nezami, Haskard, Ahokas, & Salminen, 2001).

14.5.1 Effect of lactic acid bacteria on the production of mycotoxins by toxigenic molds

When the effect of LAB on the production of mycotoxins by toxigenic molds was studied, Gourama and Bullerman (1995b) showed the inhibitory effect of a mixture of *Lactobacillus* spp. from a silage inoculant on aflatoxin production by *Aspergillus flavus* subsp. *parasiticus* without greatly affecting mold growth. They showed that

when the bacteria was grown inside of a dialysis sack with *Aspergillus parasiticus* on the outside, the amount of aflatoxin (B_1 and G_1) produced after 14 days of incubation was lower than the control. Little inhibition of toxin production was observed when the molecular weight cutoff (MWCO) of the dialysis sack was 1000, and great inhibition was achieved with MWCO of 6000–8000 and 12,000–14,000. Others have also studied the effect of LAB on the production of aflatoxins. When the antiaflatoxigenic activity of cell-free supernatants of *L. bulgaricus*, *L. acidophilus*, and silage inoculant cultures was studied by Karunaratne et al. (1990), they reported a considerable reduction on the production of aflatoxin by *A. parasiticus*. When similar experiments were performed in the presence of the LAB cells the results indicated 100% inhibition of aflatoxin production. However, even though the authors did not speculate on the reasons for the increased effect in the presence of bacterial cells compared to results obtained in their absence, it could be attributed to a binding or sequestration effect caused by the cells. The next section of this chapter will discuss further the binding effect that LAB may have on mycotoxins.

Gourama (1997) and Gourama and Bullerman (1997) reported the inhibitory activity of cell-free supernatants of *L. casei* strains on the growth of *Penicillium* spp. and the production of toxins (patulin and citrinin), and the antiaflatoxigenic activity of *L. casei* subsp. *pseudoplantarum* on *Aspergillus flavus* subsp. *parasiticus*. The active compounds in the cell-free supernatant in both reports were found to be sensitive to some proteolytic enzymes and to high temperature (Gourama, 1997; Gourama & Bullerman, 1997).

The effect of *L. rhamnosus* on growth and production of mycotoxins by *Fusarium* species, such as *F. proliferatum*, *F. verticillioides*, and *F. graminearum*, was studied by Stiles and Bullerman (2002). Fumonisin B_1 production was up to 63.2% inhibited in *F. proliferatum*, fumonisin B_2 production by *F. verticillioides* was reduced up to 43.4%, and production of deoxynivalenol and zearalenone by *F. graminearum* was affected up to 92% and 87.5%, respectively.

Interpreting the effects of LAB on the synthesis of mycotoxins by toxigenic molds is complicated and somewhat unclear because some reports show stimulation of mycotoxin production, while others show an inhibitory effect. Luchese and Harrigan (1990) reported a higher production of aflatoxin (B_1+G_1) by *Aspergillus parasiticus* when in the presence of *L. lactis*. Antifungals produced by LAB tend to be more fungistatic than fungicidal, which may cause stressful conditions for the mold, which in turn may affect the synthesis of both primary and secondary metabolites. Dalié et al. (2012) also showed that under certain conditions toxin production may be stimulated by LAB. In their experiments, when *F. verticillioides* was grown simultaneously with *P. pentosaceus* in GYEP (Glucose Yeast Extract Peptone) medium (Ponts, Pinson-Gadais, Barreau, Richard-Forget, & Ouellet, 2007) or inoculated in a 3-day-old culture of *P. pentosaceus* in GYEP medium, the amount of fumonisin (FB_1+FB_2) produced was enhanced about 13-fold after 30 days, in both inoculation conditions. However, when the LAB was first grown on MRS and a concentrated cell-free supernatant obtained and added to GYEP inoculated with the mold for growth and toxin production, the levels of fumonisin produced by *F. verticillioides* was significantly reduced when compared to controls.

Considering the potential use of LAB isolates and their metabolites as antifungal agents to be added directly into food systems, it is critical to understand how these bacteria or metabolites influence, directly or indirectly, the synthesis of mycotoxins. This will become a very important part of risk assessment plans for food systems.

14.5.2 Binding of mycotoxins by lactic acid bacteria

The ability of LAB cells to bind mycotoxin from liquid media has been extensively studied. Reports have shown that this ability, as well as the stability of the interactions involved on mycotoxin binding by LAB, are dependent on several intrinsic (i.e., bacterial strain) and extrinsic (i.e., bacterial cell density, mycotoxin load, and salt and urea concentrations) factors (El-Nezami et al., 2002; El-Nezami, Kankaanpaa, Salminen, & Ahokas, 1998a; Haskard et al., 2001).

The removal of ochratoxin A and patulin from solution by 30 different LAB was studied by Fuchs et al. (2008). Results showed that *L. acidophilus* was able to remove at least 95% of ochratoxin A from liquid medium, while *Bifidobacterium animalis* was able to bind and remove 80% of patulin in solution. Hatab, Yue, and Mohamad (2012) also studied the ability of LAB strains to remove patulin from liquid medium, in this case apple juice. All 10 strains used in the experiments were able to remove the toxin, at significantly different amounts, with best binding ability shown by *L. rhamnosus* 6224 (80.4%) and *Enterococcus faecium* 21605 (64.5%). According to the authors, the optimal mycotoxin removal was achieved at 30 °C, the binding was toxin concentration dependent (the amount of toxin removal increased with decreasing toxin levels) and the adsorption of patulin by LAB had no negative impact on the quality of the apple juice, based on various quality parameters (Hatab et al., 2012).

Lactobacillus and *Propionibacterium* strains were evaluated by El-Nezami et al. (2002) regarding their ability to remove seven *Fusarium* toxins (trichothecenes) from solution. Results showed that *L. rhamnosus* GG and *Propionibacterium freudenreichii* spp. *shermanii* JS were able to bind 18–93% of the deoxynivalenol, diacetoxyscirpenol, and fusarenon in solution, while *L. rhamnosus* LC-705 removed 10–64% of deoxynivalenol and diacetoxyscirpenol from liquid medium. When comparing the ability of lactic and propionic bacteria to remove toxin from solution, Niderkorn, Boudra, and Morgavi (2006) found that deoxynivalenol and fumonisin removal was strain specific, and that in general propionic acid bacteria was less efficient than lactic acid bacteria. The best results were achieved with *L. rhamnosus* for removal of deoxynivalenol (55%), *Leuconostoc mesenteroides* for fumonisin B_1 (about 82%), and *L. lactis* for fumonisin B_2 (100%) (Niderkorn et al., 2006).

According to El-Nezami et al. (1998a) the removal of aflatoxin B_1 from artificially contaminated liquid media by LAB was also strain dependent and very fast. Among the LAB strains studied, two *L. rhamnosus* were chosen for further testing after they removed about 80% of the toxin from solution at the beginning of the incubation time. The ability of the two strains to remove aflatoxin was shown to be dependent on temperature (maximum removal at 37 °C) and bacterial cell concentration (at least 2×10^9 CFU/ml needed for significant removal). These *L. rhamnosus* strains had their ability to remove aflatoxin from solution significantly enhanced after treatment with

1M HCl (El-Nezami, Kankaanpaa, Salminen, & Ahokas, 1998b). Among dairy strains (LAB and bifidobacteria), *L. amylovorus* and *L. rhamnosus* were the most efficient species in binding aflatoxin B_1 since they were able to bind/remove between 5% and 60% of the toxin present in liquid medium (Peltonen et al., 2001). Further investigation showed that heat and acid treatment of the cells, as observed previously by El-Nezami et al. (1998b), significantly increased their ability to remove aflatoxin from solution (Peltonen et al., 2001).

When investigating removal of deoxynivalenol from solution by LAB, Franco, Garcia, Hirooka, Ono, and dos Santos (2011) reported that both viable and nonviable cells (heat inactivated by pasteurization or sterilization) led to toxin removal. However, inactivated cells bound more deoxynivalenol from solution than viable cells, with all cells inactivated by sterilization showing a significant increase in binding compared to viable cells. According to the authors, this indicates that the main mechanism of detoxification was adsorption of mycotoxin by the cell components of LAB. Since the heat treatment most likely exposed cell wall components and plasma membranes that would not be available otherwise, an increase in adsorption by those treated cells would be expected, which was confirmed by the experiment results (Franco et al., 2011).

El-Nezami et al. (2002) also studied the binding mechanisms involved in the removal of *Fusarium* toxins from solution. In this research, *L. rhamnosus* was studied regarding its ability to either remove or degrade zearalenone and α-zearalenol from solution. Results indicated that binding rather than metabolism was also the mechanism of action used by this LAB since both viable and nonviable cells (heat and acid treated) were able to remove about 50% of the toxin from liquid medium. Furthermore, because degradation products were not detected in solution, it supported the assumption that binding was the mechanism used by the bacteria to remove toxin from solution. When the ability of *L. rhamnosus* to remove zearalenone and α-zearalenol from solution was further explored, results indicated that these toxins may share binding sites on the bacterial cell, since the ability to remove them was significantly reduced when they were both present at the same time in solution (El-Nezami et al., 2002).

Because adsorption seems to be the literature consensus regarding the mechanisms used by LAB to remove toxins from solutions, and based on their own experiment results, Bueno, Casale, Pizzolitto, Salvano, and Oliver (2007) proposed a theoretical adsorption model that is applicable to microorganisms that bind aflatoxin B_1 (AFB_1). According to the authors, the binding forces involved in this phenomenon include weak Van der Waals forces, hydrogen bonds, or hydrophobic interactions. Their model estimates the number of toxin binding sites and the system equilibrium constant, and allows for the calculation of the efficiency of cells to remove toxin from solution, which could be very helpful in selecting the most efficient microorganism to bind AFB_1 (Bueno et al., 2007).

Another important portion of the assessment regarding the usefulness of LAB to remove toxin from solutions and food systems is related to their ability to bind, but not release, the toxin easily. Haskard, Binnion, and Ahokas (2000) and Haskard et al. (2001) studied the effect of different variables on binding and releasing of aflatoxin

B_1 by *L. rhamnosus*. According to their results, around 90% of the toxin bound by the LAB was extracted with solvent from the bacterial pellet. Among the variables studied, temperature, sonication, and pH had no significant effect on the release of the toxin by *L. rhamnosus*; salt (NaCl and $CaCl_2$) concentrations showed minor effects (Haskard et al., 2001); and urea showed the greatest effect, suggesting that hydrophobic interactions play a major role in binding (Haskard et al., 2000).

A potential use for this ability shown by some LAB to bind mycotoxins would be to use them as binding agents for toxins in the gastrointestinal (GI) tract. El-Nezami et al. (1998a) and El-Nezami, Mykkänen, Kankaanpää, Salminen, and Ahokas (2000) used in vitro and in vivo experiments to evaluate the ability of lactic acid bacteria to remove toxins from liquid media and to prevent absorption of the toxin, respectively. When in vitro experiments were used approximately 80% of the initial amount of aflatoxin B_1 was removed by *L. rhamnosus*. When the same bacteria was tested using in vivo studies by the chicken duodenum loop technique, 54% (w/w) of the added aflatoxin B_1 was removed from the luminal fluid, reducing the intestinal absorption by 74% (w/w) (El-Nezami et al., 2000). Similarly to what is observed in vitro with the adsorption of mycotoxin being strain specific, in vivo experiments showed that one strain of *L. rhamnosus* reduced toxin absorption by 74% (strain GG) while another (strain LC-705) reduced toxin absorption by only 37% (El-Nezami et al., 2000).

According to Shetty and Jespersen (2006), strains of LAB with high mycotoxin-binding abilities could be used in the food industry for the fermentation of food and beverages. After fermentation, by the removal of LAB cells from the fermented product, the risk of mycotoxin contamination could be reduced. Another approach would be the use of heat-treated cells as additives without compromising the final characteristics of the product (Shetty & Jespersen, 2006). In the case of absorption in the GI tract, probiotic strains may be of value in reducing mycotoxin exposure, depending upon the stability of the complex in the GI tract and residence time (Shetty & Jespersen, 2006). However, because of all the factors influencing LAB ability to bind mycotoxins in vitro or in vivo, it becomes imperative to understand how specific organisms of interest would behave under different environmental conditions to fully evaluate their potential as mycotoxin binders in food systems or in the GI tract of humans and animals.

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Quality improvement and fermentation control in meat products

15

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15.1 Introduction

A meat product may be termed "fermented" if it is prepared by a ripening process in which microorganisms play a key role. On raw and/or salted meat cuts, microbial activity is restricted to the surface while cutting and grinding distributes indigenous and added microorganisms throughout the product. Consequently, the term "fermented meats" is often used as a synonym to "fermented sausages", and this chapter will focus on these meat products.

Some other characteristics of meat fermentations are as follows:

- Unlike milk (and wort), meat is not heat-treated before fermentation.
- Meat is low in fermentable carbohydrates and has a high buffering capacity due to proteins, phosphate-containing compounds, etc.
- Muscle cells contain plenty of membranous structures (sarcoplasmatic reticulum) with poly-unsaturated fatty acids, along with transition metals (iron, copper). Comminuting or heat treatment of meat in the presence of oxygen will therefore result in oxidative changes.

With some exceptions (e.g. slightly fermented, undried or semi-dry sausages; salami slices on pizza), products are not cooked prior to consumption.

Fermentation is a traditional way to preserve meat. However, salting meat pieces was the more common method, and only from the eighteenth century there is clear evidence of a commercial (artisanal) production of fermented sausages similar to that which we know today. According to Leistner (1995), manufacturing techniques were first developed in Italy and then spread to other European countries. On a farmyard and artisanal scale, sausages were – and still are – fermented at low temperatures, relying on the natural desired microbiota in the raw material and on the manufacturing and ripening environment. Starting in the 1960s, there was an increasing demand for convenient, ready-to-eat food of constant quality and at low prices, and there was less need for small-scale preservation of meat. At the same time, the microbiological and biochemical basis of meat fermentation was better understood, and facilities allowing ripening under defined conditions (temperature, humidity, air velocity) became affordable. This resulted in "industrialization" of the manufacture of fermented meats, including standardization of products and processes; introducing of semi-dry and/or spreadable, perishable varieties; use of defined starter cultures, etc.

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00015-3

This chapter summarizes information on the types of fermented sausages, their manufacture, the microbiological and biochemical changes during ripening, the role and composition of starter cultures and product safety. Some future developments are also discussed.

15.2 Types of fermented meats

Variables in the production of fermented sausages include:

- the animal species used and their feed (e.g. types of lipids in pig feed);
- the meat cuts used (pH value, levels and composition of fat, levels of connective tissue),
- ingoing salt;
- curing agents (nitrite, nitrate, ascorbate or none);
- fermentable carbohydrates and starter cultures;
- spices and other non-meat, especially plant-derived ingredients (e.g. paprika);
- casings (type and diameter);
- fermentation temperature;
- rate and extent of drying and ageing (time, temperature, humidity, air velocity); and
- surface treatment (smoke, mould growth).

Hence, there is a large variety of fermented sausages in different regions or even within a region (Campbell-Platt, 1995; Lücke & Vogeley, 2012). The categories and parameters for official classification of fermented sausages (if any) differ between countries. However, they generally refer to the contents of lean meat and of moisture. For example, the German "*Leitsätze*" (Codes of Practice; Anon, 2010) categorize fermented sausages as either "sliceable" ("*schnittfest*") or "spreadable" ("*streichfähig*"). Within these categories, products are classified by the content of muscle protein (total protein nitrogen × 6.25, minus collagen protein; German acronym "BEFFE"). U.S. regulations (USDA-FSIS, 2005) set the borderline between dry and semi-dry fermented sausages at a moisture:protein ratio of 1.9:1.

The water activity of *dry sausages* is below 0.91, and there are few if any microbiological changes during storage at ambient temperature. This water activity corresponds to a moisture:protein ratio of about 1.9:1 and a drying loss of about 30%, or higher if the formulation includes less fat and more lean meat (Incze, 2007). Total ripening time (fermentation + drying/ageing) exceeds 4 weeks. The water activity of *semi-dry* fermented sausages is in the range of 0.91–0.95, and acidity (pH value generally 5.0 or below) markedly contributes to their stability. Total ripening time is usually 10–20 days, and drying loss is of the order of 15–20%. The final products do not support growth of pathogenic bacteria (unless moulds growing on the surface degrade acids formed during fermentation) but are usually kept refrigerated to extend their shelf life. Spreadable fermented sausages are only briefly fermented, and drying loss is below 10%. Consequently, their water activity is in the range of 0.94–0.97, the pH is above 5.0 (mostly in the pH 5.3–5.6 range), they are distributed under refrigeration and they have only a short shelf life. Many of these sausages are traditionally eaten after cooking. A common classification of fermented sausages is given in Table 15.1.

Table 15.1 Classification of fermented sausages

Category	Final water activity	pH	Total ripening time	Drying loss	Moisture/ protein	Surface treatment	Example
Dry	<0.91	Variable, generally 5.0–5.5	>4 weeks	25–40%	<2.0	Smoke or none	German-style salami
Dry	<0.91	Variable, sometimes >6.0	>4 weeks	25–40%	<2.0	Mould	Italian salami
Semi-dry	0.91–0.94	<5.2	10–20 days	15–20%	2–3	Mostly smoke	
Spreadable	0.94–0.97	5.0–5.6	<7 days	<10%	>3	Smoke or none	Teewurst, frische Mettwurst, “sobrasada”, “sopréssa”

15.3 Principles of manufacture of fermented meats

Figure 15.1 provides a general flow diagram for the manufacture of fermented sausages. Details of the individual steps are provided in this section.

15.3.1 Purchase and selection of raw material

For product safety, the pH value of the lean meat component should not exceed 5.8 (or, with appropriately modified formulation and process, 6.0). Higher pH values favour survival and growth of pathogens and inhibit moisture removal. The microbiological quality of the raw material is important, particularly for the production of spreadable types because there is only little reduction of pathogenic bacteria during fermentation (see Section 15.6). Poor quality and/or inappropriate pretreatment of the meat and fat (e.g. prolonged storage, repeated freezing and thawing) may also increase the level of free amino acids in the sausage batter, which may result in elevated levels of biogenic amines and in off flavours (Paulsen & Bauer, 1997).

Fat deterioration limits the shelf life of many fermented sausages. It can be slowed down by selecting fat in which less than about 15% of the total fatty acids are polyunsaturated (Warnants, van Oeckel, & Boucqué, 1998). For dry sausages, this percentage is even lower (Stiebing, Kühne, & Rödel, 1993).

Other specifications for the quality of the meat and fat components of the formulation depend on the desired sensory quality, shelf life and target costs of the product and, obviously, local availability and preferences. Many varieties are made from pork only. For traditional types, meat and fat from older pigs is preferred because of the

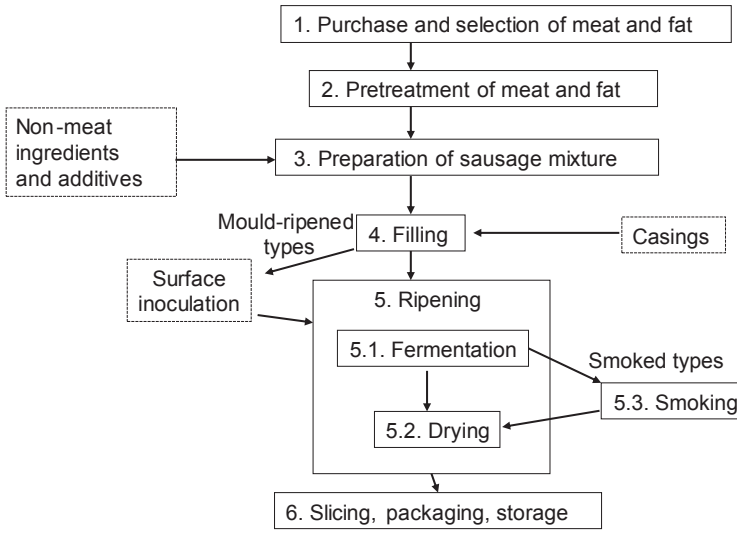

Figure 15.1 General processing scheme for fermented sausages. Modified from Lücke (2007).

firmer texture and darker colour of the lean meat. Alternatively, lean pork may be partially replaced by lean beef. In countries with Islamic traditions, meat from ruminants predominates as raw material. In traditional Turkish *sucuk*, pork back fat is replaced by sheep tail fat (Kilic, 2009), which tastes less "tallowy" than beef fat. Poultry meat may also be used, but with limitations: many cuts of poultry have high pH, a less intensive red colour and, compared to red meats, a higher level of pathogens such as salmonellae and *Campylobacter jejuni*. Moreover, poultry fat usually has a low melting point, making it unsuitable for the manufacture of dry-aged products. Hence, it is common to use pork fat in the manufacture of fermented poultry sausages.

15.3.2 Pretreatment of meat and fat

Cuts and trimmings are sorted by their fat and collagen content. To avoid undesirable changes in the microbiological and fat quality, the temperature of the raw material should be low. Since high-performance meat cutters introduce considerable amounts of thermal energy into the mix, parts of the raw material are frozen and others partly chilled to 0–2 °C. For mincing, the temperature is less critical, and some local specialities are even prepared from fresh pre-rigour meat (Lücke & Vogeley, 2012).

15.3.3 Preparation of sausage mixture

The raw material may be comminuted using a meat cutter or a mincer. Meat cutting solubilizes meat proteins, causing stronger binding between particles and a more firm texture, whereas mincing leads to more crumbly, "shorter" texture. During comminution in a meat cutter, the temperature of the batter should not rise above about 2 °C. Therefore, fat and part of the meat is processed in the frozen state. If a "grinder-filler" ("*Füllwolf*") is used, the raw material can be processed without prior freezing, which saves much energy (Irmscher, Gibis, Herrmann, Kohlus, & Weiss, 2011). Sausage varieties differ by the degree of comminution, leading to particles of different sizes. As a rule, "coarse" sausages are easier to dry and less prone to fat deterioration.

Some local specialities may contain ingredients of milk or vegetables, e.g. paprika or garlic in the Mediterranean, potatoes in Northern countries and onions in German "Zwiebelmettwurst". There is an increasing interest in replacing meat by plant protein and plant oils, and to add fibrous plant material (see Section 15.7). The use of spices and herbs depends on local preferences. White and/or black pepper is widely used; other spices may include caraway, nutmeg, mustard seeds, coriander, etc.

Addition of salt (at least 2% NaCl) is essential for the safety and sensory properties of fermented sausages. Ingoing levels are about 2.5% NaCl. With few exceptions, curing salts are also used. Nitrite is essential for the formation of curing colour, to inhibit undesired changes in fats, and, if added at levels of 100–150 mg $NaNO_2$/kg, for inhibition of certain bacterial pathogens early in fermentation (see Section 15.6). Addition of sodium ascorbate at levels between 300 and 500 mg/kg enhances the desirable effects of nitrite. Nitrate has no antimicrobial activity per se and must be reduced to nitrite by microorganisms during fermentation; nitrite levels formed are sufficient for curing colour and aroma but rarely reach antibacterial levels.

Lean meat contains less than 0.2% of fermentable carbohydrates and has a high buffering capacity. Hence, addition of about 0.3% of a fermentable sugar (glucose, maltose, sucrose or digests from starch) is necessary to lower the pH to about 5.3. Levels of 0.5–1% ingoing sugar are most common. Addition of lactic acid bacteria, along with catalase-positive cocci, became common during the last four decades, following the need to standardize product quality and processes and to save costs by shortening fermentation times without safety problems. Details of the cultures used are provided in Section 15.5.

Other additives such as binders are sometimes used for undried products or if the sausages are heated after fermentation.

15.3.4 Filling

It is important to minimize exposure of the batter to oxygen. To avoid air inclusions, vacuum-filling devices are frequently used. Casings must withstand the filling process without leaks. The choice of casings mainly depends on the desired appearance and permeability to moisture and smoke. For dry sausages, casings also must shrink during the drying process. Casings from intestines of animals (pigs, cattle, sheep) must be thoroughly cleaned; otherwise, slow drying, fat deterioration and microbiological problems may result. Casings made from modified collagen and/or cellulose are most common.

15.3.5 Ripening

The ripening process may be subdivided into fermentation (in which microbial activity is essential) and drying/ageing (where physicochemical and enzymatic processes predominate) (Figure 15.1). For traditional products, fermentation temperatures are low (10–18 °C), while in industrial sausage production, fermentation temperatures are in the range of 20–25 °C. This is close to the optimum growth temperature of the desirable bacteria (lactic acid bacteria, catalase-positive cocci) in the sausage but also to that of mesophilic pathogens. Hence, fermentation should bring the pH value down to 5.3 or below within 2 days at 25 °C or 3 days at 20 °C. This can be achieved reliably using selected lactic acid bacteria as starter cultures. Some U.S.-style semi-dry sausages are fermented at even higher temperatures (up to 40 °C). To control pathogens (particularly *Staphylococcus aureus*) under these conditions, the pH must be lowered, by use of suitable starters to 5.3 within 24 h (American Meat Institute Foundation, 1997).

During fermentation, the sausage should not dry excessively. Hence, the equilibrium humidity is adjusted to about 5–10 units (expressed as water activity × 100) below that of the sausage. After fermentation, pH values are mostly in the range between 4.6 and 5.3, depending on the ingoing levels and types of sugar and starters. Then, microbial activity is slowed down by lowering the temperature to about 12–15 °C, by lack of fermentable carbohydrates and by reducing the water activity through drying. At this stage, products may be smoked. Traditionally, smoked products prevail in regions with a wet (maritime) climate. Some traditional products such as Hungarian "*téliszalámi*" also

receive some smoke during (low-temperature) fermentation, while others are lightly smoked throughout the drying process (the so-called "*Katenrauch*" in Germany). For the production of mould-ripened sausages (common in France and Italy), starter culture preparations containing moulds are applied to the surface after fermentation.

For drying sausage, temperature is kept at 12–15 °C, and equilibrium humidity and air velocity are adjusted to ensure uniform drying until the desired water activity (or weight loss, moisture:protein ratio, etc., see Section 15.2) within adequate time, avoiding both undesired mould growth and excessive drying of the surface. Some high-quality products are aged for extended times, allowing sufficient enzymatic and physiochemical reactions to improve tenderness and colour (in particular, by the formation of Zn-protoporphyrin IX, the "Parma ham pigment"). Due to proteolysis and some degradation of acids, the pH value increases during ageing, especially in mould-ripened sausages.

15.3.6 Slicing, packaging and storage

In many countries, a major proportion of fermented sausages with large diameter are sold sliced and packaged, either under vacuum or – more frequently – under atmospheres containing about 20–30% CO_2 in nitrogen. However, packaging in the absence of air may result in off flavours of smoked and, in particular, of mould-ripened sausages. Dry-fermented sausages are, by definition, stable at ambient temperatures, while storage at 10–15 °C is sufficient to obtain the desired shelf life of semi-dry sausages. Some undried, "fresh" sausages require storage temperatures below 7 °C for microbiological safety.

15.4 Microbiological and chemical changes during meat fermentation

Changes during sausage ripening are complex because they are caused by microorganisms, meat enzymes and abiotic reactions such as fat oxidation. In the first 24 h of a typical sausage fermentation (i.e. at 20–25 °C), oxygen is consumed by meat enzymes, and nitrite (if added) reacts with myoglobin to form met-myoglobin. Salt, in conjunction with nitrite, anaerobiosis and low temperature during equilibration, inhibits growth of the gram-negative bacteria present in the raw material. As the sausage temperature increases, lactic acid bacteria form lactic and some acetic acid. This contributes to the inactivation of undesired bacteria and accelerates the reaction of added nitrite, leading to curing colour formation, and of liberation of cathepsin A, which contributes to flavour development (Molly et al., 1997). Although some strains can degrade peptides and amino acids (see, e.g. Flores and Toldrá, 2011; Sinz & Schwab, 2012), the contribution of lactic acid bacteria to the flavour of a "ripe" fermented sausage is low. Catalase-positive cocci (*Staphylococcus* spp.) reduce nitrate added as such or formed by disproportion of nitrite, help to create anaerobic conditions in the sausage by consuming oxygen and form some aroma precursors. Once a pH of 5.3 is reached,

the activity of acid-sensitive bacteria including catalase-positive cocci is markedly reduced. Hence, reduction of nitrate and microbial formation of aroma precursors should be largely completed within the first 2 days of the ripening, and high rates of acidification are detrimental to the sensory quality of the sausages. This is the case if glucono-δ-lactone is added to the batter. By hydrolysis to gluconic acid, the sausages are acidified within less than 24 h, but gluconic acid may be further fermented by lactic acid bacteria forming lactic and acetic acids, giving an unbalanced sour flavour and a strong inhibition of catalase-positive cocci.

Conditions in properly prepared and stuffed sausage batters strongly favour the development of psychotropic lactobacilli capable of growing at low water activity. Such species include *Lactobacillus sakei* and *Lactobacillus curvatus* which are frequently found on chilled meat and cold environments in butchering and processing plants. These lactobacilli ferment hexoses to lactate, and pentoses (ribose) to lactate and acetate, and *L. sakei* also gains energy from arginine degradation, a property that also may contribute to its competitiveness in meats.

Of the catalase-positive cocci, *Staphylococcus xylosus*, *Staphylococcus equorum*, *Staphylococcus warneri* and *Staphylococcus succinus* predominate in French and Mediterranean-style sausages, whereas *Staphylococcus carnosus* was reported to be more competitive in rapidly fermented smoked products (Ravyts et al., 2010). Staphylococci grow preferably close to the surface of the sausage, with little if any growth in the core. The same applies to the mycobiota of unsmoked sausages. Strains of *Penicillium nalgiovense* and of the yeast *Debaryomyces hansenii* thrive on sausage surfaces during ripening. On mould-ripened sausages they act as oxygen scavengers and degrade lactic acids and proteins, thus raising the pH value and conferring the typical appearance and flavour of such sausages.

Smoking sausages has various effects: smoke inhibits mould growth by drying the surface and by volatile antimicrobial compounds. It also contains antioxidative compounds protecting the fat from oxidative reactions and confers a typical flavour to the sausages.

Various other reactions affect the taste and aroma of fermented sausages. Many of these proceed only slowly, i.e. during drying and ageing, and are inhibited at low pH values. Hence, the sensory properties of undried or semi-dry fermented sausages are determined mainly by the meat used, the acids formed by fermentation and smoke (if applied).

15.5 Starter cultures

Table 15.2 shows the microbial species commonly added as starter cultures, as well as their effects. General selection criteria include:

- ability to grow and metabolize under the fermentation conditions (water activity = 0.95–0.97; pH < 5.8; temperatures = 20–25 °C (lower during equilibration));
- antagonistic activity against undesired competitors;
- no formation of toxins, antibiotics and biogenic amines;
- no formation of CO_2 and only little formation of acetic acid from hexoses;

- little or no net formation of reactive oxygen species (peroxides) in the presence of oxygen;
- no formation of compounds leading to off flavours; and
- ease of preservation and distribution.

In particular, there is growing concern on spreading transferable resistance to antibiotics, and starter microorganisms should therefore not carry genes encoding such features (EFSA, 2011; Talon & Leroy, 2011; Vogel et al., 2011).

In addition to the species listed in Table 15.2, other species were included in the 2012 inventory of microbial species with technological beneficial role in fermented food products (Bourdichon et al., 2012). Of these, *Staphylococcus equorum* subsp. *equorum* has potential as a starter culture for low-acid fermented meats such as Swiss-, French- and Mediterranean-type sausages (Marty, Buchs, Eugster-Meier, Lacroix, & Meile, 2012).

Table 15.2 Microbial species in starter cultures for fermented sausages

Type	Species	Main metabolic activity	Consequences
Lactic acid bacteria	*Lactobacillus sakei* *Lactobacillus curvatus* *Lactobacillus plantarum* *Lactobacillus paracasei* *Pediococcus acidilactici* *Pediococcus pentosaceus*	Formation of lactic acid and (some) acetic acid	Inhibition of pathogenic bacteria; inhibition of acid-sensitive spoilage bacteria; acceleration of drying; flavour and colour formation
Catalase-positive cocci	*Staphylococcus carnosus* *Staphylococcus xylosus* *Kocuria varians*	Reduction of nitrate; Reduction of nitrite; Degradation of reactive oxygen compounds; Degradation of peptides and amino acids	Delay of rancidity; flavour and colour formation
Yeasts	*Debaryomyces hansenii*	Oxygen scavenging; proteolysis	Delay of rancidity; flavour development
Moulds	*Penicillium nalgiovense*	Oxygen scavenging; proteolysis; Competitive exclusion of undesired moulds	Typical appearance and flavour

15.6 Microbiological safety

15.6.1 Relevant hazards and their control

Epidemiological data (summarized by Lücke, in press) indicate that the main bacterial hazards of relevance to fermented sausages are *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC). Spore-forming bacteria do not affect the safety of fermented sausages (see Lücke, 2000a; Skandamis & Nychas, 2007).

For salmonellosis, risk factors include: (1) use of pork, (2) very short fermentation without subsequent drying or ageing, (3) use of contaminated spices (e.g. for coating the surface after ripening) and (4) mould growth on the surface. This is plausible because salmonellae are more widespread on pork than on beef, and inactivation proceeds with longer ripening times. For traditional sausages made with nitrate and without starter cultures, the fermentation temperature should be below 15 °C, especially if surface pH is high due to mould growth (see Lücke, 2000a; Lücke & Vogeley, 2012).

The main risk factor for the survival of STEC during sausage ripening is use of meat from ruminants as raw material. This is not surprising because STEC occur much more frequently in ruminants than in monogastric animals. In one family-scale outbreak, pork salami stuffed in bovine casing was incriminated (Conedera et al., 2007). Poor control of the fermentation is likely to increase the risk.

Data on inactivation rates of pathogens during sausage ripening were compiled by Skandamis and Nychas (2007). Generally speaking, low pH value after fermentation, long drying and ageing times and subsequent storage at ambient rather than refrigeration temperature (Lindqvist & Lindblad, 2009) lead to lower level of pathogens in the final product. Approximate inactivation rates for salmonellae during sausage ripening are one log unit or less for spreadable sausages, two log units for products with short ageing times such as rapidly dried mini-salami (Gareis, Kabisch, Pichner, & Hechelmann, 2010) and *sucuk* (Porto-Fett et al., 2008), and 3–4.5 log units for aged products (Porto-Fett et al., 2010). For STEC, a two log but not a five log reduction required by U.S. regulations (USDA-FSIS, 2001) is achieved during the ripening of semi-dry or dry sausages with present technology (i.e. without heat or ultra-high-pressure treatment of the final product; Holck et al., 2012; Skandamis & Nychas, 2007). A permitted alternative by United States Department of Agriculture/Food Safety and Inspection Service (USDA-FSIS) is a two log reduction in combination with a systematic testing of the raw batter for absence of STEC. Data from recent outbreaks in Scandinavia (see Holck et al., 2012) indicate that a process leading to a two log reduction may be insufficient for sausages containing mutton, lamb or beef unless the slaughtering process (especially the dehiding and evisceration) is under strict control.

Dry/semi-dry fermented sausages were ranked as "low-risk" food with respect to *Listeria monocytogenes* (FDA, 2003). Regulation (EC) No. 2073/2005 stipulates a "zero tolerance" (absence in 25 g) for *L. monocytogenes* after manufacture in products supporting growth of this pathogen. In fermented sausages with pH ≤ 4.4 or

$a_w \leq 0.92$, or pH ≤ 5.0 and $a_w \leq 0.94$, or other types for which there is science-based evidence (namely, from challenge tests), *L. monocytogenes* does not grow, and a limit of 100 cells/g is tolerated if maintained until the best-before date. This basically means that neither dry nor semi-dry types support growth of *L. monocytogenes*. Only for one outbreak has there been good evidence for a fermented sausage (spreadable, *Teewurst*-type) as the agent, and five of the eight patients suffered from other diseases or were treated with immunosuppressants (Goulet, Martin, & Jacquet, 2002). Generally speaking, fermentation technology commonly used in Europe will reduce the *L. monocytogenes* count by about 1–2 log units in dry sausages and 0–1 log units in spreadable, undried sausages (see Lücke, in press; Skandamis & Nychas, 2007 for recent reviews). Since there still is a "zero tolerance" for *L. monocytogenes* in the United States (USDA-FSIS, 2003), there is much interest in anti-listerial protective cultures (see Section 15.7) and in ultra-high-pressure treatments.

Staphylococcus aureus is under control by current fermentation methods, especially by ensuring rapid fermentation rates through the use of lactic acid bacteria as starters (see Lücke, 2000a; Skandamis & Nychas, 2007 for reviews). One outbreak was traced to mould-ripened salami (Hechelmann, Lücke, & Leistner, 1988). Apparently, the temperature was raised too early after fermentation, allowing the mould to degrade acid while the water activity and temperature were high enough for growth of *S. aureus* (Rödel, Stiebing, & Kröckel, 1993; Schillinger & Lücke, 1989).

Some toxigenic moulds may grow on the surface of fermented sausages (see Leistner & Eckardt, 1981; Pestka, 1995). However, at the common drying/ageing temperatures (below 15 °C), the potential for formation of mycotoxins (in particular, ochratoxin A) and the rate of diffusion of these traces through the casing are very low (Iacumin et al., 2009; Leistner & Eckardt, 1981). Nevertheless, it is advisable to prevent undesired mould growth by drying the products at 15 °C or below until water activity is sufficiently low or if mould growth is prevented by other means such as smoking or excluding oxygen at packaging. For mould-ripened products, competitive mould starter cultures such as *Penicillium nalgiovense* should be used, or the mould strain dominating the "house flora" should be regularly checked for identity.

Some viral pathogens may survive for extended periods during sausage fermentation (see Albert et al., 2012). The transmission of human enteroviruses must be prevented by proper staff hygiene. Hepatitis E virus (HEV) is endemic in wild boars and may be transmitted to pig farms, and up to 10% of pig livers have been found to be positive for HEV (see Meng, 2011). Consumption of *figatellu*, a fermented sausage containing pork liver, common in Corsica and southern France, has been shown to cause infections (Colson et al., 2010; Meng, 2011).

Due to the meat inspection procedures at slaughter, the presence of *Trichinella* and other multicellular parasites in raw sausages is extremely unlikely, and the few cases of parasitosis reported could be traced back to unlawful slaughtering. In the United States, alternative post-slaughter intervention measures are also permitted, and *Trichinella* larvae were found to be inactivated during the manufacture of Genoa salami (see Porto-Fett et al., 2010). *Toxoplasma gondii* is rapidly inactivated during manufacture

of semi-dry and dry fermented sausage. In fresh raw sausages with very short ripening times, the risk of survival of this organism is small but not negligible (Pott et al., 2012).

Biogenic amines may be formed during sausage ripening if proteolysis liberates free amino acids and bacteria with amino acid decarboxylase activity multiply. Levels of biogenic amines vary widely (see Suzzi & Gardini, 2003; Vidal-Carou, Veciana-Nogués, Latorre-Moratalla, & Bover-Cid, 2007), especially in traditional sausages (Latorre-Moratalla et al., 2008), but generally speaking, the levels are of no major health concern (as calculated for histamine by Rauscher-Gabernig, Grossgut, Bauer, & Paulsen, 2009). Cadaverine, putrescine and histamine are mainly formed by acid-sensitive gram-negatives such as *Enterobacteriaceae*, and a rapid decrease in pH value inhibits them. In contrast, many *L. curvatus* strains can form tyramine and phenylethylamine. These bacteria grow during sausage fermentation, and use of starters suppressing them without decarboxylating amino acids themselves may reduce levels of tyramine (Paulsen & Bauer, 1997). The use of fresh meat is important to limit proteolysis by endogenous enzymes (Paulsen & Bauer, 1997).

15.6.2 Safety management

In the United States, a generic hazard analysis and critical control points system to be used by meat processors has been published by the USDA-FSIS (1999). Process steps critical for product safety (critical control points or CCPs) generally include:

1. preparation of the mix (monitoring of the added levels of salt, curing agents, sugar and starters),
2. fermentation (time, temperature and humidity), and
3. drying (time, temperature and humidity).

However, CCPs depend on the specific product and process technology and must be identified by the responsible staff at the processing plant.

Other preventative measures, in particular selection of suppliers of the raw material and ingredients, specifications and checks for incoming raw material and ingredients, selection and storage of raw material and ingredients, appropriate cleaning of natural casings, staff hygiene and training, cleaning and disinfection, waste disposal and pest control, checks on the final products and traceability, are important parts of good hygienic practice and should be specified as "Operational Prerequisite Requirement Programmes" as defined in ISO 22000 standard (ISO, 2005).

15.7 Recent and future trends

15.7.1 Protective cultures

Growth of pathogens is well controlled if up-to-date technology for the manufacture of fermented sausages is used. On the other hand, there is a trend towards low-salt, low-fat products into which alternative "hurdles" may be introduced. Moreover, there is pressure from the market and, in some countries, from official

food inspection to reduce the prevalence of pathogens further, e.g. by 5 log cycles (USDA-FSIS, 2001), which is difficult to achieve (see Section 15.6). Hence, interest in protective cultures is increased. These may be defined as cultures "that are added to foods with the aim of reducing risks by pathogenic or toxigenic microorganisms" (Vogel et al., 2011) while changing the sensory properties of the product as little as possible (Schillinger & Lücke, 1989). Candidate strains must be highly competitive during ripening and storage of meat products. In sausage fermentation, this applies to lactic acid bacteria capable of rapidly forming acids with little if any metabolic by-products. The formation of bacteriocins by some strains may reduce the levels of *L. monocytogenes* by an additional 1–2 log units as compared to the control unable to form these compounds (Lücke, 2000b). Hence, their use has been suggested, in particular, in the manufacture of briefly fermented, spreadable or semi-dry types (Erkes, 2013). However, use of protective cultures in the manufacture of cooked perishable meats appears to be more promising (Budde, Hornbæk, Jacobsen, Barkholt, & Koch, 2003; Kröckel, Dederer, & Troeger, 2011) because, unlike most fermented sausages, such products support growth of listeriae and have been incriminated in cases of listeriosis (see Section 15.6). Unfortunately, bacteriocins have little if any effect on gram-negative pathogens of relevance to fermented sausages, namely Shiga toxin-producing *E. coli*.

15.7.2 Fermented meats with enhanced nutritional value

Meat products have a poor reputation among nutritionists because of their marked contribution in salt, nitrite and fat uptake, and of some correlation between red meat ingestion and colon cancer (Cropet, 2011). Hence, there is much interest in making meat products more "healthy" (Toldrá & Reig, 2011). However, adding less than 2% sodium chloride will negatively affect the microbiological safety and sensory properties of the sausages, and replacing fat by lean meat also interferes with the formation of texture and flavour. With appropriate modification of formulations and ripening conditions, it is possible to reach a 30% reduction in fat and a 25% reduction of sodium levels in a fermented sausage (see, e.g. Corral, Salvador, & Flores, 2013; Olivares, Navarro, & Flores, 2011; Ravyts et al., 2010). It even appears feasible to reduce the fat content in German- and Spanish-style dry fermented sausages to about 15% and to replace animal fat by selected plant fats reasonably stable towards autoxidation (Mora-Gallego et al., 2013; Müller, 2006). Such sausages may be labelled with "reduced fat" and "reduced sodium", respectively, but it is not possible to develop "low-fat" or "low-sodium" sausages as defined by Regulation (EC) No. 1924/2006, in line with the Codex Alimentarius Guidelines (1997) for use of nutrition and health claims.

Regulation (EC) No. 1924/2006 also states that health claims should be used only if the food in question complies with certain nutrient profiles. Such nutrient profiles have not yet been established for meat products, but a working document of the European Commission (2009) suggests maximum levels of 0.7% sodium and 5% saturated fatty acids. It will be difficult to comply with these criteria. Despite these obstacles in labelling fermented sausages, much research has been done to improve the nutritional value of fermented sausages.

Animal fat could be completely or partially replaced by plant oils high in unsaturated fatty acids such as ω-3 fatty acids and conjugated linoleic acids. However, it is difficult to protect these fats from autoxidation without encapsulation (Josquin, Linssen, & Houben, 2012). Moreover, the sausages are softer, and drying is difficult. Hence, plant oils are, at present, mainly used in the manufacture of spreadable types such as German "*Teewurst*".

It is difficult to exclude nitrite and nitrate from the formulation of fermented sausages (see Hammes, 2012; Lücke, 2003). As outlined in Section 15.3.3, nitrite contributes markedly to inhibition of pathogens such as salmonellae early in ripening. This effect can be compensated for by using suitable starter cultures (rapidly forming acid at low fermentation temperature) and/or low ripening temperatures such as is common for traditional varieties manufactured with nitrate rather than nitrite. However, the protective effect of nitrite on fat deterioration is very difficult to compensate (Lücke, 2003).

Reviews on the use of probiotic starter cultures into the manufacturing of fermented sausages have been published by Kröckel (2006), de Vuyst, Falony, and Leroy (2008), Kołożyn-Krajewska and Dolatowski (2012), and de Macedo, Pflanzer, and Gomes (2012). Strains should be able to compete with the fermentation microbiota. Strains of the *Lactobacillus casei/plantarum* group appear most promising (Rubio et al., 2013), and strains of *Lactobacillus paracasei* (Kröckel, 2006; Sameshima et al., 1998), *Lactobacillus rhamnosus* (Työppönen, Petäjä, & Mattila-Sandholm, 2003) and *Lactobacillus pentosus* (Klingberg, Axelsson, Naterstad, Elsser, & Budde, 2005) have been commercialized. However, to date, all applications for health claims for probiotics have been denied by the European Food Safety Authority.

Functional plant ingredients (including prebiotics and dietary fibres) may also be included into sausage formulations, provided that they do not interfere with the fermentation. Moreover, bioactive peptides (with antioxidant activity or inhibitory effect on the angiotensin I-converting enzyme) may be formed by hydrolysis of meat proteins during ripening of fermented meats (Toldrá & Reig, 2011). Whether this effect can be optimized and used to develop more "healthy" meat products remains to be elucidated.

15.7.3 Ethnic/regional products

As a consequence of globalization and the pressure to develop new products, more and more regional varieties of meat products reach other markets. For example, Mediterranean-style mould-ripened fermented sausages were successfully introduced into the German market during the last few decades. This necessitates standardization and scaling-up of manufacturing processes, and the need for "autochthonous" starter cultures has been stressed (Talon et al., 2008).

15.7.4 Shortening ripening time

The costs of producing dry sausages may be reduced considerably by acceleration of the enzymatic and abiotic processes leading to the desired flavour of the products. However, due to the extreme complexity of these reactions, this is very difficult, and any

attempts in this direction had limited success. It is necessary to identify the key aroma compounds by molecular sensory science approaches (Söllner & Schieberle, 2009).

On the other hand, drying of sausages may be accelerated by freezing and slicing them after fermentation, followed by drying. This "Quick-Dry-Slice" technology has been patented (Comaposada, Arnau, Gou, & Monfort, 2004). Pathogens were inactivated at a rate similar to that of traditional processes (Stollewerk, Jofré, Comaposada, Ferrini, & Garriga, 2011). In combination with pressurization at 600 MPa, pathogens are inactivated even in the absence of added NaCl (Stollewerk, Jofré, Comaposada, Arnau, & Garriga, 2013).

15.8 Sources of further information and advice

- Books with a comprehensive account on fermented meats include: Campbell-Platt, G., & Cook, P. E. (Eds.), (1995). *Fermented meats*. Glasgow: Blackie; Toldrá, F. et al. (Ed.), (2007). *Handbook of fermented meat and poultry*. Ames, IA: Blackwell Publishing Professional (2nd edition to be published in 2014); Toldrá, F. (Ed.), (2008). *Meat biotechnology*. New York: Springer.
- Historical and socioeconomic aspects are treated in a recent article by Leroy, F., Geyzen, A., Janssens, M., De Vuyst, L., Scholliers, P. (2013). Meat fermentation at the crossroads of innovation and tradition: a historical outlook. *Trends in Food Science and Technology*, *31*(2), 130–137. Readers with appropriate language skills should consult books and Codes of Practice providing information on formulations and processing technology of regional sausage varieties. In Germany, for example, the handbook "Koch, H., & Fuchs, M. (2009). *Die Fabrikation feiner Fleisch- und Wurstwaren* (23rd ed.). Frankfurt/M: Deutscher Fachverlag" is a common reference in the meat industry.

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Quality improvement and fermentation control in fish products

16

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16.1 Introduction

Fishery products are difficult to produce premeditatedly, unlike farm products and stock farm products, especially when the quantity of fish catch widely varies each season. In addition, fish rapidly undergoes auto-digestion and decomposition after death. Historically, the most important problem related to fishery products involves ways of preserving fish and preventing fish degradation. Processed marine products such as dried fish, salt-preserved fish, fish sausage and canned fish were thus developed based on the need to prevent putrefaction. For example, canned fish and fish sausages are heat-sterilized to destroy microorganisms in fish, and then packed into sealed containers for protection against microbial contamination from the external environment. On the other hand, salt preservation, drying of fishes and soaking in vinegar inhibit the growth of microorganisms through the use of high-salt levels, low moisture and low pH.

Currently, various traditional fermented fish foods are reported in Japanese mass media, including newspapers, television and also comics. Various unique fermented foods have been attractively introduced. For example, characteristic strong smells of *Funazushi* in the Biwako Lake region and *Kusaya* in the Izu Islands are famous. Salted and fermented pufferfish ovary with rice bran, called *Fugu no mako zuke*, is exclusively made in Ishikawa Prefecture. Its detoxification mechanism remains unclear. Fish sauce products are made from salted and fermented fish not only in Japan but also in other countries in Southeast Asia (i.e., *Nampla*, *Nuok mam* and *Patis*; Table 16.1, Alexandraki et al., 2013). These Asian fish sauces are now being imported and utilized in the United States and Western countries.

Traditional fermented fish products are also made in Europe and Africa. For example, *Surströmming*, which is marketed in Sweden, is made from herring and emits a strong smell after spontaneous fermentation in cans without sterilization. *Feseekh* is made in Africa from mullet or other fishes by salting and fermentation. Since the 1950s, several studies about Southeast and East Asian culture and their traditional foods had been reported by Shinoda (1970). These reports speculate about the propagation of *Narezushi*, salted and fermented fish with cooked rice. Ishige and Ruddle (1990) indicated in their report entitled "Research on fish sauce and *Narezushi*" that the distribution of *Narezushi* and fish sauce production are correlated to paddy-rice culture in Monsoon Asia.

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00016-5

Table 16.1 Representative traditional salted and fermented fish products in Southeast/East Asia

Product	Substrates	Sensory property and nature	Country
Salted fish and fish sauce products			
Bagoong	Fish/shrimp, salt	Fish/shrimp paste	Philippines
Budu	Marine fishes, salt, sugar	Fish sauce	Malaysia, Thailand
Hoi-malaeng pu-dong	Mussel, salt	Fermented mussel	Thailand
Ika-Shiokara	Squid (muscle, liver), salt	Fermented squid	Japan
Ketjap-ikan	Fish, salt	Fish sauce	Indonesia
Myulchijeot	Small sardine, salt	Fermented small sardine	Korea
Nampla	Fish, salt	Fish sauce	Thailand
Patis	Fish, salt, food colour-optional	Fish sauce	Philippines
Gyo-shoyu (Ishiru)	Fish/squid liver, salt	Fish sauce	Japan
Saeoo Jeot	Shrimp, salt	Fermented shrimp	Korea
Lactic-acid fermented fish products			
Balao–balao	Shrimp, rice, salt	Fermented rice shrimp	Philippines
Burong isda	Fish, rice, salt	Fermented fish	Philippines
Narezushi	Fish, rice, salt	Fermented fish-rice	Japan
Pla-som	Marine fish, salt, rice, garlic	Fermented fish	Thailand
Som-fug	Fresh fish, rice, salt garlic	Fermented fish	Thailand
Sikhae	Sea water fish, cooked millet, salt	Fermented fish-rice	Korea

Source: Alexandraki, Tsakalidou, Papadimitriou, and Holzapfel (2013).

Traditional fermented fish foods can be roughly classified as follows: (1) salted and ripening type and (2) pickled (lactic-acid fermented) type. The former type is made through a ripening process using autolytic enzymes. For example, fish guts pickled in salt, called *Shiokara* in Japan, and fish sauce are of the former type. The latter type is made through a fermentation process with lactic acid bacteria (LAB). *Narezushi* made in Japan and fish *Kimchi* made in Korea are of the latter type. Researchers have enthusiastically continued to conduct systematic studies on microorganisms in both types of fermented fish products. In recent years, new genera and species have been isolated and identified using molecular techniques. This chapter presents various traditional fermented fish products, particularly those made in the Hokuriku region, which is located in the central part of Japan and facing Eurasia (China and Korea, Figure 16.1, Kuda & Yano, 2010).

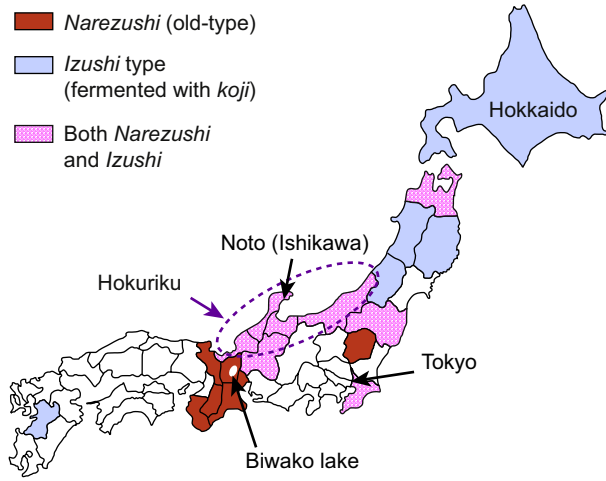

Figure 16.1 Distribution of the *Narezushi* line and *Izushi* line in Japan.

16.2 Salted and fermented fish products

16.2.1 Fish sauce

16.2.1.1 Recipe

In Noto in Ishikawa Prefecture (Figure 16.1), traditional fish sauce is prepared from whole sardines or squid guts, called "*Ishiru*", "*Ishiri*", "*Yoshiru*", or "*Yoshiri*" (Figure 16.2). *Ishiru* is made from raw materials that underwent a spontaneous ripening process in high-salt conditions from November of each year to May of the next year. To prepare fish sauce using squid guts, 18% (w/w) salt is added to the material and stirred if necessary; then the mixture is aged for approximately 2 years. To prepare fish sauce using whole sardines, 20% salt is added to the raw materials, stirred if necessary and aged for approximately 6–12 months. Halophilic LAB, *Tetragenococcus*, occasionally increase during salting and ripening (Kuda et al., 2012). *Tetragenococcus* is detected in other salted and fermented foods, such as *Miso* and *Moromi* (unrefined soy sauce). Some of the *miso* and soy-sauce brewers use *Tetragenococcus halophilus* as a starter. The exuded and accumulated liquid in the lower layer of the fermentation tank is collected using a drainage system and subjected to de-proteinization by boiling, and is used as the finished product. Fish sauce made from sardines or squid is also known as *Nampla* and *Nuoc mam* in Thailand and Vietnam, respectively.

16.2.1.2 Chemical components

Ishiru contains high amounts of nitrogen and salt. *Ishiru* made from squid has a total nitrogen content of 1.7–2.5 g/100 ml and a salinity level of 14.8–26.5 g/100 ml. *Ishiru* made from sardines has a total nitrogen content of 1.4–2.2 g/100 ml and a salinity level of 25.7–27.3 g/100 ml (Michihata et al., 2000). High amounts of various free amino acids, such as alanine (Ala), glutaminic acid (Glu), glycine (Gly), lysin (Lys) and

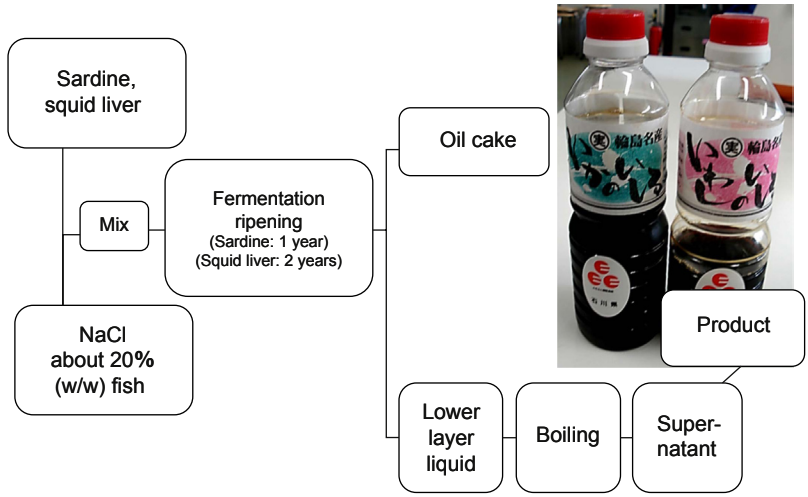

Figure 16.2 Recipe for *Ishiru*.

valine (Val), and oligopeptides consisting of Glu, Gly, aspartic acid (Asp) and Lys, are also present. Major organic acids in the fish sauce include lactic acid, pyroglutamic acid, acetic acid, formic acid, succinic acid and malic acid. It has been suggested that these ingredients are responsible for generating the *Ishiru*-specific delicate *umami* taste.

16.2.2 Shiokara: *salted fish fermented with or without their guts*

16.2.2.1 Recipe

Shiokara are basically manufactured using the following process. Salt, generally at least 10% (w/w), is added to fish and/or guts to prevent putrefaction. During ripening, the tastes are generated by the autolytic enzymes in the raw materials. It has been suggested that this specific taste is mainly due to the increase in free amino acids. There are various types of *Shiokara*, including those from squid, bonito (*Shuto*), sea urchin, sweetfish (*Uruka*), sea cucumber (*Konowata*) and salmon (*Mefun*). This section describes the preparation of squid *Shiokara*, which is the most common *Shiokara* and is therefore produced in large quantities.

The recipe for squid *Shiokara* is shown in Figure 16.3. The guts are first carefully removed to prevent the squid ink bag from bursting. The mantle and leg are then washed with water. After draining the water, the meats are chopped and placed in a large barrel. The liver is salted, minced and ripened in another barrel. The mixture is then thoroughly stirred every morning and evening. In the traditional recipe, the added amount of salt is over 10% (less than 20%) of the amount of meat, and that of the liver is around 3–10%. The rate of ripening of the squid liver depends on the amount of salt and temperature used in the process.

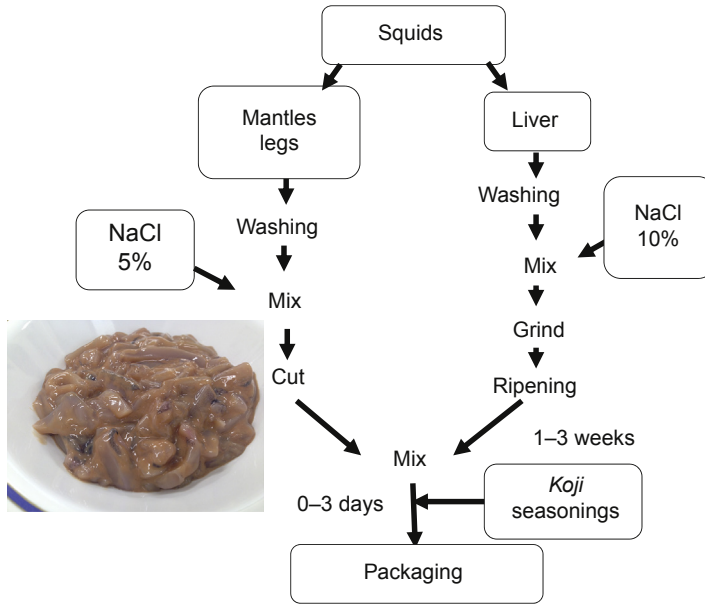

Figure 16.3 Recipe for *Shiokara*, squid pickled in salt.

During ripening of the *Shiokara*, the levels of amino acids, organic acids and volatile basic nitrogens increase. For example, the levels of the amino acids, such as Glu, leucine (Leu), Lys and Asp, increase during ripening (Fujii et al., 1994). For example, Glu levels increase from 53 mg/100 g during the early preparation stage and reach approximately 600–700 mg/100 g by the time of consumption. When squid *Shiokara* products are aged at 10 °C, these are ready for consumption within 1 to 2 weeks if pickled in 10% salt or within 1 month when pickled in 13% salt.

16.2.2.2 Low-salt squid Shiokara

Since 1975, low-salt (approximately 3–7% salt) squid *Shiokara* have gained significant acceptance over the traditional *Shiokara* with 10% and higher concentration of salt. Traditional *Shiokara* usually have a reduced risk of contamination by food spoilage bacteria. On the other hand, the low-salt *Shiokara* are usually prepared within a shorter time period to prevent bacterial growth, thus resulting in a less-intense *umami* taste due to the shortened ripening (auto-digestion) period. Instead, low-salt *Shiokara* are mixed with seasonings and require low temperature, low pH, low water activity and various types of additives to preserve quality.

16.2.2.3 Microorganisms in squid Shiokara

Most food-poisoning bacteria and putrefying bacteria cannot grow in traditional *Shiokara* because of its high-salt content. *Vibrio parahaemolyticus*, which can generally tolerate environments containing 2–3% salt, cannot grow in the pickling conditions of ≥10%

salt. *Staphylococcus aureus* has comparatively strong resistance against salt and can grow even in environments containing ≥10% salt. However, in squid *Shiokara*, bacteria other than the *Staphylococcus* genus may exist, with no congeneric *S. aureus* detected.

In September 2007, a massive food-poisoning incident occurred in Miyagi Prefecture, involving *V. parahaemolyticus* present in squid *Shiokara* and affecting a total of 620 patients. *V. parahaemolyticus* is widely distributed in coastal seawater during summer and its growth rate is high. Although food-poisoning bacteria are usually unable to grow in traditional *Shiokara*, the salt concentration of the contaminated *Shiokara* in this food-poisoning incident was only approximately 2%.

16.2.3 Nukazuke

16.2.3.1 *Common fish* Nukazuke

Nukazuke fish products are commonly called "*Heshiko*" in the Fukui Prefecture and "*Konka-zuke*" in the Ishikawa Prefecture. The recipe for common fish *Nukazuke* is shown in Figure 16.4. The region primarily recognized for *Nukazuke* production includes Obama, Fukui Prefecture and Mikawa, Ishikawa Prefecture. Even in these high-salt (15–20%) food items, LAB may be active during fermentation and ripening, in which the main lactic acid-producing bacterium is *Tetragenococcus*, which is also utilized in the preparation of *miso* and soy sauce *Moromi* (An et al., 2010; Kuda et al., 2012). In fact, the degree of lactic acid fermentation varies according to the factory, barrel and fish species. For example, some *Moromi* of *Ishiru* shows a small amount of LAB and low lactic acid levels, whereas other *Moromi* of *Ishiru* contains more than 2% (w/w) of lactic acid. The authors have observed that for a product that contains salt close to 20% (w/w), the number of LAB is generally small, and that the lactic acid level

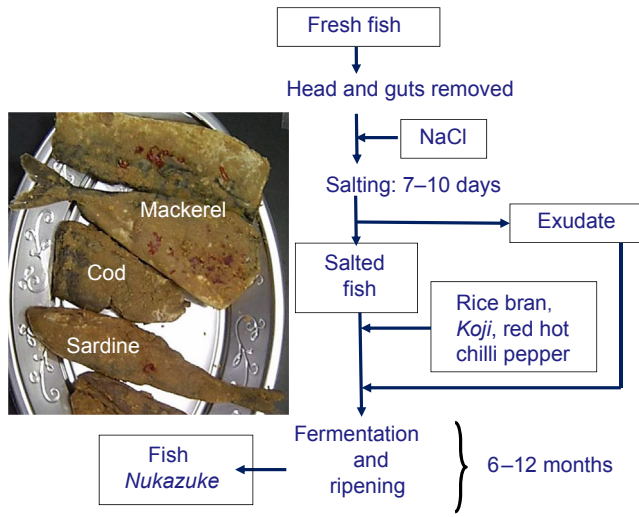

Figure 16.4 Recipe for common fish *Nukazuke*.

and the levels of free amino acid and amines are relatively low. Isolated *Tetragenococcus* strains grow well in a medium with 15% (w/v) salt, whereas they do not grow well in a medium with 20% (w/v) salt. However, these traditional foods have no reference or regulatory standard for the number of LAB, lactic acid level, salinity or pH value.

Accumulation of histamine (Hm) is occasionally a problem in *Nukazuke* fishery products and fish sauce. For example, the cumulative amount may often be more than 400 mg/l and 2000 mg/l in *Nukazuke* of sardines and *Ishiru* of sardines, respectively. Hm will be generated immediately by *Morganella morganii* and other Hm-generating bacteria when the raw fish is stored at inadequate temperature. Some of these bacteria survive in the low pH environment through the action of histidine decarboxylase. In salted foods, the halophilic LAB, *Tetragenococcus*, is involved in generating Hm. Because the actual consumption of high-salt content foods at a time is very low, the authors consider that the presence of these bacteria should not be a major problem. However, from the viewpoint of strict regulatory values established in the United States and Canada, and consumer preference for safety, technologies for practical microbiological control resulting in reduced Hm in these fermented foods should be developed.

16.2.3.2 Nukazuke *of pufferfish ovary*

Nukazuke of the ovaries of pufferfish (*Fugu no mako zuke*) is made in limited regions in Ishikawa Prefecture. The ovaries, which contain a deadly poison, are salted for at least 1 year and soaked in rice bran for 2 years (Figure 16.5). Although researchers

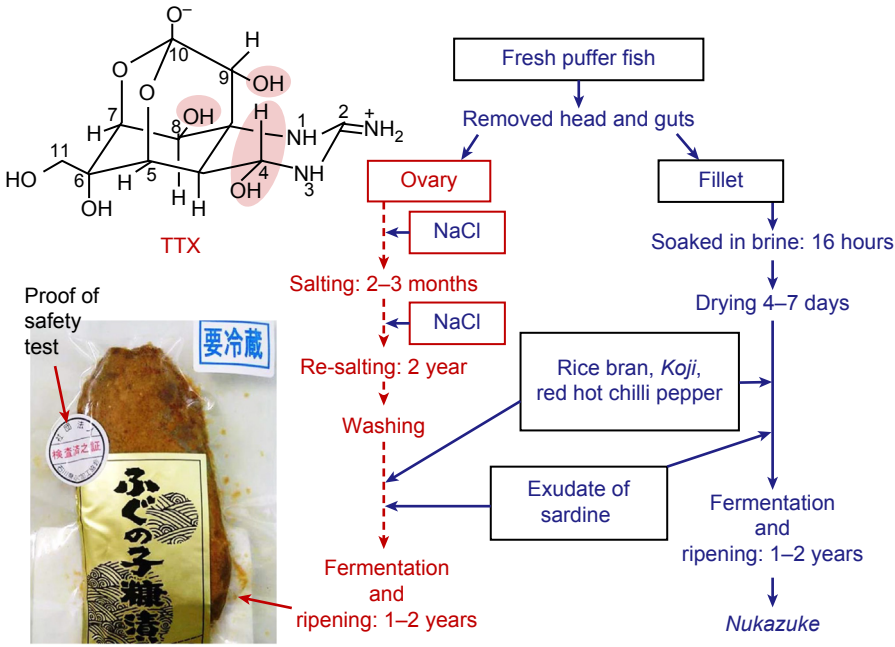

Figure 16.5 Recipe for *Nukazuke* using ovaries of pufferfish.

have challenged this mysterious detoxification mechanism for more than 30 years, no clear answer has been found. Unlike the common fish *Nukazuke*, the puffer ovary *Nukazuke* requires re-salting several times during the salting process. By re-salting, a significant amount of water-soluble *Fugu* poison (tetrodotoxin; TTX) is removed. In addition, this poison is further diluted by soaking the ovaries in rice bran. However, microbiology researchers are continuously attracted to this mysterious mechanism and consider that some kind of microbial action, including the conversion and binding, influences the amount of TTX that remains in these organs.

The Association of Puffer Fish Processing in Ishikawa Prefecture strictly requires that a *Fugu* poison test be conducted per production lot, and a certificate proving that a product passed this test is attached to every product distributed in the market.

16.3 *Narezushi*

16.3.1 Japanese Narezushi *culture*

In Southeast and East (Monsoon) Asia, traditional fermented foods are prepared from fish, salt and cooked rice (called *Narezushi* in Japan), paddy-rice. The old-type *Narezushi* is pickled in only salt, fish and rice. Currently, in Japan, the old-type *Narezushi* has been made in the Kinki region, particularly around Biwako Lake (Figure 16.1). *Funazushi* made from crucian is the most famous *Narezushi*. Furthermore, other various *Narezushi* are made from sweetfish, dace, three-lips, freshwater minnow, trout, loach and salmon. On the other hand, in northeast regions of Japan the *Izushi*-type *Narezushi* is produced, which utilizes fermentation with *Koji*, *Aspergillus oryzae* malted rice. In the Hokuriku region located between the old-type *Narezushi* line and *Izushi*-type making regions, both food cultures prevail. It is regarded that the roots of *Izushi* are in the Hokuriku region, though *Izushi* is famous as a traditional food in Hokkaido. It has also been associated with *Shihhe* (or *Sikhae*) made in Korea. Although malt is used to promote fermentation for *Shihhe*, it can also be regarded as an *Izushi*-type product.

In the Ishikawa Prefecture, *Izushi*-type fermented foods, including *Kaburazushi* and *Daikonzushi*, remain in the Kaga district, which is an urbanized region. On the other hand, old-type *Narezushi* is made in Noto, which is a rural area that follows traditional cultures. *Ishiru* is made in Noto, as previously mentioned. These facts correspond to the findings obtained by Ishige and Ruddle (1990) indicating that rice paddy cultivation is deeply associated with fish sauce and *Narezushi*.

16.3.2 Narezushi *made in Noto*

16.3.2.1 Basic recipe

The process of preparing *Narezushi* in Noto is as follows (Figure 16.6): (1) the gills, guts and eyes of the fresh fish are removed; (2) the fish is salted; (3) the fish is soaked in diluted vinegar; (4) the prepared fish and cooked rice are filled in a barrel and

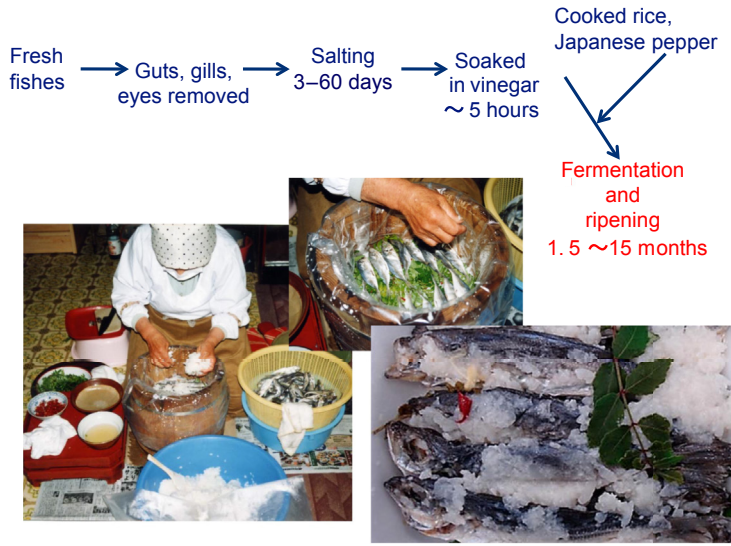

Figure 16.6 Recipe for *Narezushi* in Noto.

(5) the barrel is allowed to ripen at ambient temperature. The following values may greatly vary according to the manufacturer: salinity for salting (18–33%), duration of soaking in vinegar (ranging from several seconds to 5 h), quantity of cooked rice (ranging from half to the same amount of fish used in the recipe) and duration of ripening (ranging from 6 weeks to one or more years).

It is believed that the original *Narezushi* (*Hinezushi* in Noto) was not soaked in vinegar but took at least one year of ripening. However, currently, *Narezushi* is often eaten within two to four months after initiation of ripening.

The procedures of the filling are as follows. Rice is spread on the bottom of a barrel. Weak vinegar and liquor are used for hand-wetting. The fishes are placed in the barrel to cover all of the rice layer, and then the layer is sealed together with slight sprinkling of red pepper and Japanese pepper leaves. After the filling, which results in several layers of rice and fish, rice and Japanese pepper leaves are spread across the top layer. Then, weight stones of approximately 1.5 to 4 times the weight of the materials are placed on the lid, and the entire setup is stored in a cool place to allow ripening.

16.3.2.2 Microbiota

LAB, such as lactobacilli and lactococci are the predominant microbiota in *Aji no susu* (*Narezushi* made from horse mackerel), which is either produced in each family or was commercially available. Most of the samples (1.5 to 6 months of ripening) contained 8 log cfu/g LAB, indicating that *Aji no susu* is a typical lactic acid-fermented food (Kuda et al., 2009). In products fermented at least one year, the amount of LAB decreases to approximately 6 log cfu/g. Mold occurs on the lid of some barrels. These fungi might have a role in the development of the unique taste of *Aji no susu*.

Lactobacillus plantarum, which possesses a high fermentative ability, is isolated as the predominant bacterium from *Narezushi*. *Lactobacillus rennini* is also isolated from *Narezushi. Lactobacillus rennini* has been isolated from moderately high-salt content food, such as cheese. *Tetragenococcus*, which are halophilic LAB, have also been isolated from some *Narezushi* products. The yeast, *Debaryomyces hansenii* are commonly isolated from pickles, while *Pichia anomala*, known as the causative organism of ethyl acetate, has also been isolated. On the other hand, when genomes directly extracted from the samples were analysed by denaturing gradient gel electrophoresis (DGGE) and pyro-sequencing methods, strains of *Lactobacillus acidipiscis* or *Lactobacillus versmoldensis*, which generally cannot be detected by the described culture method, were found to be dominant (An et al., 2010; Koyanagi et al., 2011).

16.3.2.3 *Free amino acid and organic acid levels in* Narezushi

For free amino acids, at least 100 mg/100 g of Ala, Leu and Lys was included. In products subjected to 1 year fermentation, the content of various types of free amino acids was high, and the total content was two to four times higher than that in products that had been fermented for 6 months or less. The content of Glu, which is a component of the *umami* taste, greatly varied among products. The content of gamma-aminobutyric acid (GABA) generated by Glu decarboxylase (GDC) also varied based on products; some products included approximately 300 mg/100 g. This value is higher than that in germinated brown rice, which is commonly known as a high-GABA food item (approximately 10 mg/100 g), high-GABA yogurt (approximately 100 mg/100 g) and high-GABA brown rice (approximately 150 mg/100 g). Even LAB isolated from *Narezushi* contained the GABA-producing strain (related to *Lb. rennini*). This strain showed poor growth at pH 4.5 compared to other *Lactobacillus* spp. Thus, it is possible that GDC acts in response to the acidic conditions used in *Narezushi*.

The pH of *Aji no susu* is within the range of 3.9–4.3, with main organic acid as lactic acid (3.1–8.6 g/100 g). In addition, volatile short-chain fatty acids, such as acetic acid (0.1–0.6 g/100 g), were detected. The highly concentrated lactic acid is characteristic of *Narezushi* that rich in rice (carbohydrate). The concentration of lactic acid in the *Narezushi* (*Aji no susu*) made from horse mackerel and fermented for 1 year was higher than that in *Funazushi* and *Saba* (mackerel) *narezushi* made in Fukui Prefecture. Butyric acid and propionic acid, which are considered the main causative factors for the characteristic odour of *Funazushi* and *Kusaya*, were hardly detected.

Currently, Japanese *sushi* is prepared by placing a slice of fresh fish on hand-shaped vinegar rice. It is considered that *Narezushi* is an origin of the current *Sushi*. *Sushi* will have vinegar added instead of waiting for the accumulation of lactic acid.

16.3.2.4 *Aromatic compounds*

The aromatic compounds in *Narezushi* made from horse mackerel, which shows various durations of fermentation, were analysed by a Headspace GC/MS system. The content was higher and the kinds of aroma constituents were present more in the fish meat part than in the rice part. Contents of acetic acid (4100–6500 ng equivalent to BHT/g) and 1-penten-3-ol (980–2900 ng equivalent to BHT/g: dried small sardines smell)

were high. From the value of Odour Unit, which was converted from the standard threshold in humans, the ingredients that are strongly felt as an "odour" in the *Narezushi* were estimated to be aldehydes such as hexanal (raw smell), methylbutanal (almond smell), pentanal (pungent smell) and 1-octen-3-ol. These aldehydes and alcohols are reported as deterioration factors during storage and processing of meats, fish meat and milk. The long (12 months) fermented *Narezushi* showed more complications and higher odour content. It was estimated that 2-nonenal and butyric acid might be the main aroma constituents. In recent years, 2-nonenal has been associated with the distinctive body odours of older men which are expressed as orris, oil smell and raw smell.

Generally, acetic acid, propionic acid, butyric acid and amines are regarded as specific aroma compounds of *Funazushi* and other *Narezushi*. However, lipid metabolites and alcohols are major odour compounds, as previously described. Although the major factor of production of these aromas is oxidation, alcohols (i.e. 1-octen-3-ol and 1-penten-3-ol), which emit a mushroom smell and mold smell, are regarded as metabolites of fungi and bacteria. Though lactic acid, which is present in high amounts in *Narezushi*, does not have a strong smell, it might lower the pH and suppress volatilization of some alkaline compounds, such as amines and ammonia.

16.3.3 Kaburazushi *and* Daikonzushi *(Izushi-type products)*

16.3.3.1 Recipe

Kaburazushi and *Daikonzushi* are known as the winter-special products in the Hokuriku region. *Kaburazushi* is prepared using the following steps: cut turnips and yellowtail are salted for 2–3 days, and salted yellowtails are sliced and inserted into the cut turnips (Figure 16.7). These materials are fermented with *Koji* for several days (main fermentation). The preparation of *Daikonzushi* is similar to that of *Kaburazushi*. Salted Japanese radish and dried-and-rehydrated herring are fermented with *Koji*. *Kaburazushi* is commonly served as an especially attractive food to pray for a big catch and safety, and has been produced in regions near fishing ports in Kanazawa

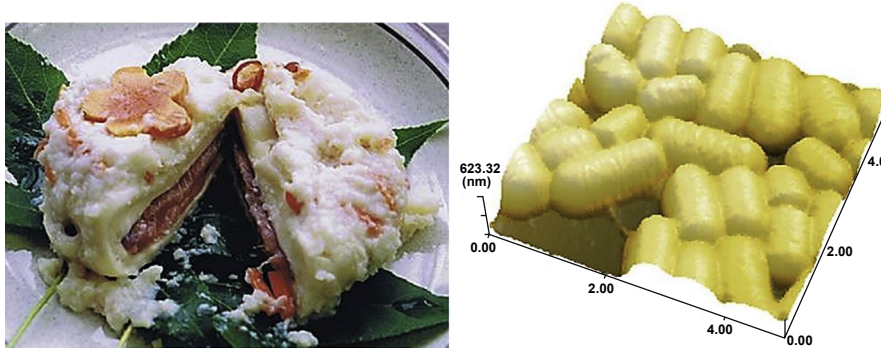

Figure 16.7 Image of *Lactobacillus plantarum* isolated from *Kaburazushi,* using atomic force microscopy.

since the Edo era (AD 1603–1867). In the past, turnips and yellowtails were expensive and harvested in the limited season, and the quantity of crop was small. Thus, *Kaburazushi* is considered a special food item in ordinary households. On the other hand, *Daikonzushi* made from Japanese radishes and herrings might be affordable and easy to obtain.

The materials and recipe of *Daikonzushi* are similar to those of other *Izushi*-type products made in the Tohoku region and Hokkaido. Originally, old-type *Narezushi* was mainly prepared from freshwater fishes in inland or mountain areas. The *Izushi*-type products are made mainly from marine fishes in plains and urban areas. From the viewpoint of the association with *Izushi*-type products, such as *Shihhe*, in Korea, this is an interesting fermented food culture.

16.3.3.2 *Microbiota flora and lactic acid fermentation in* Kaburazushi

We examined the changes in microbial population in *Kaburazushi* and *Daikonzushi* prepared by families using their own traditional recipe. The number of LAB slightly increased during the salting and then rapidly increased during the main fermentation, reaching at least 8 log cfu/g or more (Kuda & Yano, 2010). Though the lactic acid level was lower than that in old-type *Narezushi*, but reached 1.5–2.0% (w/w), the level of lactic acid fermentation was the same as for milk fermentation. It is possible that the growth of LAB is promoted by the degradation product of starch (glucose and maltose) in *Koji*, which was used in the main fermentation, and by glucose and fructose in turnips and Japanese radishes. Thus, the number of saprophytes, which were observed during the early period of fermentation, decreased, and only LAB and yeasts remained after 7 days of fermentation (time to eat).

We also examined the microbial population in commercial products. In some products, the number of LAB was less than 4 log cfu/g, and the level of acetic acid was higher than that of lactic acid. In addition, even in the products in which at least 8 log cfu/g of LAB were detected, the quantity of lactic acid was 0.26–0.46% (w/w), which was lower than that in the domestic products. Presently, because refrigerators can be used, low pH and high salt concentrations are not required for food preservation. In addition, consumers tend to prefer milder tastes and textures. Therefore, it is considered that the commercial *Kaburazushi* and *Daikonzushi* products differ in tastes from domestic and traditional products.

Similar to common vegetable pickles, various LAB have been isolated from *Kaburazushi* or *Daikonzushi*. *Lactobacillus plantarum* strains derived from *Kaburazushi* showed faster growth in Japanese radish juice than did other isolated strains and the type strain.

16.4 Functionality of lactic-acid fermented fish foods

Many researchers have described the beneficial effects of LAB isolated from traditional fermented fish products on the processing and fermentation of traditional

and new regional foods (Yin et al., 2002). For example, Desniar et al. (2013) reported on antimicrobial activity of LAB isolated from Indonesian fermented fish (*bakasam*). Some halophilic LAB (*Tetragenococcus halophilus*) strains isolated from fish-*nukazuke* suppressed histamine accumulation when used as starter cultures during fish-sauce fermentation (Kuda et al., 2012).

On the other hand, there are also several reports about a third function of LAB or lactic-acid fermented foods, which has health benefits such as normalization of the intestinal environment, cholesterol-lowering effect and antioxidative effects (Kanno et al., 2012; Kuda et al., 2012; Nakamura et al., 2012). In regions along the Biwako Lake and in Noto, some traditional medicines with *Narezushi* are recommended by suggesting "When gastrointestinal condition is not good, take a piece of rice potion of *Narezushi*". However, reports on the functionality of LAB derived from *Narezushi* are limited. As previously mentioned, highly concentrated GABA accumulates in some types of *Narezushi.* In addition, radical-scavenging activities (antioxidative properties) have been observed for the lactate ion itself.

Lactic-acid fermented foods made from fishery products, including *Narezushi* and *Kaburazushi*, might have been created as a preserved food by using anti-bacterial compounds such as salt and lactic acid, while they have been utilized as meals during celebrations for hundreds of years. On the other hand, *Nukazuke* and salted foods continue to be consumed as preserved foods. However, a large amount of *Nukazuke* cannot be consumed at a time because of the intense salty taste. LAB in these traditional fermented foods, as well as the functional ingredients generated by lactic acid fermentation, attract not only researchers but also non-researchers. The researchers should explain the functions of the fermented foods and their LAB population, with high salinity and balances of nutrients.

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Quality improvement and fermentation control in dough fermentations

17

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17.1 Introduction

Fermentation of cereals in the form of dough has been used since ancient times. It is performed by lactic acid bacteria (LAB) and/or yeasts. The ecology of the microbiota is influenced by the applied process parameters, and bacteriophages do not play an important role. The main aim of the application of sourdough fermentation is the creation of flavour. Starter cultures for dough fermentations have been available for a century, and convenient sourdough products, where the fermentation is performed by specialized ingredient producers, were developed 40 years ago. Such a split in the production process is also applied in baking, as doughs are frozen and later baked in front of the consumer. An increasing number of people suffer from coeliac disease, which requires baked goods without wheat, rye or barley. Such gluten-free doughs are optimized by application of sourdough fermentation. Lactobacilli are able to produce antifungal compounds as a base for an improved shelf life of baked goods and may reduce contamination with mycotoxins in dough. Fermentation also changes the nutritional value of baked goods. It lowers the glycemic index, improves mineral bioavailability and increases the concentration of some vitamins.

17.2 Advances in understanding of microbiota and physiology

Fermentation of doughs is applied in nearly all areas of the world. The range of products goes from Western-style bread, Chinese steamed bread or gruels for injera and kisra. The main aim of dough fermentation is leavening. Since the introduction of baker's yeast and chemical leavening agents during the industrial revolution, its application changed strongly. A second aim is the acidification of dough, originally necessary for rye processing. Figure 17.1 depicts the principles used in baking for leavening, acidification and its intermediate states. Sourdough fermentations can be classified into four classes. Type I sourdoughs are aiming at leavening in traditional bread-making processes. These fermentations are characterized by short (up to 12 h) refreshment steps at temperatures <25–30 °C, favouring the growth of yeasts and the

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00017-7

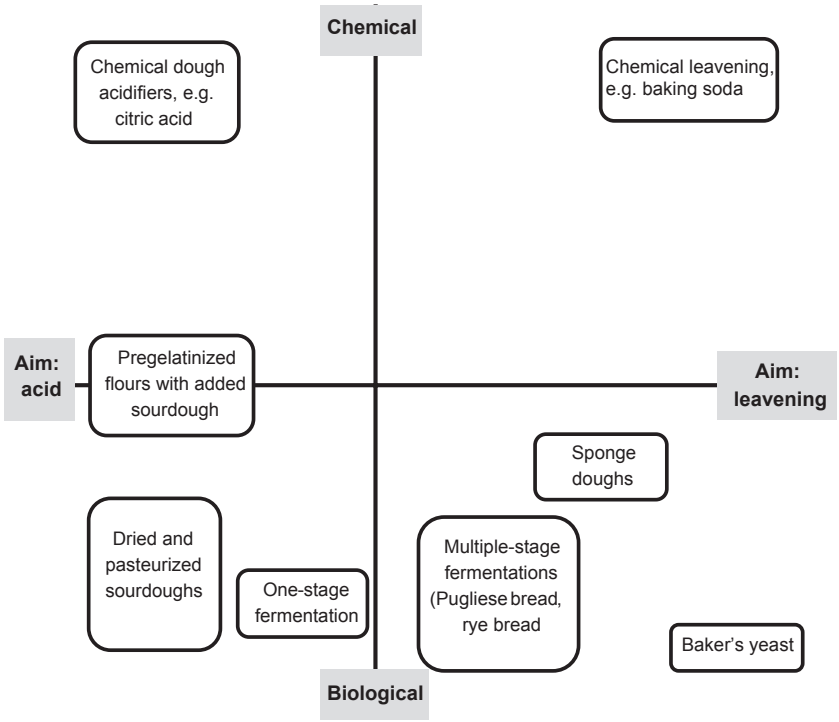

Figure 17.1 Classification of leavening and acidification agents in baking technology.

typical species *Lactobacillus sanfranciscensis*. These processes allow enough leavening only from the sourdough without using baker's yeast. Type II fermentations came up with industrialization of sourdough processing and are aiming at high acidification of the sourdough, which means that acid-resistant lactobacilli such as *Lactobacillus reuteri* or *Lactobacillus pontis* become dominant. These sourdoughs are often dried. Type III sourdoughs have special drying parameters, allowing the survival of lactobacilli. These sourdoughs are specialized products for countries where legislation requires living organisms in dried sourdoughs. Finally, type 0 sourdoughs are sponge doughs started with bakers's yeast. If the fermentation time is long enough, the pH in these doughs drops due to growth of microbiota contaminating bakers's yeast, e.g. *Pediococcus* spp. or *Leuconostoc* spp. The main characteristics of the various sourdough types are summarized in Table 17.1.

17.2.1 Microbiota

Dough fermentations are carried out on a broad range of plant raw materials as substrates and involve different processing parameters. Among food fermentations, doughs are one of the most complex systems, as the parameters influencing the microbiota change very quickly. Due to the drop in pH, the activity of endogenous enzymes changes constantly as they have different pH-optima. The pH-dependent activity has

Table 17.1 Classification of sourdoughs regarding microbial and processing effects

	Type 0	Type I	Type II	Type III
Starter (mother dough)	Baker's yeast	Sourdough (backslopping)	Sourdough (backslopping)	Laboratory culture or sourdough
Raw materials	Wheat	Wheat/rye/rice	Rye/wheat/spelt/ maize/rice/quinoa/ flax seed, etc.	Mainly rye
Fermentation conditions	One-stage process, 3–24 h	4–16 h at 25–35 °C	1–7 d at >28 °C	–
Dominant microbiota	Baker's yeast dominant, 10^6–10^8 cfu/g lactic acid bacteria	Lactobacilli and sourdough yeasts	Lactobacilli, sometimes aerobic spoilage yeasts	–
Typical final pH and TTA	Low TTA (<7), pH 3.5–5.0	pH 3.5 to 4.0, medium tta (5–20)	pH 3.2–3.8, high TTA (>20)	–
Ability to restart a fermentation by backslopping with the same technological aim, if it is harvested at the end of fermentation?	No	Yes	No	Sometimes
Technological effects	Leavening, overall bread quality	Leavening, acidification, overall bread quality	Acidification, overall bread quality, base for dried sourdoughs as flavour enhancers	Flavour enhancer with the possibility to restart a fermentation, if required from legal aspects

TTA, total titratable acidity.
Source: Modified from Böcker, Stolz, and Hammes (1995).

influence on the substrate availability for the microbes. This is in part controllable by the selection of the range of the applied process parameters.

The influence of process parameters on the composition of the microbiota by repeated refreshment (backslopping) of sourdoughs was initially investigated by Meroth, Walter, Hertel, Brandt, and Hammes (2003). Rye sourdough with two refreshment steps a day (type I) were dominated by *L. sanfranciscensis*, whereas type II sourdoughs with fermentation steps before refreshment of 24 h and longer were dominated by *L. pontis, Lactobacillus crispatus* and *L. reuteri*. Further studies by Siragusa, Di Cagno, Minervini, Gobbetti, and De Angelis (2009) and Vera, Ly-Chatain, Rigobello, and Demarigny (2012) showed that the composition of the microbiota in a backslopping process remains constant, if the process conditions are kept constant. Between rye, wheat and spelt there are no big differences in the composition of microbiota, if the usual backslopping procedures are applied (Weckx, Van der Meulen, Maes, Huys, & Vandamme, 2010). Fermentations with pseudocereals such as amaranth or quinoa were dominated by *Lactobacillus paralimentarius* (Vogelmann, Seitter, Singer, Brandt, & Hertel, 2009). For buckwheat fermentations, it is reported that yeasts are often washed out (Vogelmann et al., 2009), while *L. sanfranciscensis* has a reduced competitiveness in sorghum (Sekwatei-Monang, Valcheva, & Gänzle, 2012).

More pronounced effects on sourdough and therefore on bread quality are caused by the relative proportions of LAB and yeasts. In traditional sourdough processes, temperatures >28 °C favour growth of LAB over yeasts. The growth rate of LAB responds stronger to temperature than the growth rate of yeasts; thus at lower temperatures (<25 °C), growth of LAB is more retarded than that of yeasts (Brandt, Hammes, & Gänzle, 2004), which explains why in traditional leavened breads (Pugliese, three-stage-rye bread) without added baker's yeast, fermentation steps below 25 °C are always recommended. Another factor favouring growth of yeasts – and therefore leavening – is the amount of inoculum for starting a fermentation step. High inocula result in a low initial pH of fermentation (Brandt et al., 2004).

The genome of the key sourdough organism, *L. sanfranciscensis*, was recently sequenced by Vogel, Pavlovic, et al. (2011). Interestingly, this organism has the smallest genome among lactobacilli and the highest density of ribosomal RNA operons per Mbp genome. That contributes to the dominance of *L. sanfranciscensis in* type I fermentations, which require quick growth. *L. sanfranciscensis* was – until now – only isolated from sourdoughs. Thus, its natural habitat is still unknown. For type II fermentations, the initial starting lactobacilli, which became dominant, are originally from the intestine of rodents (Shu-Wei et al., 2012).

17.2.2 Bacteriophages

Compared to other fermented foods, bacteriophages do not play an important role in industry and starter culture selection for sourdough. Most starter cultures are mixtures of several strains and species, preventing a breakdown of acidification. The phage EV3 showed activity against 5 out of 12 strains of *L. sanfranciscensis*, the typical species of type I sourdoughs (Foschino, Venturelli, & Picozzi, 2005). Due to the relatively thick consistency of dough and the short time of fermentation until the next

refreshment step in sourdough, phages can only diffuse slowly (Foschino et al., 2005), and therefore in practice only sourdoughs with dough yields over 250 may be a noticeable target of phage attack. The presence of CRISPR (clustered regularly interspaced short palindromic repeats) loci in the genome of *L. sanfranciscensis* TMW1.1304 may also play a role in phage protection (Vogel, Pavlovic, et al., 2011).

17.3 Physiology and its impact on bread quality

The availability of hydrogen acceptors influences the energy yield from hexoses by obligate heterofermentative lactobacilli. The effects of fructose, O_2 and other hydrogen acceptors have been known for years and are reviewed by Gänzle, Vermeulen, and Vogel (2007). During the last decade, more hydrogen acceptors, which especially have an impact on dough rheology or flavour, were found. Some sourdough lactobacilli possess glutathione-reductase activity which contributes to their oxygen tolerance, as was shown by Jänsch, Korakli, Vogel, and Gänzle (2007) with the isogenic mutant of *L. sanfranciscensis* DSM20451 lacking glutathione reductase acitivity (GshR), whereas this activity is not present in *L. reuteri*. The reduction of disulfide bonds (oxidized glutathione) or of long-chain aldehydes (e.g. hexanal, nonenal) allows the regeneration of NADH and has effects on the rheology of wheat doughs. It may explain the improved extensibility of wheat dough-containing sourdoughs (Brandt, Münscher, & Hammes, 2003). The influence of LAB on the redox potential of sourdoughs is a good tool – besides pH – for monitoring fermentations (Capuani, Behr, & Vogel, 2012). The lactobacilli of sourdoughs are able to degrade a broad range of oligosaccharides (Gänzle & Follador, 2012) or to produce oligosaccharides and exopolysaccharides (reviewed by Gänzle, Zhang, Monang, Lee, & Schwab, 2009), mainly glucans and fructans. Some of these exopolysaccharides affect dough rheology (Galle et al., 2012). Although exopolysaccharides play an important role in cereal research, only dextran-containing sourdough products found their way to the market (Lacaze, Wick, & Cappelle, 2007).

17.3.1 Flavour

Reducing sugars and free amino acids are important aroma precursors, especially in bread crust. They are mainly released by endogenous enzymes of the cereal (Gänzle et al., 2007). If yeasts are present, the concentration of free amino acids is reduced, whereas lactobacilli, isolated from sourdoughs, possess strain-specific activities against the protein fractions from wheat and in the release of amino acids (Di Cagno et al., 2002; Thiele, Gänzle, & Vogel, 2001). Only a few amino acids are relevant for aroma development: leucine, isoleucine, methionine, phenylalanine, proline and ornithine (Schieberle, 1996). With the exception of ornithine, these amino acids occur naturally in flour. Ornithine is produced by the arginine-deiminase pathway (ADI) from arginine by some lactobacilli (e.g. *Lactobacillus amylolyticus*, *L. brevis*, *L. frumenti*, *L. pontis* and *L. reuteri*; Gänzle et al., 2007). Arginine is converted to citrulline and ammonium by arginine-deiminase, and citrulline is subsequently transferred

by ornithinecarbamoyl transferase to ornithine and carbamoylphosphate. Finally, carbamylphosphate is split by carbamatkinase into carbon dioxide and ammonium. In this pathway two protons are consumed, thereby supporting the acid tolerance of the lactobacilli (De Angelis et al., 2002; Rollan, Lorca, & Font de Valdez, 2003). Ornithine is the precursor of the roasty popcorn-like crust flavour of 2-acetyl-1-pyrrolin. Kang (2005) created an isogenic mutant of *L. pontis* by deletion of *arcA*-gene, which lost its ability for ornithine production. With baking experiments it was clearly shown that the roasty flavour was less intensive and 2-acetyl-1-pyrroline was below detection level as in the breads produced with the aid of the wild-type. Most flavour compounds are already present in flour (Czerny & Schieberle, 2002) and their concentration is modified during fermentation. For example, the concentration of 3-methyl-butanal (malty) is increasing fourfold during sourdough fermentation from flour. Flavour compounds present in bread dough only slightly change their concentration compared to the resulting bread crumb (Hansen & Schieberle, 2005). In general, aldehydes produced by LAB and yeasts are often reduced to the corresponding, less flavour-active alcohols by yeast. On the other hand, interesting fruity flavours are produced by specific sourdough yeasts: methyl-propyl-ester (fruity) by *Dekkera* sp. (Czerny, Schieberle, Brandt, & Hammes, 2003) or 2-methoxy-4-vinylphenol(clove-like) by *Pichia fermentans* (Opperer, Brandt, & Schieberle, 2012). Another group with a large influence on the overall flavour impression is derived from the degradation of lipids. This is often initiated by lipoxygenase from plants or autooxidation. Characteristic compounds of bread flavour are (E)-2-nonenal (cucumber), (E,E)-2,4, nonadienal (fatty-soapy) and (E,E)-2,4-decadienal(fatty, deep-fried). The concentration of lipid oxidation products strongly decreased during wheat sourdough fermentation with *L. sanfranciscensis and Candida milleri* (Czerny & Schieberle, 2002). In particular, *L. sanfranciscensis* is able to reduce (E)-2-nonenal and (E,E)-decadienal to the less flavour-intensive corresponding alcohols (Vermeulen, Czerny, Gänzle, Schieberle, & Vogel, 2007).

17.4 Developments in use of starter cultures

The most traditional technique in use for dough fermentation is backslopping. It is the inoculation of the raw material with a small amount of dough from a previous successful fermentation. Sometimes, simple starter preparations are created by addition of further flour, resulting in a reduced physiological activity of the microorganisms. These preparations can be stored for a short time, i.e. days or weeks. The only cereal fermentation where commercial starter preparations are applied is sourdough. Natural pure cultures ("Reinzucht" starters) are derived from continuously propagated fermentations; they are sold after thickening of the sourdough. Starter cultures aiming at leavening by yeasts are often sold as liquid doughs. Defined cultures are pure cultures that are propagated in microbial media and subsequently frozen or freeze dried (Vogel, Hammes, Habermeyer, Engel, & Eisenbrand, 2011). There are several criteria by which cereal starter cultures can be classified. One is the kind of substrate used for propagation. Defined starter cultures are usually grown on microbial media, and baker's yeast is usually (with the exception of some "organic" yeasts) propagated

on molasses supplemented with ammonium. On the other hand, "Reinzucht"-type starters are propagated on a cereal substrate. This type of culture has a shelf life of around 4–8 weeks, whereas frozen or dried cultures are stable up to 2 years. In most areas of the world, either parts of a previous fermentation are used as starters for cereal fermentations or spontaneous fermentation is applied. In industrialized countries, due to improved reproducibility of fermentation and resulting bread quality, mainly commercial starter cultures are in use. While it is necessary for a starter culture to contain active microorganisms, a stable sourdough product with a long shelf life can only be achieved if lactic acid bacteria and yeasts are brought to a physiologically inactive state. This is performed by drying or pasteurization of the sourdough, or by autosterilization of the dough over time (Brandt, 2007). Such convenient products are used in direct dough processing for flavour enhancement or regulation of dough acidity.

17.4.1 Frozen dough

Fresh breads are products with a short shelf life, due to staling and aroma losses over storage. One way to solve this is the continuous baking of fresh bread. The processing of bread always needs at least 3 h time, if the baked goods are leavened biologically. In the late 1980s, the commercialization of interrupted baking by freezing, either the unfermented or the fermented dough pieces, began. It allows dividing the production process into a part for professionals (mixing and fermentation) and the baking step, directly in front of the eyes of the consumer with always a fresh taste.

This market is growing, and in Germany 451,000 tons of frozen breads and rolls were produced in 2010 (Tiefkühlinstitut). There is one major challenge in the development of such frozen doughs, and that is maintaining the gassing power of yeast during frozen storage. In addition, lysates from dead yeast cells contain reducing substances, e.g. gluthathione, which affects the rheology of wheat doughs negatively (Ribotta, Leon, & Anon, 2003). Several studies suggest the use of *Torulaspora* yeast instead of *Saccharomyces*, due to their improved osmotolerance (Rosell & Gomez, 2007), but until now, these yeasts have not been dominant on the market. Baker's yeast producers try to improve the freezing tolerance of the yeast *S. cerevisiae* by modifying fermentation conditions leading to accumulation of proline (Sasano et al., 2012; Takagi, 2008) or trehalose (Gelinas, Fiset, LeDuy, & Goulet, 1989).

17.4.2 Gluten-free sourdoughs

The ingestion of gluten and related proteins causes an immune-mediated enteropathy known as sprue or coeliac disease in genetically susceptible individuals. Epidemiological studies have shown that 1 in 100 people worldwide suffer from this disease. A huge prevalence of coeliac disease was reported for the Sahawari poeple (5.6%; Catassi & Yachha, 2009). The only efficient therapy is the avoidance of the gluten-containing cereals such as wheat, rye, spelt and barley in all foods. Oat can be tolerated by most, but not all, people intolerant to gluten. Therefore, oat may be allowed on a national level, if it is not contaminated with wheat, rye or barley (Codex Alimentarius). Generally, food containing less than 20 mg/kg of gluten can be labelled as "gluten-free".

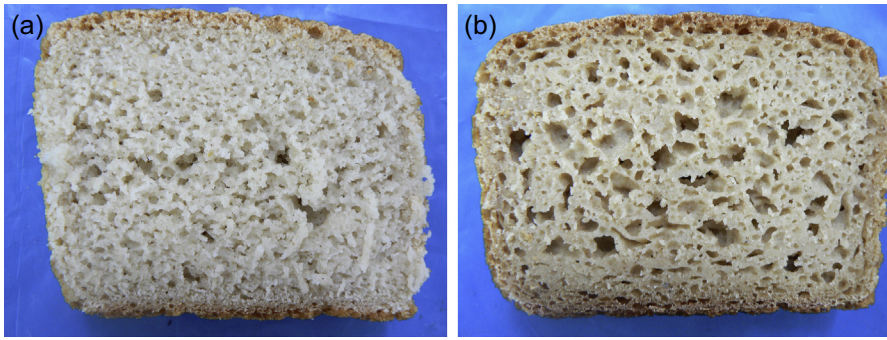

Figure 17.2 Structure differences in gluten-free rice breads. (a) With 10% buckwheat flour. (b) With 10% dried buckwheat sourdough. Other recipe ingredients are the same: 90% rice flour, 2% salt, 3% baker's yeast, 1% hydroxypropylmethylcellulose, dough hydration 100%.

As the structure-giving gluten is missing in gluten-free doughs, it has to be replaced by hydrocolloids or other water-binding compounds, e.g. guar gum, xanthan or hydroxypropylmethylcellulose. Usually, a gluten-free bread recipe contains higher amounts of starch and is processed with higher amounts of water compared to wheat and rye doughs, resulting in a batter-like consistency. As a consequence, in general the nutritive value of these breads is not well balanced. They are rich in carbohydrates and lack of dietary fibre (Hager, Axel, & Arendt, 2011). Over the last years gluten-free breads have improved significantly, and sourdough plays an important role, especially for flavour, taste and texture (Moroni & Arendt, 2009). Sourdough fermentation can be performed with cereals and pseudocereals used in gluten-free bread baking, e.g. rice, millet, sorghum, amaranth, quinoa or buckwheat. Due to the higher water content, gluten-free breads are subjected to faster microbial spoilage than wheat breads, but this may be minimized by application of sourdough fermentation. Sourdough has a positive influence on the crumb (Schober, Bean, & Boyle, 2007), which, in contrast to wheat, is not limited to fresh sourdoughs. Even the application of dried sourdoughs, especially in rice breads, results in a more coherent structure with a bigger pore-size, as shown in Figure 17.2. In the same bread recipe, buckwheat was used as flour and as a fermented and dried sourdough. In general, the advantages of using sourdoughs in bread processing are even more evident in gluten-free bread processing than in wheat breads.

17.5 Quality and safety issues

17.5.1 Antifungals

Mould spoilage is the main factor determining the microbial shelf life of breads and other baked goods. The main spoilage organisms are *Penicillium roquefortii, Eurotium* sp., *Paecilomyces variotii*, *Aspergillus niger*, other *Penicillium* species (Lund, Filtenborg, Westall, & Frisvad, 1996) and yeasts such as *Pichia anomala* or

Saccharomycopsis fibuligera (Legan & Voysey, 1991). Breads prepared with the aid of sourdough fermentation mould later than breads without, even if they were chemically acidified to the same level (Barber, Ortola, & Spicher, 1990). The main antifungal effect is the production of acetic acid by heterofermentative lactobacilli. Moreover, during the last years, efforts have been made to identify antifungal metabolites from sourdough bacteria. The widely used preservative propionic acid can be produced by *L. reuteri*; *L. diolivorans* is able to convert 1,2 propandiol to propionic acid; and *L. buchneri* produces 1,2 propandiol from lactate (Krooneman et al., 2002; Oude Elferink, Krooneman, Gottschal, & Faber, 2001). With a co-fermentation *of L. buchneri* and *L. diolivorans* in sourdough, not only was propionic acid production demonstrated but also sourdoughs were produced where the concentration of acetic acid exceeded that of lactic acid (Zhang, Brandt, Schwab, & Gänzle, 2010). Further antifungal compounds may be produced in sourdough. For example, phenyllactate and 4-hydroxyphenyllactate act against a broad range of moulds and are produced from phenylalanine and tyrosine (Lavermicocca et al., 2000; Lavermicoccca, Valerio, & Visconti, 2003). Still, the amounts produced during sourdough fermentation are not enough for a convincing effect (Vermeulen, Gänzle, & Vogel, 2006). The same is true for cyclic dipeptides (Schnürer & Magnusson, 2005). They are produced during baking at high temperatures up to 100-fold more than by fermentation (Ryan, Dal Bello, Arendt, & Köhler, 2009). A promising tool for enhancement of shelf life of breads is the conversion of linolenic acid to monohydroxy-octadecanoic acid by *Lactobacillus hammesii* (Black, Zannini, Curtis, & Gänzle, 2013), which acts as antifungal compound.

17.5.2 Mycotoxin decontamination

Whereas antifungal compounds produced during fermentation may reduce the spoilage of resulting breads and the spoilage is usually clearly visible, mycotoxins are the main safety aspect of bread production and cannot be recognized by the consumer. The main mycotoxins playing a role in dough fermentations are aflatoxins, deoxynivalenol, fumonisins, ochratoxins and zearalenone. In principle, decontamination of food caused by mycotoxins is possible by (1) prevention of contamination, (2) decontamination of mycotoxin-containing food and (3) inhibition or reduction of mycotoxin absorption of consumed food into the digestive tract (Halasz, Laszity, Abonyi, & Bata, 2009). In dough fermentation, only the decontamination and biodegradation of mycotoxins are applicable, but are presently not being used systematically. Several studies report the binding (adsorption) of mycotoxins to yeast and lactobacilli (Dalie, Deschamps, & Richard-Forget, 2010; Piotrowska & Zakowska, 2005; Shetty, Hald, & Jespersen, 2007). In this case, the mycotoxins remain in the food and are not relevant to dough fermentations. Reports on the fate of deoxynivalenol during fermentation and baking differ (Abbas, Mirocha, Pawlowsky, & Pusch, 1985; Lancova et al., 2008; Neira, Pacin, Molto, & Resnik, 1997); in general, its concentration seems more or less independent of fermentative microorganisms in the dough. Ochratoxin A is reduced during dough proof at about 30% by baker's yeast (Valle-Algarraet al., 2009) and significantly by lactobacilli in a type II sourdough fermentation. More details on mycotoxin degradation are presented in Chapter 14.

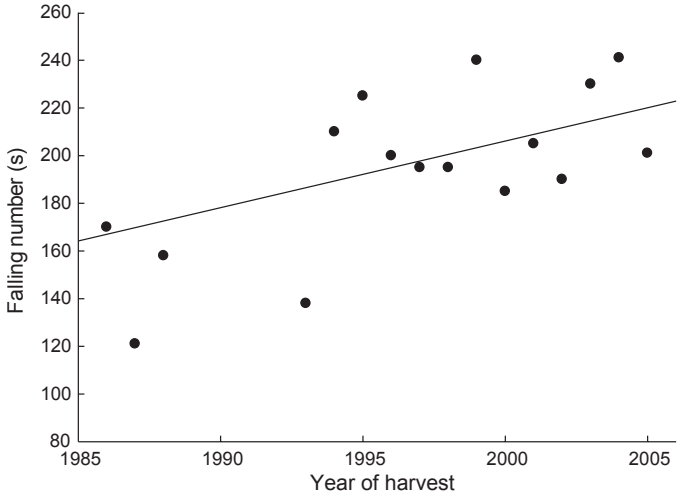

Figure 17.3 Development of average falling numbers, as indicator for amylase activity, of rye during two decades in Germany.

17.5.3 Shifts in the quality of cereal flours

Another important factor mainly influencing the quality of fermented doughs and the resulting baked goods is the quality of cereal raw materials. From a technological point of view, nowadays it is not necessary to use acidification in rye processing in order to reduce the activity of amylases during dough baking (starch gelatinization). Success in breeding has lead to strongly reduced sprouting on the field in the rye plant. Figure 17.3 shows the development of falling numbers after harvest in Germany during the last two decades. The falling number is the time needed for a stirrer to fall through a gelatinized flour sample and is therefore an easily applicable parameter for the activity of α-amylase. Low values are characteristic for sprouting on the field. Thus sourdough is not necessary at present for rye baking, but the quality of unacidified rye breads is unsatisfactory, especially in crumb elasticity (Brandt, Rabe, & Brümmer, 2007).

17.6 Health benefits

In his *Natural History* (1826), Pliny the Elder stated that "those persons who are dieted upon fermented bread are stronger in body", which is an early statement on the health benefits of sourdough breads. Whole meals of cereals are a good source of minerals. Their bioavailability is limited, as they are often bound to phytic acid (myo-inositol-hexophospate). Wheat and rye contain about 1.2% of dry matter (Fretzdorff & Brümmer, 1992). With the drop in pH during sourdough fermentation, endogenous phytases of the cereals are activated and the bound minerals are released (Fretzdorff & Brümmer, 1992). Lactic acid bacteria and yeasts also possess phytase

activity, but their role in phytate degradation is negligible compared to endogenous activity (Türk, Sandberg, Carlsson, & Andlid, 2000; Reale, Konietzny, Coppola, Sorrentino, & Greiner, 2004). The status of several vitamins changes during fermentation. Folates increase due to synthesis by yeasts. Although consumed by lactic acid bacteria during fermentation, taken together with yeasts this has no impact (Jägerstad et al., 2005; Kariluoto et al., 2006). Nicotinamid increases 10-fold during rye sourdough fermentation (Mihhalevski, Nisamedtinov, Hälvin, Oseka, & Palme, 2013). Strains of *L. reuteri* are able to produce cobalamin (Taranto, Vera, Hugenholtz, de Valdez, & Sesma, 2003), which is detectable up to 0.2 µg/100 g in dried type II sourdoughs, if such strains were used.

Easily digestible carbohydrates such as sugar and starch play a significant role in the development of type II diabetes and obesity. As an indicator of the digestibility of carbohydrates, the concept of the glycemic index was introduced. It characterizes the increase of blood glucose after digestion of a meal. It has been shown that sourdough fermentation lowers the glycemic index of several bread varieties (Liljeberg & Björck, 1996; Liljeberg, Lönner, & Björck, 1995). The mechanism is unclear, but the formation of organic acids, especially lactic acid, plays a significant role. Neither the rate of starch hydrolysis during fermentation nor the presence of fibres influence the glycemic load (Scazzina, Del Rio, Pellegrini, & Brighenti, 2009).

17.6.1 Gut health

The gut microbiota play an important role in nutrient metabolism and contribute to fitness of the immune system. Sourdough modulates the dietary fibre complex and its subsequent fermentation patterns (Poutanen, Flander, & Katina, 2009). In addition, several sourdough lactic acid bacteria produce exopolysaccharides such as glucans or fructans. Levan produced by *L. sanfranciscensis* possesses prebiotic properties (dal Bello, Walter, Hertel, & Hammes, 2001). Cell-wall components of lactobacilli show immunomodulatory effects (Wells, 2011), thereby indicating that for some effects it is not necessary that lactobacilli reach the intestine alive (Adams, 2010; Chuang et al., 2007). Thus, sourdough breads have a good potential for promoting gut health. Research on the immunomodulatory effects of dead lactobacilli in sourdough bread has started, but results have not been published yet.

It was shown by Di Cagno et al. (2002) that coeliac-toxic peptides can be degraded by proteolytic sourdough lactobacilli. Nevertheless, if coeliac peptides are degraded, the technological function of the gluten proteins is also degraded. Thus, this approach should only be used to decrease the risk of gluten contamination in production of gluten-free products (De Angelis et al., 2006). Initial gluten content in a gluten-free recipe with 400 ppm was reduced to 20 ppm. Although it is possible to produce wheat and rye sourdough with a gluten content of <20 ppm, it is not very well accepted by patients suffering from coeliac disease.

In addition to coeliac disease and wheat allergy, a gluten sensitivity also exists where gluten ingestion has negative effects on the body, these being neither related to an allergy nor to an autoimmune sickness. Gluten sensitivity has a prevalence of 5–10% in the Western population, and the therapy is also the abstinence of gluten. One

of the triggers of that disease was recently identified by Junker et al. (2012) as amylase-trypsin-inhibitors (ATIs) in wheat. They regulate the activity of amylases in the growing plant. Amylase inhibitors may be degraded by specific sourdough organisms, for example by *C. milleri* (Brandt, 2001).

17.7 Future trends

The increasing number of people who renounce eating wheat will lead to further intensity of research and development of "alternative" cereals and pseudocereals for bread making. Cereals as such are an important staple food in all parts of the world, and their impact on health will be studied in detail with the help of metabolomics. The application of these methods will also allow a more detailed understanding of the natural habitats and the relating physiology of sourdough organisms. Sourdough underwent a renaissance during the last two decades, and is now used not only in countries with a sourdough tradition but also in areas where long-time dough fermentations are unusual. This might be interrelated with an exchange of bakery recipes all over the world; on the other hand, the application of sourdough may enable the omission of some baking additives such as acidifiers, preservatives or reducing agents.

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Quality, safety, biofunctionality and fermentation control in soya

18

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18.1 Introduction

Soya beans (*Glycine max* (L.)) are the most important source of plant-derived dietary protein, oil and biofunctional components. In various publications, the spelling differs from soybean, soya bean, soya, soy, etc. In this chapter, we will keep to soya, except when specific mention is made of the beans to distinguish them from other parts of the plant.

Soya is well known as a source of human and animal nutrition (American Soybean Association, 2012a), as well as a raw material for industrial purposes. A range of commercial products is prepared from soya as the starting ingredient (American Soybean Association, 2012b). In this chapter, we will limit the focus to the significance of soya to the human diet and health.

For food uses, soya derivatives are used as ingredients in a wide range of consumer foods. Examples are soya oil, lecithin, soya flour in bakery products, soya protein isolates, etc. This chapter will not deal with these ingredients, but rather focus on food products made by fermentation processes.

The region of origin of soya fermentation is Asia, and in particular Southeast Asia and East Asia. It is not only the widespread use of soya that makes this region unique, but also the use of filamentous fungi in food preparation. Whereas in many other cultures, filamentous fungi (moulds) are associated with spoilage and even toxicity – caused by mycotoxins – the Asian household has discovered the capability of certain filamentous fungi to produce flavour and texture that form the basis of nutritious and savoury foods and condiments. In this chapter we will discover some of these wonderful products.

18.2 Fermented soya products

A range of fermented soya food products is known (Chen et al., 2012), and Table 18.1 illustrates some important representatives that will be discussed in this chapter. There are several ways to distinguish fermented soya products, for example, by their consistency, their salt content or the type of microorganisms used for their fermentation. In Table 18.1, the products are listed according to consistency: sauces are liquid, pastes are semi-solid and another group comprises solid or firm food products. The salt content depends very much on the manufacturing process. Salt has been used traditionally

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00018-9

Table 18.1 Overview of some major fermented soya foods and condiments

Group	Name	Origin	Salt added	Reference
Sauces	Shoyu	Japan	Yes	Aidoo et al. (2006)
	Kecap	Indonesia	Yes	Röling et al. (1996)
Pastes	Miso	Japan	Yes	Li and Ma (2003), Kim, Hong et al. (2010)
	Doujiang	China	Yes	Lim (1991), Kim, Hong et al. (2010)
	Doenjang	Korea	Yes	Kim et al. (2009)
	Chongkukjang	Korea	Yes	Nout et al. (2014)
Solid-fermented soya foods	Natto	Japan	No	Murooka and Yamshita (2008)
	Kinema	Nepal and India	No	Sarkar et al. (1994)
	Tempe	Indonesia	No	Nout and Kiers (2005)
	Sufu	China	Yes	Han, Rombouts et al. (2001)
	Douchi	China	Yes	Zhang et al. (2007)

for preservation purposes and, of course, for taste. Salt continues to play an important role in the process because some maturation steps take several weeks or months at ambient temperatures and the product needs to be protected against pathogenic or toxin-producing spoilage microorganisms. Salt also facilitates some enzymic conversions that enhance digestibility and flavour development.

18.2.1 Soya sauce

Soya sauces are found all over South and East Asia, and local differences exist in process conditions and ingredients used, catering for the preferences and expectations of the consumer (Chen et al., 2012). Japanese soya sauces have been exported all over the world, and their production was industrialized and mechanized more than 50 years ago. Soya sauces are used for cooking, for marinades and as a savoury ingredient. In principle, Japanese soya sauce or shoyu is made from soya beans, wheat, water, salt and microorganisms. The essential steps in the process are outlined

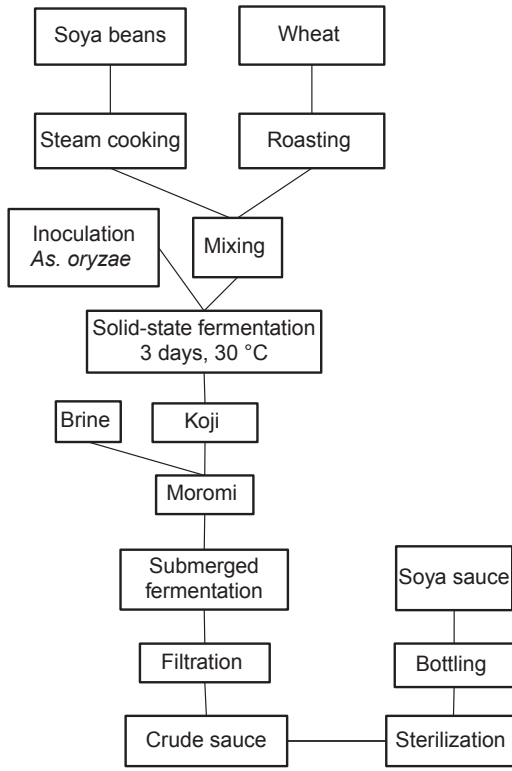

Figure 18.1 Soya sauce production process. A mixture of cooked soya beans and crushed roasted wheat is inoculated with *Aspergillus oryzae* and incubated to obtain koji. Koji is aged in brine to obtain crude soya sauce, which is filtered and bottled.

in Figure 18.1. Soya beans are cooked to facilitate their degradation by enzymes during later stages. The cooking can be done at ambient atmospheric pressure, but pressure cookers are used at industrial scale. Soya protein is denatured, moisture content is increased, and some undesirable components are inactivated. Major undesirable components of raw soya beans include the trypsin inhibitor, which is a protein that blocks soya digestion in the human gastrointestinal tract, and lipoxygenase, an enzyme that can generate bitter-tasting lipid derivatives during soaking of soya beans in water. Cooking thus improves digestibility and helps to avoid generating off-taste.

The objective is to allow a solid-state fermentation (SSF) of the cooked soya beans with filamentous fungi. However, the moisture content of the cooked soya beans is rather high for fungal SSF, and soya beans also lack fermentable carbohydrate that could serve as a source of carbon for fungi.

For that reason, the Japanese process includes wheat as an ingredient (Fukushima, 1989). Wheat kernels are roasted by hot air treatment, and coarsely milled to gritty particles. The roasting causes the wheat protein and starch to be accessible for enzymic degradation. The wheat grits are mixed with the cooked soya beans to achieve the optimum water activity for fungal SSF, and the mixture is inoculated with tane

koji, a traditional powdered starter consisting of conidia of selected *Aspergillus oryzae* strains. *Aspergillus oryzae* is selected for vigorous growth and enzyme production, mainly proteases, carbohydrases, phosphatases and other enzymes. Inoculation rates usually are 10^6 propagules per gram of soya–wheat mixture. After inoculation, incubation takes place at 30 °C for 3 days, during which period the fungal mycelium grows and a start of sporulation becomes visible. When the moulded mixture turns yellowish-green because of the pigmented fungal conidia, the SSF has resulted in koji, a mass of partially degraded soya and wheat containing active hydrolytic enzymes.

The next step in the process is a submerged fermentation of koji particles in brine, a phase that is referred to as moromi. The brine has a salt concentration of about 20%, and the ratio of koji to brine is approximately 1:8. In the moromi fermentation, which can take 6–12 months at temperatures around 15 °C, the solid soya and wheat particles are gradually broken down to water-soluble molecules such as amino acids, free fatty acids, saccharides, etc. As a result of the availability of easily assimilable carbon and nitrogen, opportunistic bacteria and yeasts will slowly develop. These are halotolerant microorganisms, so a rather specific mixed microbiota consisting of *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* usually predominates. Since these improve the taste by the formation of organic acids, esters and other flavour compounds, they are nowadays added to the moromi to ensure a constant and predictable fermentation outcome.

After the moromi fermentation, the raw sauce is obtained by filtration; the filtered sauce is pasteurized and bottled. Table 18.2 illustrates the chemical composition of soya sauce (Fukushima, 1989). It should be noted that of all nitrogen present, approximately half is present as amino nitrogen. This implies that the soya protein has been broken down to a very large extent into low-molecular-mass water-soluble compounds.

18.2.2 Soya pastes

Soya pastes have a semi-solid consistency, because they have been ground to a smooth paste. These pastes are used as a side-dish, or as soup ingredients to provide a savoury taste.

Table 18.2 Chemical composition of soya sauce

	Content (g/100 ml)
Water	70
Protein	7.4
Total nitrogen	1.7
Amino nitrogen	0.83
Crude lipid	0.6
Carbohydrate	7.2
Ash	18.4
NaCl	16.8
Sodium	0.5
Alcohol	2.3
pH	4.7

Source: Data from Fukushima (1989) and Nout, Lee, and Han (2014).

18.2.2.1 Miso

Miso is a paste made from moulded rice, cooked soya beans and salt (Shurtleff & Aoyagi, 2001a; Wood, 1982). The principle of the manufacturing process is shown in Figure 18.2. A koji is prepared almost similar to the koji for soya sauce, using selected strains of *As. oryzae*. After mixing three parts koji, salt, and five parts steamed rice, the mixture is homogenized, packed and fermented for several months depending on consumers' demand. Some amount of previous miso is mixed with the mash and packed in a container for fermentation. Instead of adding previous miso, pure cultures of the yeast *Z. rouxii* can be added. Table 18.3 illustrates the major chemical constituents of miso. Miso is a good source of protein, minerals and B-vitamins.

18.2.2.2 Doujiang

Doujiang is a Chinese soya bean paste containing semi-solid and partially intact soybeans, made from soya beans, wheat, salt, potable water and naturally occurring/cultivated microorganisms, sometimes with additional ingredients such as grains and/or flour, *Lactobacillus* sp., distilled alcohol derived from agricultural products, sugars,

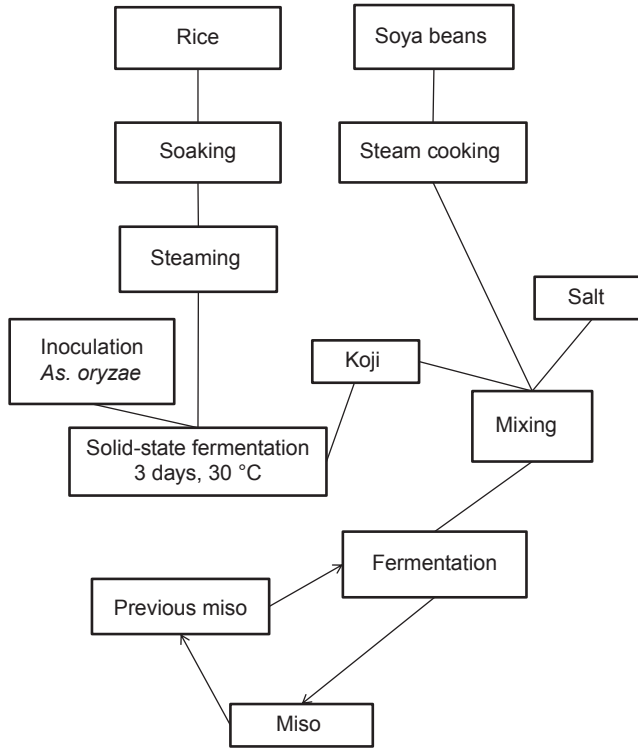

Figure 18.2 Miso process. Steamed rice is fermented with *Aspergillus oryzae* to obtain koji, which is mixed with cooked soya beans and salt for fermentation to obtain miso.

Table 18.3 Constituents of miso

Water (g/100 g)	41.5
Crude protein (g/100 g)	11.8
Crude lipid (g/100 g)	6.1
Carbohydrates (g/100 g)	28.0
Crude fibre (g/100 g)	2.5
Energy (kJ/100 g)	865
Ca (mg/100 g)	66
Fe (mg/100 g)	2.74
Na (mg/100 g)	3647
Zn (mg/100 g)	3.32
Thiamin (mg/100 g)	0.1
Riboflavin (mg/100 g)	0.25
Niacin (mg/100 g)	0.86
Vitamin B_6 (mg/100 g)	0.22
Folacin (mg/100 g)	0.033

Source: Data from Wood (1982) and Chen et al. (2012).

starch syrup and naturally flavoured raw materials (Kim, Lee, Park, & Kim, 2010; Zhao et al., 2009). Raw soya beans are soaked, and steam cooked at 121 °C for 20 min. Wheat flour and cooked soya beans are mixed at a ratio of 2:3, cooled to 40 °C, and inoculated with *As. oryzae* starter, and allowed to ferment for 4 to 7 days. When fungal conidia appear, the mixture is mashed and immersed into brine, which is agitated weekly. The submerged fermentation can take up to 12 weeks (Zhao et al., 2009).

Doujiang is fermented by naturally occurring or cultivated microorganisms (Kim, Lee, et al., 2010), including *Bacillus amyloliquefaciens*, *Bacillus megaterium*, *Lactobacillus fermentum* and *Lactobacillus plantarum*, and yeasts such as *Candida humilis*, *Kluyveromyces lactis*, *Williopsis saturnus* and *Z. rouxii*. In industry the natural fermentation can be simulated by using a mixed starter of *Z. rouxii* and *L. plantarum;* these starter cultures obviously do not fully represent the microbial community in the traditional production (Zhao et al., 2009).

Japanese- and Chinese-fermented soya bean pastes differ by their dominant microbiota. Whereas *As. oryzae* and *Z. rouxii* are major fungi present in most of the Chinese and Japanese fermented soybean pastes (Kim, Lee, et al., 2010), the bacterial microbiota of Japanese paste contain *T. halophilus* and *Staphylococcus gallinarum* but the Chinese paste is dominated by *Bacillus* spp.

18.2.2.3 Doenjang

Doenjang is a soya bean paste originating in Korea. A starter called meju is obtained by SSF with *Bacillus subtilis* and *As. oryzae* for 1–2 months at 20–30 °C (Kim, Hong, Song, Shin, & Kim, 2010; Kim et al., 2009). Whereas this may suggest a pure-culture fermentation, the actual microflora is quite complex (Kim et al., 2009; Nam, Lee, & Lim, et al., 2012) and differs between traditional and industrial doenjang. This meju

undergoes a submerged maturation in brine with 20–25% salt content. At this salinity, microbial activity will be low but the enzymes formed in meju will be able to degrade the meju mass. After several months, a liquid stands on top of a sediment. The latter is mixed again with cooked rice and a small amount of meju and mixed. This mixture is ripened in jars for a few weeks. It may be safely assumed that part of the rice will be assimilated by the microorganisms from meju, and that the meju residue will contribute protein fractions to form the umami taste (Rhyu & Kim, 2011) of the paste.

18.2.3 Solid fermented soya foods

18.2.3.1 Natto and kinema

Natto and kinema are discussed in combination because they are made by similar processes (Sarkar, Tamang, Cook, & Owens, 1994; Wei & Chang, 2004). Figure 18.3 outlines the essential steps in making natto and kinema. We can distinguish four phases: preparation, inoculation, incubation and finishing. The preparation for natto involves soaking whole soya beans at 21–23 °C for 20 h, draining, steaming at 121 °C for 40 min and cooling to about 50 °C for inoculation (Wei & Chang, 2004). For kinema, whole raw soya beans are soaked in water overnight at an ambient temperature of about 25 °C, and then cooked until easily crushed (Sarkar et al., 1994) and pounded to coarse particles while hot.

The inoculation of natto is done with a pure culture of spores of *B. subtilis natto* that will germinate very well at 50 °C. For kinema, no microbial starter is added, as this is a natural fermentation allowing bacterial spores that survived the cooking process to germinate. This can be stimulated by sprinkling some wood-ash on the pounded soya beans.

In both types of fermentations, the beans are covered and kept at temperatures around 50 °C for 1–3 days. During this incubation period, bacilli multiply to about 10^9–10^{10} colony-forming units (cfu) per gram. In the natto process, the fermentation takes place in the packaging tray that will be sold off the supermarket shelves. Kinema is brought to the market in bulk, where it is measured out according to customer's demand and packed in plant leaves.

Microbiota predominating the kinema fermentation were studied. The functional microorganisms are *Bacillus* species, *B. subtilis*, *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus circulans*, *Bacillus thuringiensis* and *Bacillus sphaericus* (Sarkar, Hasenack, & Nout, 2002). In addition, some bacteria are found frequently that have their origin in post-contamination after cooking. These include *Enterococcus faecium* and yeasts such as *Candida parapsilosis* and *Geotrichum candidum* (Sarkar et al., 1994).

18.2.3.2 Tempe

Tempe (in literature also spelled as "tempeh") is a sliceable, cake-like product made of dehulled cooked soya beans, penetrated and fermented by a mixed microbiota dominated by filamentous fungi (Nout & Kiers, 2005). Tempe is used as a side dish with rice as a staple. It can be prepared in many ways (Shurtleff & Aoyagi, 2001b).

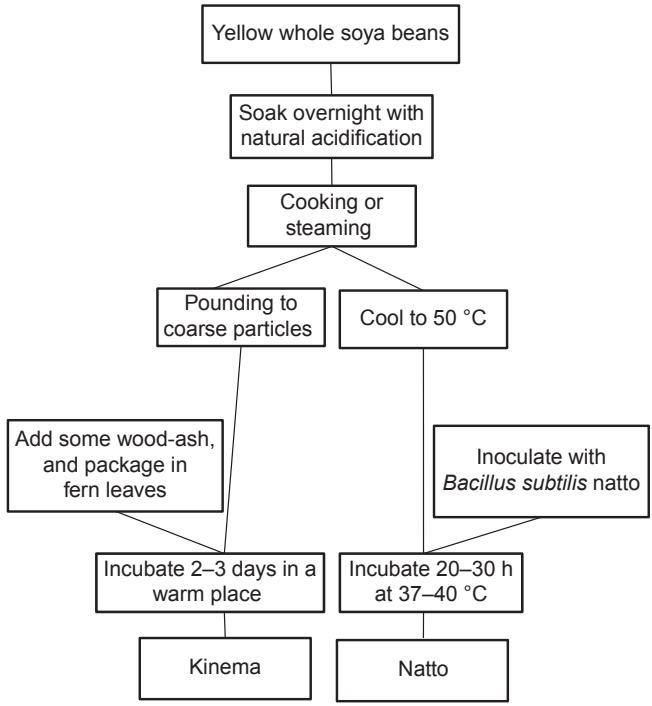

Figure 18.3 Natto and kinema processes. Natto and kinema processes are similar in principle, with the difference that natto is obtained with a pure-culture fermentation whereas kinema undergoes a natural fermentation with a mixed microflora.

Figure 18.4 shows the principles of the tempe-making process. Whole raw soya beans are soaked in water overnight at an ambient temperature of 28 °C, after which they are dehulled. The dehulled split beans (cotyledons) are cooked in water for 30–40 min, after which the cooked water is drained and the cotyledons are spread out to cool. During the cooling, the adhering water evaporates, enhancing the cooling and drying the cotyledons surface. At about 30 °C the cotyledons are inoculated with 10^6 propagules per gram of sporangiospores of filamentous fungi, mostly *Rhizopus microsporus* (Rombouts & Nout, 1989; Thanh & Nout, 2004). These can be added as pure culture, but in many small-scale production units a traditional starter called "usar" is used. Usar contains a mixed microbiota of filamentous fungi and other microrganisms (Nout, Martoyuwono, Bonné, & Odamtten, 1992). The incubation takes place in packages that offer limited access to atmospheric oxygen (De Reu, Rombouts, & Nout, 1995; Han & Nout, 2000), at 30–40 °C during 1–2 days (the higher the temperature, the shorter the incubation) until the fungal mycelium is dense and binds the cotyledons firmly together into a solid mass. The fresh tempe is cut and sold fresh in Indonesia. In other parts of the world, the fermentation takes place in perforated packages corresponding to the weight or size units for the consumer market (Ko & Hesseltine, 1979; Nout & Rombouts, 1990). During the fermentation, fungal metabolism causes changes

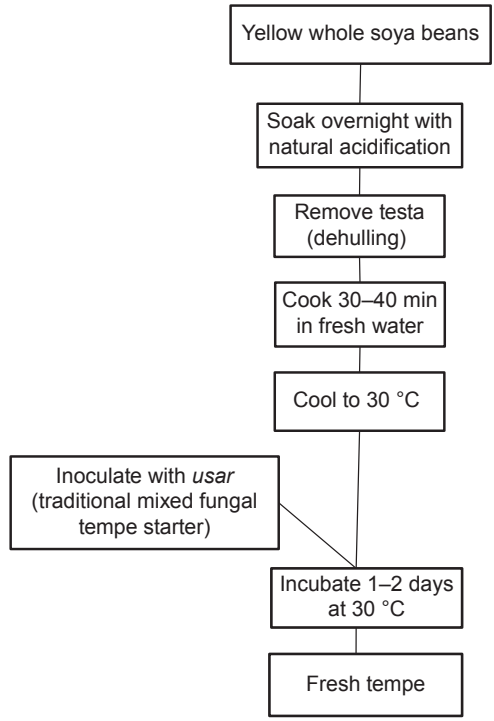

Figure 18.4 Tempe process. Tempe is made in two fermentation stages. The first stage is a natural acidification taking place during overnight soaking. The second stage is a mixed fungal–bacterial solid-state fermentation of the cooked soya beans.

in the composition of tempe, as shown in Table 18.4. Due to protein degradation, water-soluble nitrogenous substances (peptides, amino acids) are increased. Likewise, the lipid degradation results in decreased crude lipid and concomitant increase of free fatty acids. Such enzymic degradations generally result in increased amounts of low-molecular-mass water-soluble solids. The softening of the soya beans in tempe (De Reu, Linssen, Rombouts, & Nout, 1997) is in accordance with decreased hemicellulose levels. On the other hand, dietary fibre levels are increased due to the formation of fungal mycelial polysaccharides including cellulose and chitin.

18.2.3.3 Sufu and tofu

Sufu is a product from China, but is hardly ever known by that name (Han, Rombouts, & Nout, 2001). The Chinese call it "Fu-ru". The appellation "sufu" was used in the 1960s in international scientific literature and since then this name has persisted. We will therefore continue the tradition.

Sufu could be described as fungal fermented and matured tofu. Sufu is a popular breakfast ingredient in China. It gives flavour and taste to the bland soya milk, vegetables and fried dough of breakfast. The process could be divided in stages: preparing

Table 18.4 Some chemical modifications during soya bean tempe fermentation

	Cooked beans before fermentation (g/100 g)*	Tempe fermented for 48 h (g/100 g)*
Water-soluble nitrogen	0.5	2.0
Crude lipids	22.5	14.1
Free fatty acids	0.5	21
Hemicellulose	2.0	1.1
Fibre	3.7	5.8†
Water-soluble solids	13	21

*Dry weight basis.
†Fibre content increases during fermentation due to the formation of fungal mycelium.
Source: From Ko and Hesseltine (1979).

tofu, preparing pehtze, brining of pehtze, mixing with several ingredients and maturing (Han, Rombouts, et al., 2001).

Tofu is a protein-rich coagulate made from soya milk. The coagulation is done by addition of salts such as Ca_2SO_4 (gypsum) to soya milk, and the coagulate is collected and pressed to obtain a firm consistency. Slabs of tofu are cut to cubes (dices), which are inoculated by spraying them with a conidiospore suspension of the mucoraceous fungus *Actinomucor repens*. The dices are placed on needles so that all sides are exposed to the air, and incubated at 15–20 °C for 5–15 days (Han, Rombouts, et al., 2001). The resulting product is called pehtze. In principle, pehtze could be compared with koji, because it contains a range of fungal enzymes of *Ac. repens* that can modify the product during a later stage. Pehtze is salted with solid salt or brine to add preservation and to enhance enzyme activity. The cubes of brined pehtze are transferred to glass jars, which are topped up with maturation mix containing spices, red kojic rice (a biocolorant), sometimes rice wine and other ingredients. In the jars, the product will gradually mature: its composition will equilibrate; protein, lipid and carbohydrate fractions will be modified (Han, Ma, Rombouts, & Nout, 2003; Han, Rombouts, & Nout, 2004) to result in a product with a strong pungent smell and a delicious flavour.

18.2.3.4 Douchi

Douchi is a solid fermented soya bean product, consisting of individual black beans. These are used in soups, sauces, etc., as a savoury ingredient. Several types of douchi can be distinguished according to the microorganisms dominating the fermentation, i.e. bacteria or filamentous fungi. The principle of the douchi manufacturing process is shown in Figure 18.5. Soya beans are washed, soaked 3–4 h, steamed for about 50 min and cooled to 30 °C. The beans are inoculated with *Aspergillus egypticus* (Zhang, Tatsumi, Fan, & Li, 2007) and kept at 30 °C for 3–4 days to form koji (called "qu" in Chinese). The koji is then washed with water and mixed with 16% salt, 10% water, ginger and various mixed powdered spices, and stored in closed jars for several weeks at 35 °C. Although this process seems similar to miso making, douchi is not mashed (the product consists of whole beans) and its moisture content is lower than in Miso.

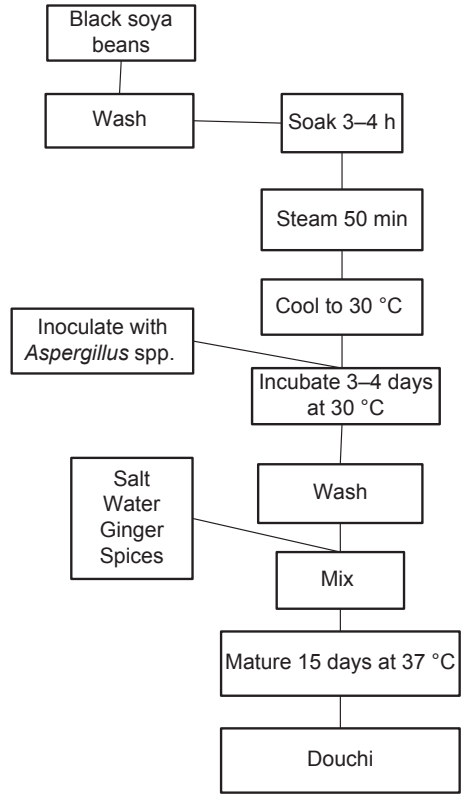

Figure 18.5 Douchi process. Black soya beans are washed, soaked, steamed and inoculated with *Aspergillus* spp. after cooling. The beans undergo solid-state fermentation and are then washed and matured in a mixture of brine and spices.

The chemical composition of douchi is summarized in Table 18.5. During koji formation, crude lipid and reducing sugars decrease and acidity is doubled. During maturation, fungal enzymic activities (neutral protease and β-glucosidase) caused increased amino nitrogen and organic acid levels, and degradation of isoflavones (Zhang et al., 2007).

18.3 Quality and food safety aspects

18.3.1 Ecology of microbiota

Soya fermentation processes have a strong impact on the evolution of microbes in the product. This may be positive in the case of natural fermentations, but sometimes it may constitute a hazard. Knowledge of the parameters and microorganisms involved is useful in controlling optimum quality and consumer safety. Environmental factors (Nout, De Dreu, Zuurbier, & Bonants-Van Laarhoven, 1987) that impact

Table 18.5 Douchi: chemical modifications during the fermentation of koji and the maturation of douchi

	Koji (Qu)		
	0 h	**96 h**	**Douchi**
Crude protein (%)	36.0	39.6	
Crude fat (%)	23.8	19.9	
Reducing sugars (mg/g)	11.0	5.4	
Total acids (mg/g)	0.65	1.30	
Amino nitrogen (%)	0.15	0.40	1.7
Organic acids (acetic, lactic, L-pyroglutamic) (mg/g)	1.5	4.3	27.2

Source: Data from Zhang et al. (2007).

microbiological events are (1) soaking in water overnight at ambient temperature, (2) cooking, (3) brining or salting and (4) use of defined or natural mixed starter cultures.

1. Soaking causes a diffusion of low-molecular-mass molecules such as saccharides and soluble proteins into the soaking water. Under (sub)tropical conditions, these offer ideal conditions for the growth of bacteria, first rapid-growing *Enterobacteriaceae*, followed by slower-growing lactic acid bacteria (LAB) that will acidify the water and the soya beans (Yan, Wolkers-Rooijackers, Nout, & Han, 2013). This situation of natural fermentative acidification has been shown to be favourable for safety as it will prevent the establishment of spoilage and pathogenic bacteria. In colder climates where a natural acidification is consequently too slow, acidification could be enhanced by "back-slopping" (Nout, De Dreu, et al., 1987), i.e. addition of some fermented soakwater from a previous batch, containing a natural enrichment of active LAB (Yan et al., 2013).
2. Cooking at 100 °C for 20 min or longer will kill all vegetative microbial cells, but not bacterial endospores. On the other hand, if pressure cooking is applied at 121 °C for 20 min, most bacterial endospores will be killed as well. In both cases the cooked product will be susceptible to bacterial overgrowth if not protected by hermetic packaging, or by inoculating it with a sufficient concentration of starter microorganisms to out-compete unwanted overgrowth. If soya beans have been acidified prior to cooking, an additional "hurdle" (low pH) helps to prevent or delay overgrowth by acid-sensitive spoilage bacteria. During a cooking step at 100 °C, bacterial endospores will be activated to germinate as soon as the cooked beans have cooled down to about 50 °C, enabling them to grow rapidly and colonize the cooked beans (Nout, De Dreu, et al., 1987).
3. Brining or salting increases the NaCl concentration to levels that are inhibitory to most microorganisms (Han, Sesenna, Beumer, & Nout, 2005). Only halotolerant or halophilic bacteria or yeasts would have the opportunity to (slowly) grow and cause effects.
4. The type of starters used can vary from defined single-strain starters to traditional mixed starters. Defined single or mixed starters, such as used for natto and sufu, have been selected for their specific properties, and in order to draw appropriate profit from their addition, they need to be provided with optimum conditions for growth including absence of competing microbiota and the control of temperature, pH and water activity. Natural mixed microbiota

are cheaper; they have adapted to the prevailing environment and substrate and are therefore robust, but their composition and their performance are not predictable.

18.3.2 Fungal functions and hazards

The range of functional fungi is rather limited. Of the filamentous fungi, the family *Mucoraceae* is important because it contains genera such as *Mucor*, *Rhizopus* and *Actinomucor*, which are all involved in soya fermentations (Han, Kuijpers, Thanh, & Nout, 2004). The function of these moulds is to provide texture and formation of hydrolytic enzymes. Several species of *Rhizopus*, namely *Rhizopus oryzae*, *Rhizopus rhizopodiformis*, *R. microsporus*, *Rhizopus oligosporus* and *Rhizopus homothallicus*, were categorized as potentially pathogenic because of their ability to grow at 37 °C (Scholer & Müller, 1971). Therefore at industrial level, precautions should be taken to avoid inhalation of sporangiospores. This group of fungi is not known to produce mycotoxins; the mycotoxin "rhizonin" that was reported previously is not a fungal metabolite but it is produced by bacterial endosymbionts, such as *Burkholderia* sp. (Partida Martinez et al., 2007).

Of the Fungi Imperfecti, the genus *Aspergillus* plays an important role in soya koji. It was established that the most frequently used species *As. oryzae* does not produce mycotoxins and is safe for human consumption (Barbesgaard, Heldthansen, & Diderichsen, 1992). However, the fact that this fungus belongs to the same section (*Aspergillus* Flavi section) as *Apergillus flavus* and *Apergillus parasiticus*, both potent producers of aflatoxins, has caused much research into the safety of fungal strains. It was demonstrated that genotypes of *As. oryzae* used in koji making were not diverse, and had a considerably smaller genome than *As. flavus* (Wicklow, McAlpin, & Yeoh, 2007).

Of the Ascomycetes, the genus *Monascus* is important in the production of red kojic rice, also called "angkak". This product is used as a biocolourant for sufu, and more recently it is used as a health food-supplement. *Monascus* spp. are able to produce the mildly carcinogenic mycotoxin citrinin (Lee, Lee, & Pan, 2010; Mornar, Sertić, & Nigović, 2013). *Monascus*-fermented commercial soya products in Korea and China were analyzed for their citrinin content. In China (Liu, Wu, Su, Chung, & Yu, 2005), the cytotoxicity of the fermented products was considered a hazard requiring control, whereas in Korea (Kim, Ji, & Lee, 2007), most tested products had citrinin levels below the allowed level of 50 µg/kg. Selection of starter strains that produce only low levels of citrinin has been reported (Pattanagul, Pinthong, Phianmongkhol, & Tharatha, 2008), and their use would contribute to food safety.

Yeasts occur in fermented soya foods, but have not been reported in connection with hazardous situations.

18.3.3 Bacterial functions and hazards

Several genera of bacteria have positive effects on the quality of fermented soya foods. Lactic acid bacteria (LAB) are important for flavour as well as for protection against pathogenic contaminants. The acidification by LAB was shown to inhibit the growth

of *B. cereus* in tempe (Nout, Beernink, & Bonants-Van Laarhoven, 1987), whereas their potential to form bacteriocins may contribute to the protection against *Listeria* sp. *Klebsiella* spp. are found in, for example, kinema and soya soak water. This genus is known for its ability to produce vitamin B_{12}. The occurrence of *Bacillus* spp. in various products such as natto, kinema and douchi was mentioned. This genus is a strong producer of hydrolytic enzymes that enable digestibility of fermented soya foods. It was also shown to produce nattokinase, which may protect against thrombosis (Wang et al., 2006).

However, the occurrence of contaminations by *B. cereus* in fermented products dominated by *B. subtilis* could be a matter of concern. The ability of *B. cereus* to form toxins was evaluated in kinema, which is normally dominated by *B. subtilis*. It was demonstrated that *B. cereus* can produce hazardous levels of toxins when proliferating as a pure culture on soya beans. However, in the presence of a competing dominance of *B. subtilis*, such as in kinema, only insignificant amounts of toxin were formed (Nout, Bakshi, & Sarkar, 1998). In tempe, protein-degradation fractions inhibited the germination of *B. cereus* endospores (Roubos-van den Hil, Dalmas, Nout, & Abee, 2010).

A similar phenomenon was observed in sufu, contaminated on purpose with enterotoxin-producing *Staphylococcus aureus*. Although *S. aureus* was able to grow in the high salinity of sufu, it was not able to form its enterotoxins (Han et al., 2005).

Concluding, it may appear that under fermentation conditions dominated by strongly competing fermentation microorganisms, toxin-producing bacteria have only limited possibilities to produce their toxins. Nevertheless, fermented soya foods require control for chemical and microbiological safety (Han, Beumer, Rombouts, & Nout, 2001), based on Hazard Analysis Critical Control Points (HACCP) concepts, because post-processing contaminations with *Salmonella*, *Listeria* and other pathogens can occur at later stages in the food production chain.

18.4 Biofunctionality and health aspects

18.4.1 Digestibility

The digestibility of soya is limited because of the presence of some anti-nutritional factors such as trypsin inhibitors, lectins and flatulence-causing oligosaccharides, and because of structural barriers (cell walls) that are poorly digestible and that inhibit diffusion of digestive enzymes.

Fermentation processes have been shown to enhance digestibility significantly (Kiers, Nout, & Rombouts, 2000; Kiers, Van Laeken, Rombouts, & Nout, 2000). As an example, fungal SSF of soya beans is illustrated. Digestibility can be measured in several ways. The most expensive but representative approach is an in-vivo feeding experiment (Kiers et al., 2003) monitoring weight gain and feed conversion ratio as criteria for digestibility. Such experiments are usually conducted to confirm previous laboratory-based in-vitro experiments that can be carried out under controlled conditions and that require only small quantities of samples.

In-vitro digestibility tests (Kiers, Nout, et al., 2000) involve the use of artificial saliva, gastric and pancreatic juices during an optimized protocol of incubation periods and pH values; after enzymic digestion, the digest is the liquefied portion and this can be evaluated for its composition, in relation to that of the undigested starting material. Table 18.6 shows the effect of SSF on the in-vitro digestibility and accessibility of soya nutrients. This table illustrates that 22.3% of soaked and cooked soya can be digested by human digestive enzymes into water-soluble breakdown products. This is called in-vitro digestibility. The in-vitro accessibility refers to the smaller compounds that can pass a 12–14 kDa cut-off dialysis membrane simulating the intestinal mucosa barrier. The essential information of the table is that the fermentation with *R. microsporus* causes a strong relative increase of in-vitro accessible compounds, from 4.8% to 16.4%.

In-vivo tests can be done with human volunteers, but in some cases it is more convenient and statistically more significant to test with farm animals such as piglets. These can be selected for genetic homogeneity, and they can be given the same controlled diet (Kiers et al., 2003). In the context of combating porcine weaning diarrhoea, in-vivo digestibility tests were done, and weight gain and feed conversion were measured. This gives more information than in-vitro digestion tests because here the uptake and metabolism of nutrients are all included in the outcome, i.e. weight gain. Table 18.7 shows data comparing the performance of a standard animal feed containing either toasted soya, cooked soya or cooked and fermented (*R. microsporus*) soya. Daily weight gain, feed intake and feed conversion ratio with cooked and cooked-fermented soya did not differ significantly, but both were significantly better than toasted soya. Cooked-fermented soya gave a significant mitigation of *Escherichia coli*–induced diarrhoea (Kiers et al., 2003).

18.4.2 Formation and bioaccessibility of functional molecules

Due to the activity of the functional fermentation microbiota, molecules can be formed in fermented soya foods that have been proven and/or suggested to have positive influences on human health. Folic acid or folate, and vitamin B_{12} or cyanocobalamin are vitamins that can be synthesized by microorganisms (Mo et al., 2013). Isoflavones in soya are glycosides and as such, poorly accessible. By enzymic action these

Table 18.6 Effect of solid-state fermentation of cooked soya beans on in-vitro digestibility and accessibility

	Digestibility (%)	Accessibility (%)
Soaked and cooked soya beans	22.3	4.8
Soaked, cooked and fermented with *Rhizopus microsporus* LU582, 24 h at 30 °C	26.2	16.4

Accessibility was estimated as soluble solids passing through a 12–14 kDa cut-off dialysis membrane.
Source: Data from Kiers, Nout, et al. (2000).

Table 18.7 In-vivo weight gain, feed intake, feed conversion and severity of *Escherichia coli*-induced diarrhoea in piglets fed on animal formula containing iso-energetic levels of toasted, cooked or cooked and solid-state fermented soya

	Toasted	Cooked	Cooked and SSF with *Rhizopus microsporus* LU573 for 48 h at 37 °C
Average daily weight gain (g)	334 ± 14[a]	376 ± 14[b]	393 ± 15[b]
Average daily feed intake (g)	463 ± 17[a]	501 ± 18[b]	523 ± 19[b]
Feed conversion (intake: weight gain ratio)	0.72 ± 0.01[a]	0.75 ± 0.01[b]	0.74 ± 0.01[b]
Average diarrhoea severity	2.3 ± 1.1[a]	1.8 ± 0.8[a,b]	1.7 ± 0.6[b]

[a,b]Values with different superscript letters differ significantly ($p < 0.10$).
24 piglets per treatment: diarrhoea severity: 0 = firm and formed faeces, 2 = shapeless faeces, 4 = liquid faeces and 6 = watery diarrhoea. SSF, solid-state fermentation.
Source: Data from Kiers et al. (2003).

glycosides could be hydrolyzed into saccharides and isoflavone aglycons (Champagne, Tompkins, Buckley, & Green-Johnson, 2010), the latter being easier to be taken up in the human intestinal tract.

18.4.2.1 Folate and vitamin B_{12}

Table 18.8 shows folate and vitamin B_{12} in fermented soya (Mo et al., 2013). Folate and vitamin B_{12} are vitamins formed by microorganisms. Whereas folate also occurs naturally in soya beans, soya does not contain vitamin B_{12} naturally. During soaking and cooking, some of the natural folate from soya beans is lost in soaking and cooking water. However, these losses are easily compensated by the synthesis of folate by fungal fermentation. The table shows that the level of folate produced differs with the fungal starter culture used, but is not strongly affected by cooking of the tempe. For vitamin B_{12}, the levels depend on bacteria present during the fermentation. Some authors were able to obtain high vitamin B_{12} levels by using selected starter cultures in tempe fermentation (Okada, 1989). Cooking of tempe lowers the vitamin B_{12} level.

18.4.2.2 Isoflavones and aglycons

Table 18.9 shows the formation of the isoflavone aglycons daidzein and genistein during the soaking, cooking and fungal fermentation of soya beans (Mo et al., 2013). These aglycons are formed by degradation of the corresponding glycosides daidzin and genistin. Whereas the glycosides are not bio-accessible, the aglycons show a very high (about 100%) accessibility. Isoflavones have been associated with reduced

Table 18.8 Folate and vitamin B_{12} in raw, soaked, cooked and fermented soya (µg/kg dry matter)

	Folate	Vitamin B_{12}
Raw soya beans	2671	ND
Soaked with biological acidification, 24 h at 30 °C*	1915	ND
Cooked beans, 20 min at 100 °C	1340	ND
Fermented with *Rhizopus microsporus* at 30 °C for 24 h	1889	0.86
Fermented with *R. microsporus* at 30 °C for 48 h	3904	0.85
Cooked fermented product	3153	0.34
Fermented with traditional mixed tempe starter at 30 °C for 24 h	1577	0.63
Fermented with traditional mixed tempe starter at 30 °C for 48 h	1625	1.06
Cooked fermented product	1619	0.72

ND, not determined.
*Soaked water was inoculated with *Lactobacillus plantarum* LU852, 10^6 cfu/ml water.
Source: Data from Mo et al. (2013).

Table 18.9 Formation of isoflavone aglycons daidzein and genistein in SSF soya (mg/kg dry matter)

	Daidzein	Genistein
Raw soya beans	56	69
Soaked with natural fermentation 24 h at 30 °C	406	397
Cooked beans, 20 min at 100 °C	562	678
Fermented with *Rhizopus microsporus* at 30 °C for 48 h	684	863
Cooked fermented product	1391	1388

SSF, solid-state fermentation. Aglycons (daidzein, genistein) were 100% accessible in vitro.
Source: Data from Mo et al. (2013).

prevalence of breast and prostate cancer, cardiovascular diseases and osteoporosis (Mo et al., 2013). The data show that the formation of aglycons starts already during the soaking, resulting from the activity of endogenous glucosidases of raw soya beans. During early warming stages of cooking, these enzymes continue their action. In cooked beans, fungi will continue to form glucosidases, which will liberate aglycons during the incubation stage and during the cooking of tempe.

18.4.2.3 Obesity control

Some products such as doenjang have been associated with weight loss in obese persons (Cha et al., 2012). A study demonstrated that daily supplementation of 9.9 g dry doenjang/day for 12 weeks reduced body weight and visceral fat in overweight adults.

Although the study did not aim to elucidate the mechanism of action, it was suggested that isoflavone aglycons are associated with the expression of human lipid metabolism genes.

18.4.3 Anti-diarrhoea effect of tempe and tofu

Diarrhoea can have many causes. One type affecting young humans and animals is called "infant weaning diarrhoea", and it involves babies and piglets that have to switch from mother's milk to solid food. This is a stressful event because the intestinal epithelium and mucosa have to adapt to different chemical composition of its content. Due to stress, the infant has a higher susceptibility to intestinal infections with pathogens such as rotavirus and bacteria such as *E. coli*. Such pathogens, singly or combined, can disturb the mineral and fluid balances of the infant and lead to rapid dehydration and interrupted growth. In human babies, weaning diarrhoea is one of the causes of "under-five mortality", whereas in piglets it causes economic losses due to delayed growth performance.

Traditionally it had been observed in Indonesia that tempe given during infant diarrhoea can reduce its severity and shorten its duration (Karyadi & Lukito, 1996; Mahmud, Hermana, & Karyadi, 1985; Soenarto, Sudigbia, Hermana, Karmini, & Karyadi, 1997). This prompted a systematic study of the anti-diarrhoea effect of fermented soya, and the mechanism of this effect (Roubos-van den Hil, Nout, Beumer, van der Meulen, & Zwietering, 2009).

First, in-vitro approaches were used to determine the effect of the soya fermentation by various microorganisms such as LAB, *Bacillus* spp. and filamentous fungi on the digestibility of soya beans. It became clear that LAB do not have a significant impact on digestion, but that *Bacillus* spp. and filamentous fungi cause a considerable increase in the generation of water-soluble low-molecular mass molecules in the digest (Kiers, Nout, & Rombouts, 2000).

Such artificial digests were investigated further in-vivo in piglets, at the small intestine level (Kiers et al., 2006). Digests were perfused in segments of the small intestine of anaesthetized piglets. Studies were made of the effect of such digests on the nutrient uptake and fluid balance under controlled healthy conditions, and were compared with situations of stress that induced diarrhoea. Such stresses were induced by previous exposure to diarrhoea-causing *E. coli* and rotavirus, singly or combined.

In particular, digests of tempe caused a significant protection against fluid loss (read: diarrhoea), and this was confirmed in a larger-scale piglet feeding trial under controlled conditions during which the piglets were not intentionally stressed by exposure to diarrhoea-causing pathogens. Tempe-fed piglets had better weight gain and feed conversion, and were less affected by weaning diarrhoea (Kiers et al., 2003).

Subsequent investigations aimed at elucidating the mechanism of this protective effect and finding the molecules responsible. This was approached by a series of chemical separations and extractions, to detect which class of compounds (proteins, enzymes, carbohydrates, etc.) were the most effective protectants. In order to measure and compare small samples in a laboratory setting, cell-line experiments were developed.

Since the hypothesis was that diarrhoea is caused by *E. coli* and that attachment of *E. coli* cells to intestinal epithelial cells is a first requirement for its pathogenesis, the attachment or adhesion was considered a crucial target. The anti-adhesion effect of tempe fractions was tested in piglet cells (Kiers, Nout, Rombouts, Nabuurs, & Van der Meulen, 2002) and in human cell lines (Caco-2) (Roubos-van den Hil et al., 2009). Both experimental approaches were used and eventually led to the finding that the most effective protectants are carbohydrate fractions (Kiers, Nout, Rombouts, Nabuurs, & van der Meulen, 2007) of a molecular mass >30 kDa (Roubos-Van Den Hil, Schols, Nout, Zwietering, & Gruppen, 2010), which are not present in cooked soya beans but are gradually formed by fungal enzyme activity. The chemical composition of this carbohydrate (oligosaccharide) fraction revealed a relative dominance of arabinose (Roubos-Van Den Hil, Schols, et al., 2010), which indicates that their origin must be in the arabinan or arabinogalactan side chain of the pectic cell-wall polysaccharides of the soya beans.

Whereas previously described experiments had all been focused on tempe, we discovered later that tofu (Mo, Zhu, & Nout, 2012) has an anti-adhesive effect as well, which is of much interest because the world consumption of tofu is many times larger than that of tempe.

18.4.4 Salt reduction

Public health authorities worldwide are concerned about the relation between salt consumption, particularly sodium, and diseases such as hypertension. There is a need to avoid excessive salt intake, and in this context, food-producing enterprises are urged by international (WHO, 2012) and national health and consumer organizations (Dötsch et al., 2009) to reduce sodium chloride in their products. This is not an easy task, because salt is a functional ingredient in many foods. It is connected with consumer acceptance (taste), texture (through its interaction with, for example, proteins) and shelf-life (salt reduces the water activity and has its specific antimicrobial effect). Nevertheless, it is appropriate to experiment with decreasing levels of added NaCl in product formulations. Another option is to replace some of the NaCl by other salts such as KCl, $(NH_4)Cl$, $CaCl_2$, etc. Each of these has its own specific properties, such as different taste, hygroscopicity, and effect on water activity. Whereas several food-producing enterprises are actively pursuing sodium reduction or substitution, there is still much scope for further implementation and control.

In view of fermented soya bean products, and referring to Table 18.1, many candidates for salt reduction are certainly present. Some industrial products have been modified already at an experimental scale, such as soya sauce (Yanfang, 2010) and douchi (Gao & Liu, 2010).

In the context of sufu manufacture, the functionality of salt was investigated. The amount of sodium chloride added to pehtze for further maturation could be successfully reduced from 11% (control) to 8% (Han, Cao, Rombouts, & Nout, 2004). This resulted in a faster modification of protein fractions (Han, Wang, Rombouts, & Nout, 2003), so this may bring an advantage of the final product being available earlier for the consumer. At lower NaCl concentrations (5%), however, it was noticed that the

preservative effect of salt was lost, as evidenced by product spoilage caused by acidifying bacteria (Han, Cao, et al., 2004).

Nowadays food producers do not need to rely anymore on preservation by salt alone. Combinations of reduced salt with other preservative "hurdles" such as acidity, bacteriocins, mild pasteurization, modified atmosphere packaging and high hydrostatic pressure are certainly worth trying.

18.5 Future trends and research needs

With the rapid development of the digital age and of molecular biology, sophisticated methods of microbiological and chemical analysis gradually become affordable, enabling their use on a routine basis for monitoring and control purposes. This is of obvious importance in the continuous battle against food frauds, and to assure food safety. It is also a stimulus for research and development, as it allows the construction of a knowledge base of complex microbiota and their ecology in fermented soya foods.

Based on detailed knowledge and understanding of the robustness of microbial consortia, an even better definition can be drawn of the required properties of starters for specific fermentations. Such a "starter signature" could include its genome, metabolome, transcriptome and other novel "omes".

Another topic of future attention will certainly be the "functionality" of microorganisms and chemical substances in terms of public health and product quality in general. Apart from the issue of whether authorities treat medical claims in a different way from health food claims, it is essential in all cases that perceived or expected favourable effects of microorganisms or food components are substantiated in vivo and that their mechanism of action is explained and proven.

As mentioned earlier, the reduction of sodium intake to healthy levels is an issue of world-scale. Clearly, several fermented soy foods are vehicles of sodium intake, and reduction of their salt content will benefit humans and environment alike.

Finally, fermented foods may be ancient, but they will remain popular in the future because they are part of our life and culture and, in addition, their health benefits are increasingly being recognized and appreciated. We should devote more attention towards their updating.

18.6 Sources of further information and advice

Several books covering food fermentation and fermented foods in general are worth mentioning for further reading. They include works of Rose (1982), Steinkraus (1989, 1996), Wood, Adams, et al. (1985), Wood, Beddows, et al. (1985), Boekhout and Robert (2003) and Adams and Nout (2001).

A review dealing with fermented foods in general by Campbell-Platt (1994) should not be missed. In addition, the functional properties of fermented foods have been

reviewed, such as the beneficial effects of *Monascus* fermented foods (Lee & Pan, 2012), pre- and probiotic effects of fermented foods (Mohammadi & Mortazavian, 2011; Peres, Peres, Hernández-Mendoza, & Malcata, 2012), bioactive phenols in fermented foods (Martins et al., 2011) and other functional fermented foods (Marotta et al., 2012).

Specifically on soya bean fermentations, reviews on Thua Nao (Chukeatirote, Dajanta, & Apichartsrangkoon, 2010), the use of okara (soya milk residue) (O'Toole, 1999) and the functionality of soya oligosaccharides (Choct, Dersjant-Li, McLeish, & Peisker, 2010) are recommended.

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The microbial dynamics of wine fermentation

19

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19.1 Introduction

The production of wine entails a complex series of microbial conversions of grape components into metabolites that both enhance and stabilize the finished product. In this regard it is one of the world's oldest biotechnologies. There are several stages of the production process that select for different microbial communities, including the grape surface pre- and post-harvest, fermentation, and aging. The microbial populations at each of these stages are distinct but the organisms present at each stage influence subsequent evolution of microbial populations at the later stages. Processing decisions impact the presence, persistence, and contributions of the different members of the microbial communities of wine (King & Beelman, 1986; Renouf et al., 2006; Zott, Miot-Sertier, Claisse, Lonvaud-Funel, & Masneuf-Pomarede, 2008). The organisms present during wine production can arise from the vineyard or grape, from biofilms found on harvesting or winery equipment, from the air or airborne by insects, from deliberate commercial inoculation, or from other components such as fining agents introduced into the wine during processing or bottling.

The fermentation of grapes can be viewed as supporting distinct microbial specialists in the utilization of the primary energy sources present: sugars (fermentation) and substrate movements or partial oxidation reactions (respiration) to generate energy via the protonmotive force. The sugar fermentation phase is dominated primarily by *Saccharomyces cerevisiae*, a yeast that has been extensively studied as a model eukaryote resulting in a wealth of information on the biological activities of this organism. It was the first eukaryotic organism to have its genome sequenced (Goffeau et al., 1996; Oliver, Winson, Kell, & Baganz, 1998). The lactic acid bacteria (LAB) dominate the use of proton gradients and protonmotive force as a source of energy, and the acetic acid bacteria (AAB) dominate the phase of use of organic substrates in oxidation reactions for electron capture and energy generation. Several of these and other microbes involved in the wine production process have also been sequenced (Curtin, Borneman, Chambers, & Pretorius, 2012; Kleerebezem et al., 2003; Makarova et al., 2006; Matsutani, Hirakawa, Yakushi, & Matsushita, 2011; Mills, Rawsthone, Parker, Tamir, & Makarova, 2005; Piskur et al., 2012; Sakurai, Arai, Ishii, & Igarashi, 2011), and the availability of this information in combination with microbial community profiling technologies has enabled a detailed examination of the microbial dynamics of wine production. These studies have demonstrated the impact of vineyard site effects, climate and vineyard management practices on the organisms entering the winery, and

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00019-0

identified factors important in the persistence of these organisms. The establishment and dynamic changes in the microbiota of wineries have also been explored during the production process and the impact of fermentation conditions, sanitation practices, use of defined inocula, nutrient supplementation, and use of microbial inhibitors has been delineated. The contributions of these fermentation organisms and of those present during the aging of wine to wine flavor and aroma has also been explored, with the goal of being better able to manage and predict the appearance and levels of these important components in order to achieve a desired wine style (Fleet, 2008; Styger, Prior, & Bauer, 2011; Sweigers, Bartowsky, & Henschke, 2005).

Wine fermentations are never sterile and support a community of microorganisms that changes over the life of the wine. Analysis of the impacts of the microbial community on *Saccharomyces* has revealed the establishment of a novel prion state that restructures the metabolism of this organism. The process of wine production is uniquely suited to the delineation of changing microbial dynamics and energy source depletion.

19.2 Overview of the winemaking process: from vineyard to bottle

Wine production is a fundamentally simple process, with the potential to be highly nuanced (Figure 19.1). Following harvest, grapes are crushed and the resulting must (juice + seeds + skins) is used either directly for fermentation (red wines) or pressed to release the juice from the skins and seeds (white and blush wines). Grapes are not

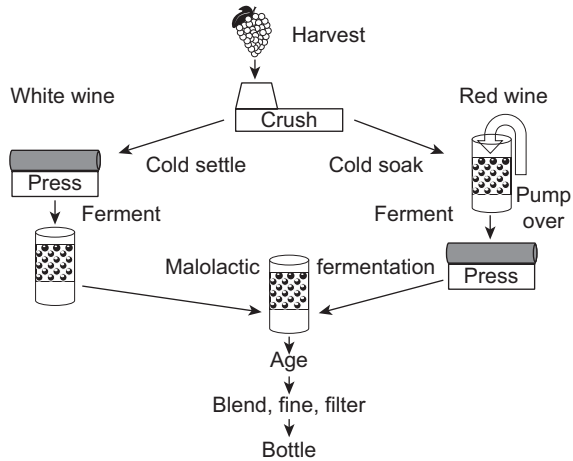

Figure 19.1 Schematic of the wine production process. Red and white wine production differs with respect to the timing of removal of the juice from the skins. Red wines are made from grapes fermented on the skins to allow for extraction of pigmented compounds. The post-fermentation steps of wine production vary by the style of the wine to be produced and its risk for microbial or oxidative spoilage.

washed prior to crushing, and the crushing operation often contains materials other than grapes, such as soil, leaves, and insects, that may also be a source of organisms. The juice or must is then transferred to and fermented in wood, stainless steel, or plastic bins or concrete vats with or without temperature control. Red wines are traditionally fermented on the skins as the skins contain the red pigments of grape, the anthocyanins, that give red wine its color and that are released from grape skin cells during the fermentation process. Once fermentation initiates and carbon dioxide is being evolved, the skin cells and other insoluble material become buoyant and form a layer or cap on the surface of the wine. In order to efficiently extract skin-cell components, the cap is forcibly submerged into the tank (referred to as a "punch down") or flushed with fermenting juice from the bottom of the tank (referred to as a "pump-over"). The process of pumping-over or punching down achieves a mixing of the tank to facilitate diffusion of cellular components from grape cell debris and also introduces oxygen to the microbial species present and redistributes nutrients and end-products throughout the tank. In addition, microbial activity is often highest in the cap, and therefore the cap attains a higher temperature than the rest of the tank due to the heat released from metabolism, so the mixing process serves to reduce the temperature of the cap and increase the temperature of the rest of the tank. The higher temperatures of the cap aid in extraction of components trapped within cell material and often enrich for thermotolerant microbial species. Thus in red wine fermentations there are multiple phases present: the liquid phase of the juice, the solid phase of the grape skins and seeds, the surfaces of the containers that can house development of biofilms, and the exposure to air on the surface of the tank. Tanks also develop localized environmental stratification, meaning juice conditions in one section of the tank may vary in temperature, nutritional content, inhibitors, and redox potential, thus fostering development of non-uniform microbial communities. The accumulating ethanol enhances the extraction of the skin-cell components as well as inhibits the growth of sensitive microorganisms. The creation of a carbon dioxide blanket on the surface of the tank by fermenting microbes inhibits the activities of aerobes that may be on the exposed surface of the cap. Although various mixing strategies are used, none are typically performed under sterile or anaerobic conditions in red wine production. The type and frequency of mixing impact oxygen availability for the yeast and the nature and number of other microbes present. Fermentations of red wines are conducted at temperatures ranging from 18 to 32 °C, depending upon the variety of grape and style of wine desired. Once fermentation is complete, the red wine is pressed off of the skins and seeds and subjected to the aging program of the winery.

White wine fermentations are restricted to the juice extracted from the grapes immediately upon crushing and pressing. White juices often undergo the process of "cold settling", in which the juice is held at a low temperature (4–10 °C) for 24–48 h to allow the solids from the grape berries to settle to the bottom of the tank. The juice is then separated from the solids and fermented at a warmer temperature, typically 12–16 °C. The white fermentations are often conducted at temperatures lower than those of red fermentations in order to retain volatile flavor components. In some situations, red musts are also held at low temperatures in a process referred to as a

"cold soak" to extract the readily soluble anthocyanins and other components (Sacchi, Bisson, & Adams, 2005). These low-temperature holds of juices and musts allow the development of cyrotolerant microbial species early in the production of wine (Fleet, 2008; Hierro, Gonzalez, Mas, & Guillamon, 2006; Zott et al., 2008).

The fermentations may be inoculated with selected yeast or left to ferment by the native microbiota present on the grapes and in the winery. In either case, the fermentation is eventually dominated by *Saccharomyces*. This yeast is a single celled eukaryotic fungus that reproduces by budding. Two species of *Saccharomyces*—*S. cerevisiae* and *S. bayanus*—are commonly found in wine fermentations. Many commercial strains and native isolates are hybrids of these and other species of *Saccharomyces* (Belloch et al., 2009; Bisson, 2012; Dequin & Casaregola, 2011; González, Barrio, Gafner, & Querol, 2006; González, Barrio, & Querol, 2008; Masneuf, Hansen, Groth, Piskur, & Dubourdieu, 1998; Naumova, Naumov, Masneuf-Pomarede, Aigle, & Dubourdieu, 2005).

Depending upon the style, the wine is either immediately ready for bottling following completion of fermentation or undergoes post-fermentation processing such as aging in wood or stainless steel. A secondary bacterial phase, called the malolactic fermentation, that involves LAB can also occur, and may be encouraged by the winery depending upon the style of wine (Osborne & Edwards, 2005). Fining to remove undesirable components or filtration to remove hazes and precipitates can also be conducted. Finally the wines may be blended to achieve greater complexity and style. The last step in the process is bottling, which can be done under sterile conditions but frequently is not. One of the goals of wine production is to achieve microbial stability of the wine prior to bottling, such that sterile conditions are not required for preservation of the wine in the bottle. It is important to emphasize that wine production is not a sterile process. It takes advantage of the ability of *Saccharomyces* to dominate a mixed-culture fermentation and produce sufficient ethanol to inhibit other microbes. Similarly, the malolactic fermentation relies on the activity of a single lactic acid bacterium, *Oenococcus oeni*, for the evolution of desired bacterial flavors and for the consumption of nutrients that would otherwise be available for spoilage organisms (Bartowsky & Henschke, 2008; Osborne & Edwards, 2005).

The proliferation of other organisms at either of these stages can lead to the appearance of spoilage characteristics that detract from wine quality and that if severe enough will make the wine unmarketable. For example, there are strict limits imposed on the levels of acetic acid allowable in wine, and if acetic acid-producing microbes gain a foothold in the wine and the wine exceeds these legal levels, it cannot be sold as wine. If wine is left to age without intervention, the ultimate microbial outcome once all nutrients have been exhausted will be vinegar. Alternately, the deliberate inoculation with non-*Saccharomyces* yeast to obtain a greater diversity of aromatic compounds is also being explored (Fleet, 2008; Lema, Garcia-Jares, Orriols, & Angulo, 1996; Viana, Gil, Genovés, & Manzanares, 2008). Thus astute management of the microbial populations at each stage of the winemaking process is therefore essential to attain the desired positive contributions to wine aroma and prevent or mitigate the appearance of any spoilage characteristics.

From a microbial perspective of energy metabolism, the microbial dynamics of wine can be broken down into three energy source-driven phases: use of carbon-based

energy sources, use of substrate decarboxylation and movements to generate a protonmotive force, and use of partial oxidations to likewise generate a protonmotive force. The yeast dominates in the first phase, the fermentative use of sugar substrates. The LAB of wine specialize in the use of substrate decarboxylation and proton movements and generation of a protonmotive force across the plasma membrane for energy production, while the AAB utilize partial oxidation reactions for electron capture and biological energy generation. Thus the production of vinegar from wine grapes follows a series of evolutions of microbial communities best able to utilize the predominant available energy substrates that are present. In some cases these communities co-exist and in others there is a progression of use: carbon energy substrates, followed by proton movements, followed by partial oxidation reactions. At any point if there are substrates left from a previous phase, spoilage communities may bloom to complete the depletion of available energy sources.

19.3 Pre-fermentation microbiota

Wine production is therefore a continuum with respect to the emergence and disappearance of specific classes of microbes during processing of the fruit and wine. The first microbes present in the process arise pre-fermentation from the grape or winery surfaces (Bisson & Joseph, 2009). The grape berry surface is initially dominated by aerobic fungi, yeasts, and bacteria that will not persist and flourish under winemaking conditions. As the fruit matures on the vine, these species are displaced by fermentative organisms as a consequence of the greater seepage of grape components from the grape due to dehydration (Bisson & Joseph, 2009). Some of these organisms will be present upon crushing of the fruit and will be able to metabolize berry components in the absence of inhibition.

The second point of entry of microorganisms into the winemaking process is from the winery flora. Several studies have examined the impact of winery microbiota in wine production, and as would be predicted, this impact depends upon sanitation and inoculation practices and when the study was conducted during the production season as microbial populations build up on winery surfaces as fruit continues to be received. Thus the local winery population impacting the first fruit to arrive at the winery is fewer in number than the local winery population impacting fruit harvested and processed much later in the same season. The nature of the community also differs. As the season progresses, the winery microbiota becomes dominated by *Saccharomyces* (Bisson & Joseph, 2009; Renouf, Perello, Strehaiano, & Lonvaud-Funel, 2006).

The grape surface contains a community of bacteria, yeast, and other fungi (Barata, Malfeito-Ferreira, & Loureiro, 2012; Bisson & Joseph, 2009; Fleet, 2008). Most important among these are the non-*Saccharomyces* fermentative yeast species, as these organisms have the potential to persist in the fermentation. In general the yeast are found in the lowest percentages and numbers (Barata et al., 2012; Bisson & Joseph, 2009). Members of the AAB, *Gluconobacter* spp. and *Acetobacter* spp., and LAB (*Lactobacillus* spp., and occasionally *Pedicoccus* spp. and *O. oeni*) can also be found on the surfaces of the grapes and may impact wine fermentation and may appear

in the wine post-yeast fermentation (Barata et al., 2012). Grape surfaces also have been shown to be colonized by aerobic bacteria that do not persist in the grape juice fermentation: *Enterococcus* spp., *Enterobacter* spp., *Bacillus* spp, *Burkholderia* spp., *Serratia* spp., and *Staphylococcus* spp. (Barata et al., 2012), although occasionally members of these species may be found in wine.

The diversity of yeast species on grapes has been examined in vineyards worldwide (Barata et al., 2012; Barnett, Delaney, Jones, Magson, & Winch, 1972; Bureau et al., 1982; Combina et al., 2005; Davenport, 1974; Garijo et al., 2011; Goto & Yokotsuka, 1977; Martini, Ciani, & Scorzetti, 1996; Mercado, Dalcero, Masuelli, & Combina, 2004; Nisiotou & Nychas, 2007; Parish & Carroll, 1985; Prakitchaiwattana, Fleet, & Heard, 2004; Raspor, Milek, Polanc, Smole Mozina, & Cadez, 2006; Renouf, Claisse, & Lonvaud-Funel, 2007; Rosini, Federici, & Martini, 1982; Sabate, Cano, Esteve-Zarzoso, & Guillamon, 2002; Sapis-Domercq, Bertrand, Mur, & Sarre, 1977; Yanagida, Ichinose, Shinohara, & Goto, 1992), and previous reviews have covered this topic (Fleet, 1993; Fleet, Prakitchaiwattana, Beh, & Heard, 2002; Kunkee & Bisson, 1993). Aggressive washing techniques suggest a concentration of 3×10^5 yeast cells/cm^2 on the berry surface (Rosini et al., 1982). Other studies report a range of 10^4 to 10^6 yeast cells/cm^2 on the grape surface (Fleet et al., 2002).

The factors impacting the genera and species that are found have also been assessed. The methodologies have differed, but there is a striking similarity of the main genera and species detected. The change in species on the surface of grapes that occurs during ripening follows a pattern of early dominance by the basidiomycetous yeasts, *Aureobassidium*, *Cryptococcus*, *Rhodosporidium*, and *Rhodotorula* pre-veraison and during early ripening, giving way to the ascomycetous yeast, particularly *Hanseniaspora*, *Metschnikowia*, and *Candida*, as the fruit ripens. Berry damage that occurs later in ripening due to physical or biological factors enriches the ascomycetes including *Saccharomyces*. The presence of other yeast genera depends upon regional and climactic influences, the grape variety, disease pressure and level of damage of the grapes, and vineyard practices (Barbe, de Revel, Joyeux, Bertrand, & Lonvaud-Funel, 2001). Many factors in addition to stage of ripening have been identified that impact the presence and numbers of yeasts on the surface of grapes (Kunkee & Bisson, 1993).

The three principal ascomycete genera found on grapes — *Hanseniaspora uvarum* (anamorph: *Kloeckera apiculata*), *Metschnikowia pulcherrima* (anamorph: *Candida pulcherrima*), and *Candida stellata*—vary in relative and absolute numbers across different vineyard sites. In some reports, *Hanseniaspora* is the dominant species (Beltran et al., 2002; Combina et al., 2005; Hierro et al., 2006) and in others it is *Candida* (Clemente-Jimenez, Mingorance-Carzola, Martinez-Rodriguez, Las Heras-Vazquez, & Rodriguez-Vico, 2004; Torija, Rozes, Poblet, Guillamon, & Mas, 2001). *Candida* strains have been shown to be able to complete the alcoholic fermentation in some cases (Clemente-Jimenez et al., 2004). Several of the *C. stellata* isolates from wine have been subsequently identified as *Candida zemplinina* (Csoma & Sipiczki, 2008). In one study of grapes from cooler climates (Yanagida et al., 1992), the basidiomycetes *Cryptococcus* and *Rhodotorula* dominated in number over the ascomycete yeasts. In another the dimorphic fungus, *Aureobasidium*, was found as the dominant yeast on grape surfaces

in addition to *Cryptococcus,* followed by *Rhodotorula* and *Rhodosporiduim,* depending upon the grape variety (Prakitchaiwattana et al., 2004).

In addition to common soil and plant bacteria that do not persist in wine formation, members of the lactic and acetic acid bacterial families can be found on the surface of the grape. These bacteria can pose a problem in wine fermentation, particularly if an antimicrobial agent like sulfur dioxide is not used or if the yeasts are inhibited in their ability to dominate the fermentation, allowing other organisms to proliferate. The metabolic activities of these bacteria often lead to high acetic and organic acid concentrations that can be inhibitory to yeast.

A key factor influencing the species present on the grape surface is the amount of damage to the fruit. The leakage of sugar and other substrates either through physical damage mediated by insects, birds, or invasive fungal species, or as a consequence of berry aging and dehydration, enriches for the ascomycetes (Fleet et al., 2002; Parish & Carroll, 1985; Prakitchaiwattana et al., 2004). Damaged grape clusters support a much higher population of organisms than intact fruit and the type of damage also impacts the species present (Barata, González, Malfeito-Ferreira, Querol, & Loureiro, 2008). The common fungal rot caused by *Botrytis cinerea* leads to the evolution of species that are different than what is typically seen upon mechanical damage or "sour rot" (Barata et al., 2008). Fermentative organisms tend to dominate in rot situations, including wild vineyard species of *Saccharomyces.* The presence of these organisms and their metabolites will impact the organisms subsequently conducting the alcoholic fermentation or that might be present as spoilage organisms in the winery. The amount of natural seepage varies with different grape varieties and the tightness of the clusters, and some studies have seen a strong correlation of variety with the biodiversity of the fruit surface (Yanagida et al., 1992). Some of the variation in species identified in different studies is a function of the physiological ripeness and integrity of the grapes when harvested for the analysis or the underlying level of berry damage and rot. Sulfur dioxide is often added to grapes at the stage of crushing to block both chemical oxidation reactions and to inhibit microbial activity. The fermentative yeasts are more tolerant of sulfur dioxide than are the aerobic yeast, bacteria, and molds present on the berry surface. Other types of processes, such as thermal treatments or pH reduction, can also be used to reduce microbial populations.

Other yeasts may also be found on grape surfaces although they are not as universal. *Saccharomyces* can be detected, but is present on grape surfaces at very low levels (Prakitchaiwattana et al., 2004; Martini et al., 1996), and in some studies has been undetectable (Combina et al., 2005; Raspor et al., 2006). In a comprehensive study using direct DNA profiling of grape surface microbes, 52 species of yeast were identified from the following 22 genera: Auerobasidium, Auriculibuller, Brettanomyces, Bulleromyces, *Candida*, Cryptococcus, Debaryomyces, Hanseniaspora, Issatchenka, Kluyveromyces, Lipomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, *Saccharomyces*, Sporidiobolus, Sporobolomyces, Torulaspora, Yarrowia, Zygoascus, and *Zygosaccharomyces* (Renouf et al., 2007). Other researchers have also found *Hansenula* (Heard & Fleet, 1985; Longo, Cansado, Agrelo, & Villa, 1991; Mora & Mulet, 1991) and *Saccharomycodes* (Combina et al., 2005). *Saccharomyces* is more commonly isolated from heavily damaged grapes (Mortimer & Polsinelli, 1999).

Analyses of the yeast populations found on winery surfaces, equipment, and in winery air have also been conducted (Blanco, Orriois, & Losada, 2011; Ciani, Mannazzu, Maringeli, Clemente, & Martini, 2004; Garijo et al., 2008; Garijo et al., 2009; González-Arenzana, Santamaria, López, Tenorio, & López-Alfaro, 2012; Haas et al., 2010; Martini, 2003; Mercado et al., 2004; Ocón, Gutiérrez, Garijo, López, & Santamaria, 2010; Ocón et al., 2013; Renouf et al., 2007; Santamaria, López, López, Garijo, & Gutiérrez, 2008). These studies have demonstrated the role that the winery microbiota are a significant source of pre-fermentation microbial activity in juice and must (Fleet & Heard, 1993; Renouf et al., 2007). Following grape processing, the numbers of *Saccharomyces* found per unit volume can increase by three orders of magnitude or more (Boulton, Singleton, Bisson, & Kunkee, 1996). Biofilms readily form on winery surfaces (Joseph, Kumar, Su, & Bisson, 2007). Stainless steel is commonly used for fermentation, but juices are also fermented in more porous containers such as wooden barrels and vats. These are notoriously difficulty to clean, let alone sanitize, and cannot be sterilized without loss of integrity. Microorganisms often also coat walls, outer barrel surfaces, hoses, and drains, particularly during barrel aging, as this is typically done under conditions of humidity to prevent evaporative loss of wine volume. Sanitation practices vary widely, as does the practice of supplementation with nutrients. All of these factors impact winery microbiota. Analysis of the surfaces of barrels indicated high numbers of *Saccharomyces*, with *Candida*, *Cryptococcus,* and *Brettanomyces* also commonly present, although in lower concentrations (Renouf et al., 2006, 2007). Bacteria and molds can be more commonly found on winery surfaces except during active fermentation, when the populations of yeasts can be high. There is considerable diversity of mold species present in wineries (Picco & Rodolfi, 2004).

19.4 Fermentation microbiota

Numerous yeast genera and species are found initially in fermenting grape juice. The low pH of wine, high-sugar content, rapidly generated anaerobic conditions, and presence of phenolic compounds creates the ideal environment to support the growth of fermentative yeasts and enriches for these organisms. The metabolic activities of yeast can have a profound impact on the composition of the wine and therefore of its aroma and flavor properties (Ciani et al., 2004; Fleet, 1993; Gil, Mateo, Jimenez, Pastor, & Huerta, 1996; Lema et al., 1996; Romano, Fiore, Paraggio, Caruso, & Capece, 2003; Romano, Capece, Serafino, Romaniello, & Poeta, 2008). Some wine styles in fact depend upon the metabolites of specific yeasts for their characteristic compositions. The yeasts that impact the composition of the wine can come in with the grapes from the vineyard, can be residents of the winery microbiota, or can be spread by insect vectors such as fruit flies, bees, and wasps (Fleet et al., 2002). The organisms found in wine can also be derived from direct inoculation using commercial yeast preparations (Boulton et al., 1996). Berry pH, which is generally between pH 3.0 and 4.0, has a strong impact on the activity and survivability of the grape surface microbes; and the lower the pH, the fewer the number of species that will be present in an active state.

Over 20 yeast genera have been identified from wine fermentations (Renouf et al., 2007). In addition to this species diversity, there is also significant biodiversity within a given species (Cavalieri et al., 1998; Sabate, Cano, Querol, & Guillamon, 1998; Schuller, Alves, Dequin, & Casal, 2005; Sipiczki, 2002, 2006; Valero, Cambon, Schuller, Casal, & Dequin, 2007; Versavaud, Courcoux, Roulland, Dulau, & Hallet, 1995; Vezinhet, Hallet, Valade, & Poulard, 1992). The extent and persistence of the diverse yeast populations is influenced by the winemaking conditions employed. For example, holding of must at low temperatures to increase extraction from the skins, termed a "cold soak" results in a bloom of yeast species tolerant of low temperatures (Fleet & Heard, 1993). The presence of these yeasts can then influence the metabolic behavior of the principle agent of the yeast fermentation, *Saccharomyces*, in addition to directly contributing aroma impact compounds to the wine.

Wine fermentations can be divided into two types: directly inoculated and uninoculated. Uninoculated fermentations are also called "native flora", spontaneous, natural, or (more correctly) autochthonous fermentations, and rely on the indigenous microbiota of the grapes and winery for fermentation, with winery strains of *Saccharomyces* often dominating these fermentations (Blanco et al., 2011). In both cases, following crushing of the grapes, the must displays high concentrations of the yeasts present on the grape berry (Blanco et al., 2011; Clemente-Jimenez et al., 2004; Fleet et al., 2002; Santamaria et al., 2008; Schuller et al., 2005). These yeasts initiate the bioconversion of grape juice into wine. How long the non-*Saccharomyces* yeast persists depends upon the winemaking conditions and relative levels of the major species present. The factors affecting the yeasts found in autochthonous fermentations are similar to those affecting the microbiota on the berry, such as the maturity of the fruit, age of the vineyard, variety, use of antifungal agents, climate, and vineyard location (Fleet, Lafon-Lafourcade, & Ribereau-Gayon, 1984; Ganga & Martinez, 2004; Longo et al., 1991; Martini, Federici, & Rosini, 1980; Parish & Carroll, 1985; Regueiro, Costas, & Rubio, 1993; Rosini et al., 1982; Valero, Schuller, Cambon, Casal, & Dequin, 2005; Van der Westhuizen, Augustyn, Kahn, & Pretorius, 2000). The use of antifungal agents in the vineyard results in increased populations of *Metschnikowia* (Regueiro et al., 1993) and decreased populations of *Saccharomyces* (Valero et al., 2007). In addition, harvesting techniques can also impact the yeasts present in the fermentation, particularly if the berries are damaged during harvest and microbial growth occurs during shipping to the winery (Boulton et al., 1996).

19.5 Post-fermentation microbiota

The process of alcoholic fermentation does not completely deplete the wine of carbon and energy sources. Other organisms are able to persist and proliferate, particularly if oxygen is available. This stage in the evolution of the wine can be divided into two categories based on the type of microbial activity occurring. The first category is the malolactic fermentation, the conversion of malic acid to lactic acid conducted by members of the LAB. In the process the bacteria obtain energy from the ensuing protonmotive force generated by the conversion of malate to lactate (Figure 19.2).

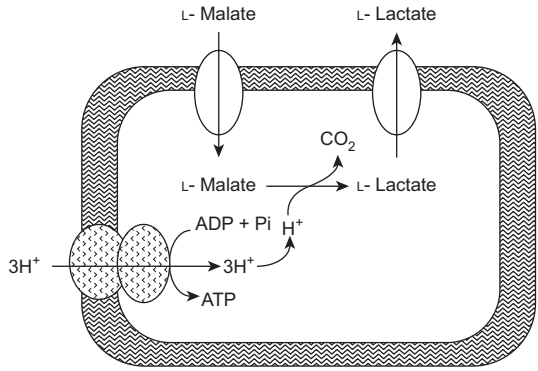

Figure 19.2 Generation of a protonmotive force via the malolactic fermentation. The decarboxylation and fixation of a proton on lactic acid with subsequent translocation of the lactic acid out of the cell leads to the generation of a protonmotive force across the plasma membrane that can be used in the generation of ATP.

The second category of post-fermentation microbial activity generally concerns the appearance of unwanted or spoilage yeast and bacteria (Loureiro & Malfeito-Ferreira, 2003). After fermentation, ethanol concentration is high and available as a carbon and/or energy source. Residual sugars, organic acids, and amino acids may also be present, some of which may have been released by the yeast upon entry into stationary phase or subsequent autolysis (Boulton et al., 1996). These energy sources are available for use by other microorganisms that can proliferate under the seemingly harsh conditions of wine. In addition, wine is rich in potential substrates for partial oxidation reactions. If wine is exposed to oxygen, organisms such as the AAB can bloom on the surface of the wine and convert ethanol to acetic acid. Flor yeast can also bloom at this time and convert the readily available ethanol to acetaldehyde, the process that underlies sherry formation.

19.5.1 The malolactic conversion microbiota

The malolactic conversion refers to the biological process by which members of the LAB convert the dicarboxylic malic acid found in grape juice to the monocarboxylic lactic acid (Liu, 2002; Osborne & Edwards, 2005). In this process, energy is obtained by the microbes, the pH is increased as protons are "fixed" on the lactic acid, and overall acidity is reduced by the conversion of dicarboxylic acids to monocarboxylic acids (Bartowsky, 2005; Davis, Wibowo, Fleet, & Lee, 1988; Herrero, Mayo, González, & Suárez, 1996; Marcobal & Mills, 2009). The pH may increase by 0.1–0.2 units following the malolactic conversion. The decarboxylation of malate and fixation of a hydrogen ion on lactic acid and subsequent excretion of lactic acid generates a protonmotive force across the bacterial membrane that is used to provide energy for growth, survival, and metabolism (Figure 19.2) (Cox & Henick-Kling, 1989, 1995; Olsen, Russell, & Henick-Kling, 1991; Salema, Lolkema, Romão, &

Dias, 1996). The malolactic fermentation is most often conducted by the bacterium *O. oeni*, as this bacterium tolerates the low pH (below 3.5) and high ethanol found in finished wines and is more tolerant of sulfur dioxide than other LAB (Bartowsky, 2005; Dicks & Endo, 2009). Higher pH wines (above 3.5) can support the growth and metabolism of a wider array of LAB (Boulton et al., 1996). In a survey of indigenous malolactic fermentations, the organism present in over 70% of the fermentations was *O. oeni*, but significant genetic diversity of the strains was observed (González-Arenzana, López, Santamaria, Tenorio, & López-Alfaro, 2011) suggesting that winery-specific genotypes might develop. Multilocus sequencing of strains of *O. oeni* demonstrated that strains also show some clustering by region of origin (Bridier, Claisse, Coton, Coton, & Lonvaud-Funel, 2010). Comparative genome hybridization and genomic sequence comparisons indicate significant genetic diversity among winery strains of *O. oeni* (Borneman, Bartowsky, McCarthy, & Chambers, 2010). Borneman et al. (2010) estimated that up to 10% of the coding sequence of the *Oenococcus* genome was specific to the strain analyzed. Thus this bacterium appears to have a core of common genetic elements with a high degree of plasticity across strains.

Heterofermentative, facultatively heterofermentative, and homofermentative LAB can all be found in wine (Bartwosky, 2005; Dicks & Endo, 2009; König & Fröhlich, 2009; Unden & Zaunmüller, 2009). Heterofermentative bacteria catabolize sugars using the pentose phosphate pathway, and in addition to lactic acid produce carbon dioxide, acetic acid, ethanol, and acetaldehyde as end-products (Bartowsky, 2005). The homofermentative LAB catabolize hexoses via the glycolytic pathway and produce much higher levels of lactic acid (Bartowsky, 2005). The major heterofermentative species reported are *O. oeni*, *Lactobacillus hilgardii*, and *L. brevis*. The facultatively heterofermenative lactobacilli *L. plantarum* and *L. pentosus* have also been found in wine (Rodas, Ferrer, & Pardo, 2005). In addition to these organisms, other lactobacilli have also been regularly isolated from wines: *L. buchnerei*, *L. fructivorans*, *L. fermentum*, *L. vini*, and *L. linderii* (Dicks & Endo, 2009). "Ferrocious" lactobacilli, *L. kunkeei* and *L. nagelli*, so named because of an ability to dominate early in juice and as a consequence inhibit the yeast fermentation, are also found in wine (Edwards, Haag, Collins, Hutson, & Hyang, 1998; Edwards, Collins, Lawson, & Rodriguez, 2000; Dicks & Endo, 2009). *Pediococcus inopinatus*, *P. parvulus,* and *P. pentosaceus* are occasionally identified in wine (Bartowsky, 2005; Dicks & Endo, 2009). *P. damnosus* has been isolated from spoiled wines numerous times (Bartowsky, 2005; Dicks & Endo, 2009). More distantly related *Leuconostoc mesenteroides* and *Weisella paramesenteroides* have also been found in wine (Dicks & Endo, 2009). What organisms are found depends upon the vineyard site and adjacencies, the level of rot in the vineyard, the pH of the juice, the use of sulfur dioxide as an antimicrobial agent, presence of inhibitors and bacteriocins, availability of nutrients and juice, and wine temperature (Boulton et al., 1996). Lactic acid bacterial populations are high in air samples of wineries during crush, during the malolactic fermentation, and during transfer or processing of wines containing the bacteria (Garijo et al., 2008). Yeast and fungi can also be found in the air during processing activities (Garijo et al., 2008), and these airborne microbes can be a source of inocula for other fermentations and during bottling.

Wine is not considered stable against lactic acid bacterial growth until the malate has been converted to lactate. However, this is not the only energy source that can be consumed by these organisms. They are able to metabolize residual hexoses in the wine should the yeast not completely degrade these components, and can use pentoses untouched by the yeast, although there is some debate as to whether pentoses can function as substrates for these organisms under wine conditions. When growing on sugar substrates, these bacteria are capable of utilizing external electron acceptors, creating sugar alcohols in the medium (Unden & Zaunmüller, 2009). The LAB likewise can use a variety of substrates to generate a protonmotive force across the plasma membrane (Vincenzini, Guerrini, Mangani, & Granachi, 2009; Zaunmüller, Eichert, Richter, & Unden, 2006; Unden & Zaunmüller, 2009). Lactic acid bacteria are more fastidious in growth requirements than *Saccharomyces* but can utilize a broader array of grape substrates to meet those needs (Bartowsky, 2005; Fernández & Zúñiga, 2006; Marcobal & Mills, 2009). Amino acids can be obtained directly from the growth medium, but the LAB can also degrade peptides and proteins found in wine that are not metabolized by *Saccharomyces* (Manca de Nadra, Farias, Moreno-Arribas, Pueyo, & Polo, 1999). The conversion of amino acids into their corresponding amines via decarboxylation, prominent among these organisms (Lonvaud-Funel, 1999, 2001), has been proposed as an additional mechanism of generation of a protonmotive force across the bacterial cell membrane for the generation of energy (Fernández & Zúñiga, 2006; Vincenzini et al., 2009).

Many factors impact the types of LAB present in wine and on the surface of the grapes. Of obvious importance is the initial wine pH, and *O. oeni* can withstand lower pH values than other LAB (Bartowsky, 2005). Antagonistic reactions between and among LAB in wine are well known (Lonvaud-Funel & Joyeux, 1993), and some end-products of yeast metabolism, such as sulfur dioxide, may also be inhibitory to the proliferation of the LAB. Finally, the temperature of the cellar is the most important factor in the development of active populations of LAB (Bartowsky, 2005).

19.5.2 Spoilage microbiota

During wine aging, microbial activity may bloom in the winery or in the bottle post-bottling (Bartowsky & Henschke, 2008; Du Toit & Pretorius, 2002). The types of microorganisms present during aging depends upon the type of vessel used, use of antimicrobial agents like sulfur dioxide, and winery sanitation practices. Both stainless steel and barrel surfaces can support microbial biofilm formation (Joseph et al., 2007). Stainless steel is easier to sanitize than porous wooden surfaces, which tend to build up significant numbers of yeast and bacteria over the years of use. The *Saccharomyces* and non-*Saccharomyces* yeasts found during fermentation can persist through aging, although these yeasts are usually not biologically active (Boulton et al., 1996). Species of *Candida*, *Pichia*, and particularly *Brettanomyces* can be found in wines in the barrel and can lead to cosmetic (film) or organoleptic defects in the wine (Kunkee & Bisson, 1993; Heresztyn, 1986; Rankine, 1966; Renouf et al., 2007). Significant diversity is found among isolates of *Brettanomyces* as well (Conterno, Joseph, Arvk, Henick-Kling, & Bisson, 2006). *Zygosaccharomyces*, due to its

tolerance of both sulfur dioxide and sorbate, can also be found as a contaminant of wine (Thomas & Davenport, 1985).

The AAB can also be a significant source of wine spoilage (Adachi et al., 2003; Bartowsky & Henschke, 2008; Du Toit & Pretorius, 2002; Ehrenreich, 2009; Guillamón & Mas, 2009; Joyeux, Lafon-Lafourcade, & Ribereau-Gayon, 1984). These organisms are able to obtain energy from partial oxidation reactions that create electron movements within a membrane and subsequent exclusion of protons, creating an electrochemical gradient that can be used for ATP generation (Figure 19.3) (Deppenmeier & Ehrenreich, 2009; Matsushita, Toyama, & Adachi, 1994; Matsushita, Inoue, Adachi, & Toyama, 2005), and will proliferate on the surfaces of wine if oxygen is available. The AAB oxidize ethanol to acetic acid via acetaldehyde in the presence of oxygen, enabling growth of these organisms in finished wine following both the alcoholic and malolactic fermentations. The oxidative reactions can occur either on the surface of the cell using membrane-bound dehydrogenases, or within the cytoplasm of the organism (Ehrenreich, 2009). Cytoplasmic dehydrogenases are unable to translocate protons but are able to feed electrons to the membrane-associated quinones in the electron transport chain (Ehrenreich, 2009). *Acetobacter* spp. are fairly universal in wineries and will produce enough acetic acid to make the wine unmarketable as such (Bartowsky & Henschke, 2008). The main species of AAB found in wine are *Acetobacter aceti* and *A. pasteurianus* (Bartowsky & Henschke, 2008), in addition to a newly identified species, *A. oeni* (Silva et al., 2006). Other species of *Acetobacter*, *Gluconobacter*, and *Gluconoacetobacter*

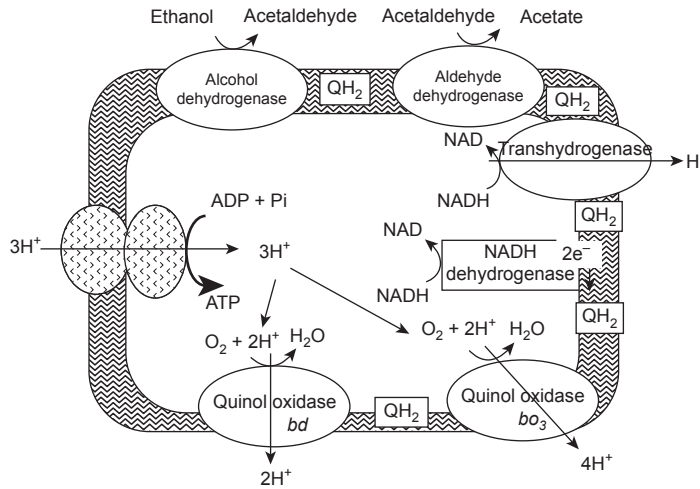

Figure 19.3 Energy generation in acetic acid bacteria. The oxidation of ethanol leads to the release of electrons captured by quinones (QH_2) in the plasma membrane of acetic acid bacteria. The transfer of electrons within the membrane can be used for the release of protons from integral membrane dehydrogenases or from membrane-associated dehydrogenases. The removal of protons by the quinol oxidases from the membrane creates a protonmotive force that can be used in the generation of ATP.

are found on the surfaces of the grape, can increase in damaged grapes (Bartowsky & Henschke, 2008), and may be present and active initially in grape juice or must. These organisms can tolerate lower temperatures than those typical for the LAB, and their numbers have been reported to increase in red musts during cold soak (Du Toit & Lambrechts, 2002).

The two most important spoilage yeasts are *Brettanomyces/Dekkera* and *Zygosaccharomyces* (Boulton et al., 1996). *Zygosaccharomyces* is resistant to sulfur dioxide and able to initiate fermentation in the bottle in the presence of residual sugar, leading to carbon dioxide evolution and cell turbidity (Boulton et al., 1996). Recent analysis of wine isolates of *Saccharomyces* have revealed introgressions of the *Zygosaccharomyces* genome (Galeote et al., 2011) with important impacts on cellular physiology. *Brettanomyces/Dekkera* are adept at colonizing wineries, particularly wooden fermentation vats, and produce a variety of spoilage characters (Boulton et al., 1996). These yeasts are quite diverse and produce an array of aroma impact compounds (Conterno et al., 2006). Analysis of the *Dekkera* genome has revealed a complex and variable genetic structure across wine isolates and documented chromosome ploidy abnormalities (Curtin et al., 2012; Piskur et al., 2012). The availability of these genomes and those of other yeasts associated with wine has enabled comparative biology analyses that are enhancing our understanding of the processes of evolution and specialization (Sherman et al., 2009).

Microorganisms may also bloom in the bottle post-bottling if conditions favor growth. Three general types of spoilage can arise in the bottle and they can often co-occur: turbidity or haze due to microbial growth or polysaccharide production, the appearance of off-characters negatively impacting wine aroma or flavor, and the development of carbon dioxide gas undesired in still wine production. The malolactic fermentation can occur post-bottling, producing cloudiness due to cell growth and fizziness due to carbon dioxide evolution. This occurs if the wine has not undergone the malolactic fermentation prior to bottling or if the wine has not been sterilely filtered prior to bottling and sterilely bottled. Sufficient oxygen may leak in from the closure to support the growth of these bacteria and in some cases support development of AAB in the bottle. If residual sugar is present, even *Saccharomyces* may bloom in the bottle and generate carbon dioxide and cell turbidity. If sufficient carbon dioxide is produced, the closure may fail or the bottle may break, neither of which is desirable. Thus the level of residual nutrients in combination with the level of residual microorganisms is an important consideration in bottling to assure the integrity of the wine until consumption.

19.6 Methods of diversity assessment

A critical factor in the analysis of yeast biodiversity concerns the methodology used to identify the microbes present. A recent comprehensive review has appeared on this topic (Bokulich, Bamforth, & Mills, 2012). Often, yeasts are cultured prior to identification by subsequent physiological or molecular analyses. The act of growing yeast colonies in isolation prior to characterization and taxonomic identification may result

in failure to detect some species that are present or skew the relative numbers of different yeasts as minor populations are missed given their under-representation among the colonies. Many wine microbes enter viable but non-culturable states and will be undetected using plating methods (Millet & Lonvaud-Funel, 2000). Direct plating on non-selective rich media favors the faster-growing yeasts such as *Saccharomyces* and may limit the growth of more slowly growing yeasts so that they are not observed. Inclusion of conditions or inhibitors to prevent or limit the growth of fast-growing yeasts often prevents or limits the growth of other yeast species and strains present. One of the most frequently used methods to identify *Saccharomyces* versus non-*Saccharomyces* yeasts is plating on Lysine Agar (Egli, Edinger, Mitrakul, & Henick-Kling, 1998; Fleet, 1993; Ganga & Martinez, 2004). *Saccharomyces* should not grow on lysine as a sole nitrogen source; therefore only non-*Saccharomyces* yeasts will grow on these plates. Direct selection by plating a wine on media containing cycloheximide, a standard selection for *Brettanomyces* yeast (Boulton et al., 1996), has also been used to select for other non-*Saccharomyces* yeasts (Renouf et al., 2006). Researchers attempt to select for a wide range of organisms by plating on non-selective media such as Wallersteins Laboratory (WL) nutrient agar and identifying yeast by colony morphology and dye uptake (Pallmann et al., 2001). However, this may select against yeast that grow slowly on WL. To get around these issues, people often plate on several different media that select for different types of yeast (Nadal, Carro, Fernandez-Larrea, & Pina, 1999; Nisiotou & Nychas, 2007). However, it is generally accepted that plating technologies introduce a selection for a subset of the organismal diversity present and at best offer a partial view of the microbiota at any given stage of the winemaking process. Patterns of nutrient utilization and production of secondary metabolites, as well as sporulation and morphological characteristics, were traditionally used to identify organisms after isolation (Kurtzman & Fell, 1998). However, given the extent of the natural diversity within species, spontaneously arising mutations can alter phenotypic properties such that the yeast is misidentified. As a consequence these methods have been almost entirely replaced by molecular techniques (Bokulich et al., 2012).

Initially, molecular techniques were used to identify yeast isolates after isolation and growth in pure culture. Many different techniques have been used for this purpose, including polymerase chain reaction (PCR) of the 26S ribosomal DNA (Kurtzman & Robnett, 1998) and sequencing and PCR and restriction enzyme digestion of internal transcribed spacers (ITS) from the 5.8S ribosomal DNA (Guillamón, Sabate, Barrioj, Cano, & Querol, 1998). These techniques still contain the bias inherent in the initial plating and isolation of the organism to be identified. To avoid this type of bias, direct DNA sampling methods coupled with molecular characterization of the consortium DNA and identification of different marker sequences are being used to determine the numbers and types of yeast in an environmental sample (Prakitchaiwattana et al., 2004; Bokulich, Joseph, Allen, Benson, & Mills, 2012). Techniques such as PCR combined with denaturing gradient gel electrophoresis (DGGE) (Cocolin, Bisson, & Mills, 2000), quantitative PCR (q-PCR) (Phister & Mills, 2003), and direct high-throughput sequencing (Bokulich et al., 2012) have been used with great success to study the ecological succession of microbes during fermentations and to identify spoilage organisms in wine. These methods allow the identification of

organisms that do not grow on a given medium under given conditions. However, these methods also have their limitations. Analysis of DNA cannot distinguish between viable and non-viable cells, the methods often are limited to finding organisms only if they occur above a certain threshold frequency in the population, and depending upon the technique used, are frequently limited to finding only those types of organisms that have been previously characterized molecularly. PCR-based methods typically rely upon specific primers that select only organisms of a certain genus and/or species. If an organism is present that is not expected to occur in the specific environment under examination, it may not be detected using specific primers.

19.7 Factors impacting the presence and persistence of microbes

Numerous studies have categorized the changes and persistence of non-*Saccharomyces* strains during inoculated and uninoculated fermentations (Beltran et al., 2002; Constanti, Poblet, Arola, Mas, & Guillamon, 1997; Gutierrez, Lopez, Santamaria, & Sevilla, 1997; Gutierrez, Santamaria, Epifanio, Garijo, & Lopez, 1999; Hierro et al., 2006; Querol, Barrio, & Ramon, 1994; Renouf et al., 2006; Schutz & Gafner, 1993, 1994; Torija et al., 2001; Van der Westhuizen, Augustyn, & Pretorius, 2000; Van Keulen, Lindmark, Zeman, & Gerlosky, 2003; Vezinhet et al., 1992; Xufre, Albergaria, Inacio, Spencer-Martins, & Girio, 2006). Several factors have been found to affect the persistence of the non-*Saccharomyces* yeasts and bacteria during fermentation. Sanitation practices can have a dramatic effect on the organisms present during fermentation. In one study, wineries with poorer sanitation practices had higher levels of the fermentative yeasts, presumably because these yeasts had colonized winery equipment (Regueiro et al., 1993). Surprisingly, sulfur dioxide, used as an antimicrobial agent typically added to juice upon crushing of the fruit, does not show a significant effect on the wild fermentative yeast species (Henick-Kling, Edinger, Daniel, & Monk, 1998). Other studies have seen a slight effect in a decrease in yeast cell numbers with use of sulfite, but have not seen an effect on the aroma profile of the resulting wines (Egli et al., 1998). In contrast, the basidiomycetous yeasts seem to show a greater sensitivity to sulfite, with one study reporting decreases of these yeasts up to 90% (Rementeria et al., 2003).

The pH and temperature of fermentation can impact the persistence of the yeast species present (Charoenchai, Fleet, & Henschke, 1998; Heard & Fleet, 1985). Members of the genera *Hansenula*, *Issatchenkia*, and *Saccharomyces* decreased dramatically while *Hanseniaspora* and *Candida* species increased upon incubation of the juice at low temperatures during cold settling (Mora & Mulet, 1991). In a similar study using a cold soak of must from a red grape variety, again *Hanseniaspora* and *Candida* species persisted during this incubation at low temperature; however, these species showed increased dominance during the alcoholic fermentation (Hierro et al., 2006) than was observed with white grape processing. Interestingly, this study also showed that during the fermentation, *Pichia* emerged along with *Saccharomyces*. Thus the

changes in microbial populations accompanying the cold settling altered the microbial dynamics much later during the fermentation. The variation in persistence of yeast species during fermentation is also dependent upon the grape variety (Clemente-Jimenez et al., 2004). One factor that does impact the persistence of non-*Saccharomyces* flora is the inoculation with commercial strains of *Saccharomyces*. Inoculation with *Saccharomyces* leads to a faster domination of the fermentation and more rapid inhibition of the other yeasts present (Egli et al., 1998; Ganga & Martinez, 2004).

19.8 Dynamics of yeast during the primary fermentation phase

19.8.1 Overview of fermentation

Grape juices are variable in composition, depending upon the varietal, time of harvest, nutritional status of the vine, and seasonal influences (Amerine et al., 1980). The juice of ripe grapes is high in sugar content present as an equimolar mixture of glucose and fructose. During fermentation, the sugar is converted to ethanol and carbon dioxide, leading to a dramatic change in osmolarity and, as ethanol increases, a dramatic decrease of the specific gravity of the matrix. High osmolarity is itself a stressor and induces a stress response in *Saccharomyces* (Gasch et al., 2000). *Saccharomyces* populations may be greatly outnumbered by other organisms on the surface of the grape but have evolved metabolically to enable domination of high-sugar substrate fermentations. There are a number of factors that enable *Saccharomyces* to bloom under these conditions: rapid adaptation to high osmolarity, rapid uptake of sugar, rapid switch to a fermentative mode of metabolism, production of ethanol and heat, rapid consumption of oxygen, and micronutrient and macronutrient depletion. The whole-genome duplication evident in the evolutionary history of *Saccharomyces* and related fermentative yeasts (Kellis, Birren, & Lander, 2004; Scannell et al., 2007; Wolfe & Shields, 1997) is credited with enabling the specialization in anaerobic fermentation (Conant & Wolfe, 2007). There is a bias in the retained duplicated genes toward the process of glycolysis, enabling faster fermentation rates (Conant & Wolfe, 2007). It is also likely that the availability of multiple copies of the same activity allowed proteins to further specialize to adapt to the surrounding cytoplasmic environment and to evolve proteins functioning in aqueous as well as in ethanolic environments.

Yeast fermentation behavior has been difficult to model kinetically, given the number of parameters involved and the varying composition of grape juice (Cramer, Vlassides, & Block, 2002). A typical fermentation profile is presented in Figure 19.4. Initially there is a lag in sugar consumption due to the adaptation and growth of the yeast in the starting juice. Once the maximum cell density has been attained, fermentation will progress at its maximal rate. At some point in the fermentation, usually when adaptation to increasing ethanol forces a reduction in fermentation rate, there is a transition to a slower fermentation rate, as shown in Figure 19.4. Eventually the

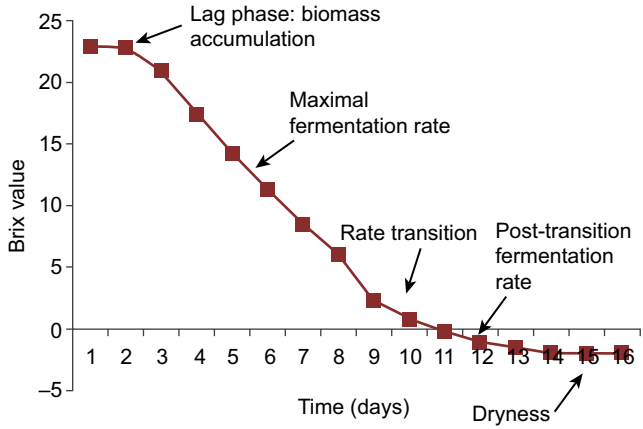

Figure 19.4 Typical Chardonnay fermentation profile. Soluble solids (Brix) measurement follows complex kinetics. Fermentation lag is due to the growth phase and buildup of new cells. Once maximal cell density is attained, fermentation proceeds at its fastest rate. At some point the accumulation of ethanol and other stressors leads to a reduction in fermentation rate, often accompanied by a dramatic transition in fermentation rate as shown. The fermentation then continues at the stress-adapted rate to completion.

sugar is depleted and the fermentation is considered to be dry. The position of the transition point is quite diagnostic for fermentation progression. The earlier the transition, the more likely the fermentation will arrest before all of the available sugar has been consumed. Although nitrogen is most often the limiting nutrient, the kinetics of carbon utilization does not correlate well with nitrogen levels, especially toward the end of fermentation (Insa, Sablayrolles, & Douzal, 1995; Maginot, Roustan, & Sablayrolles, 1998). Fermentation rates likewise do not correlate well with cell number, because the fermentation capacity of individual cells can vary. The challenges in modeling fermentation kinetics likely reflect the significant role of ethanol tolerance as a key driver of metabolic behavior. Ethanol impacts numerous biological functions such as passive proton flux and membrane–protein interactions, and displaces water of hydration, thereby disrupting protein complexes and active sites. Adaptation to steadily increasing ethanol concentrations has been suggested to be the major factor impacting gene regulation and expression during fermentation (Marks et al., 2008). Energy reserves also appear to be a critical factor, with higher levels of glycogen and trehalose being associated with improved survival (Benaroudj, Lee, & Goldberg, 2001; Thomsson, Gustafsson, & Larsson, 2005). The impact of the presence of other microorganisms in the environment on fermentation kinetics has not been well characterized. Such studies are often difficult to conduct. However, there are known instances of negative interactions wherein the early appearance of a specific antagonistic microbe, such as *Lactobacillus kunkee*, can have a profound inhibitory effect on subsequent fermentation progression (Edwards et al., 1998).

Most fermentation of grape juice is conducted by non-growing cells. Current understanding of metabolically active but non-proliferative states in yeast is limited. In the

case of wine, in addition to the presence of stressors, growth may be limited by the attainment of terminal cell density (Bisson, 1999). Cells immediately resume growth with no appreciable lag if the cell number in the non-proliferative condition is reduced. Ethanol-inhibited cells likewise immediately commence growth as soon as the ethanol content decreases (Marks et al., 2008). Cells under these conditions have not entered a classic resting state but instead remain primed to grow immediately upon restoration of permissive conditions. Grape juice also contains variable levels of nitrogen and phosphate, and one or both of these substrates may be limiting, especially if non-*Saccharomyces* microbes are present in high concentration during the early stages of fermentation (Bisson, 1999; Fleet et al., 2002). Micronutrients can also be limiting and exacerbated by non-*Saccharomyces* yeasts, which have been shown to deplete these components from the medium (Bisson, 1999).

Heat is also an end-product of yeast fermentative metabolism. For every 100 g of sugar consumed, the temperature increases by 1.3 °C (Boulton et al., 1996). Depending upon the volume and type of fermentation vessel, ambient temperature, or the use of refrigeration, temperature increases of 12–15 °C or higher can be common during batch fermentation. Temperature stratification may develop if mixing is inadequate. The carbon dioxide produced during fermentation allows some mixing and redistribution of yeasts and nutrients throughout the fermentation vessel. Changes in membrane and cell wall composition required for adaptation to high temperature are different from those required for adaptation to ethanol, and the simultaneous presence of both stressors prevents an optimal adaptation to either (Bisson & Block, 2002).

The presence of ethanol affects the tolerance of the cell to hydrogen ions (Kudo, Vagnoli, & Bisson, 1998). Ethanol increases the passive proton flux into the yeast cells, and growth inhibition occurs when the influx of protons exceeds the capacity of the plasma membrane ATPase to maintain the cytoplasmic pH (Bisson, 1999). The lower the pH, the less tolerant a cell is to increase ethanol concentrations, unless other counterbalancing ions are present (Kudo et al., 1998). The pH of grape juice is generally between 3.0 and 4.0, varying in this range depending upon the metabolic activities of yeast and the other microbes present. As the pH rises above 3.5, a multitude of bacteria that were inhibited at lower pH values can begin to grow, increasing both competition for nutrients and levels of potentially inhibitory end-products (Boulton et al., 1996).

Another factor that may be limiting during grape juice fermentation is oxygen. Grape juice fermentations rapidly become anaerobic due to microbial metabolism and to the activity of grape-derived polyphenol oxidase, which consumes molecular oxygen (Bisson, 1999). The absence of oxygen limits metabolic options for the organism; indeed, nutrient starvation under anaerobic conditions has been shown to be fundamentally different to starvation under aerobiosis (Thomsson et al., 2005). Under aerobic conditions, yeast tolerates limitation of carbon more than of nitrogen, but under anaerobic conditions the opposite is true (Thomsson et al., 2005).

Grape juice also contains a wide array of phenolic compounds such as benzoic and cinnamic acids, flavanols, and anthocyanidins, the composition and levels of which vary dramatically with the grape variety, from trace amounts to mg/L concentrations (Amerine et al., 1980). Many of these compounds have been shown to be bioactive in

humans, and it is likely that they play roles in microbial biology as well. Some of these compounds are predicted to be able to enter the cell and affect redox status and be capable of interfering with protein function. Phenolic compounds may be stimulatory or inhibitory; their presence has been shown to influence yeast metabolic activities (Cantarelli, 1989). The members of the multidrug resistance transporter family, one of the largest in *Saccharomyces* (Goffeau et al., 1997), may play a critical role in the export of toxic phenolic compounds or their derivatives.

19.8.2 Microbial dynamics during fermentation: impact of yeast strain diversity

Two primary species of *Saccharomyces* are found during the alcoholic fermentation: *S. cerevisiae* and *S. bayanus* (formerly *S. uvarum*), with *S. cerevisiae* being the more prevalent (Sipiczki, 2002). Occasionally, *S. pastorianus* can be found, as can hybrids of these yeasts (Naumov, 1996). Sequence comparisons between *S. cerevisiae* and *S. bayanus* indicate approximately 80% identity of coding sequences and roughly 74% identity of non-coding sequences (Cliften et al., 2003). Significant genetic diversity exists among wine strains of both *S. cerevisiae* (Baleiras Couto, Eijsma, Hofstra, Huis in't Veld, & vander Vossen, 1996; Briones, Ubeda, & Grando, 1996; Gallego, Perez, Nunez, & Hildago, 2005; Khan, Augustyn, Van der Westhuizen, Lambrechts, & Pretorius, 2000; Lopes, van Broock, Querol, & Caballero, 2002; Sabate et al., 1998; Schuller et al., 2005; Schutz & Gafner, 1993, 1994; Valero, Cambon, Schuller, Casal, & Dequin, 2006; Van der Westhuizen, Augustyn, & Pretorius, 2000; Van der Westhuizen, Augustyn, Kahn, et al., 2000; Versavaud et al., 1995) and *S. bayanus* (Sipiczki, 2002). Analysis of over 1600 isolates of *S. cerevisiae* from 54 spontaneous fermentations demonstrated the existence of 297 unique strains (Schuller et al., 2005). In a more limited study, 13 out of 16 isolates (81%) were determined to be unique strains and the 4 identical strains were isolated from the same location (Baleiras Cuto et al., 1996). Even higher ratios of unique genotypes have been found: 87.5% (Valero et al., 2007), 81–91% (Gallego et al., 2005), and 91–96% (Schuller et al., 2005), depending on the technique used. The greatest numbers of genotypes in all of these studies are represented by a single isolate, indicating that the true extent of the diversity present in the wild is still being underestimated. Significant strain diversity exists within the same vineyard environment, suggesting the importance of localized conditions for the selection of genetically modified strains or, alternately, the existence of factors driving genetic change. One such factor may be exposure to ultraviolet light. Metabolites produced either by the plant or by other microbes in the environment, such as mycotoxins, may also serve to accelerate the appearance of genetic differences in the absence of any direct selective pressure.

Not surprisingly, the fitness of strains for specific fermentation niches also has been found to vary. In some cases, only one or a few strains dominate throughout fermentation (Versavaud et al., 1995). In contrast, other researchers have found that different strains dominate at different stages of the fermentation (Sabate et al., 1998), or that several strains of *Saccharomyces* appear to be simultaneously present in equivalently high numbers (Torija et al., 2001; Vezinhet et al., 1992). Presumably, the biodiversity

of wine strains in the environment results in these different patterns of dominance in fermentations. Strains that are dominant in one environment may not show the same degree of dominance in another, because the strain attributes conferring dominance may be best suited to the fermentation conditions of a specific winery or vintage. As conditions of production change, different strains may become dominant. Assessment of strain diversity across vintages has shown that different strains are present each year (Gutierrez et al., 1999; Schuller et al., 2005).

The genetic diversity of wine yeasts has also been documented using genomic sequence comparisons and functional genomic analysis of transcript profiles (Borneman, Forgan, Pretorius, & Chambers, 2008; Dunn, Levine, & Sherlock, 2005; Fay, McCullough, Sniegowski, & Eisen, 2004; Gresham et al., 2006; Legras, Merdinoglu, Cornuet, & Karst, 2007; Liti, Barton, & Louis, 2006; Liti et al., 2009; Schacherer, Shapiro, Ruderfer, & Kruglyak, 2009; Townsend, Cavalieri, & Hartl, 2003; Tsai, Bensasson, Burt, & Koufopanou, 2008; Winzeler et al., 2003). Strains that are undistinguishable from each other by genomic or mitochondrial DNA profiling may carry mutations leading to changes in important enological phenotypes, particularly if the genetic differences are targeted to high-impact genes (such as transcription factors) or genes involved in flavor modification or production. Indeed, analyses of the presence of single nucleotide polymorphisms (SNPs) suggest that they exist across populations of *Saccharomyces* with a frequency of approximately 2.8 SNPs per kilobase of DNA (Schacherer et al., 2009). Borneman et al. (2008) found in the sequence comparison of a wine strain AWRI1631 to S288c an SNP frequency of 1 per 150 base pairs or roughly 7 SNPs per kilobase. SNPs occur less frequently in genes located near the centromere and more frequently for genes located in subtelomeric regions (Schacherer et al., 2009). Deletions of genetic material also occur (Schacherer et al., 2009), but are found at a very low frequency in the essential genes.

Analysis of the population genomics of commercial winery and vineyard isolates in comparison to non-wine isolates of *S. cerevisiae* indicates a high degree of relatedness among the wine strains regardless of the analytical methodology used (Legras et al., 2007; Liti et al., 2006, 2009; Schacherer et al., 2007; Schacherer et al., 2009). These analyses indicate that wine strains appear to have derived from a single common ancestor, and although some geographically isolated lineages can be observed, there is a strong influence of human migration patterns on the yeast population diversity (Legras et al., 2007; Liti et al., 2009; Schacherer et al., 2009). The majority of vineyard and winery isolates appear to be homothallic diploids, and the main mode of cellular reproduction appears to be clonal rather than sexual (Legras et al., 2007). Roughly 28% of the over 600 wine and vineyard isolates examined were homozygous, suggesting that sporulation and self-diploidization occur in the wild (Legras et al., 2007).

Commercial and native yeast isolates display greater genomic and genetic instability than laboratory strains (Ambrona, Vinagre, & Ramirez, 2005), and aberrations in the number of some chromosomes are common (Bakalinsky & Snow, 1990). They also display high levels of heterozygositiy, chromosomal polymorphisms and rearrangements, and karyotype instability (Carro & Pina, 2001; Codon, Benitez, & Korhola, 1998; Hughes et al., 2000; Izquierdo Canas, Ubeda Iranzo, & Briones Perez, 1997; Johnston,

Baccari, & Mortimer, 2000; Landry, Oh, Hartl, & Cavalieri, 2006; Landry, Townsend, Hartl, & Cavalieri, 2006; Longo & Vezinhet, 1993; Mortimer, 2000; Myers, Dunham, Kung, & Troyanskaya, 2004; Oshiro & Winzeler, 2000). The dynamic nature of the genome likely poses a distinct advantage in the environment, as evidenced by the extensive diversity observed among native isolates from the same site (Hauser et al., 2001). The biodiversity of wine strains of *Saccharomyces* is likely a consequence of both natural selection and random mutagenesis and accumulation of mutations. Wild yeasts show elevated rates of spontaneous mutagenesis, which if followed by sporulation and diploidization, can lead to the rapid creation of significant diversity across a population. The return to a homozygous state has been termed "genome renewal" (Ambrona & Ramirez, 2007; Mortimer, Romano, Suzzi, & Polsinelli, 1994).

19.9 Advances in understanding yeast fermentation physiology

A significant amount of research has been conducted with the aim of understanding the physiological and biochemical changes occurring in yeast during the process of fermentation (Bisson, Karpel, Ramakrishnan, & Joseph, 2007). These studies have employed transcript, protein, or metabolite profiling in order to obtain a complete picture of the metabolic activities of the yeast at each stage of fermentation: adaptation to the high osmolarity of grape juice, transit from active growth to the non-proliferative fermentation stage, cell death, and population decline accompanying exhaustion of sugar and accumulation of ethanol. Analysis of yeasts subjected to the stresses found during fermentation such as nitrogen limitation, presence of acetic acid, other inhibitors, and temperature shocks have also been conducted. In addition, metabolomic studies have been undertaken with the goal of delineating the contribution of yeast metabolic activity to the aroma and flavor profile of the wine (Rossouw, Naes, & Bauer, 2008; Schoondermark-Stolk et al., 2006). This area is of intense interest given the high positive and negative impacts yeasts can have on wine aroma.

19.9.1 Profiling of fermentation progression

Transcript profiling studies of wine strains of *S. cerevisiae* have largely focused on three areas: the profiling of the time course of a permissive grape juice fermentation in both synthetic media and actual juices, analysis of the impact of normally occurring stress conditions on the wine yeast transcriptome, and, as biodiversity has become more appreciated, the comparison of wine strains to laboratory strains and to each other under various growth and environmental conditions. A permissive fermentation is one in which the strain is able to complete the fermentation under the given nutritional and environmental conditions as evidenced by the complete consumption of available sugar and the concomitant production of ethanol. Although environmental and biotic stressors may be present under these conditions, the cell is able to adapt to those conditions and maintain metabolic activity. The impact of specific types of stress

on wine yeast has also been examined, generally by imposition of the stress followed by analysis of the response to that stress.

S. cerevisiae is uniquely tailored to dominate natural grape juice fermentations. Analysis of gene expression profiles in this environment should provide a more extensive understanding of the biology of this important model organism. Several investigators have profiled yeast expression patterns using global analyses or assessment of specific genes in natural grape juices or in synthetic juice media under nutrient sufficient conditions to assess the transcriptional changes accompanying normal growth and the transition to non-proliferative fermentation (Backhus, DeRisi, & Bisson, 2001; Godard et al.. 2007; Marks, van der Merwe, & van Vuuren, 2003; Marks et al., 2008; Puig & Perez-Ortin, 2000; Riou, Nicaud, Barre, & Gaillardin, 1997; Rossignol, Dulau, Julien, & Blondin, 2003; Varela, Cardenas, Melo, & Agosin, 2005; Zuzuarraegui et al., 2005; Zuzuarregui, Monteolivia, Gil, & del Olmo, 2006; Zuzuarregui & del Olmo, 2004). Even though different transcript profiling platforms were used and a range of commercial and native isolates analyzed, a common portrait of gene expression during fermentation of synthetic or actual grape juices has consistently emerged. Initial growth arrest in these studies was attributed to eventual nutrient restriction, to attainment of terminal cell density, or to the inhibitory effects of accumulated ethanol. Each of these studies demonstrates a global remodeling of ribosomal composition and translation and mRNA processing upon entry into a non-proliferative state. These responses likely signal exit from active growth and occur regardless of the cause of growth cessation. Initial adaptation to loss of the ability to proliferate is characterized by a gene expression profile that indicates that the cells remain primed to resume growth as soon as the limitation is alleviated. Metabolic activities are maintained while those associated with net growth display decrease in expressed mRNA content. If the stressors are not alleviated, the cell progresses into a highly adapted quiescent state that requires a period of incubation in permissive media in order for growth to resume. As fermentation continues, ethanol stress increases, activating a stress response within the cell. This response appears to be a graded response with a gradual decrease in the expression of genes involved in biosynthesis, and global changes in transport proteins and membrane lipid composition (Aguilera, Peinado, Millán, Ortega, & Mauricio, 2006; Henderson, Lozada-Conteras, Jiranek, Longo, & Block, 2013). Changes in global gene expression patterns indicate that the cells undergo a gradual and continual adaptation to the disruptive effects of ethanol (Marks et al., 2008).

There is also an increased expression of genes involved in the oxidative stress response. This may appear paradoxical given that these fermentations are largely anaerobic. However, acetaldehyde, an oxidizing agent, is an intermediate in ethanol production and may be responsible for the need to induce these pathways. Further, hydrogen peroxide can be produced from reactions between phenolic compounds and molecular oxygen. Thus, even in the absence of respiration, reactive oxygen species may be present. Osmotic shock has also been shown to generate reactive oxygen species, and there is a relationship between anaerobicity and osmotolerance (Krantz et al., 2004). Increases in expression of some genes known to be involved in ethanol tolerance are observed, but interestingly, expression of other genes that have been shown to lead to ethanol sensitivity when mutated is not affected (Marks et al., 2008).

This observation underscores the challenges associated with using time of expression to define the cellular function and physiological role of the genes expressed. Genes involved in glycogen, trehalose, and glycerol metabolism also increase in expression, and these components have been shown to be important in survival of ethanol stress (Benaroudj et al., 2001).

Transcript profiling has revealed many features of the non-proliferative, metabolically active late fermentation stage of *S. cerevisiae*. Upon attainment of maximal cell density or an inhibitory concentration of ethanol, further growth ceases and fermentation rate is maximal (Rossignol et al., 2003). Fermentation rate then gradually decreases as ethanol continues to accumulate in the environment. It is not known if this is due to inhibition by ethanol or to an adaptation of energy generation to match the reduced energy needs of the cell. Genes associated with cell growth and amino acid biosynthesis are also increasingly downregulated as fermentation progresses, consistent with the disruptive effects of ethanol and concomitant risks associated with attempting cell division under these conditions. The physiological changes needed to resist the disruptive effects of ethanol likely preclude continued growth (Bisson & Block, 2002).

19.9.2 Assessment of problem fermentations

A major difficulty in wine production is the failure of the yeast to completely convert all of the available sugar to ethanol. This is referred to as an incomplete, arrested, or stuck fermentation. The high levels of residual sugar left in the wine are problematic on two accounts. First, if the sugar level is over the threshold of detection, the wine will be perceived as sweet and not dry. The second problem associated with high residual sugar is microbial spoilage. Carbon substrates left behind can be utilized by spoilage organisms later in the life of the wine, placing the wine at high risk of becoming unmarketable. A related problem is a slow or sluggish fermentation. In this case the sugar is eventually consumed, but the slow progression ties up tank space and can also foster spoilage if the carbon dioxide blanket is not maintained or other organisms are able to bloom that further inhibit fermentation progression. Slow and incomplete fermentations are chronic problems in wine production worldwide.

Since fermentations often arrest due to the presence of stressors in the environment, one of the major areas of research interest in wine strains of *S. cerevisiae* is the analysis of response to stress. The principal stressors encountered by yeast under these conditions are high osmolarity, high ethanol, extremes of temperature, nutrient limitation, and presence of inhibitory metabolites, as mentioned already (Bisson, 1999), and may occur at different stages of wine production. Genomic analysis of the response to each of these types of stress has been conducted in laboratory and in wine strains (Alexandre, Ansanay-Galeote, Dequin, & Blondin, 2001; Aranda & del Olmo, 2004; Backhus et al., 2001; Beltran et al., 2006; Erasmus, van der Merwe, & van Vuuren, 2003; Jimenez-Marti & del Olmo, 2008; Kuhn, DeRisi, Brown, & Sarnow, 2001; Marks et al., 2003, 2008; Mendes-Ferreira, del Olmo, Garcia-Martinez, Jimenez-Marti, Leao, et al., 2007; Mendes-Ferreira, del Olmo, Garcia-Martinez, Jimenez-Marti, Mendes-Faia, et al., 2007; Novo et al., 2006; Rep, Krantz, Thevelein, & Hohmann,

2000; Rossignol, Postaire, Storai, & Blondin, 2006; Sahara, Goda, & Ohgiya, 2002). Several excellent reviews on the yeast stress response have appeared (Gasch, 2003; Gasch & Werner-Washburne, 2002; Gray et al., 2004; Siderius & Mager, 2003).

Existing environmental and growth conditions can profoundly influence the stress response (Siderius & Mager, 2003). Plasma membrane composition at the time of stress can impact detection of stress and signal transduction, and the availability of nutrients can be important for the synthesis of stress response factors. Even under permissive growth conditions, the stress response on rich yeast extract peptone dextrose medium varies from that on minimal yeast nitrogen base medium (Siderius & Mager, 2003). Environmental conditions may also mitigate a stress response. Cells were more heat tolerant under anaerobic conditions than under aerobiosis (Davidson & Schiestl, 2001). This observation suggests that a primary consequence of heat exposure is the release of reactive oxygen species that accompanies disruption of metabolically active mitochondrial membranes.

Several studies have focused on the impact of nitrogen limitation and addition on transcript expression profiles (Backhus et al., 2001; Marks et al., 2003; Jimenez-Marti & del Olmo, 2008; Jimenez-Marti, Aranda, Mendes-Ferreira, Mendes-Faia, & del Olmo, 2007; Mendes-Ferreira, del Olmo, Garcia-Martinez, Jimenez-Marti, Leao, et al., 2007; Mendes-Ferreira, del Olmo, Garcia-Martinez, Jimenez-Marti, Mendes-Faia, et al., 2007). Although different strains and growth conditions were used, some remarkable similarities in response to nitrogen limitation were observed. Cultures grown on ammonia as principal nitrogen source display elevated expression of enzymes involved in amino acid biosynthesis as compared to fermentations on a mixture of amino acids (Jimenez-Marti & del Olmo, 2008). In this study, nitrogen catabolite repression appeared stronger with the mixture of amino acids than with ammonia alone. Arginase activity appears to be a good indicator of the status of nitrogen metabolism during fermentation (Jimenez-Marti et al., 2007). Arginase activity increases as ammonia and other preferred nitrogen sources are consumed, but is reduced upon supplementation with other nitrogen sources; thus, it would serve as a good indicator of the metabolism of the cells if a baseline of activity is known. Expression levels of the *ACA1* gene also seemed well correlated with nitrogen metabolism (Jimenez-Marti et al., 2007). These authors also observed effects of the nitrogen source and supplementation on the aroma profile of the cultures. Another study also used transcriptome analysis to identify potential markers of nitrogen deficiency (Mendes-Ferreira, del Olmo, Garcia-Martinez, Jimenez-Marti, Leao, et al., 2007; Mendes-Ferreira, del Olmo, Garcia-Martinez, Jimenez-Marti, Mendes-Faia, et al., 2007). Thirty-six genes were identified that seemed to be responsive to the nitrogen status of the medium. However, most of these genes were also known to be regulated in response to other factors, such as carbon source limitation or stress, and would not be specific to nitrogen limitation. Nitrogen limitation in general seems to lead to increased expression of genes involved in oxidative metabolism and in ribosome remodeling (Backhus et al., 2001; Mendes-Ferreira, del Olmo, Garcia-Martinez, Jimenez-Marti, Mendes-Faia, et al., 2007) regardless of whether the high nitrogen condition was due to arginine (Backhus et al., 2001) or to ammonia (Mendes-Ferreira, del Olmo, Garcia-Martinez, Jimenez-Marti, Mendes-Faia, et al., 2007).

19.9.3 Prion formation and other adaptive responses

The causes underlying problem fermentations have been well characterized, as described earlier. However, an emerging area of interest is in understanding how environmental stresses imposed before or early in fermentation can lead to sluggishness or arrest later during the course of fermentation. Some of this molecular memory is likely due to differences in expression levels of stress response genes that persist during subsequent cell divisions (Ivorra, Perez-Ortin, & del Olmo, 1999). The discovery of specific stress-induced yeast prions, which are protein "switches" capable of drastically altering phenotype via heritable protein conformational states without altering the genotype of a cell, offers an additional insight into the establishment of populations of cells with fundamentally different metabolic properties (Halfmann & Lindquist, 2010). Brown and Lindquist (2009) showed that under selective stress, a well-known transcriptional regulator of *HXT* gene expression, Std1 (Kim, Brachet, Moriya, & Johnston, 2006; Lakshmanan, Mosley, & Ozcan, 2003; Santangelo, 2006), is involved in a conformational change in the primary plasma membrane ATPase, Pma1, resulting in a stable, dominant, heritable prion complex. This Pma1/Std1 complex protects Std1 from glucose-induced degradation, allowing it to bind with Rgt1, a co-regulator of *HXT* expression. Despite the presence of glucose, this transcription regulatory complex enters the nucleus and is responsible for a specific 36-fold decrease in *HXT3* gene expression upon induction of the prion state (Brown & Lindquist, 2009). Thus, the normal glucose-mediated increase in *HXT3* expression is blocked once this complex has been formed. This prion is termed "GAR" for resistant to "glucose associated repression" with brackets denoting its non-Mendelian inheritance (Brown & Lindquist, 2009). While cells harboring the (*GAR*$^+$) prion display a range of associated physiological phenotypes, the (*GAR*$^+$) state is defined as the ability of a cell to utilize alternative carbon substrates in the presence of glucose. In a winemaking context, this early stress appears to be due to the presence of microorganisms, particularly members of the LAB (Walker and Bisson, unpublished observations). While prion states are usually spontaneously induced in low frequencies in response to stress, the presence of inducing microorganisms can induce (*GAR*$^+$) in an entire yeast population, potentially causing the yeast to shift from a dominant mode into an almost commensalism mode of metabolism that increases their longevity while decreasing their fermentative capacity.

The prion complex forms a dynamic regulatory relationship that links the plasma membrane ATPase (*PMA1*), which is largely responsible for maintenance of ethanol tolerance, with the *STD1* transcription factor responsible for regulation of the *HXT3* gene. The highly abundant Pma1 protein responds to environmental conditions and metabolic activity by pumping protons out of the cell in order to maintain pH homeostasis of the cytoplasm (Figure 19.5). Ethanol increases the flux of protons from the medium into the cell, and protons also arise from sugar catabolism and amino acid transport. Although some buffering of pH can occur in the vacuole and cytoplasm, the proton-pumping capacity of the Pma1 pump is critical to maintaining pH homeostasis and is saturable. A role of this pump in downregulation of metabolism under stressful cytoplasmic pH conditions assures continued cell

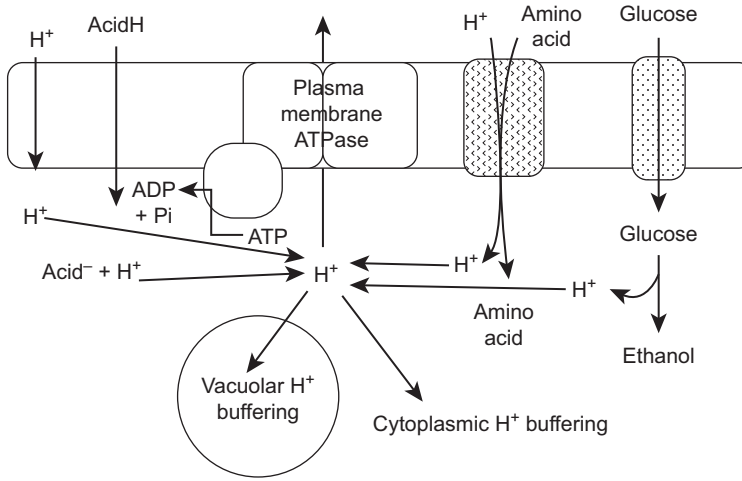

Figure 19.5 Central role of the plasma membrane ATPase in the maintenance of proton homeostasis within the cytoplasm. Protons gain entry into the cytoplasm through passive proton diffusion, which can increase with increasing ethanol; from the uptake of protonated acids; from symport with amino acids; or from metabolism. The cytoplasm and vacuole can buffer proton levels, but the main buffering activity is provided by the action of the ATPase. Saturation of the ATPase can lead to cell death; thus metabolic activities are tightly coordinated with ATPase activity.

viability albeit at the expense of continued metabolism. The existence of the Pma1/Std1 complex therefore links environmental stressors to fundamental changes in the metabolic response of the cells to the presence of external glucose. The dominant heritable nature of the prion complex means that newly formed cells not exposed to the conditions originally leading to the creation of this complex will nonetheless inherit the prion complex. The formation of this prion constitutes a novel means by which the pre-history of a culture of cells can be passed on to future generations and defines (*GAR*$^+$) as an intriguing new type of prion-based epigenetic mechanism of inheritance. It is likely that future studies will reveal even more prion-like adaptation events occurring during fermentation that enable inheritance of a response to past stressors without leading to genomic change.

19.10 Future trends

The application of enhanced microbial community profiling tools will greatly facilitate our understanding of the complex process of wine fermentation and the organisms involved. As more winery and fermentation microbiota are described, new tools aimed at developing specific aroma and flavor profiles of wine will emerge. In addition, the more the information gained regarding the biology and fermentation physiology as well as the diversity of *Saccharomyces*, the better will be our ability to manage the primary

fermentation. In parallel, as we gain a greater understanding of the genomic complexity and metabolic capabilities of the LAB, the use of these organisms in quality wine production will likewise improve. Finally, the genetic tractability of *Saccharomyces* and stability of hybrid genomes portends an era of judicious genetic manipulation of this organism to broaden its metabolic capabilities leading to the production of even more diverse wines (Fleet, 2008; Pretorius, Curtin, & Chambers, 2012).

19.11 Sources of further information and advice

There are numerous resources available on *Saccharomyces* and its application to wine production. Two excellent volumes have appeared devoted to the microbiota of wine: *Biology of Microorganisms on Grapes, in Must and in Wine* (König, Unden, & Frölich (Eds.), (2009). Springer) and *Wine Chemistry and Biochemistry* (Moreno-Arribas & Polo (Eds.), (2009). Springer). These books cover many of the topics mentioned in this text. The Génolevures project has sequenced partially or in full the genomes of several important organisms in wine production (http://genolevures.org/). The field of wine microbiology is dynamic and broad and new advancements are being made not only in the technology of microbe analysis but also in the application of those technologies to industrial fermentations.

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Quality improvement and fermentation control in beer

20

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20.1 Introduction

Beer is one of the oldest fermented beverages in mankind's history, and the brewing process can be considered as very traditional, without significant changes since its ancient origin. The process is based on natural enzymatic activity occurring during the malting of grain, mashing of grist and fermentation of wort (Bamforth, 2009). Although the details of the brewing process can vary depending on the specific beer type, it always consists of these stages: malting, production of wort, fermentation, maturation and downstream processing, which may include stabilization, pasteurization, filtration and packaging, as necessary (Figure 20.1).

The purpose of malting is to produce/activate enzymes and to cause modification in the barley kernel. In order to initiate the processes, water has to be supplied to the kernel (steeping) and the barley is made to germinate. When the modification of the green malt (cytological, chemical) has proceeded far enough, kilning is performed in order to increase the storage capacity of malt (drying) and to form colour and flavour compounds. The conversion of the insoluble components of the malt into soluble and particularly fermentable sugars is the purpose of wort production. The process consists of three main steps: mashing (production of extract by the action of enzymes), lautering (removal of insoluble malt fraction) and wort boiling (extraction of hop components, sterilization, etc.). To transform wort into beer, active brewer's yeast cells have to be pitched into the cooled and oxygenated wort. The conventional fermentation process consists of two phases: main fermentation (usually 5–8 days) and maturation (usually 7–30 days). During main fermentation, the conversion of fermentable sugars (e.g. glucose, maltose, maltotriose) into ethanol and CO_2 takes place, together with the production of other metabolic by-products (esters, higher alcohols, aldehydes, organic acids). Some of the by-products (vicinal diketones, acetaldehyde) impart undesirable flavours to green beer and therefore maturation is required to reduce the concentration of undesirable flavour compounds as well as to saturate the final beer with CO_2. The filtration, pasteurization and stabilization of the beer are carried out in order to achieve microbial, colloidal and flavour stability so that no visible and perceptible changes occur in the final product for a long time.

Although the basic brewing procedures remain still the same, the technological development and the implementation of modern industrial equipment has gradually changed breweries from the age of craftsmanship to present-day fully automated, highly hygienic, energy/water recuperating industrial facilities. However, it was always imperative that the alterations of the technological process did not lead to

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00020-7

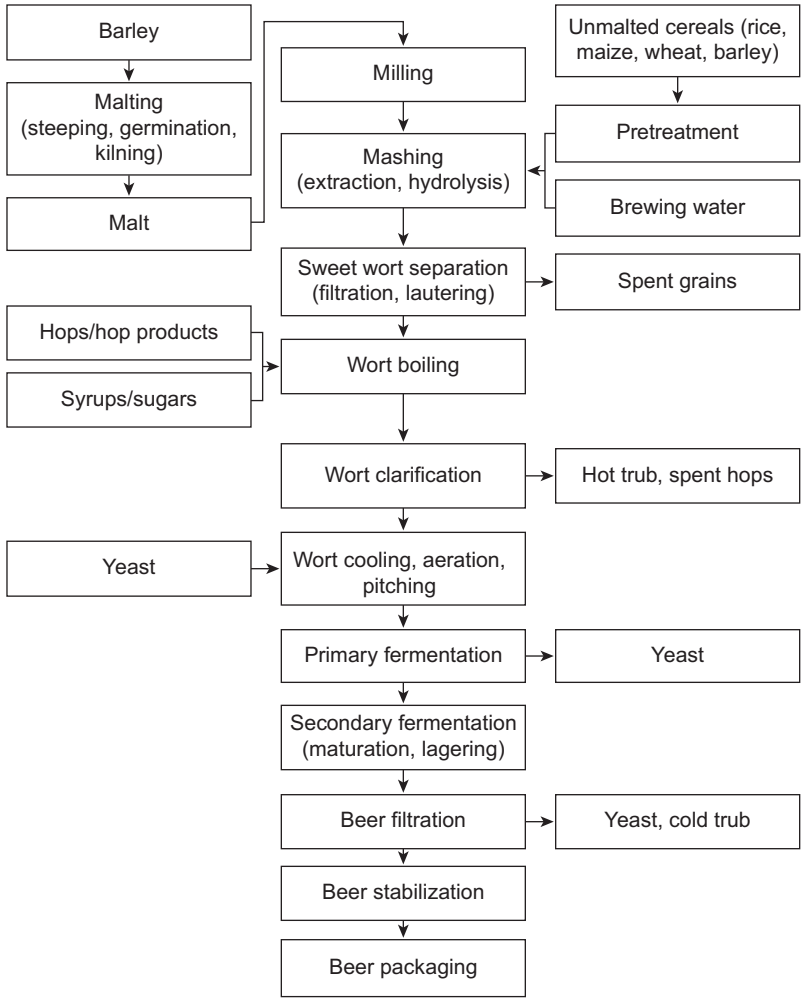

Figure 20.1 Schematic outline of the brewing process.

changes in the quality of the final product negatively rated by the consumers. Thus the introduction of technological innovations usually requires an extensive preceding investigation of its influence on the sensorial quality of the product. Given the fact that beer is a complex aqueous solution containing CO_2, ethanol, inorganic salts and hundreds of organic compounds, this is not always an easy task.

Developments in the brewing industry have touched each and every facet of this traditional multidisciplinary biotechnology. It is not within the scope of this chapter to describe all the interventions into the brewing process of the last decades. Instead, the authors will focus on selected topics in genetics, engineering, process hygiene, product development and health issues, and give updates of the latest developments based on fundamental knowledge in corresponding areas.

20.2 Genetic improvement of brewer's yeast

Yeast has played an important role as a fermenting agent throughout the whole history of brewing, although the nature and underlying biochemical processes of fermentation had been unknown until the nineteenth century (Saerens, Duong, & Nevoigt, 2010). Ancient brewers thus unconsciously domesticated and selected yeast strains with characteristics suitable for beer fermentation (Lodolo, Kock, Axcell, & Brooks, 2008).

Brewer's yeast, like a number of other microorganisms, has undergone many reclassifications, and thus various names were introduced in the literature. Generally, yeasts are classified as fungi (Boulton & Quain, 2001). Brewer's yeast can be defined according to European Brewery Convention as yeast culture used for production of the bottom-fermented (lager) or top-fermented (ale) beers (European-Brewery-Convention, 2005). Top-fermenting yeasts tend to rise to the beer foam in larger quantities during fermentation (simultaneously they also sediment), while bottom-fermenting yeasts usually flocculate and settle to the bottom of a vessel at the end of the main fermentation (Kunze, 1996; Kurtzman, 2003). Brewer's yeasts are considered members of the genus *Saccharomyces*. The latest classification of lager yeasts based on DNA relatedness is *Saccharomyces pastorianus* (formerly *Saccharomyces carlsbergensis*) and ale yeasts *Saccharomyces cerevisiae* (Boulton & Quain, 2001).

Nowadays, pure yeast cultures are predominantly used for fermentation in the brewing industry. However, a simple selection of the most suitable yeast strains may not be enough for the changing requirements of the brewing industry (high-gravity wort fermentation, continuous fermentation, low alcohol formation, etc.). Consequently, research focused on the improvement of technological properties of brewer's yeast is relevant. The following strategies may be used for this purpose: conventional breeding techniques, protoplast fusion, random or targeted mutagenesis and genetic engineering (Piddocke & Olsson, 2010). The main objectives of yeast strain development in brewing are to increase the productivity of the fermentation process while preserving or improving the quality of the final product. Most of the research papers concerning improvement of brewer's yeast have studied bottom-fermenting yeasts due to the fact that lager beers represent the overwhelming majority (90%) of the beer market (Saerens et al., 2010).

In general, the basis of brewer's yeasts selection is to find or prepare (e.g. by breeding techniques, protoplast fusion, mutagenesis and genetic engineering using genetic manipulation) a strain that is better equipped to achieve a desired task (Boulton & Quain, 2001). The most important technological properties of brewer's yeast are the following:

- Efficiency to transform sugars into ethanol (rate of fermentation, degree of attenuation)
- Stress tolerance (primarily caused by temperature, osmotic pressure, ethanol toxicity or hydrostatic pressure in CCVs)
- Genetic stability
- Resistance to contamination
- Vitality and viability
- Flocculation and sedimentation characteristics
- Formation of flavour-active compounds
- Rate of diacetyl elimination (Boulton & Quain, 2001; Piddocke & Olsson, 2010).

Early attempts to apply conventional breeding approaches faced a number of problems such as polyploid or aneuploid genomes that lead to a very poor sporulation and a low degree of spore viability of lager yeast (Andersen, Hoffmann, Grifone, Nilsson-Tillgren, & Kielland-Brandt, 2000; Polaina, 2002). Nevertheless, some spores with mating capability were isolated and a few novel brewer's yeast strains with enhanced properties were obtained (Bilinski & Casey, 1989; Saerens et al., 2010).

Protoplast fusion is a technique that enables the asexual combination of two complex genomes. However, similarly to conventional breeding, the final result is uncertain because of unpredictable gene transfer with little or no understanding of the specific genetic perturbations (Piddocke & Olsson, 2010). As an example of protoplast fusion, the conversion of non-flocculent brewer's yeast to a flocculent one by electrofusion can be cited (Urano, Sahara, & Koshino, 1993; Urano, Sato, Sahara, & Koshino, 1993).

Generally, mutagens such as UV radiation or mutagenic chemicals can be used to induce mutations in yeast DNA. This random mutagenesis is a technique with a low probability of obtaining the desired phenotype, especially in the case of polyploid (aneuploid) lager yeast. In addition, the frequency of generating deleterious gene alterations is relatively high (Saerens et al., 2010). Despite all difficulties, several brewer's yeast strains with desired phenotypes were obtained, e.g. mutants of lager yeast with improved fermentation performance under high-gravity conditions (Blieck et al., 2007), enhanced production of flavouring compounds (Lee, Villa, & Patino, 1995) or an ale mutant with lowered production of acetic acid and enhanced productivity of ethanol (Mizuno, Tabei, & Iwahuti, 2006).

Fast progress in molecular biology has opened new possibilities in improvement of the production properties of brewer's yeast. Genetic engineering allows direct manipulation with genetic information (gene deletion, overexpression or modulation) and considerable reduction of the risk of losing the positive traits present in the host strain or accumulating negative traits (Saerens et al., 2010). Even though genetic modification techniques are currently not acceptable due to legal obstacles and negative attitudes of consumers, a number of successful metabolic engineering attempts focused on increasing ethanol yield, enhancing sugar utilization during fermentation, decreasing the production of undesirable flavour compounds, improving flocculation properties, etc., were carried out (Piddocke & Olsson, 2010). However, the complexity of the genetic engineering approach lies in the often observed necessity to modify several genes (structural, regulatory) simultaneously in a coordinated manner to achieve the desired phenotypic response (Saerens et al., 2010).

20.2.1 Saccharide utilization

The requirement of low-carb beers is the reduced content of carbohydrates. Therefore genetic engineering has aimed to develop yeast strains with the ability to hydrolyse residual dextrins in wort. Many studies have dealt with transfer of genes encoding amylolytic enzymes into brewer's yeast (Piddocke & Olsson, 2010). Genes encoding amylolytic properties have been mostly obtained from *S. cerevisiae* var. *diastaticus* (Dequin, 2001), but other donors such as several *Aspergillus* species (Dequin, 2001),

Schwanniomyces occidentalis (Piddocke & Olsson, 2010), *Bacillus amyloliquefaciens* (Steyn & Pretorius, 1991) and *Lipomyces starkeyi* (Zhang, Wang, He, Liu, & Zhang, 2008) were also used. The successful novel strains showed in varying degrees of improved capability to utilize dextrins or even starch and increased production of ethanol, while the product's flavour and aroma profile was retained. Another topic related to saccharides is the reduction of glucose repression of maltose and maltotriose utilization, which is achieved through the improvement of their transport proteins. This is of particular interest for high-gravity brewing with very high glucose concentrations in wort (Piddocke & Olsson, 2010).

20.2.2 Ethanol production

The popularity of alcohol-free beers is growing, and in addition to other production strategies, genetic engineering allows constructing of strains with lower ethanol production (Branyik, Silva, Baszczynski, Lehnert, & Silva, 2012). However, reduction of ethanol was found to be accompanied by an increased formation of fermentation by-products (Saerens et al., 2010). Nevoigt et al. (2002) decreased the ethanol production by overexpressing the gene *GPD1*, which encodes the key enzyme in glycerol formation, glycerol-3-phosphate dehydrogenase, in an industrial lager yeast. But besides glycerol, other undesirable fermentation by-products, such as acetoin, diacetyl and acetaldehyde, were also produced in elevated concentrations.

20.2.3 Flavour profile

Yeasts produce a lot of various flavour active by-products (e.g. esters, higher alcohols, aldehydes, ketones, organic acids), which participate in the final sensorial character of beer (Pires, Teixeira, Branyik, & Vicente, 2014). Some of these by-products are undesirable because they impart beer off-flavours/aromas (Kunze, 1996). Particularly interesting topics are the reduction of diacetyl, dimethyl sulphide (DMS) or hydrogen sulphide and an enhanced production of sulphur dioxide or esters (Piddocke & Olsson, 2010; Saerens et al., 2010). Diacetyl is a typical undesirable compound in lager beer responsible for buttery off-flavour at low concentrations (ca. 0.15 mg/l). It is formed by an extracellular non-enzymatic decarboxylation of α-acetolactate (Takashi, 2008). To avoid diacetyl formation, a non-yeast α-acetolactate decarboxylase gene can be inserted into the yeast genome, leading to α-acetolactate degradation directly into acetoin without diacetyl accumulation (Fujii et al., 1990). Another solution is to increase the flux from α-acetolactate towards amino acid synthesis. This has been achieved by yeast modification of the genes *ILV3* (encoding dihydroxyacid reductase) and/or *ILV5* (encoding acetohydroxyacid reductoisomerase) (Mithieux & Weiss, 1995; Omura, 2008).

20.2.4 Flocculation

Yeast flocculation is a reversible and asexual process of cell aggregation, which plays an important role in sedimentation to the bottom of the fermentation vessel. Adhesins or flocculins, the cell-wall glycoproteins, are responsible for flocculation, together

with a whole range of physiological and environmental factors, and are encoded by the *FLO* genes. Well-timed flocculation is welcome in beer fermentation, and it is used for the most efficient removal of yeast biomass from beer. However, many different yeast strains used in industry do not have optimal flocculation properties (flocculation instability, premature flocculation, etc.). Therefore, many research papers have dealt with improvement of this phenomenon by means of genetic engineering. There have been good results in improving the yeast flocculation by overexpressing specific *FLO* genes or replacing their native promoters (Saerens et al., 2010; Vidgren & Londesborough, 2011).

20.3 Anaerobic beer contaminants

It has been postulated that improvements in the downstream processing technology included the reduction of the oxygen level in packaged beer together with replacement of bottle pasteurization by filtration techniques, which made the growth of strictly anaerobic bacteria in beer possible (Vaughan, O'Sullivan, & van Sinderen, 2005). Therefore, Gram-negative, anaerobic bacteria have attracted particular attention in recent decades. The Gram-negative, strictly anaerobic beer-spoilage bacteria are currently identified as belonging to the genera *Megasphaera*, *Pectinatus*, *Selenomonas* and *Zymophilus*. Phylogenetic analyses, supported by chemotaxonomic markers, placed these bacteria in the *Sporomusa* sub-branch of the class *Clostridia* in the phylum *Firmicutes* (Helander, Haikara, Sadovskaya, Vinogradov, & Salkinoja-Salonen, 2004). The taxons responsible for ~30% of all spoilage incidents of finished and packaged beer worldwide are *Pectinatus* and *Megasphaera* (Vaughan et al., 2005).

There are three species in the *Pectinatus* genus associated with beer spoiling: *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis* and *Pectinatus haikarae*. *Pectinatus* mainly spoils unpasteurized beers by producing turbidity and large quantities of propionic acid, some acetic acid and sulphur compounds (H_2S, methyl mercaptan). The spoiled product smells like rotten eggs, making it unfit for consumption (Vaughan et al., 2005). Many factors control the growth of *Pectinatus* species in beer, including oxygen and ethanol levels and acidity. Although *P. frisingensis i*s more oxygen-tolerant than *P. cerevisiiphilus*, both species were able to grow at low oxygen levels present in beer (up to 0.3 mg/l) and wort (up to 0.96 mg/l) (Chowdhury, Watier, & Hornez, 1995). *Pectinatus frisingensis* grew well in commercial beers with 3.7–4.4% (w/v) alcohol, but not in strong beers (≥5.2%, w/v). *Pectinatus* species are the most acid-tolerant beer spoilers in the *Sporomusa* sub-branch (*P. frisingensis* grew optimally at pH 4.1–5.1). All species tolerate hop bitter acids at levels normally found in beer. *Pectinatus* species are mesophiles that grow at 15–40 °C with an optimum at 30–32 °C. Heat resistance studies indicated that the treatments applied in the brewing process are sufficient to inactivate *Pectinatus* cells (Suzuki, 2011).

The beer-spoilage group *Megasphaera* includes three species: *Megasphaera cerevisiae*, *Megasphaera paucivorans* and *Megasphaera sueciensis*. *Megasphaera* mainly affects unpasteurized low-alcohol beers by producing C5 and C6 fatty acids, H_2S and turbidity. The growth rate of *M. cerevisiae* is reduced above 2.1% (w/v) and pH below 4.1.

The growth temperature of this taxon ranges from 15 to 37 °C. *Megasphaera* is able to grow in beer with less than 0.3 mg/l of dissolved oxygen and its tolerance to hop bitter acids is comparable to that of *Pectinatus* (Back, 2005; Suzuki, 2011).

The occurrence of *Pectinatus* and *Megasphaera* is largely unknown outside the beer-brewing environment, while they appear to be common inhabitants in brewery bottling hall deposits (Suzuki, 2011). Therefore, the need for rapid detection and identification methods to prevent spoilage incidents is required. Multiplex polymerase chain reaction (PCR) methods have been developed to identify all six *Pectinatus* and *Megasphaera* species. In this method, multiple primer sets specific to these microorganisms are mixed in one single reaction tube, making the PCR procedure less laborious and time consuming (Iijima, Asano, Suzuki, Ogata, & Kitagawa, 2008). In accordance with this, the knowledge of the adhesiveness of *Pectinatus* and *Megasphaera* species to different solid materials (bottling machinery, conveyor belts, floor covering, etc.) could help to identify the risks associated with biofilm deposition. Moreover, investigation of the multispecies biofilm communities could help to create a true picture concerning the contamination mechanism and survival of anaerobic beer-spoiling bacteria in the environment of bottling halls.

20.4 New trends in fermentation

Fermentation and maturation are the most time-consuming steps in the production of beer. In a competitive environment such as the beer market, the potential time savings offered by shortened fermentation has always been an attractive challenge. Nevertheless, only time savings are not sufficient to promote a radical transition of the conventional technology. Capital investment, operating costs, space requirements, quality and marketing analysis are among the perspectives that have to provide additional justification of the intended change in fermentation technology.

Traditional beer fermentation and maturation processes use open fermentation and closed lager tanks. These vessels, previously been considered indispensable, have been replaced during the last decades in many breweries by large-scale fermenters (bioreactors) called cylindroconical vessels (CCVs) or tanks (Figure 20.2). These have proved to be successful both providing operating advantages and ensuring the quality of the final beer. In addition, the productivity of fermentation in CCVs was increased by high-gravity wort brewing, a technology requiring particularly the use of special brewer's yeast strains. Another promising technology, namely continuous beer fermentation using free or immobilized brewing yeast, by contrast, has found only a limited number of industrial applications. Nevertheless, continuous fermentation technology still has a potential to be exploited.

20.4.1 Improvements in fermentation vessels

Growing market competition and an effort to achieve greater profitability led the brewing companies to implement large fermentation vessels (CCVs), in which intensified fermentation and maturation processes take place (Figure 20.2). The CCVs

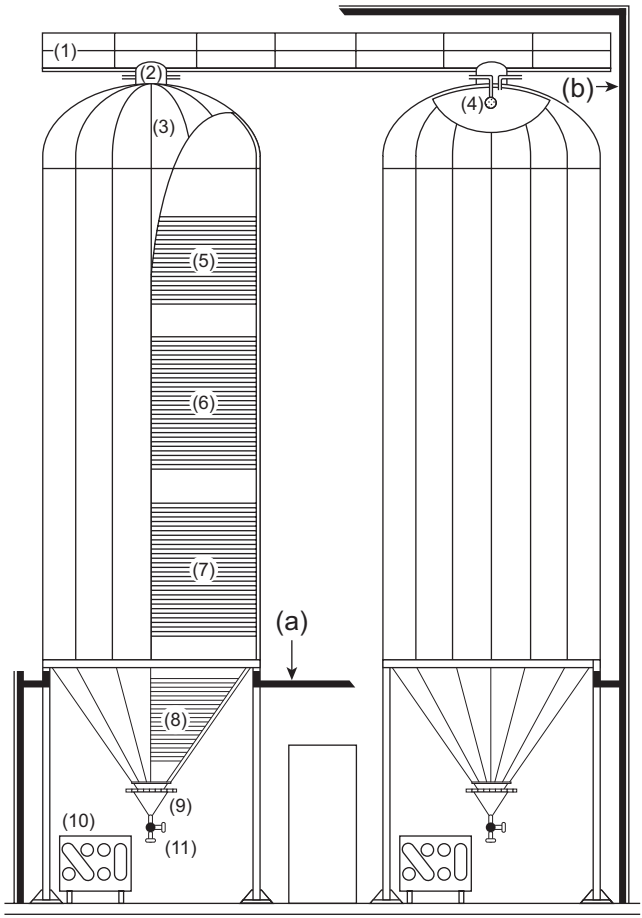

Figure 20.2 Installation of dual-purpose outdoor (a) and indoor (b) cylindroconical vessels (CCVs) suitable for fermentation and lagering. (1) Servicing platform. (2) Tank dome with fittings. (3) Insulation. (4) Cleaning in place inlet and spray ball. (5) Cooling zone for lagering. (6, 7) Cooling zones for fermentation. (8) Cone cooling zone. (9) Cone base. (10) Distribution panel with connection pipes. (11) Multi-way valve block.

are built with a cylindrical upper part and a cone-shaped lower part (angle 60–90°), which allows the yeast to be collected easily at the bottom. They are made of chrome nickel steel (304) with small surface roughness preventing bacterial colonization and allowing easy cleaning. At high chloride content in brewing liquors, more resistant steel (316) can be used (Briggs, Boulton, Brooks, & Stevens, 2004). CCVs are built almost in any size (100–6000 hl) with a corresponding number of cooling zones (one always in the cone). These large-scale fermenters have usually a very simple design, without any internal structures (agitators, baffles, spargers), but require reasonably swift filling, fermentation control (cooling) and cleaning as well as selection of yeast strains. However, the advantages of CCVs prevail and most breweries consider them

Table 20.1 Advantages and disadvantages of cylindroconical vessels (CCVs)

Advantages	Disadvantages
Lower construction costs and space requirement	Increased demands for the removal of trub since the collapsed foam cannot be collected
Reduced requirements for operation and maintenance (possibility of full automation and process control)	Increased demands on yeast resistance to stress (higher temperature and pressure)
Reduced beer losses	Prolonged filling with subsequent brews (wort) can lead to inhomogeneity of the batch and deterioration of beer quality
Reduced losses of bitter substances	
Reduced risk of contamination	
Increased utilization of amino acids	
Improved cooling possibilities	
Effective automatic cleaning and sanitation	
Efficient CO_2 collection and recovery	

indispensable (see Table 20.1). They not only provide operating advantages but also ensure the quality of the beer during fermentation and maturation (Kunze, 1996).

Beer fermentation is traditionally done without the use of mechanical agitation. Mixing in CCVs is provided mainly by the CO_2 evolved plus a contribution from convection as a result of cooling (Takamoto & Saito, 2003). However, there is very little CO_2 evolution at the start of the batch or at the end when the nutrients become depleted. In accordance with this, significant temperature, wort and yeast biomass heterogeneity have been noted in large-scale CCVs, particularly during the early phase of fermentation. For instance, yeast sedimentation began at a time when wort was only 60% attenuated, and more than about 90% had sedimented when some 25% fermentable sugars still remained. This suggests that the bulk of yeast was little involved in fermentation and diacetyl reduction (Boulton, Wilson, Peters, & Wright, 2005). In addition, due to the poor mixing, the temperature of yeast slurry in the cone of the CCVs was 2–3 °C higher than the set point, which can negatively affect yeast quality. The mechanical homogenization of CCV using a three-bladed impeller resulted in a 1.5-day shorter and more consistent fermentation time without damaging the viability of yeast (Boulton, Pricen, & Peters, 2007). However, the retrofitting of a mechanical stirrer in a CCV is expensive and may compromise sterility (Nienow, Nordkvist, & Boulton, 2011).

Mixing in a vessel can also be achieved by using a jet of the fluid in the tank. The rotary jet mixer (RJM) is a modified jet head used for cleaning-in-place (CIP) in breweries. It can therefore be easily installed by using the same pipeline and used for both mixing and CIP. In large-scale (5000 hl) high-alcohol lager fermentation with RJM (250 hl/h), there were a marked reduction in fermentation time (~50%), impressive

consistency of the batch time, lower levels of residual fermentable sugars and higher normalized yield of ethanol. In addition, sensorial analysis (400 consumers) found no difference between unstirred fermentations and those in which RJM was used (Nienow et al., 2011).

20.4.2 High-gravity and very-high-gravity wort fermentation

Today's need to produce good-quality beer in a short time and in the least expensive way has prompted many breweries to use high-gravity (HG, 16–18% (w/v)) or very high-gravity (VHG, >18% (w/v)) wort fermentation. (V)HG wort fermentation technology is a very simple method, allowing the brewery to increase its volumetric productivity (by up to 50% for VHG) and reduce production costs without significant investments required (Puligundla, Smogrovicova, Obulam, & Ko, 2011). While the use of HG wort has become common practice in many breweries, the use of VHG worts (20–25% (w/v)) has not been widely adopted by the brewing industry so far. The reason for this is that VHG wort fermentation has drawbacks related to yeast physiology (osmotic stress, increased ethanol and CO_2 concentration, nutrient limitation), which may reflect in disproportionately high production of esters, extended lag-phase duration, decreased foam stability, increased residual sugars in beer and reduced fermentation potential of yeast crops (Gibson, 2011; Piddocke, Kreisz, Heldt-Hansen, Nielsen, & Olsson, 2009). The industrial implementation of (V)HG wort fermentation requires optimization of process parameters (pitching rate, temperature), improvement in stress tolerance of yeast strains and/or improved fermentation performance through wort supplementation.

Increasing the wort concentration usually results in an increase in fermentation time. By an appropriate increase of the pitching rate and fermentation temperature, it is possible to achieve only a small increase in fermentation times. This forms the basis of (V)HG wort fermentation strategies, in which concentrated worts are fermented and subsequently diluted to sales gravity. One of the tools to improve the fermentation performance of (V)HG worts is to increase pitching rate. It was shown that it results in higher fermentation and ethanol production rates, somewhat higher concentration of fusel alcohols and increased diacetyl level in green beer (Nguyen & Viet Man, 2009; Verbelen et al., 2008).

The strategy of wort supplementation with metal ions, lipids and "yeast foods", with the aim to improve fermentation performance, is reviewed in detail by Gibson (2011). Among metal ions, zinc and magnesium were identified as elements with a crucial role in wort fermentation, the supplementation of which led to reduced attenuation time (Zn, Mg), increased uptake of trisaccharides (Zn) and increased ethanol production (Zn) and tolerance (Mg). It was also found that zinc has an indirect effect on the synthesis of higher alcohols and esters as well as on foam properties. Calcium supplementation of wort may only be beneficial when Ca concentration is limited in the local water supply (Gibson, 2011).

High-gravity worts usually require higher oxygen supply to ensure that the fermentation proceeds appropriately. Oxygen is essential for yeast growth and for unsaturated fatty acid (UFA) and sterol synthesis. Therefore, the supplementation with UFA

(oleic acid 18:1, linoleic acid 18:2) and ergosterol acts as a form of oxygen credit in the case of (V)HG worts, in which solubility of oxygen is limited (Gibson, 2011; Pham, Doan, & Le Van, 2010).

Increased C:N ratio as a result of high-gravity adjunct brewing often results in lower growth and amino acid uptake rates (Lekkas, Stewart, Hill, Taidi, & Hodgson, 2007). In these cases, improved fermentation performance, higher yeast viability and more complete sugar consumption can be achieved by supplementation with yeast extracts or extract-based yeast foods. However, the positive effect of yeast foods cannot be ascribed solely to increased availability of nitrogen, but the presence of vitamins and metals (Zn, Mg) may contribute as well (Gibson, 2011). The decision to include yeast food in wort will depend not only on the desired fermentation performance but also on the flavour profile of the final beer. Beer produced from 40% rice adjunct wort supplemented with yeast extract had sensorial quality comparable to all-malt beer (Le Van, Strehaiano, Nguyen, & Taillandier, 2001). Nutrient supplementation can also combine various compounds (yeast extract, ergosterol, oleic acid), as was shown by Dragone, Silva, Silva, and Lima (2003).

Improving the performance of individual brewer's strains by searching VHG-tolerant variants can use the approaches of evolutionary engineering in situations where genetically modified organisms are not desired (Cakar, Seker, Tamerler, Sonderegger, & Sauer, 2005). Mutagenized industrial yeasts were put through a VHG wort fermentation and then incubated anaerobically in the resulting beer while maintaining the maltose or maltotriose concentration at about 10–20 g/l until most of the cells had died. The survivors fermented VHG worts 10–30% faster and more completely, but the sedimentation behaviour and profiles of yeast-derived flavour compounds of the survivors were similar to those of the parent strains (Huuskonen et al., 2010). Breeding lager yeast spore clones with laboratory or ale yeast strains also enhanced the stress-resistance and fermentation profile of the resultant hybrid strains (Sanchez, Solodovnikova, & Wendland, 2012).

20.4.3 Continuous immobilized cell fermentation

The application of immobilized brewing yeasts for continuous beer fermentation and maturation is a challenging opportunity for the brewing industry. The attractiveness of the continuous beer fermentation system lies mostly in accelerated transformation of wort into beer. Immobilized yeast cell technology allows the production of beer to be accomplished in as little as 2–3 days. Nevertheless, the continuous mode of reactor operation, immobilization, ageing and mutation of immobilized cells provokes different physiological responses when compared to batch free-cell systems. Given the complexity of these systems, the alterations in yeast physiology often have an unpredictable impact on the flavour profile of the produced beers (Branyik, Vicente, Dostalek, & Teixeira, 2008; Willaert & Nedovic, 2006). Therefore, in spite of the economic advantages, the continuous process has found only a limited number of pilot-plant or industrial-scale applications.

When evaluating continuous beer fermentation with immobilized cells, a clear distinction has to be made between the processes of primary and secondary fermentation.

Primary fermentation is biochemically a rather complex process accompanied by intensive biomass growth, heat and carbon dioxide evolution. This imposes significant technical demands on the immobilized cell reactor design (homogeneous phase distribution, sufficient mass and heat transfer, removal of excess yeast and CO_2, prevention of clogging and channelling, etc.) Consequently, the large-scale applications of continuous immobilized cell systems for primary fermentation have not been permanently successful (Branyik, Vicente, Dostalek, & Teixeira, 2005). Two examples of large-scale use are briefly described in the following text; for further details, see Willaert and Nedovic (2006) or Mensour, Margaritis, Briens, Pilkington, and Russell (1997).

The Japanese Kirin Brewery developed a multistage system where the first stage (stirred-tank) is utilized for an aerobic fermentation (stimulates yeast growth and free amino nitrogen uptake), while the following stages (packed-bed reactors) are used for anaerobic fermentations (Yamauchi et al., 1994). Beer of acceptable quality was produced in this system within 3–5 days. It was operational at pilot-scale (100 hl) for 2 years in a restaurant brewery (Willaert & Nedovic, 2006).

Meura Delta, a Belgian brewing equipment manufacturer, developed a system based on a silicon carbide cartridge loop bioreactor for partial attenuation and a free-cell stirred-tank reactor for complete attenuation and flavour maturation. It is claimed to have an overall productivity of 350 hl of beer per hl of reactor volume per year (four times that of conventional CCV) and has been found to be stable over a period of more than 6 months. Two industrial-scale Meura systems have been installed in Belgium (Mensour et al., 1997).

The goal of secondary fermentation (maturation) is to balance the final beer flavour, especially to reduce diacetyl and its precursors by conversion into acetoin and 2,3-butanediol. During maturation, beer also reaches final attenuation, which is accompanied by a moderate cell growth comparing to primary fermentation. Secondary fermentation represents, from an engineering point of view, a simpler process, allowing the application of stationary particle reactors where the medium is passed either upwards or downwards through the bioreactor packed with immobilized yeast (Branyik et al., 2005).

The system developed by Cultor (Finland) is industrially available and has been operational at industrial scale (1 million hl per year) since 1993. This accelerated maturation system is based on heat treatment of green beer (10 min at 90 °C for complete conversion of α-acetolactate to diacetyl) followed by reduction of diacetyl to acetoin during maturation with a continuous immobilized yeast system operating at a retention time of 2 h (Pajunen, 1996). This packed-bed immobilized yeast bioreactor system has found industrial application also in continuous alcohol-free beer production (Mensour et al., 1997).

Although the volumetric productivity of traditional batch fermentation is lower than that of the continuous process, the traditional batch process still overwhelmingly prevails over continuous fermentation technology. The main goals of the current research on continuous beer fermentation using immobilized cell systems are to mimic the traditional physiological state of the brewing yeast in the continuous systems; to select (breed) brewer's yeast strains suited to continuous fermentation process; and to further reduce the investment costs, for instance by using cheap carrier materials such as wood chips of spent grains (Branyik et al., 2006; Linko et al., 1997).

20.5 New products: finding profitable niches

Our post-modern era is characterized by rapid changes in almost all fields of life. Although traditional beer types still dominate the market, the trend of this era also pushes breweries to the development of novel products. The motivation is on both sides: customers usually like to discover novelties, while producers compete with each other for customers inter alia by offering new taste and quality enhancement. In recent years, the sales of low-alcohol and alcohol-free beers increased significantly. Although other special beer products (enriched beers, gluten-free beer, ice beer or beer-based mixed drinks) still have a low significance in the overall market, they have an interesting potential. These novel products also require particular technologies for their production.

20.5.1 Alcohol-free beer

In recent years, the consumption of alcohol-free beer (AFB) has been rising significantly due to the fact that it represents an alternative to standard soft drinks. People can also enjoy a refreshing beer taste in situations when drinking alcoholic beverages is restricted (during working time, driving, medical treatment, pregnancy and breast-feeding or for religious reasons) (Branyik, Silva, et al., 2012; Burberg & Zarnkow, 2009). The legally admitted maximum ethanol content in AFB is 0.5% by volume or 3.945 g/l in the majority of countries. There are several technologies that enable the production of beer with such low ethanol contents. One possibility is based on the gentle removal of alcohol from the normally fermented alcoholic beer by thermal methods (e.g. vacuum distillation) or using membrane separation techniques (i.e. reverse osmosis, dialysis). However, the simplest approach lies in limited or interrupted fermentation with suppressed alcohol formation, which can be reached using a combination of several strategies.

Decreasing the original gravity of wort (5–8% (w/w)) and altering the mashing process directly influence the concentration of fermentable sugars in wort, which in turn determines the alcohol level in beer (Branyik, Silva, et al., 2012; Burberg & Zarnkow, 2009). Lower sugar content in wort can be achieved by inactivation of the starch-saccharifying enzyme β-amylase by high mashing temperature (75–80 °C) (Muller, 2000).

Fermentation itself can be stopped when the desired alcohol content is reached, either by removal of yeast (filtration, centrifugation) or by their deactivation (pasteurization). A further possibility is to create fermentation conditions, which suppress yeast metabolism (Narziss, Miedaner, Kern, & Leibhard, 1992). The most practical tool to suppress yeast metabolism is low temperature. This method is called the "cold contact process (CCP)", which ensures very slow ethanol production; while other metabolic processes, such as formation of higher alcohols and esters or reduction of carbonyls, may exhibit moderate activities (Perpete & Collin, 1999). After interrupting the fermentation at an alcohol content less than 0.5% vol., the AFB is usually matured for at least 10 days at 0–1 °C to enrich flavour and improve the colloidal stability. Then the product is filtered, carbonated, stabilized and sterilized (Branyik, Silva, et al., 2012; Burberg & Zarnkow, 2009).

For the production of AFB, special yeasts that produce lower amounts of ethanol or no ethanol at all can be used as well. This can be achieved either by selection of proper yeast strains that are not able to ferment maltose and maltotriose (e.g. *Saccharomycodes ludwigii*) or by intentional modification of brewing yeast by random mutation or genetic engineering (Branyik, Silva, et al., 2012; Burberg & Zarnkow, 2009).

The quality of the final product depends on the selected procedure. Thermal processes represent some risk of thermal damage and loss of aroma, body and carbonation. Using membrane dealcoholization leads to AFB with fuller and better taste; however, losses of harmony and body were also registered (Branyik, Silva, et al., 2012; Burberg & Zarnkow, 2009). Limited or interrupted fermentation produces AFB with a high fraction of non-fermented residual extract that causes a sweet and worty character of the product. A number of additional techniques were developed to improve the flavour impression, such as addition of dark or pale caramel malt, wort dilution after instead of before wort boiling, acidification with acid malt or lactic acid (Narziss et al., 1992), elimination of oxidized malt substances by addition of ascorbic acid and washing out undesirable volatiles (e.g. sulphur compounds, carbonyls) with CO_2 or N_2 (Montanari, Marconi, Mayer, & Fantozzi, 2009). The special yeast strains that are unable to ferment maltose and maltotriose give rather sweet beers; however, the typical worty flavour of AFB is masked due to an elevated formation of higher alcohols and esters especially when wort acidification is ensured (Narziss et al., 1992).

20.5.2 Special beers: enriched beers

Enriched beers are usually understood as highly hopped beers, but this indication can include increased levels of other substances such as polyphenols, vitamins or various minerals (Brohan, Jerkovic, & Collin, 2011; Jeney-Nagymate & Fodor, 2007; Shindo, 2003; Stacey & Sullivan, 2003; Wunderlich, Zurcher, & Back, 2005; Yonkova, Surleva, & Ginova-Stoyanova, 2012). Usually the beers are enriched with those components that have a positive impact on the consumer's health, thereby increasing the attractiveness of the product.

In terms of polyphenols, a number of microbreweries in the Akita region in the north of Japan have released a range of polyphenol-enriched beers (Shindo, 2003), while Brohan et al. (2011) suggested a use of red sorghum for production of stilbenoid-enriched beers. However, the most promising are beers with an elevated content of xanthohumol (XN), a prenylated chalcone. XN has been recently found as a very attractive hop substance on account of its wide variety of biological effects that are presently frequently being studied by medical researchers as well as by brewing experts (Colgate, Miranda, Stevens, Bray, & Ho, 2007; Guerreiro et al., 2007; Wang, Ding, Liu, & Zheng, 2004; Wunderlich, 2007). Although XN is the main prenylflavonoid of hops (0.2–1.1%, (w/w)), standard beers contain very low concentrations (<0.2 mg/l) of this phenolic compound. The reason is a loss of large amounts of XN during conventional brewing. During wort boiling, XN is isomerized into isoXN, adsorbed on trub and/or insufficiently extracted from hop. Further losses occur during fermentation, filtration and stabilization (especially with PVPP) (Burberg & Zarnkow, 2009; Magalhaes, Dostalek, Cruz, Guido, & Barros, 2008; Wunderlich, 2007;

Wunderlich et al., 2005). Hence, it is necessary to introduce a special brewing procedure in order to obtain XN-enriched beer. Adjustment of technology basically lies in a very late and high dosage of hop rich in XN and in rapid cooling of wort. A wort temperature below 80 °C causes inhibition of the XN isomerization (Burberg & Zarnkow, 2009; Wunderlich, 2007; Wunderlich et al., 2005). This technology leads to increased concentration of XN up to 3 mg/l in pale unfiltered beer (Wunderlich, 2007). However, using roasted malt makes it possible to achieve even higher XN levels in beer, above 10 mg/l (Burberg & Zarnkow, 2009; Magalhaes et al., 2008; Wunderlich, 2007). It was found that the roasting process generates substances that prevent the XN isomerization and thus support its transfer from hop to beer (Walker, Lence, & Biendl, 2003; Wunderlich et al., 2005).

Alternative strategies to increase the nutritive value of beer have also been presented. Jeney-Nagymate and Fodor (2007) studied beer fortification/enrichment with vitamins E and C, the antioxidant properties of which are valuable both for the consumer's health as well as for the improvement of beer shelf life. Similarly, thiamin may be added to beer in high concentrations (up to 27 mg/100 ml of beer) without negative sensory influence (Stacey & Sullivan, 2003). Further, several studies on beer enriched with various minerals were reported, e.g. fluoride-enriched beer was designed to have radioactive decontaminative properties (Yonkova et al., 2012); in selenium-enriched beer, selenium bounded in organic substances is suggested to be valuable for the human body (Sanchez-Martinez et al., 2012).

Bioactive/functional properties of beer can be improved by using unusual raw materials such as medical mushroom *Ganoderma lucidum* (Leskosek-Cukalovic et al., 2010) or algae (Branyik, Bittner, & Jung, 2012).

20.5.3 Beers designated for consumers with specific health requirements

Normal beer contains some components to which a significant percentage of the population is sensitive, intolerant or allergic. Hence, these people cannot drink beer produced by traditional technology. Gluten-free beers present an option for people who suffer from celiac disease (i.e. intolerance to gluten). Standard beer is widely produced from barley or wheat, which are sources of gluten. Nowadays, the most discussed, studied and used strategy of gluten-free beer production is the use of gluten-free starchy materials such as sorghum, millets, rice and maize, or the pseudocereals amaranth, buckwheat and quinoa (Agu et al., 2012; Burberg & Zarnkow, 2009; Chiba et al., 2012; De Meo et al., 2011; Russell, 2009; Zweytick & Berghofer, 2009). The alternative raw grain can be used in malted or unmalted form. Unmalted raw grain does not contain sufficient amounts of amylolytic enzymes; thus the addition of exogenous enzymes has to be considered (Burberg & Zarnkow, 2009; Zweytick & Berghofer, 2009).

Other health requirements are associated with carbohydrates and their reduced content in beer, which led to products such as low-carb or light beers. These products are designated for people who are on a slimming or other diet or for diabetics (Burberg & Zarnkow, 2009). Production of beer with a low content of carbohydrates requires

several technological peculiarities. The typical one is a very high degree of attenuation, which leads to a higher ethanol production. Therefore an alcohol reduction or blending with brewing water usually follows after the brewing process. The complete attenuation can be achieved by the mashing process, which directly influences the concentration of fermentable sugars in wort. Thus, very low mashing-in temperatures and longer rests are used during wort production to support the activity of both amylolytic enzymes. Alternatively, exogenous enzymes might be added during mashing (Briggs et al., 2004; Burberg & Zarnkow, 2009).

20.6 Beer in relation to nutrition and health

Beer is a complex mixture consisting of hundreds of different compounds (more than 450 constituents have been characterized). Some of these originate from raw materials; others are formed during the brewing process (Briggs et al., 2004; Preedy, 2009). Basically, beer is composed of water, ethanol and residual extract, which includes approximately 75–85% carbohydrates, 6–9% nitrogenous compounds, inorganic compounds, phenolic compounds, bitter substances and also other compounds that, despite their low concentration, can have an important impact on the sensorial properties, nutritional value and health beneficial effects (see Table 20.2) of the final beer (Preedy, 2009). In general, different beers contain different proportions of the same constituents depending on the quality and amount of raw materials used and production technology (Bamforth, 2002; Briggs et al., 2004).

Water comprises the major beer component, which forms more than 90% of the final product (Preedy, 2009) and the medium that extracts and dissolves constituents from the raw materials (Briggs et al., 2004). The mineral composition of brewing water substantially influences sensorial and nutritional characteristics of the final product (Preedy, 2009).

Ethanol content of beer ranges between less than 0.05% (v/v) up to about 12.5% (v/v), however, most beers contain 3–6% (v/v). In recent years, numerous research papers have been published dealing with health benefits of moderate drinking of alcoholic beverage, including beer. In this context, a frequently discussed topic is the reduction of the risk of cardiovascular diseases by ethanol (Russell, 2006) and the influence of moderate alcohol consumption on decreasing the stress load (Bamforth, 2002). Moreover, there are evidences that moderate alcohol consumption may be associated with (1) better cognitive function in old age (Cervilla, Prince, Joels, Lovestone, & Mann, 2000), (2) improved lipoprotein metabolism (Walzl, 2009) and (3) protection against infection by *Helicobacter pylori*, which causes inflammation and other disorders of the gastrointestinal tract (Brenner, Rothenbacher, Bode, & Adler, 1999; Walzl, 2009). Bamforth (2002), in a review, critically discusses many medical research papers dealing with the pros and cons of moderate alcohol drinking.

Conventionally fermented beers may contain about 25% of the original starch in non-fermentable form (dextrins) and only low levels of fermentable sugars (i.e. glucose, fructose, sucrose, maltose and maltotriose). The total saccharide content of beers ranges between 0.89% and 5.98% (w/v) expressed as glucose (Bamforth, 2002; Briggs et al., 2004).

Table 20.2 An overview of the health-beneficial effects of beer and bioactive beer constituents in moderate drinking

Health benefits	Beer/bioactive beer constituents	References
Reduced risk of cardiovascular diseases	Ethanol Phenolic compounds Bitter compounds B vitamins	Mayer Simon & Rosolova, (2001) Preedy (2009) Russell (2006)
Anticancer activities	Prenylflavonoids	Preedy (2009) Walzl (2009)
Regulation of blood glucose levels	Beer	Preedy (2009)
Improvement in lipoprotein metabolism	Ethanol	Walzl (2009)
Stimulation of gastric acid secretion	Non-alcoholic components	Preedy (2009)
Protection against infection caused by *Helicobacter pylori* (gastrointestinal tract disorders)	Ethanol	Brenner et al. (1999) Walzl (2009)
Prophylactic effect against the formation of kidney stones	Beer	Borghi et al. (1999) Krieger et al. (1996) Preedy (2009) Walzl (2009)
Prevention of Alzheimer's disease	Beer	Gonzalez-Munoz et al. (2008)
Lower risk of the development of Parkinson's disease	Beer	Hernan, Chen, Schwarzschild, & Ascherio, (2003) Walzl (2009)
Psychosomatic effects (decreasing stress, supporting relaxation)	Ethanol Hop compounds	Bamforth (2002) Preedy (2009) Russell (2006)
Stimulation of cognitive function in old age	Ethanol	Cervilla et al. (2000) Dufouil, Ducimetiere, & Alperovitch, (1997) Walzl (2009)
Sedative and hypnotic effect	Bitter hop compounds	Bamforth (2002) Preedy (2009)
Phytoestrogenic properties	Isoflavonoids	Bamforth (2002) Preedy (2009)
Antioxidant effects	Polyphenols Maillard compounds Ascorbic acid	Preedy (2009)
Isotonic drink	Beer	Walzl (2009)
Source of minerals (e.g. potassium, magnesium, selenium, dietary silicon)	Minerals	Bamforth (2002) Preedy (2009) Walzl (2009)
Source of soluble fibre	Beer	Preedy (2009) Walzl (2009)

For comparison, fully attenuated low-carbohydrate beers designed for diabetic patients contain 0.4–0.9% (w/v) carbohydrates (Briggs et al., 2004).

Beer is well known as a source of several vitamins, in particular B vitamins. Nevertheless thiamine (B_1) content is quite low (Bamforth, 2002) and this relative thiamin deficiency can lead to stimulation of alcohol consumption (Forsander, 1998). Some beers may contain considerable level of ascorbic acid (vitamin C), particularly if it was used during beer production as an antioxidant agent (Preedy, 2009).

The major ions in beers are the potassium, sodium, calcium and magnesium cations and the chloride, sulphate, nitrate and phosphate anions (Briggs et al., 2004). The higher ratio of potassium to sodium (typically 4:1 in beers) is favourable to human health and consistent with a low-sodium diet. This ratio provides a significantly greater diuretic effect of beer in comparison with water. The excess of nitrate anions is undesirable due to the risk of their reduction and subsequent reaction with amines to form carcinogenic *N*-nitrosamines (Briggs et al., 2004). Beer is frequently cited as a significant source of selenium, a trace element nutrient for humans (Bamforth, 2002). Similarly, beer is a rich source of dietary silicon (Preedy, 2009), which affects the uptake of aluminium in the digestive tract and decelerates its accumulation in the body. Thus moderate beer drinking is considered as a protective factor in preventing Alzheimer's disease (Gonzalez-Munoz, Pena, & Meseguer, 2008). Further, yeasts are a source of a chromium-containing complex that is associated with regulation of blood glucose levels (Preedy, 2009).

Beer contains several nitrogenous compounds such as amino acids, peptides and polypeptides, proteins, nucleic acid fragments, amines and heterocyclic compounds, the total concentration of which ranges from 0.3 to 1.0 g/l. These substances have a wide range of nutritional, regulatory and immunostimulatory effects. In terms of biogenic amines, it is necessary to point out that patients who are treated for depression with monoamine oxidase inhibitors must not drink beer because of the risk of accumulating toxic levels of tyramine (Briggs et al., 2004; Preedy, 2009). Further, secondary amines of beer, such as dimethylamine, can react with oxides of nitrogen to form carcinogenic *N*-nitrosamines, as mentioned above.

A number of various polyphenols that originate either from malt (two-thirds) or from hop (one-third) have been found in beer (Preedy, 2009). They serve as very valuable antioxidants and may help to protect against coronary heart diseases (Bamforth, 2002). For example, it has been proved that ferulic acid, ranging in beer between 0.52 and 2.36 mg/l, is readily assimilated by the human body (Bourne, Paganga, Baxter, Hughes, & Rice-Evans, 2000; Preedy, 2009). A noteworthy group of phenolic compounds are also isoflavonoids, thanks to their phytoestrogenic and other health-promoting properties. However, it was found that beer contains low levels of these substances; thus, the risk of adverse modification of the hormonal status of men is negligible (Bamforth, 2002).

Beer is considered to be particularly valuable also due to hop-bittering compounds, to which sedative and hypnotic effects were attributed (Bamforth, 2002).

The nutritional value of the foods and their impact on human diet and health is a general topic reflected in the lifestyle of many people (Tepper, Choi, & Nayga,

1997). This trend also concerns alcoholic beverages including beer (Wright, Bruhn, Heymann, & Bamforth, 2008). Moreover, beer – due to the fact that it is one of the most widely consumed alcoholic beverages worldwide – has come into the focus of medical research and social surveys (Preedy, 2009; Walzl, 2009). The main conclusion of extensive research is that drinking moderate amounts of beer has positive effects on human health and contributes to a longer life (Walzl, 2009). It is also noteworthy that moderate beer consumption has been found to have health-positive effects comparable to those of red wines (Walzl, 2009; Wright et al., 2008).

The energetic value of beer is calculated from its ethanol, carbohydrate and protein contents (Briggs et al., 2004). It is substantial to note that ethanol has a significantly higher energy contribution than saccharides, i.e. 7 kcal/g and 3.75 kcal/g, respectively. Hence the beers with reduced levels of saccharides may not necessarily have lower energy values (Bamforth, 2002; Briggs et al., 2004; Preedy, 2009). Since beer contains only trace amounts of lipids, their energy value is insignificant (Preedy, 2009). Beer is an isotonic drink that replenishes water, which supplies valuable energy through readily available carbohydrates, plus vitamins and minerals (Walzl, 2009). The alcohol-free beers also claim beneficial effects of healthy beer components with a simultaneous effect of the lower energy intake and complete absence of negative impacts of alcohol consumption (Branyik, Silva, et al., 2012).

20.7 Future trends

Breweries are constantly trying to improve the efficiency of the production process while trying to preserve or improve the quality of the product. At the same time, they are developing new products with other flavours or with enhanced sensorial characteristics. A promising direction for the development of new products appears to be the use of different non-traditional yeast strains able to enrich the beers with new flavours with added value. These yeast strains can be combined with traditional brewer's yeast and used at different stages of the fermentation process. Simultaneously with improving the sensorial properties, some non-traditional yeasts (e.g. *Saccharomyces boulardii*) would also be able to increase the nutritional value of low-alcohol beers in particular.

In the engineering field of the brewing industry, a significant breakthrough could be achieved by successful application of continuous wort manufacturing and fermentation. However, this is not on the actual agenda, and therefore the engineering progress in brewing will rather manifest in the accuracy/reliability of measuring and control devices, extensive data acquisition and automation (typical for large breweries) as well as further decrease in energy/water consumption.

Acknowledgement

The authors would like to thank the Grant Agency of the Czech Republic (project GAČR P503/12/1424) for financial support.

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Coffee: fermentation and microbiota

21

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21.1 Introduction

The name "coffee" is derived from the word *Kaffa*, which is the name of an Ethiopian province. Wild coffee plants occur in various regions distributed across the African continent, which emphasizes the fact that coffee is native for Africa. From Ethiopia coffee was brought to Arabia, where coffee beans were roasted and brewed for the first time. Coffee, called "gahwah", was a popular drink in the Arabian world in the fifteenth century. From Arabia, coffee was brought to Italy and from there to Western Europe, so that coffee became a very popular drink in Europe and was consumed in the so-called coffee houses. The origin of coffee industry can be traced to the Dutch, who were the first people who cultivated coffee plantations during the seventeenth century (Schillinger, Ban-Koffi, & Franz, 2010). The coffee plant belongs to the family *Rubiaceae,* also called "coffee family". This family belongs to the angiosperms and comprises about 611 genera and more than 13,000 species (Stevens, 2001). The coffee plant is a woody perennial evergreen shrub or small tree (Figure 21.1). Although more than 100 different species of the genus *Coffea* are known, the most popular species are *Coffea arabica*, *Coffea canephora* var. *robusta*, *Coffea liberica* and *Coffea excelsa*, but only *C. arabica* (Arabica coffee) and *C. canephora* var. *robusta* (Robusta coffee) are used for the majority of coffee production, accounting for 56% and 44%, respectively, of the world's production in 2011, reaching 134 million bags (Cagliani, Pellegrino, Giugno, & Consonni, 2013). *Coffea arabica* is typically cultivated in highland regions with an altitude ranging from 600 to 2400 m and has a sweety, fruity aroma. Thus *C. arabica* is mainly native to the highlands of southwestern Ethiopia with additional populations in south Sudan (Boma Plateau) and north Kenya (Mount Marsabit), whereas *C. canephora* is planted in flat (low-lying) regions, has a more strong cocoa flavour and is more resistant to disease. *Coffea canephora* grows in certain swamp areas, but it also particularly grows in western Africa, from Liberia to Sudan and Uganda, with high genetic diversity in the Democratic Republic of the Congo (Mishra & Slater, 2012). The production of Robusta coffee can be easily mechanized, which leads to cheaper prices. The price gap between the Arabica and Robusta coffee has significantly widened within the last years (Cagliani et al., 2013). While it would be desirable to combine their genetic traits for robustness and quality, traditional plant breeding techniques to accomplish this have been largely unsuccessful, since *C. arabica* is tetraploid whereas *C. canephora* is diploid (De Los Santos-Briones & Hernández-Sotomayor, 2006).

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00021-9

Figure 21.1 Coffee tree with ripe cherries.
Photo courtesy of Dr Louis Ban-Koffi.

Coffee plants are cultivated in more than 80 countries around the world, with 70% being produced by smallholder farmers (De Los Santos-Briones & Hernández-Sotomayor, 2006). More than 100 million people in the coffee-growing areas worldwide derive their income directly or indirectly from coffee production (Mishra & Slater, 2012). Brazil is the largest producer and exporter of *C. arabica* (ABIC, 2010; Silva et al., 2013), followed by Colombia, Paraguay, Venezuela, Indonesia, Ethiopia, India and Mexico. Coffee plantations cover about 10.6 million ha of land, mostly in the tropics (Clay, 2004), and the coffee trade has an annual turnover of close to 10 billion US$, making it the second most important commodity traded in the world next to petroleum (Silva et al., 2013).

21.2 Coffee processing

Coffee fruits are the so-called drupes, with a pulpy mesocarp and lignified endocarp (De Castro & Marraccini, 2006). Two coffee seeds (beans) are located within the drupes, being the most inner part of the coffee berry. They are surrounded by the silver skin, followed by a hard cover, the pericarp, which consists of the endocarp (parchment), mesocarp (pulp) and exocarp (skin). The mesocarp is also called mucilage and consists of sticky, sugar-like substances. The exocarp is usually dark when the coffee cherries are ripe. The colour of the berries thus changes during the 12-month ripening period from green to yellow and then red. The ripe coffee berries can either be harvested selectively by hand, or the whole amount of berries can be harvested at one

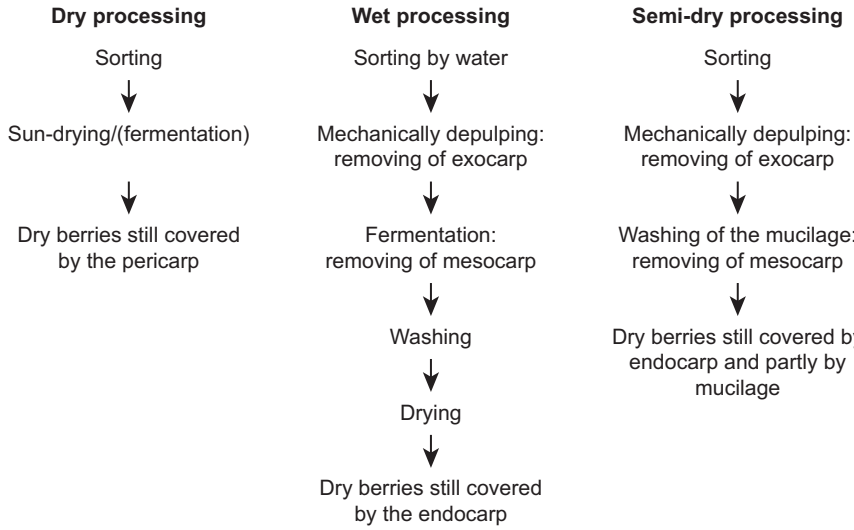

Figure 21.2 Three different processing methods of coffee berries: dry, wet and semi-dry processing.

time, often mechanically. These two possibilities result in distinct differences in quality. Depending on the quality of the coffee, the berries are sorted after harvesting to select unripe cherries. The presence of unripe cherries during the processing may lead to increased stringency (Clarke, 1987). The quality of coffee beans can be improved by sorting ripe and unripe fruits before processing. However, in order to minimize costs, most producers harvest all berries (ripe and unripe) at once. It is quite a common habit that all berries are processed and dried together, after which the beans for the high-quality coffee are sorted out. The beans of lesser quality are then mixed together with other low-quality beans and sold to reduce the economic loss of the producer (da Silva Dias et al., 2012).

The coffee fruits have to be processed to remove the pulp in order to obtain the green coffee beans, which are then dried and roasted. The processing can be done using three different methods (dry, wet or semi-dry) to obtain the seeds (Figure 21.2). The difference between the three distinct processing methods is not only the performance, but also the resulting products (parchment beans with and without leftovers of mucilage or beans still covered by the complete pericarp). The easiest and oldest way is *dry processing*, which results in the so-called unwashed or natural coffee (Silva, Batista, Abreu, Dias, & Schwan, 2008). This method is often used in countries with less rainfall and long periods of sunshine (Silva et al., 2008), e.g. in Brazil (Vilela, Pereira, Ferreira Silva, Batista, & Schwan, 2010), Ethiopia, Haiti, Indonesia and Paraguay (Silva et al., 2008). Dry processing is without microbial fermentation and is generally used for Robusta coffee (Silva, Schwan, Dias, & Wheals, 2000).

For dry processing, the berries are often left on the coffee plants until overripe before harvesting. The berries are then dried in the sun to a moisture level of about 10–11%. Alternatively, the berries are dried directly after harvest by spreading in a

Figure 21.3 Coffee drying.
Photo courtesy of Dr Louis Ban-Koffi.

~10 cm thick layer on the ground during the day and heaping together for the night (Figure 21.3). This process is a combination of fermentation and drying, which lasts about 10–25 days, depending on weather conditions (Mutua, 2000; Silva et al., 2000; Sakwari et al., 2013). After the dry processing, the berries are dry, leathery fruits still covered by the pericarp and are mechanically peeled. The dry method is used to produce cheaper coffee brands. In the *wet method*, which leads to a coffee of higher quality, the berries are fermented. Using the wet method, the coffee cherries are sorted by dumping into water, in which the unripe fruits float and are removed, whereas the ripe cherries sink to the bottom. After this, the cherries are mechanically depulped to remove the exocarp, while the mesocarp is removed by submerged fermentation for 12–36 h, followed by washing and drying for 5–10 days, depending on weather conditions (Batista et al., 2009; Sakwari et al., 2013). The remaining moisture content of the dried beans is 12% (Silva et al., 2000). Wet-processed coffee cherries are also called washed-coffee cherries in contrast to dry-processed ones, which are not washed. The resulting beans after wet processing are covered by a crumbling parchment skin.

The third method, the *semi-dry method* or "pulped natural" method, is a hybrid technique of wet and dry processing, in which the berries are mechanically depulped, followed by drying, while the beans are still covered partly by mucilage. Here, the fermentation step is omitted (da Silva Dias et al., 2012; Vilela et al., 2010). The beans still contain a parchment skin and leftovers of the mucilage. Using the wet or semi-dry processing seems to reduce the negative effects of the presence of unripe beans, because the drying is more uniform with less black, green and sour beans, producing

Figure 21.4 Hulled, green coffee beans.
Photo courtesy of Dr Louis Ban-Koffi.

coffee with a higher commercial value (Borem, 2008). After dry, wet or semi-dry processing the beans are hulled, which removes the hard pericarp, the remaining mucilage or the parchment skin to obtain green coffee beans (Figure 21.4). Processing of Arabica coffee using the wet method is done in Brazil, Colombia, Central America and Hawaii (Schwan & Wheals, 2003; Silva et al., 2000). Wet-processed Robusta coffee is produced only in a few countries (India, Ivory Coast, Vietnam, Indonesia and Uganda), and the product represents a specific niche in the international market (Velmourougane, 2013). Coffee quality is not only influenced by the presence of unripe green beans but also by the presence of other defective beans, e.g. brown or black ones, which are overmature and shrivelled, sour or damaged by insects and by the presence of extraneous material (e.g. stones, twigs) (Oliveira, Franca, Mendonça, & Barros-Junior, 2006). The New York Coffee and Sugar Exchange devised a type of classification in which coffee beans are classified into types of different quality depending on the number of defects per 300 g coffee sample (Oliveira et al., 2006).

21.3 The microbiology of coffee fermentation

21.3.1 Microbiota present in coffee fermentations

The microbiota present during the fermentation of coffee beans is dependent on the variety of the plant and bean moisture, processing method, competition of substrates, enzymatic capacity of the colonizing species, their antimicrobial activity and

environmental factors (e.g. humidity, temperature and microbiota of the soil) (Batista et al., 2009; Silva et al., 2008; Vilela et al., 2010). The most important change during the coffee fermentation is the breakdown of pectin by the microbiota and the concomitant production of organic acids (Silva et al., 2000). A too long a fermentation time, due to unsuitable environmental circumstances, e.g. lack of sun or high humidity, may favour the growth of spoilage microorganisms and mycotoxin-producing fungi (Silva et al., 2000). Differences in the microbiota during wet processing and dry processing exist, and the microbiota associated with dry processing are much more variable and complex than those associated with wet processing (Silva et al., 2008). Generally, the fermentation microbiota are represented by a succession of bacteria, yeast and filamentous fungi (Silva et al., 2008). The numbers of bacteria involved in the fermentations vary from 10^4 to 10^9 CFU/cherry (Silva et al., 2000) and the microbiota involved are quite diverse. Hundreds of strains of bacteria, yeasts and fungi can be isolated from a coffee fermentation. In the past, several new species and genera could be detected in ecosystems associated with coffee. For example, the new lactic acid bacterial species *Leuconostoc holzapfelii* (De Bruyne et al., 2007) and the two novel acetic acid bacterial species *Gluconacetobacter johannae* and *Gluconacetobacter azotocaptans* (Fuentes-Ramirez et al., 2001) were isolated from coffee and coffee fermentations. New filamentous fungi were also isolated from ecosystems association with coffee, e.g. *Penicillium brocae*, which was isolated from the coffee berry borer in Mexico (Peterson et al., 2003), and *Aspergillus sclerotiicarbonarius* and *Aspergillus aculeatinus* (Nooim et al., 2008) from Thai coffee beans. It can be expected that in the future even more new species, and possibly also genera, will be discovered from coffee fermentations. A succession study of Silva et al. (2008) showed that more than 78% of microorganisms present during a dry-processed coffee fermentation were represented by bacteria, first Gram-negative, then Gram-positive from day 0 until day 6, whereas yeasts and filamentous fungi were present at numbers below 20% in this time period. The Gram-negative bacteria present in the initial stages of coffee fermentation belong to the *Enterobacteriaceae,* and in wet coffee fermentations from Ethiopia *Enterobacter cloacae*, *Klebsiella oxytoca* and *Hafnia alvei* were common isolates (Holzapfel & Müller, 2007). In the wet coffee fermentations from Ethiopia, the bacterial counts increased from an initial 10^2–10^3 CFU/g up to 10^8 CFU/g in the first 24 h. Lactic acid bacteria (LAB) became predominant and decreased the pH of the fermenting material, thus preventing the growth of spoilage bacteria (Holzapfel & Müller, 2007). This was clearly different from the Brazilian fermentation, where LAB did not predominate. In the early stages of Ethiopian coffee fermentation, the genera of *Leuconostoc* and *Weissella* were present in high numbers, while homofermentative and heterofermentative lactobacilli, as well as enterococci, were also isolated from the fermentation. Among these heterofermentative cocci, the genera *Leuconostoc mesenteroides*, *Leuconostoc citreum*, *Leuconostoc pseudomesenteroides*, *Weissella cibaria* and *Weissella soli* could be identified (Schillinger et al., 2008), while a novel species, *Leuc. holzapfelii* could be newly described (De Bruyne et al., 2007). *Lactobacillus plantarum* and *Lactobacillus brevis* isolates predominated among the lactobacilli isolates (Holzapfel & Müller, 2007). From days 8 to 14 (Silva et al., 2008), the numbers of bacteria decreased from less than 50% to ~10%, whereas the numbers of yeasts

increased to nearly 80%. Yeasts occur at high numbers during coffee fermentation, with total counts between 4×10^4 and 5×10^7 CFU/g having been reported in the wet processing of *C. arabica* in Tanzania, with the numbers increasing with the time of fermentation (Masoud, Cesar, Jespersen, & Jakobsen, 2004).

The median value of microorganisms present on coffee cherries processed by the dry method in Brazil was log 7.2 CFU/cherry (Silva et al., 2000). In the study of Silva et al. (2000), a total of 754 strains of bacteria, yeasts and fungi were isolated and identified to at least genus level. Bacteria, filamentous fungi and yeasts represented 43.8%, 40.1% and 16.1%, respectively. The most abundant group (46.5%, 118 isolates) of bacteria present was the group of gram-positive, non-spore formers, containing species of the genera *Cellulomonas*, *Arthrobacter*, *Microbacterium*, *Brochothrix*, *Dermabacter* and *Lactobacillus*. Silva et al. (2008) reported a lower incidence of *Enterobacteriaceae* ($<10^4$ CFU/g) when compared to spore-forming gram-positive bacteria during the initial stage of dry coffee fermentation in Brazil. Approximately 80% of the latter belonged to the genus *Bacillus* (predominantly *Bacillus polymyxa* and *Bacillus subtilis*), while among the *Enterobacteriaceae* the genera *Enterobacter* and *Serratia* dominated. Microorganisms belonging to the genera *Pseudomonas*, *Klebsiella* and *Acinetobacter* were also isolated (Silva et al., 2008). The most frequently isolated yeast genera determined by Silva et al. (2000, 2008) were *Pichia*, *Debaryomyces*, *Candida*, *Arxula* and *Saccharomycopsis*, and the most frequently isolated and characterized fungi included the genera *Cladosporium*, *Fusarium*, *Penicillium*, *Aspergillus*, *Pestalotia* and *Paecelomyces*. An interesting result of the study of Silva et al. (2008) was that the number of fungi was inversely proportional to the number of yeasts; for example, the yeast *Debaryomyces hansenii* supported a decrease of the fungal population. *D. hansenii* may play a role in preventing degradation of stored fruits and grains (Payne & Bruce, 2001).

21.3.2 Enzyme activities of the fermentation microbiota

The fermentation of pectinaceous sugars by the microbiota of coffee fermentation leads to the production of ethanol and acetic, lactic, butyric and other higher carboxylic acids (Silva et al., 2000). Cellulolytic *Bacillus* species produce a variety of extracellular enzymes, which contribute to the degradation of cellulose and pectin (Coughlan & Mayer, 1991), that are present in the skin, pulp and mucilage of the coffee berries (Silva et al., 2008). Silva et al. (2008) showed that none of the bacteria isolated from dry-processed coffee fermentations in Brazil possessed the ability to produce polygalacturonase, an enzyme that is responsible for breakdown of polygalacturonic acid. However, isolates of the Gram-negative bacterial species *Tamutella ptyseos*, *Pseudomonas putrefaciens*, *Enterobacter aerogenes*, *Acinetobacter* sp. and *Providencia mirabilis* produced pectin lyase, which may accelerate the fermentation by degrading the pectin present in the mucilage (Silva et al., 2008). The yeast species *Pichia burtonii*, *Debaryomyces polymorphus*, *Arxula adeinivorans*, *Pichia holstii* and *Pichia anomala* isolated from dry-processed coffee fermentations were also reported to possess pectin lyase activity (Silva et al., 2008). In a study on pectin-degrading enzymes from yeasts involved in coffee fermentation, *P. anomala*,

Pichia kluyveri and *Hanseniaspora uvarum* strains were shown to produce polygalacturonase but not pectin esterase or pectin lyase (Masoud & Jespersen, 2006). *Leuconostoc mesenteroides*, the most predominant lactic acid bacterial species in coffee fermentation, did not show pectinolytic activity in a study of Avallone, Brillouet, Guyot, Olguin, and Guiraud (2002), whereas only the rarely isolated species *L. brevis* possessed polygalacturonase activity. In the study of Avallone et al. (2002), the most frequently isolated pectolytic species were *Erwinia herbicola* and *Klebsiella pneumoniae*, which produced pectate lyases, an enzyme that can only depolymerize de-esterified pectins and not the highly methyl-esterified coffee pectic substances (Avallone, Guyot, Brillouet, Olguin, & Guiraud, 2001). Avallone et al. (2002) thus suggested that mucilage degradation was due to acidification rather than to microbial pectinolytic enzymes.

21.4 Towards the use of starter cultures to optimize fermentation

Quality is the most important aspect impacting the marketing of coffee. The organoleptic and visual quality characteristics are the accumulation of various parameters that affect coffee during processing. The intrinsic quality is established at the farm level, with the processing method probably having the greatest impact on quality (Velmourougane, 2013). Faulty or unsanitary processing practices (maturity of fruits, hygiene of fermentation vats, length of fermentation, quality of water used for processing) can produce sensory defects. Correct fermentation thus is of great importance to coffee quality. Firstly, fermentation can decrease acids and decrease the time for demucilation, which is especially important in production of Robusta coffee cherries with thicker mucilage when compared to Arabica cherries (Velmourougane, Shanmukhappa, Venkatesh, Prakasan, & Jayarama, 2008). Fermentation may not only serve to aid in removal of the thick mucilage layer, but has also been reported to be important for flavour development (Velmourougane et al., 2000). Furthermore, some yeasts are able to inhibit the growth of mycotoxinogenic filamentous fungi. It is well known, for example, that coffee is a favourable substrate for ochratoxin A (OTA) production by *Aspergillus* or *Penicillium* species (Mantle & Chow, 2000; Silva et al. 2013). Masoud, Poll, and Jakobsen (2005) showed that the main esters and alcohols produced by the yeasts *P. anomala, P. kluyveri* and *H. uvarum* inhibiting *Aspergillus (A.) ochraceus* growth were ethyl acetate, isobutyl acetate, 2-phenyl ethyl acetate, ethyl propionate and isoamyl alcohol. Amongst these, the most effective inhibiting compound was 2-phenyl ethyl acetate.

A controlled coffee fermentation process with a controlled microbiota may guarantee a standardized quality and reduce the economic loss for the producer. In the literature, only a few studies have been published towards the use of starter cultures for coffee fermentations, although the attempt to control coffee fermentation has existed for over 40 years. A pectolytic yeast (Agate & Bhat, 1966; Avallone et al., 2002) and

waste-water of a previous fermentation (Avallone et al., 2002; Butty, 1973; Calle, 1957, 1965) used as inocula were early attempts to utilize starters in coffee fermentations. In more recent studies, Silva et al. (2013) used a multicomponent starter preparation that included pectinolytic yeasts and bacteria. Fifteen strains were preselected based on their ability to produce pectin lyase and organic compounds and included *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Candida parapsilosis*, *Pichia carribica*, *Pichia guilliermondii* and *S. cerevisia*. Of these, *S. cerevisiae*, *P. guilliermondii* and *C. parapsilosis* were finally selected based on their aroma compound profile as possible future starter cultures for Brazilian dry fermentation coffee processing (Silva et al., 2013). Massawe and Lifa (2010) used a starter combination of six yeast strains (three *P. anomala* strains and three *P. kluyveri* strains) and six lactic acid bacterial strains (*Leuconostoc*, *Weissella*, homo- and heterofermentative lactobacilli and *Enterococcus* strains) in coffee fermentation to inhibit two OTA producing *A. ochraceus* strains. These authors could show that there was no growth of moulds after fermentation and drying periods and that the starter combination could be successfully used as a biocontrol agent against *A. ochraceus* (Massawe & Lifa, 2010). Even today, little is known today about the contribution of yeasts or bacteria towards coffee flavour, and the use of starter cultures for field-sized controlled fermentations is still in its infancy. This clearly requires further study. Before effective starter cultures can be developed, however, there is also a need for more in-depth studies on the microbial ecology of coffee fermentations, preferably by culture-independent molecular biological techniques. Only a detailed overview on the types and roles of microorganisms associated with the fermentation will yield information about the predominant bacteria in the different stages of fermentation and will allow the selection of appropriate functional starter strains.

21.5 Mycotoxin production

Many of the fungi naturally occurring on coffee beans are potentially capable of mycotoxin formation. Ochratoxin A is the most important mycotoxin in coffee. Ochratoxin A, which is produced by strains of *Aspergillus* and *Penicillium,* is nephrotoxic, carcinogenic, teratogenic and immunosuppressive (Bucheli & Taniwaki, 2002; WHO, 2001). OTA presence in green coffee beans was reported by Levi, Trenk, and Mohr (1974) for the first time and it was generally accepted that OTA is degraded during the roasting process until Tsubouchi, Terada, Yamamoto, Hisada, and Sakabe (1988) found OTA in commercial roasted coffee beans. OTA can be detected in green coffee beans in concentrations ranging between 0.2 and 360 µg/kg and it is mainly produced by *A. ochraceus* and *Aspergillus carbonarius* (Bucheli & Taniwaki, 2002).

In Thailand, the most important fungi with the potential to produce ochratoxin A were reported to be *Aspergillus westerdijkiae* for the Northern Arabica coffee and *A. carbonarius* for Southern Robusta coffee (Schillinger et al., 2010). A high moisture content (>20%) represents a particular risk for the growth of OTA-producing

fungi; i.e. factors that favour OTA contamination are not only inappropriate storage and transport of coffee beans but also the processing step (Bucheli, Kanchanomai, Meyer, & Pittet, 2000; Bucheli & Taniwaki, 2002). The critical points of risk along the production chain are cherry maturation, cherry processing, coffee grading, transport and storage (Bucheli & Taniwaki, 2002). OTA-producing fungi may be present on the cherries before or during the ripening process and show a tendency to be more present on overripe and damaged cherries than on green and ripe cherries (Bucheli & Taniwaki, 2002).

The drying process in a humid environment is probably the processing step with the highest risk of OTA contamination. Before drying, the cherries contain ca. 59–63% water with free sugars as an easily accessible carbon source. These environmental factors represent ideal growth conditions for OTA-producing fungi during the initial 3–5 days of drying on the outer part of the cherries (Bucheli & Taniwaki, 2002; Schillinger et al., 2010). Wet processing appears to be less susceptible to infection by ochratoxin-producing moulds, probably because of the removal of the fruit pulp, which was found to be an excellent substrate for OTA-producing *A. carbonarius* strains (Joosten, Goetz, Pittet, Schellenberg, & Bucheli, 2001). A water activity below 0.8 will prevent OTA production in green coffee and the conditions of 10–12% MC and 50–70% RH are considered appropriate for safe storage of green coffee without quality loss (Schillinger et al., 2010). Filamentous yeasts are killed during the coffee roasting process, while a high concentration of mycotoxins is not affected by the heat. As mentioned earlier, the use of starter preparations, especially those containing yeasts, is therefore important for controlling the growth and hence mycotoxin production of fungi during coffee fermentation when mycotoxins are potentially produced. The use of starter preparations may be especially important in regions where environmental conditions such as high humidity, little sunshine and high rainfall favour the growth of mycotoxinogenic filamentous fungi (Silva et al., 2008).

21.6 Conclusion

Although numerous studies have dealt with the microecology of coffee fermentation, many of these studies did not use state-of-the-art molecular biological methods for exact determination and profiling of the microorganisms present in the fermentation. Because of this and the differences in production methods in the various coffee-producing areas in the world, there is still a great need for basic microecological studies in the area of coffee fermentation. It is hoped that ultimately detailed understanding of the composition and differences of the microbiota associated with different types of fermentation will make it possible to gain a little more insight on what effect processing parameters have on shaping the microbial community and how this effects quality. This in term may aid further efforts towards production of starter cultures, which currently are still in their infancy but may in the future be important to control the production of coffee so that products with consistent quality or products with specific quality parameters are achieved.

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Quality improvement and fermentation control in vegetables

22

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22.1 Introduction

Fermentations belong to the oldest methods of preserving plant materials while retaining their nutritive value. As can be seen from Table 22.1, fermentation of fruits and vegetables may be effected by various groups of microorganisms. However, it is remarkable that in Europe and America preferentially lactic acid bacteria (LAB) and yeasts are associated with vegetable fermentations, whereas in Asian countries a great number of foods are fermented by fungi. But the most important procedure for biopreservation of plant material is lactic acid fermentation (LAF), which will be the topic of discussion in this chapter. Due to the development of efficient heat sterilizing and refrigeration systems, LAF lost its importance as a preservation method in the industrialized countries. Nevertheless, in recent years this type of vegetable fermentation has gained importance. The reason for that is that fermentations today are more than just preservation methods. In addition to preserving fruit and vegetables, according to Buckenhueskes (2001), they are used to:

- Develop characteristic organoleptic properties, i.e. taste, aroma and texture.
- Destroy naturally occurring toxins and undesirable components of the raw material, e.g. reducing the linamarin content of cassava (Giraud, Gosselin, & Raimbault, 1992) or the glucosinolates in cabbage (Gail-Eller & Gierschner, 1984).
- Improve the digestibility, e.g. of some legumes by reduction of oligosaccharides causing flatulence (Camacho et al., 1991).
- Enrich products with desired microbial metabolites, e.g. L(+)-lactic acid or amino acids.
- Create new products for new markets, e.g. fermented fruit juices (Wiesenberger, Kolb, Schildmann, & Dechent, 1986) or tempeh-like products from *Vicia faba* beans (Berghofer & Werzer, 1986).

22.2 History and present product range

The production of lactic acid fermented food of plant origin was known in virtually all historical periods and cultures and is still a technology practised all over the globe. Generally it is believed that LAF of vegetables was first practised in China, and from there it is said to have been brought by the Mongols and Tatars to Europe

Advances in Fermented Foods and Beverages. http://dx.doi.org/10.1016/B978-1-78242-015-6.00022-0

Table 22.1 Important types of vegetable fermentation

Type of fermentation	Product	Raw material	Major microorganisms involved
Lactic acid fermentation	Lactic acid fermented vegetables	See Table 22.2	*Leuconostoc mesenteroides* *Lactobacillus plantarum* *Lactobacillus brevis* *Lactobacillus sakei* (previously *Lactobacillus bavaricus*)
	Lactic acid fermented vegetable juices	Sauerkraut, carrots, celery, tomatoes, red beets	*Lb. plantarum* *Lactobacillus paracasei* *Lactobacillus xylosus*
Acetic acid "fermentation"	Vinegar	Grapes, potatoes, various fruits after alcoholic fermentation	*Acetobacter europeus* *Acetobacter aceti* *Acetobacter pasteurianus* *Acetobacter hansenii* *Gluconobacter oxydans*
Alcoholic fermentation	Beer	Cereals (malt)	*Saccharomyces cerevisiae* *Brettanomyces bruxellensis* *Lb. brevis*
	Wine and fruit wines	Grapes, various fruits	*S. cerevisiae* *Oenococcus oeni*
	Spirits	Potatoes, various fruits and vegetables	*S. cerevisiae* *Kluyveromyces marxianus*
Mixed fermentations	Sourdough	Rye flour, wheat flour	*S. cerevisiae* *Lactobacillus sanfranciscensis* *Lactobacillus pontis*
	Cacao	Cacao beans	Yeasts, lactic acid bacteria, acetic acid bacteria
	Coffee	Coffee beans	*Enterobacteriaceae,* lactic acid bacteria, yeasts
	Soya sauce	Rice, wheat, soya beans	*Aspergillus oryzae* *Lactobacillus* spp. *Pediococcus* spp. *Zygosaccharomyces rouxii*

Source: From Buckenhueskes (2007).

(Vickers & Bourne, 1976). However, Barrau (1983) assumes that even Stone Age people must have known a kind of "sour cabbage", since they had probably eaten the fermented stomach contents of their hunted animals and they also probably preserved leaves and plants in the stomachs, which would then certainly undergo a slight fermentation.

Pliny the Elder in the first century AD is said to be the first who described the production of sauerkraut by preservation of the so-called salted cabbage in earthen vessels. It is certain that under the conditions described, the cabbage was fermented by microorganisms that, for the major part, were located in the pores of the vessels and that originated from a former fermentation. However, heads of white cabbage (*Brassica oleracea* var. *capitata for. alba* L.), the raw material of today's sauerkraut, seem to have been known at the earliest in the eighth century AD (Fritz & Stolz, 1980, p. 77). Lind (1772) was the first to describe sauerkraut manufacture comparable to contemporary processing. By the beginning of the nineteenth century, fermentation of vegetables was mainly done in households, until 1830 when the first company was established for the production of sauerkraut. An important year in the history of sauerkraut was the year 1775, when Captain James Cook was awarded the Great Copley Medal for his observations and conclusions about sauerkraut as an effective source for prevention of scurvy, which up to that time was feared as the plague of the sea (Eichholtz, 1975).

As Table 22.2 shows, there are numerous lactic acid fermented vegetables commercially available on the European market up to the present day. This range is complemented by different mixtures of fermented vegetables. Of general economic importance, however, for Europe and the United States, are only three products; in the order of quantity produced, these are fermented table olives, sauerkraut and fermented cucumbers. The best-known lactic acid fermented vegetable in Asia is kimchi, which is especially famous as a typical Korean product but is also produced in other Asian countries. Because of its special features, kimchi will be described separately in Section 22.8.

22.3 Food fermentations: complex networks

The fermentation of plant substrates in itself constitutes a very complex network of independent and interactive microbiological, enzymatic, chemical, biochemical and physical processes and reactions. The essential correlations of such a network are presented in Figure 22.1, illustrated by the example of the fermentation of white cabbage to sauerkraut. The figure shows clearly the immense importance of lowering the pH caused through the microbial acid formation on the one hand, as well as the added sodium chloride on the other, so that these aspects are emphasized separately later. However, this already complex process is not an autonomous system. To complicate matters, fermentation is influenced by a multitude of exogenous factors, which ultimately have an effect on the quality of the final products. These factors can be divided into four groups: (1) technological factors (2) the nature and amount of admixed ingredients and optional additives; (3) the quality of the raw materials used, which in turn depends on numerous agricultural factors and (4) last but not least, the nature of the microbiota introduced with the raw material.

Table 22.2 Raw materials for lactic acid fermented products in Europe

Vegetable	Scientific name
(Globe) Artichoke	*Cynara scolymus* L.
Asparagus	*Asparagus officinalis*
Capers	*Capparis spinosa* L.
Carrots	*Daucus carota* L. subsp. *sativus (Hoffm.)*
Cauliflower	*Brassica oleracea* L. *convar. Botrytis* var. *botrytis L.*
Celery	*Apium graveolens* L. var. *rapaceum (Mill.)*
Cucumbers	*Cucumis sativus* L.
Eggplants	*Solanum melongena* L.
Fungi	Not specified
Green/waxy pepper	*Capsicum annuum* L. var. *grossum*
Green beans	*Phaseolus vulgaris*
Green tomatoes	*Lycopersicon lycopersicum* L. (unripe fruits)
Kohlrabi	*Brassica oleracea* var. *Gongylodes L.*
Lupinus beans	*Lupinus polyphyllus*
Melons	*Citrullus lanatus*
Okra	*Abelmoschus esculentus*
Olives	*Olea europea* L. subsp. *europaea*
Peas	*Pisum sativum*
Red beets	*Beta vulgaris* L. subsp. *vulgaris* var. *Conditiva (Alef.)*
Red cabbage	*Brassica oleracea* L. *convar. Capitata L.* var. *capitata L. f. rubra*
Silver-skinned onions	*Allium fistulosum*
Swedes	*Brassica napus* L. var. *napobrassica L.*
Tomato-shaped paprika	*Capsicum annuum convar grossum (L.) Terpo provar. Tetragonum (Mill.) Terpo conc. Rubrum (Aug.)* (Red and green fruits)
Turnips	*Brassica rapa* L. var. *rapa L.*
White cabbage: whole heads and sauerkraut	*Brassica oleracea* L. *convar. Capitata L.* var. *capitata L. f. alba*

Source: Modified from Buckenhueskes et al. (1990).

22.4 Technological factors

22.4.1 Production of fermented vegetables

Under conditions of adequate preparation and flawless application of the available technological knowledge it can be assumed that probably all known types of vegetables can be subjected to lactic acid fermentation. The botanical, physical, chemical and textural properties of the various fruits and vegetables differ widely, causing several differences of the applied technology. Since it is not possible to discuss the individual working steps in detail, a highly simplified flow sheet for

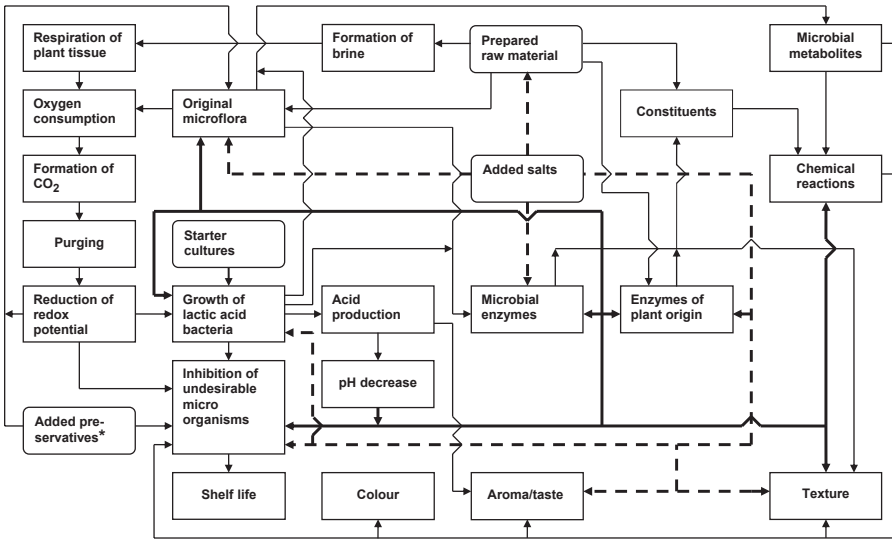

Figure 22.1 Significant reactions and their most important interactions that take place during the lactic acid fermentation of cabbage to sauerkraut. The bold dotted lines indicate the reactions influenced by the added salt. The bold solid lines indicate the influence of lowering the pH caused through the microbial production of acids on the entire process. *Only permitted in a few countries.

Buckenhueskes (2007).

manufacturing lactic acid fermented vegetables is given in Figure 22.2. According to Buckenhueskes (2001), the essential requirements for the entire process can be described as follows.

To produce high-quality fermented products, vegetables and fruits must be sound, undamaged and at the proper stage of maturity. After delivery, mechanically damaged, bruised or diseased fruits, dirty or withered leaves as well as unripe and overripe fruits must be removed. For the production of sauerkraut, the core of the cabbage must be removed. Raw materials that are to be fermented whole must be suitably size graded to achieve a homogeneous fermentation.

The further pretreatment depends on the particular vegetable and may include working steps like peeling (e.g. carrots, beetroot and celery), blanching (e.g. green beans), cooking (e.g. beetroot, to modify the texture as well as to destroy the characteristic earthy flavour) or an alkaline treatment (green Spanish-style olives) (Figure 22.2). If the respective vegetable is to be fermented in an altered form, it may be pierced (e.g. cucumbers, tomatoes) or cut into pieces, slices or fibers, a treatment that leads to the release of substances from the tissue into the brine (see Section 22.4.4).

For fermentation, the pretreated vegetables are then placed in suitable fermentation vessels. Due to the respective product, the plant size and the applied technology, these vessels may range from 100-l drums to tanks or silos with up to 100-ton capacity. Filling must be done very carefully. In order to achieve favourable

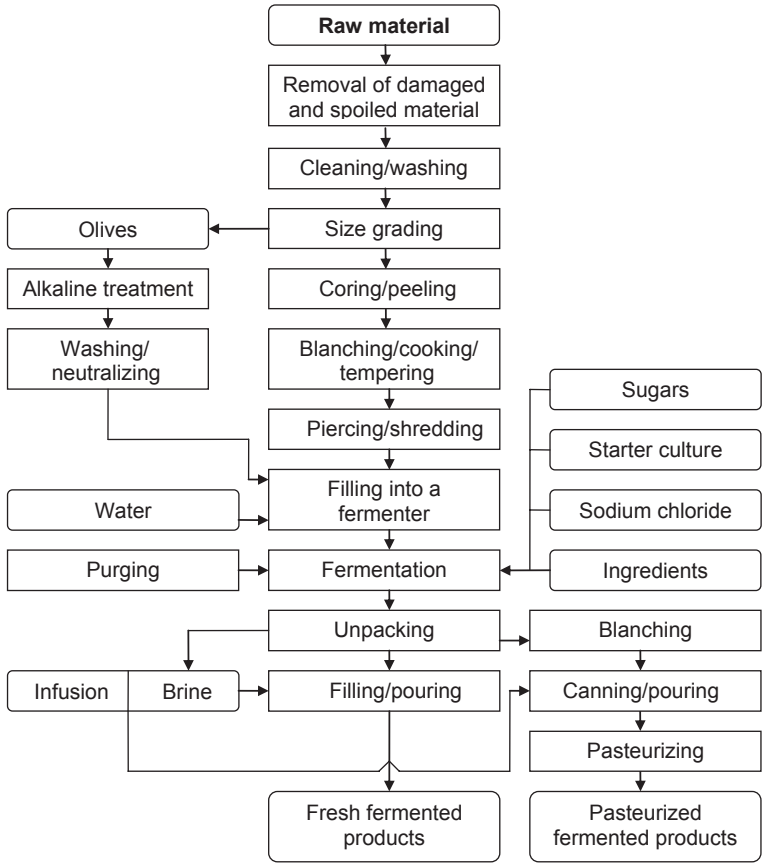

Figure 22.2 General flow sheet for the production of fermented vegetables. Buckenhueskes (2007).

fermentation conditions, vegetables like shredded cabbage must be salted very homogenously on the conveyor belt between the shredding machines and the tanks or during filling of the fermentation vessels. Whole fruits like cucumbers, olives or tomatoes must be placed into vessels containing some brine in order to prevent mechanical damages and to displace the air between the vegetable parts. In addition to the salt, other additives optionally may be necessary, such as spices, sugars or starter cultures. The filled vessels must be sealed in such a way that the plant material is totally covered by brine. To achieve anaerobic conditions, open tanks or silos are normally covered with wooden plates weighted with stones or by water-filled balloons in order to submerge the material and thus to exclude oxygen and microorganisms from the air.

Under these conditions, a spontaneous fermentation process usually begins immediately. The best products will be achieved at fermentation temperatures between 15 and 20 °C. The fermentation time depends primarily on the temperature, the type of

vegetable, the degree of disintegration and the desired acidity of the final product. During fermentation, the fermentation vessels must be carefully monitored and in particular controlled for the development of total acidity and pH. Sometimes it is necessary to remove yeasts and moulds that have grown on the surface of brines. *Debaryomyces hansenii* and *Pichia membranifaciens* are among the most common spoilage yeasts in brine. To prevent microbial spoilage of fermenting vegetables, but especially the growth of yeasts and moulds on the surface of the brine or within the fermenting product, the addition of sorbic acid, tartaric acid, acetic acid and/or benzoic acid is permitted in several countries (Buckenhueskes, Aabye Jesen, Andersson, Garrido Fernandez, & Rodrigo, 1990).

The final fermented product (pH < 4.1) may either be distributed fresh, packaged or unpackaged, or pasteurized in pouches, cans or jars. In the latter case, the fermented vegetables are first blanched and then filled into the container, poured and after closing subjected to a gentle pasteurization. For victuals that easily soften, the original fermentation liquid should be replaced by a brine containing certain amount of sodium chloride and lactic acid in order to top the containers.

If the fermented fruit and vegetables are to be distributed unpasteurized, it is essential that all fermentable carbohydrates have been metabolized. Otherwise, a secondary fermentation may occur caused by yeasts and resulting in gaseous spoilage, brine turbidity and probably an alcoholic fermentation. In the case of cucumbers, secondary fermentation may cause bloater formation (Daeschel & Fleming, 1984).

In the case of pasteurized products, fermentation may be interrupted as soon as the pH value reaches ≤ 4.1. For that reason the goods are blanched, canned, topped with fermentation liquid or salt and sometimes herbs, spices and/or vine containing brine, and pasteurized. In order to achieve less heat treated and therefore better-quality products, as well as to save energy, the traditional pasteurization methods of trial and error should be replaced by evaluated and defined pasteurization processes using *P*-value calculation. However, the application is still difficult, due to the lack of information especially about the heat inactivation kinetics of quality-relevant enzymes like pectinolytic enzymes or lipoxygenases (Buckenhueskes, Gierschner & Hammes, 1988).

22.4.2 Ecological conditions in the fermentation vessel

In general it can be stated that each particular fruit or vegetable species provides a specific environment in terms of type, availability and concentrations of substrate, buffering capacity, competing microorganisms and perhaps natural plant antagonists (Daeschel, Andersson, & Fleming, 1987).

The ecological factors in a fermentation vessel at the beginning of the fermentation process can be described as follows (Buckenhueskes, 2001):

- The fermenting substrate is characterized by solids in a liquid environment (brine). Whereas the microorganisms as well as the salt and the released nutrients can be distributed through the entire fermentation vessel by circulating the brine, mass transfer within the vegetable pieces can only occur by diffusion.
- Due to the liquid environment, microorganisms originating from the raw material are distributed throughout the fermenting mass.

- Pathogens such as salmonellae, clostridia or listeriae, as well as other undesirable microorganisms, are present in all probability.
- In general, the raw materials used are rich in nutrients, growth factors and minerals; however, for microbial growth, these substances must be released from the plant tissue into the surrounding liquid.
- Water activity (a_w value): 0.95–0.99.
- pH: 5.9–6.5 for cabbage; 8.5 is maximum for olives.
- Temperature: $t = 5–20\,°C$ for vegetables, 25–30 °C for vegetable juices.
- Sugar content: 25–100 g/kg of vegetable.
- Buffering capacity: 0.15–0.90 g of lactic acid/100 g of vegetable.
- Sodium chloride content: 0.6–2% for sauerkraut, 5–10% for brine of cucumbers.

The compilation of these data shows that wounded or disintegrated plant tissues make evident that they provide an excellent substrate for bacterial growth at the beginning of fermentation and that they are extremely perishable, which raises the risk of growth of pathogenic microorganisms. It is therefore necessary that the LAB are selectively promoted so that fermentation can start quickly and reliably.

22.4.3 Redox potential and anaerobiosis

As long as the vegetables are not subjected to a stronger heat treatment prior to filling into the fermentation vessels – or in the case of olives, are not subjected to any alkaline treatment – and apart from mechanically damaged cells caused by peeling or cutting, the plant tissue consists mainly of biologically intact cells, which have continued to run the normal biochemical post-harvest reactions.

In cases like sauerkraut production, the addition of sodium chloride enhances the release of tissue fluids from the vegetable cells during filling into fermentation vessels. This liquid or the brine, which for example in cucumber fermentation is directly given into the fermentation vessel, spreads between the vegetable particles, so that the air is largely driven from the fermentation substrate. The oxygen then still available, which is partly dissolved in the brine, is consumed by the plant cell respiration and the activity of aerobic and facultative anaerobic microorganisms. At the same time, various gases are formed. Among these, carbon dioxide is of the greatest significance. Carbon dioxide passes through the fermentation mass to the surface of the fermentation vessel. It acts as a gas, scrubbing and tearing gas bubbles with it that are located in the fermentation substrate. Outside the fermentation vessel, these processes can be perceived by a more or less intensive foaming. In the fermenting mass they cause an increase in anaerobiosis and thus a significant decrease in the redox potential – a further necessary prerequisite for the growth of LAB.

22.4.4 Release of nutrients and place of fermentation

A necessary condition for a successful fermentation is sufficient availability of all nutrients and growth factors, necessary for the growth of the fermenting microorganisms. With most of the various types of vegetables, this should be the case, but the nutrients are well protected inside the vegetable cells. Therefore they must be made

available to the microorganisms, which are effected by mechanical wounding in form of piercing, shredding or slicing.

Stitching is done to injure the plant skin in order to improve mass exchange, which otherwise can only occur by the relatively slow-running diffusion. A higher degree of wounding the tissue results in an accelerated decrease of pH during fermentation. Since the fermentation mainly occurs in the liquid phase of the entire system, this phenomenon can be explained by a faster release of nutrients from the vegetables resulting in a faster acid production and finally in an accelerated penetration of acid from the brine into the vegetable tissue (Buckenhueskes & Gierschner, 1987).

This example suggests it reasonable to assume that fermentation only occurs in the liquid phase of the fermenting substrate. Although it was rather improbable that microorganisms are able to invade the naturally germ-free plant tissue and to grow there, Daeschel and Fleming (1981) could show that this could happen, e.g. with LAB in fermenting cucumbers. They assume that the bacteria invade cucumbers together with the brine through the stomata and are then distributed over the vessel system. By using electron microscopy, colonies of *Pediococcus pentosaceus* could be detected directly below the epidermis. On the other hand, yeasts, which had been added to the brine for fermentation, were not able to penetrate into the pickles, presumably because of their larger size. Today, LAB located in the tissue is also known in other fermented vegetables, as in peppers, tomatoes and olives, where they are sometimes referred to as "yeast spots" (Buckenhueskes & Bohrer, 1991; Vaughn, Stevensen, Dave, & Park, 1972).

22.5 Ingredients and additives

22.5.1 Sodium chloride

For the preservation of vegetables, there are two methods in which the added salt (sodium chloride) is crucial: brining (or salting) and fermentation. Although both methods are closely related to each other, they are based on different principles of conservation. For our discussion, this is essentially a question of the salt concentration. In the case of salting, with equilibrium salt contents above 10%, the stabilization is based solely on the non-specific decrease of the water activity (a_w value) and the high ionic activity. In the case of LAF, the preserving effect, however, is primarily based on the formation of acids in the product leading to a decreasing pH value. Here the effect of the added salt on the a_w value is only of secondary importance. Brining is rarely practised today owing to problems of salt removal and decreased consumer acceptance of high-salt foods.

As the production of silage for animal feeding indicates, the LAF of plant material may be feasible even without any addition of salt. But since the added salt possesses several technological functions (see Figure 22.1), the danger of undesirable fermentations increases, and so sodium chloride will be the most important ingredient:

- A key function of salt is its contribution to flavour. Decision on the salt concentration is determined by the vegetable type and consumer demands. Typically, e.g. the salt content

of canned sauerkraut in Germany ranges around 11.3 g/kg as compared to 16.7 g/kg in the United States (Buckenhueskes, Gessler, & Gierschner, 1988).

- The development of anaerobic conditions in fermentation vessels is also promoted by salt. By increasing the osmotic pressure, salt will accelerate the release of tissue fluids in the production of vegetable materials such as cabbage, and in particular from shredded cabbage during filling into fermentation vessels. Fermentation capacity is raised by removing a part of the brine, thus providing additional space for the shredded cabbage, and moreover enhancing oxygen exclusion and growth of LAB.
- The selective effect of salt on microorganisms occurring naturally on vegetables forms the basis for controlling a desirable fermentation in vegetable products. The growth of detrimental bacteria and fungi is inhibited by increasing amounts of salt, while at the same time it will selectively allow the growth of beneficial LAB. Especially heterofermentative LAB such as *Leuconostoc* spp. are favoured by low salt concentrations of approximately 1% in sauerkraut, whereas they are generally inhibited at 3%. Homofermentative LAB species such as *Lactobacillus plantarum* are better adapted to higher amounts of salt by which fermentation will be accelerated. This selective effect may also have hygienic advantages, but will lead to "unbalanced" fermentations dominated by homofermentative LAB strains resulting in sauerkraut with poor flavour due to either absence or low concentrations of desirable metabolic by-products including acetic acid. On the other hand, there appears to be general agreement on the negative effects of salt concentrations below 0.8% frequently resulting in undesirable developments in fermentation including softening of sauerkraut.
- The softening defect may be explained by an adverse effect of salt on the texture of the final product, including several plant and microbial enzyme systems (see Figure 22.1). Enzymes such as pectinesterase are a major cause of softening of cabbage, cucumbers, olives and other fruits and vegetables. Activities of these pectinolytic enzymes are highly pH-dependent, with an optimum around pH 6.0 and a strong decline at pH ≤ 4.0. Control by elevated salt concentrations may only show a significant effect at >3% NaCl (Omran, Buckenhueskes, Jäckle, & Gierschner, 1991). Cell wall degradation usually starts with an enzyme-catalysed de-esterification of carboxyl groups of pectin molecules, followed by hydrolysis of the polysaccharide chain by polygalacturonases, active at the de-esterified positions within the chain.

Even when these processes typically accelerate disintegration of the cell wall constituents, resulting in subsequent softening of the plant tissue, de-esterification may also improve the texture of fermented fruits and vegetables. Basic to this is the linking of bivalent cations such as calcium with de-esterified carboxyl groups of adjoining pectin molecules. In cucumber fermentation this can be achieved by stimulation of pectinesterase, the inhibition of polygalacturonases and the simultaneous addition of calcium chloride to the fermented product (Meurer, 1991). Pectinesterase may be stimulated in cucumber fermentation either by using raw materials of optimal maturity and/or by processing control steps including adjustment of sodium chloride and calcium chloride concentrations. Polygalacturonases may be inhibited either by addition of sodium chloride or by heat treatment before fermentation.

A causative role for LAB as the dominant factor in the softening of fermented vegetables has not been established. Pectinesterase and endopolygalacturonase activities have been reported for *Lb. plantarum* (Sakellaris & Evangelopoulos, 1989; Sakellaris, Nikolaropoulos, & Evangelopoulos, 1989); however, other researchers have failed thus far to confirm these enzymes.

22.5.2 Carbohydrates

LAF appears to be the dominating type of fermentation of plant materials, both for food and feed purposes, and includes a wide range of fermented vegetables, while also comprising an ensilage process of various crops for animal feed. The most essential requirement is the availability of sufficient amounts of fermentable carbohydrates for fermentative conversion into lactic acid and acetic acid/or ethanol. The buffering capacity of the particular product is positively correlated with its protein content and will determine the extent of the acid-related pH decrease in the fermented plant material (Buckenhueskes & Gierschner, 1985). If the buffering capacity is known, it is possible to calculate the minimum amount of fermentable carbohydrates required for sufficient acid production to reach a pH of 4.1. For sauerkraut the desired pH reduction was shown to require around twofold the calculated amount of carbohydrate. On this basis, an estimation of the amount of fermentable carbohydrates such as sucrose or glucose required to achieve a desired fermentation can be made. In doing so it appears that the naturally occurring levels of fermentable carbohydrates is not sufficient in all types of vegetables, such as in the case of cauliflower or celery. In such cases, appropriate amounts of sugars must be added to the fermenting vegetables.

22.5.3 Other additives

Other food additives may be applied in vegetable fermentations, but will depend on the particular product, while regulatory restrictions may be posed in numerous countries. Ascorbic acid is frequently added to sauerkraut, mainly with the purpose of preventing gray or brownish discolourations of fresh or canned products. In some countries citric acid or sulphur dioxide is permitted for preventing discolourations.

Microbial spoilage, and in particular growth of yeasts and moulds on surfaces of brines and fermenting vegetables, may be prevented by sorbic acid, which is permitted in several countries. In addition, several Eastern European countries have approved the use of defined amounts of tartaric acid, benzoic acid or acetic acid (Buckenhueskes et al., 1990).

22.6 Microbiology of fermentation

22.6.1 LAB and plants

Depending on factors such as vegetable species, the used plant organ, agricultural measures or climatic conditions during the growing season, fresh vegetables harbour numerous and various types of microorganisms. The total bacterial counts of vegetables reach values of 10^4–10^8 CFU/g, and sometimes even more (Garcia-Villanova Ruiz, Galvez Vargas, & Garcia-Villanova, 1987). These microbiota are dominated mostly by Gram-negative species and Gram-positive spore formers; yeasts and moulds are also always present, but in significantly lower numbers. In the case of sauerkraut production, the analysis of 30 different samples of white cabbage from four growing seasons has shown that the microbiota normally are dominated by aerobic

bacteria (e.g. pseudomonads, enterobacteria and coryneforms) and yeasts, while LAB represents less than 1% (between 0.15% and 1.5%) of the total bacterial population (Schneider, 1988). According to older literature, yeasts should play an important role in flavour formation; however, their share in the total microbial count is normally less than 0.1% (Buckenhueskes, Schneider, & Hammes, 1986).

Although an extremely small population of LAB is present, it is assumed that plants are the natural habitat for some species. According to Daeschel et al., 1987, certain species are always to be found, namely:

- *Lactobacillus* spp.: *Lb. brevis, Lb. buchneri, Lb. casei, Lb. fermentum, Lb. plantarum, Lb. curvatus, Lb. s akei.*
- *Leuconostoc* spp.: *L. mesenteroides* subsp. *mesenteroides.*
- *Pediococcus* spp.: *P. acidilactici, P. pentosaceus (*formerly *P. cerevisiae).*
- *Enterococcus* spp.: *E. faecalis, E. faecalis* var. *liquefaciens, E. faecium.*
- *Lactococcus* spp.: *Lc. lactis.*

It seems that these organisms belong mainly to those that use the so-called Mn(II) mechanism, to protect themselves against toxic O_2^- radicals. The Mn(II) mechanism means that these organisms are capable of intracellular Mn^{2+} accumulation in order to neutralize endogenous O_2^- radicals according to the formula:

$$O_2^{-\bullet} + 2H^+ + Mn^{2+} \rightarrow H_2O_2 + Mn^{2+}$$

Since this mechanism has only been found in LAB, one may speculate that plants serve as a reservoir for accumulation of manganese by epiphytic LAB (Daeschel et al., 1987).

The numbers of LAB on living plants are restricted by many factors, including UV light, temperature and available nutrients; however, it appears that LAB coexist with plants. High numbers of LAB have been reported on damaged parts of plants. It is assumed that this is not only due to the nutrients then available. It is rather believed that certain LAB may protect plants from pathogenic microorganisms by producing antagonistic substances such as acids and bacteriocins (Daeschel et al., 1987).

22.6.2 Microbial sequences

The naturally occurring LAF of plant materials is distinguished from that of other raw materials such as meat and milk, in that the latter initially has a comparatively lower number of microorganisms of less diversity. The key to a successful fermentation is to enhance the activity of the desired fermentation microorganisms and to suppress the growth of pathogenic and spoilage microorganisms (Daeschel et al., 1987). The microbial population in general undergoes considerable changes during the course of vegetable fermentation. The spontaneous fermentation of cabbage, for example, has been categorized into four distinct stages, in which this succession is a consequence of the changing environmental conditions within the fermenting substrate:

- 1st Stage: Early initiation of fermentation occurs even during the filling of cabbage into the vessels. Tight "packaging" or compressing of the shredded cabbage will remove air and thus enhance anaerobic conditions, thereby rapidly inhibiting the numbers of strictly aerobic bacteria

such as *Pseudomonas* and *Flavobacterium* (cf. Buckenhueskes, 2007). Respiration by the plant tissue and, in addition, oxygen utilization by facultatively anaerobic enterobacteria, will further serve to increase anaerobiosis during the first 2 or 3 days. Concomitantly with oxygen deprivation, the pH decreases due to the fermentative production of organic acids such as lactic, acetic, formic and succinic acids. Undesired foam formation may result from carbon dioxide production during the early fermentation stages. The role of the mixed population of microorganisms in flavour development during this early stage of fermentation has not been clarified.

- 2nd Stage: The facultatively anaerobic LAB soon dominate this stage as a result of the selectively beneficial effect of a more anaerobic atmosphere, the lower redox potential, the added salt and the reduced pH. Thereby the non-LAB will be increasingly inhibited. Thanks to its excellent adaptation and its universal association with plant substrates such as cabbage, this fermentation stage is initiated and generally dominated by *Leuconostoc mesenteroides*, even when it is not as acid-tolerant as many other LAB species. When optimum conditions exist such as in cabbage juice, it may reach maximum cell numbers of $>10^8$ cfu/ml after 12–14 h incubation (Stamer, Stoyla, & Dunckel, 1971). In many plant fermentations, *L. mesenteroides* may be considered as a typical "pioneer" organism, as it produces lactic and acetic acids resulting in a rapid decrease of the pH. Moreover, being heterofermentative, it also produces carbon dioxide (in addition to lactic and acetic acids) by which replacement of air (oxygen) is promoted and thus the enhancement of anaerobic conditions. Such conditions are beneficial for the stabilization of vitamin C (ascorbic and dehydro-ascorbic acid) and the natural colour of the cabbage. In addition to *L. mesenteroides*, other *Leuconostoc* species such as *Leuconostoc fallax* are also associated with the 2nd stage of sauerkraut fermentation (Barrangou, Yoon, Breidt, Fleming, & Klaenhammer, 2002), with similar attributes to the fermentation process as *L. mesenteroides.*
  Typical of the microbial succession in sauerkraut fermentation, the so-called "betabacteria" (heterofermentative lactobacilli such as *Lb. brevis* with higher acid and salt-tolerance than *L. mesenteroides*) will gradually dominate the population accompanied by a reduction in viable *Leuconostoc* numbers. The length of the first two stages of sauerkraut fermentation is determined by the temperature, but typically ranges from 3 to 6 days, and is associated with an increase in lactic acid up to around 1% (Müller, 1988).
- 3rd Stage: A further shift in the lactic population towards the predomination of homofermentative lactobacilli characterizes this stage. This change is the combined result of changes in intrinsic conditions, in particular increased anaerobiosis, reduced pH and an elevated salt concentration. Minor populations such as streptococci (most probably enterococci) and pediococci may comprise <10% of the total LAB population, as compared to homofermentative *Lactobacillus* strains (formerly grouped under the "subgenus" *Streptobacterium*), represented mainly by *Lb. plantarum.* Some strains have formerly been referred to as *Lactobacillus cucumeris* (Pederson, 1936). More recent studies have shown *Lb. plantarum* to comprise only between 30% and 80% of the so-called "streptobacteria" during the third stage of fermentation, characterized by LAB populations of up to 10^9 CFU/g and rapidly declining populations of aerobic bacteria and yeasts (Buckenhueskes, 2007). High population numbers, particularly for the species *Lb. sakei* and *Lb. curvatus,* have meanwhile also been reported for this stage of sauerkraut fermentation. Of special interest is the occurrence of "*Lactobacillus bavaricus*", which is characterized by the exclusive formation of L-(+)-lactic acid (Kandler, Hammes, Schneider, & Stetter, 1986), but which has been shown to be a subjective (racemase-defective) synonym of *Lb. sakei* (Kagermeier-Callaway & Lauer, 1995). The major part of the available carbohydrates (glucose, fructose and sucrose) is converted by the LAB into organic acids, predominantly lactic acid. During this fermentation stage, the total acidity (calculated as lactic acid) increases to a level of 1.5–2.0%.

Consumers' preferences in Europe for milder (acid and salt) have prompted the un-packaging and pasteurization of sauerkraut upon reaching a pH of 3.8–4.1.

- 4th Stage: This stage is characteristic of unpasteurized sauerkraut typically distributed as "fresh". Extended fermentation results in additional lactic acid formation by gradually dominating strains of *Lb. brevis* and other heterofermentative LAB species able to metabolize pentoses such as arabinose and xylose. Free pentoses are usually not present in living plant material but are liberated from hemicellulose by acid-induced hydrolysis after harvesting (Dewar, McDonald, & Whittenbury, 1963). The strong lactic acid production, mainly by *Lb. brevis*, may increase the total acid content up to 2.5% with a concomitant decrease of the pH down to 3.4.

Successive population dynamics of various LAB, typical of vegetable fermentations, is strongly influenced by factors such as the initial microbial population, growth rate of individual microorganisms and their tolerance to salt and acid. High levels of heterofermentative *L. mesenteroides* growth are especially undesirable during cucumber fermentation. This is because such growth increases production of CO_2 and so may play a role in gaseous spoilage. A similar point applies to the fermentation of Spanish-style olives. Freshly harvested olives contain a bitter-tasting glucoside called oleuropein. The olives are therefore treated with sodium hydroxide (sometimes referred to as lye) before the fermentation process begins. Once this has had time to work, and so to make the olives less bitter, the sodium hydroxide is then removed through washing and neutralization. Unfortunately, this results in a loss of nutrients.

22.6.3 Bacteriophages

Thus far, neither in spontaneous fermentations nor after application of starter cultures have bacteriophages been described as causing a problem in fruit and vegetable fermentations. Nevertheless, in 2002 Yoon et al. isolated nine different phages from industrial sauerkraut fermentations. These were representatives of the *Siphoviridae* and *Myoviridae* families that are active against strains of *L. mesenteroides* and *Lb. plantarum* from the same fermentation silo. Due to the diversity of the discovered phages they indeed represent a fundamental risk potential, but thus far, such problems have not been described in practice. This is not surprising, because pure-culture fermentations seldom occur. If a starter or a naturally fermented batch is infected with phages, other strains of naturally occurring LAB will become dominant and carry out the fermentation. This leads normally to a delay in the fermentation, but it is not endangered. To what extent possibly occurring phages can be distributed in the fermenting substrate depends on the product, the time of phage attack and the technology used. Thus, a distribution in a sauerkraut silo will be much more difficult than for example in the fermentation of cucumbers or olives, in which the brine sometimes is circulated or purged with nitrogen. Both treatments cause a homogenous dissemination of phages in the brine.

22.6.4 Starter cultures for lactic acid fermented vegetable products

Except for lactic acid fermented vegetable juices, fermentation of the vast majority of vegetables is done by naturally occurring LAB (i.e. spontaneous fermentation) rather

than by defined starter cultures. In principle it may be assumed that all species of microorganisms that reach microbial counts of 10^6–10^7 CFU/g or more may have a noticeable influence on the sensory characteristics of the final product. As a consequence, in terms of taste expression, a broad range can be expected, ranging from cutting-edge products on the one side to products with no more tolerable off-flavour notes on the other side. The assumption that sensorially high qualitative end-products can arise only under the condition of microbial diversity and the described sequence of fermentation stages has long contributed to the belief that the use of starter cultures for a controlled and safe fermentation would not be possible in this kind of product (Buckenhueskes & Hammes, 1990).

On the other hand, the requirement of certification according to ISO 9000-9004, modern consumer demands and economical reasons necessitate the development of controlled fermentation processes in order to provide safe products on a defined and consistently high-quality level. Given the numerous influencing factors, the fermentation of fruit and vegetables is difficult to control, primarily on account of their shapes, the large number of naturally occurring microorganisms and the variability in nutrient content. Since it is not possible to eliminate the natural microbiota by appropriate methods, pure-culture fermentation cannot be realistically achieved.

One promising approach might be the application of defined starter cultures that are capable of growing rapidly and that are highly competitive in the environmental conditions under which the product is kept. Therefore, numerous attempts have made especially to ferment cabbage to sauerkraut, but also to ferment cucumbers and olives by defined starter cultures. Especially in the case of sauerkraut, most of the experiments performed with pure cultures or mixtures of pure cultures led indeed to the desired uniform, sufficient and rapid acidification of the products. But they lacked the typical rounded sauerkraut flavour, which essentially is due to the lack of certain fermentation by-products or the insufficient decomposition of compounds characteristic for the respective raw material (Kandler, 1981). Sauerkraut with perfect sensorial characteristics was obtained with selected strains of *L. mesenteroides,* which simultaneously improved the uniformity of sauerkraut from different batches. However, even in this case, so far no such benefits could be achieved compared to spontaneous fermentation that would economically justify the cost of the production of starter cultures (Buckenhueskes & Hammes, 1990).

But nevertheless, it can be expected that the use of starter cultures in the manufacture of lactic acid-fermented fruit and vegetable products will gain importance in the future. Some reasons for this are as follows (Buckenhueskes & Hammes, 1990):

- Ensuring the product quality at a high level (standardization), whereby an optimized technical management is required.
- Improving the hygienic safety (e.g. prevention or reduction of pathogenic microorganisms and biogenic amines (BA)).
- Economical production by shortening the fermentation time. However, there are natural limitations since the microbial processes must run in a certain harmony in parallel to the enzymatic and chemical processes of flavour and texture formation.
- Improved utilization of the raw materials used.

- Targeted exploitation of certain metabolic activities to optimize product quality. An example is the reduction of the nitrate content of lactic acid-fermented vegetable juices. Depending on the plant species, the part of the plant utilized, several horticultural factors and processing, the nitrate content of fermented vegetables can vary considerably. Microorganisms present during the early fermentation stage may convert nitrate into nitrite (Andersson, 1984). The acidic environment may then encourage a reaction between the nitrite and amides or secondary and tertiary amines. Such reactions produce nitrosamines, which are dangerous because of their strong, organ-specific carcinogenic properties. To avoid this serious problem, one can try to reduce the amount of nitrate contained in the initial materials. A biotechnological approach is the use of LAB and other microorganisms with the potential to reduce nitrate. For example, Kerner, Mayer-Miebach, Rathjen, and Schubert (1990) report that *Paracoccus denitrificans* has the ability to decrease the nitrate content of a commercial carrot juice (Kerner et al., 1990). A number of studies have reported that the metabolic activity of LAB leads to a decrease in the nitrate and nitrite levels in plant products. For example, Wolf and Hammes (1987) show that an MRS agar, which contains nitrate and molybdenum, can be used with *Lactobacillus pentosus* to bring about a nitrate reductase. However, in studies focused on cabbage juice, the nitrate was not reduced to a sufficient level. Only 10% of the initial nitrate underwent reduction, and so a significant amount of nitrate was still available for conversion to nitrite. Certain strains of *Lb. pentosus*, *Lb. plantarum* and *L. mesenteroides* are also able to produce nitrite reductases, with the final product of ammonium. However, the acidic environment prevents the reduction of nitrate or nitrite from being complete. The process is not, therefore, suitable for the production of fermented vegetables (Emig, 1989).
- Targeted exploitation of certain metabolic activities to form functionally active substances in the product. Commercially available examples are the so-called L-(+)-sauerkraut and most fermented vegetable juices, except sauerkraut juice. L-(+)-sauerkraut is directly produced within small jars (300–400 ml) using a *Lb. sakei* (formerly *Lb. bavaricus*) strain as a starter culture. This strain mainly produces L-(+)-lactic and it is highly competitive so that the initial *Leuconostoc* population is suppressed and even the homofermentative and racemase-possessing LAB are not able to compete. In freshly (not pasteurized) distributed sauerkraut, the L-isomer represents more than 90% of the total lactic acid content. The suppression of the heterofermenters leads to a less-developed flavour, which, however, is accepted by the health-food customers. Such sauerkraut is produced in Germany and distributed directly in the fermentation jars through health food stores.
  The majority of lactic acid fermented vegetable juice is manufactured according to the "lactoferment process" (Buckenhueskes & Gierschner, 1989). In that case, the initially produced mash or raw juice is pasteurized prior to fermentation so that pure-culture fermentation can be achieved through the addition of a starter. LAB described for this purpose include *Lactobacillus* species (*Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Lb. plantarum*, *Lactobacillus salivarius*, *Lactobacillus xylosus*, *Lb. brevis* and *Lb. casei*), *Lc. lactis* and *L. mesenteroides*. The species most often used today are commercially available strains of *Lb. plantarum* and sometimes *Lb. casei*.
- Facilitating the fermentation in cases where the raw material must be subjected to a heat treatment prior to fermentation. This could for example be necessary to inactivate enzymes causing discolouration or aroma deviations (e.g. chlorophyllase in green vegetables), to destroy or inactivate plant origin toxins (e.g. phasin in beans) or to influence the texture of the vegetable (e.g. beetroot, carrots).
- Access to new products.

Depending on the particular material to be fermented as well as the desired quality criteria of the final product, starter cultures have to possess a range of characteristics.

Table 22.3 Traits considered relevant in starter cultures for vegetable fermentation

Criteria	Sauerkrauts	Cucumbers	Olives	Vegetable juices*
Technologically relevant criteria				
Rapid and predominant growth	++	++	++	+
Homofermentative metabolism	-	++	++	++
Salt tolerance	+	+++	++	o
Acid production and tolerance	++	++	++	+
Inability to metabolize organic acids	++	++	++	+
Growth at low temperature	++	++	++	o
Few growth factors required	o	o	+	o
Tolerance of phenolic glycosides	o	o	++	o
Formation of dextrans	-	-	-	-
Pectinolytic activities	-	-	-	-
Formation of bacteriocins	+	+	+	o
Bacteriophage resistance	o	+	+	++
Sensorially relevant criteria				
Heterofermentative metabolism	++	-	-	-
Formation of flavour precursors	++	++	+	o
Nutritionally relevant criteria				
Reduction of nitrate and nitrite	+	o	o	++
Formation of L(+)-lactate	+	+	o	++
Formation of biogenic amines	-	-	-	-

++, important; +, advantageous; o, not relevant; -, detrimental.
*Except sauerkraut juice.
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Selection criteria for starters that should be used in the fermentation of sauerkraut, cucumbers, olives and vegetable juices are compiled in Table 22.3.

Starter cultures currently on the market for the production of lactic acid-fermented vegetable products comprise strains of *Lb. plantarum*, *Lb. xylosus*, *Lb. cellubiosus* and *Lb. sakei* (strains formerly designated as *Lb. bavaricus*).

22.7 Faulty products and spoilage

22.7.1 Biogenic amines

Some microorganisms associated with fruit and vegetable fermentations may cause the enzymatic formation of BA from free amino acids by the activity of substrate-specific amino acid decarboxylases. BA are small biologically active organic bases and are both physiologically/chemically and pharmacologically of great interest. They interfere in

numerous biological processes; they are precursors to vitamins, phosphatides, coenzyme A and a large number of alkaloids; and are also probably of importance to act as growth factors. BA do not constitute a fundamental threat to human health because our body has regulatory mechanisms towards amines, both endogenously produced and exogenously supplied. The most important mechanism of inactivation is the oxidative deamination by mono- and diaminooxidases, a mechanism that may fail under different conditions, for example as a result of taking certain medications (monoamine oxidase inhibitors). If the capacity of the regulatory mechanisms is overwhelmed, it may come to intoxication leading in the simplest case to skin redness, but also headaches, cardiovascular symptoms, gastrointestinal disorders, urticaria and even shock phenomena, in extreme cases with fatal result (Buckenhueskes, Sabatke, & Gierschner, 1992). The best-known intoxication is caused by histamine, which at levels of more than 50 mg/100 g product may cause toxic effects or allergy-like symptoms.

Since fruit and vegetables normally are fermented by undefined spontaneous microbiota, decarboxylase-positive microorganisms are typically involved. Genera of *Enterobacteriaceae* and *Bacillaceae* that are present at the beginning of the fermentation, as well as species of *Lactobacillus*, *Pediococcus* and "*Streptococcus*" (probably *Enterococcus*), known from fermenting plant material, are reported to be capable of decarboxylating one or more amino acids. BA (and their precursors) found in fermented vegetables are ethanolamine (serine), putrescine (ornithine), cadaverine (lysine), spermidine (reaction of putrescine with a propyl-amine residue that descends from methionine), phenylethylamine (phenylalanine), tyramine (tyrosine) and histamine (histidine).

Which microorganisms are involved primarily in the formation of BA in the case of sauerkraut is still not really understood. The previously published data merely indicate that cadaverine and putrescine are formed virtually from the very beginning, while tyramine and histamine occur at elevated levels only from about the fourth day of fermentation (Halás, Baráth, & Holzapfel, 1999; Künsch, Schärer, & Temperli, 1989). As producers of the BA, partly organisms of the first fermentation phase are discussed, such as *Proteus morganii*, *Enterobacter aerogenes* and *Hafnia alvei* (Peterson & Fred, 1923). On the other hand, the occurrence of BA is often thought to be associated with the presence of *Pediococcus damnosus* (formerly *P. cerevisiae*) (Künsch, Schärer, & Temperli, 1990; Mayer & Pause, 1972; Mayer, Pause, & Vetsch, 1973). Taylor, Leatherwood, and Lieber (1978) suggested that histamine production mainly occurs during the first fermentation phase. This explains why histamine is not generally considered as a typical major BA in sauerkraut. The histamine concentration in sauerkraut has been reported to fall within the range of 9–200 mg/kg (Mayer et al., 1973; Mayer & Pause, 1972), although typical amounts seem to range between 12 and 78 mg/kg (Buckenhueskes et al., 1992). Average values of 174, 146 and 50 mg/kg have been reported for tyramine, putrescine and cadaverine, respectively, in household and commercial sauerkraut from the Czech Republic and Austria, with the lowest concentrations in the household product (Kalac, Spicka, Krizek, Stewidlova, & Pelikanova, 1999).

Experiments have shown that the formation of BA by the use of starter cultures (*Lb. plantarum*) cannot be eliminated but nevertheless could be substantially reduced and thus controlled (Halás et al., 1999). According to Künsch et al. (1989), in a high-quality sauerkraut the following values for BA should not be exceeded:

histamine, 10 mg/kg; tyramine, 20 mg/kg; 2-phenylethylamine, 5 mg/kg; putrescine, 50 mg/kg; and cadaverine, 25 mg/kg. To enable this, all measures should be taken to ensure a rapid and reliable onset of fermentation, which, however, after reaching a pH of 4.0–3.8, should be stopped through pasteurization. An additional security would be offered by the use of starter cultures (Askar & Treptow, 1986; Buckenhueskes & Hammes, 1990). Strains of *Lb. plantarum* selected for their low amino acid decarboxylase activity have shown promising results as starter cultures for reducing BA formation during sauerkraut fermentation (Halás et al., 1999).

22.7.2 Microbial spoilage

Table 22.4 provides a helpful overview of potential problems in the fermentation process of fruits and vegetables and during storage. The emphasis is on those problems that are economically significant. Some of these difficulties can be averted through good manufacturing practice. However, even so, there is an unfortunate lack of understanding about their microbial and molecular origins.

During cucumber fermentation, the formation and damage associated with bloaters is a serious problem. The problem increases with larger cucumbers, higher temperatures for fermentation and higher CO_2 levels in the brine. It is therefore important to keep the CO_2 content in the fermentation mixture as low as possible. This can be done by, for example careful control of any bacteria and yeasts that produce carbon dioxide (McDonald, Fleming, & Daeschel, 1991), removing CO_2 from the brine using

Table 22.4 Problems that may occur during production and storage of lactic acid fermented fruits and vegetables

Vegetable	Discolouration	Softening	Other problems
Cucumbers	White or grey internal spots; surface discolourations	+++	Bloater formation, off flavour, butyric acid fermentation
Sauerkraut	Grey, pink or brown	+++	Off flavour
Olives	Grey, brown or yellow spots	+++	Off flavour, butyric acid fermentation, propionic acid fermentation
Cauliflower	Yellow or pink		
Green tomatoes	Grey	++	
Paprika	Grey	+++	Bitter taste
Carrots	Bleaching		Oxidized taste
Celery	Brown		
Green beans		++	Off flavour
Swedes	Grey or pink		

++, relevant; +++, serious problem.
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nitrogen gas (Fleming, Etchells, Thompson, & Bell, 1975), circulation of the brine or limiting the growth of heterofermentative LAB.

Homofermentative LAB can also produce CO_2 in sufficient quantities for bloater damage. This occurs through malolactic fermentation, where CO_2 and lactate are produced from malate. As recorded by McFeeters, Fleming, and Thompson (1982a, 1982b), malic acid is the chief naturally occurring organic acid in fermenting cucumbers, with a concentration of between 0.2% and 0.3% in a group of six cultivars. Daeschel, McFeeters, and Fleming (1985) and McDonald, Shieh, Fleming, McFeeters, and Thompson (1994) have explained that *N*-methyl-N′-nitro-*N*-nitrosoguanidine mutagenesis can be used to produce *Lb. plantarum*, which does not possess the ability to generate CO_2 from malic acid, and how this mutant form of *Lb. plantarum* can then be employed to solve the problem described above. However, this technique is as yet unexploited in commercial situations.

22.8 Kimchi

According to the Codex Alimentarius standard 223-2001, kimchi is a product predominantly prepared from Chinese cabbage (*Brassica rapa pekinensis*; syn. *Brassica pekinensis* Rupr) as well as from other cleaned, sliced, salted and spiced vegetables subjected to a fermentation.

Kimchi is considered the most typical Korean food and is an integral part of nearly every meal. Depending on the available raw material, the region, the season and the technology used, there are almost 200 different kimchi products today, with water kimchi ("*mul*-kimchi"), cucumber kimchi and radish kimchi as the most famous ones.

Kimchi is usually produced as follows. First, the Chinese cabbage and other vegetables (especially radish, cucumber and/or scallions) are salted in order to reduce the water activity (a_w value). In a second step, the salted vegetables are washed with fresh water, drained and then mixed with the desired spices, especially with chili or hot pepper powder, ginger and other edible *Allium* species such as garlic and horseradish. According to the Codex standard the following ingredients can be additionally used in the production of kimchi: fruits, rice or wheat paste, nuts, salted or fermented seafood, sesame seeds and sugars. In the final step the so-prepared vegetables are filled into prepared containers – traditionally glazed pottery – in which they are subjected to spontaneous lactic acid fermentation, with the aim of ripening and preservation. Kimchi is considered matured as well when it is fermented for 3 weeks at 4 °C or 4 days at 15 °C.

Kimchi fermentation is primarily a lactic acid fermentation, but also a certain growth of aerobic bacteria, yeasts and moulds is always observed. As with other lactic acid-fermented vegetables, some stages of fermentation can be observed in kimchi fermentation, including the initial growth of aerobic microorganisms and the risk of surface skin formation at the end of the fermentation due to the growth of yeasts. For a good fermentation the most important species are considered *Lb. plantarum*, *Lb. brevis*, *E. faecalis*, *L. mesenteroides* and *P. pentosaceus*. *Lb. brevis* is considered of special importance for the maturation process (Kwon & Young-Kyung, 2003).

However, due to the diversity of the recipes and ingredients used for kimchi manufacture, a wide range of species from different LAB genera, comprising *Leuconostoc*, *Lactobacillus*, *Weissella*, *Lactococcus* and *Pediococcus*, are considered as key organisms in kimchi fermentation (Park et al., 2012). Using pyrosequencing confirmed the initial (day 0) presence of diverse genera such as *Leuconostoc*, *Lactobacillus*, *Pseudomonas*, *Pantoea* and *Weissella*, but showed predominance of *Leuconostoc* strains commencing from day 3 until the end of kimchi fermentation (day 100) (Jeong, Jung, Lee, Jin, & Jeon, 2013). However, Jung et al. (2013) also found strains of *Lb. sakei* and *Weissella koreensis* to predominate during later fermentation stages. Designated as "one of the five healthiest foods in the world" (Lee et al., 2011), exploiting the health benefits of kimchi has become a special challenge for present-day research. Beneficial effects such as enhancing of intestinal health and anticarcinogenic effects (Kong et al., 2005) have been suggested for kimchi. Typical probiotic characteristics (Chang, Shim, Cha, & Chee, 2010) and specific anti-obesity effects and cholesterol reduction have been reported for some LAB strains associated with kimchi (Ji et al., 2012; Yoon et al., 2011, 2013). Technically, strains of *L. mesenteroides* and *Weissella* show promise as starter cultures (Lee & Lee, 2011; Park et al., 2013), while particularly strains of *Lb. sakei* and *Lb. plantarum* have been selected for implementation on the basis of their functional (health-promoting/probiotic) properties (Ji et al., 2013).

22.9 Future trends

LAF has a long tradition in the production of many kinds of preserved fruit and vegetable products. It is a highly valuable tool, since most of the essential physiological properties for such fermentation are inherent in it. Moreover, it is trusted by consumers who are more suspicious of other methods of preserving foods, including the extension of a food's shelf-life through gene modulation. In light of this, it is doubtful whether genetic engineering can be helpfully employed to further develop the process of food fermentation. Although many LAB properties depend upon plasmid-encoded traits that facilitate genetic modulation, a respect for current public opinion indicates that, for the present, it would be expedient to focus on the range of naturally occurring strains of LAB rather than to invest in the genetic modification of microorganisms.

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