Arnold E. Morton
Editor

Fermented Foods

Sources, Consumption and Health Benefits

Food Science and Technology

FOOD SCIENCE AND TECHNOLOGY

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EDITOR

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PREFACE

In recent years, there has been a growing trend in the consumption of functional foods. Functional foods are those that when consumed regularly produce a specific beneficial health effect beyond their basic nutritional properties. In this book, the authors focus on providing an overview of the current knowledge on technical approaches for the manufacturing of fermented dairy foods, as well as aspects concerning nutrition and health; the effects of supplementation of yogurt with appropriate plant materials for developing novel functional yogurt with antioxidant properties; the role of probiotics applications in fermented foods and the application of probiotic bacteria in foods for promoting health benefits; and finally, the monitoring of microbial volatile organic compounds in traditional fermented foods as well as the strategies of preservation and innovation paths in the field of traditional fermented foods.

Chapter 1 – Bioactive compounds formed *in situ* or added during manufacture of fermented dairy foods, such as galacto-oligosaccharides (GOS), caseinophophopeptides (CPP) and conjugated linoleic acid (CLA) have shown to exert various biological activities affecting digestive, cardiovascular, immune and nervous systems. In particular, the prebiotic capacity of GOS, anticariogenic activity of CPP and anti-carcinogenic and anti-adipogenic effects of CLA, are some of the most important. GOS, non-digestible carbohydrates comprised of galactose and glucose, are produced by the activity of β-galactosidases enzymes on lactose. In turn, CPP are derived from caseins by the action of proteolytic enzymes (trypsin, quimotrypsin), and CLA is synthesized by microbial activity from its precursor fatty acid (mainly linoleic acid). This chapter presents an overview the current knowledge on technological approaches for manufacturing of fermented dairy foods enriched in GOS, CPP and CLA, as well as aspects concerning nutrition and health.

Chapter 2 – Probiotic application in food product development is one of the major industries worldwide. The whole concept of probiotic is not new and probiotics in the form of fermented foods have been consumed by humans for thousands of years. Originally, probiotic delivery was mainly associated with fermented dairy foods such as yoghurt and, even today fermented dairy foods play a significant role in delivering probiotics to humans. Probiotic delivery has also moved progressively towards the non-dairy fermented foods including fruits, vegetables, cereals and meat and, even as nutraceuticals in the form of capsules. Despite the mode of delivery and the type of carrier food, fermented probiotic foods have been considered to promote the health and well-being of consumers. Inclusion of probiotics with known health features into the food matrices before the fermentation process is one of the most common practices in manufacturing fermented probiotic foods. Other than the nutritional and health benefits, probiotics may also positively influence the sensory

characteristics such as texture, aroma and taste of the final product and may responsible for extending the shelf life of the fermented product. This chapter focuses on discussing the role of probiotics applications in developing fermented foods, quality characteristics of both dairy and non-dairy fermented foods and their therapeutic effects in human nutrition and health.

Chapter 3 – Numerous fermented foods (fermented milk, fish, meat, vegetables,and plant products) are consumed around the world. These food products are prepared by the fermentation of various raw materials. Lactic acid bacteria (LAB) from the genera *Lactobacillus (Lb), Lactococcus (Lc), Leuconostoc (Ln), Pediococcus(P)* and *Weissella (W)* are the most important bacteria in desirable food fermentations. These bacteria are involved in the flavor, aroma compounds, γ-aminobutyric acid (GABA), and antimicrobial substances formation during fermentation. Several fermented fruit and vegetable products that arise from lactic acid fermentation are important for the nutritional requirements of a large proportion of the world's population. Diversity and probiotic potentials of LAB, *Lb. plantarum*, *Lb. paraplantarum*, *Lb. pentosus*, *Lb. sakei, Lb. casei*, *Lb. fermentum*, *Lb. paracasei*, *Lb. brevis*, *Lb. buchneri*, *Lb. parabuchneri*, *Lb. pantheris*, *Lb. harbinensis*, *Lb. kimchi*, *Lb. fallax*, *Lc.lactis*, *Ln. mesenteroides*, *Ln. pseudomesenteroides*, *P. pentosaceus, P. acidilactici, W. confusa*, *W. koreenis* and *W. cibaria* isolated from plant products have been reported. LAB produce bacteriocins with strong antibacterial activity to *Listeria*, *Bacillus* and *Staphylococcus*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, and *Carnobacterium*. LAB, *Lb. brevis*, *Lb. plantarum*, *Lc. lactis* subsp. *lactis*, *En. casseliflavus*, and *Streptococcus thermophilus* are found to produce GABA. Traditions and economic factors that limit the use of dairy fermented products in some developing countries promote the idea of reduction of milk components as vehicles for probiotic agents. Recently, the development of non-dairy probiotic products, including fruit, vegetables, and cereals, has been widely studied. Lactic acid fermentation contributes to improvement in the nutritive quality of vegetable juices. Beverages such as fruit and vegetable juices would be the food category where the healthy probiotic bacteria will make their mark. Fruit and vegetable juices and cereals, the potential substrate for the production of probiotic beverages by *Lb. plantarum*, *Lb. casei*, and *Lb. delbrueckii*, *Lb. rhamnosus* and *Lb. acidophilus*, are evaluated for their ability to survive during storage. These beverages could be a good vehicle for delivery of probiotic LAB to consumers, and have the potential to become a commercial product. Consumption of these probiotic products and pickles that contain GABA for reducing blood pressure has been associated with good health and longevity. In addition, acetic acid bacteria play role in vinegar fermentation. Antioxidant, antimicrobial, mineral, volatile, physicochemical, and microbiological characteristics of traditional vinegars have been characterized. Vinegar ingestion favorably influences biomarkers for heart disease, cancer, and diabetes. Traditionally fermented plant products not only have served as food supplements but have also had numerous health benefits attributed to them.

Chapter 4 – Recently, probiotic products were developed and rapidly increase in the functional food market. Commercial probiotic products include primarily dairy products (fermented milks, yoghurts, ice cream, cheeses), non-dairy products (beverages, breakfast cereals, fermented meats, dry-foods) and dietary supplements. The application of probiotic bacteria in foods for promoting health benefits is based on the concept that the maintenance of a healthy gut microflora provides protection against gastrointestinal disorders including infections and inflammatory syndromes of the bowel. The viable cell concentration of the probiotic bacteria in a food product or nutraceutical formulation should be as high as possible

(at least 10^6-10^7 CFU per gram of product at the time of consumption) because a significant number of bacterial cells die during storage and passage through the stomach and the small intestine. Therefore, identifying the factors influencing probiotic survival in food and developing ways to enhance probiotic survival during storage is an important area of research with considerable impact for the food industry.

It is possible to make probiotic cells more robust to external conditions. From an industrial perspective, the application of encapsulation has helped to increase the incorporation of probiotics in various foods. However, in general, before such a product reaches the market organoleptic assessment of the product needs to be carried out to ensure consumer acceptability. The sensory quality of the product is a challenge for probiotic-containing products.

Chapter 5 – Several studies have shown that free radicals extant in the human organism cause oxidative damage to different molecules such as lipids, proteins and nucleic acids and thus are involved in the beginning stage of some degenerative diseases. Biological antioxidants are substances which are able to delay or inhibit the oxidative damage of different biomolecules associated with several diseases including cancer, liver disease, aging, inflammation, diabetes, hypertension, Parkinson's disease and atherosclerosis. Plants present a good source of nutrients and antioxidants compounds. One of the important global market trends is searching for unique food ingredients and flavours with enhanced health properties. Yogurt is slow lactic fermentation of lactose from milk by thermophilic lactic acid bacteria, and is one of the most consumed fermented foods in many countries. It is widely recognized as functional food because of the nutritional properties, being a good vehicle to deliver probiotics to consumers. Incorporation of plant materials in yogurt during fermentation is an effective strategy to increase antioxidant intake and therapeutic value that may help to reduce the risk of developing chronic disease. The aim of the present study is to review the effects of supplementation of yogurt with appropriate plant materials for developing novel functional yogurt with antioxidant properties.

Chapter 6 – The bio-preservation of perishable edible raw materials through fermentations represents the first biotechnological application and one of the first forms of food processing in human history. Nowadays, this millenary tradition declined in many geographical contexts by reason of differences in raw materials, environmental conditions and in traditional knowledge. Flavour is one of the factors mainly characterizing the degree of typicality of fermented products. Many volatile organic compounds (VOCs) responsible for flavour (and off-flavour) perception are of microbial origin. Hence, microbial volatile organic compounds (mVOCs), by influencing the quality of food, affect consumer preference on fermented food. The huge number of geographical, compositional, microbiological and technological variables highlights the important need for tailored technologies to improve consumer acceptance of traditional fermented foods. Direct-Injection Mass Spectrometric (DIMS) technologies, associate time resolution with high sensitivity and robustness, thus offering interesting insights in the field. In particular, Proton Transfer Reaction Time of Flight Mass Spectrometry (PTR-ToF-MS) is an analytical tool for the detection and quantification of very small quantities of VOCs with the possible simultaneous real-time monitoring of these VOCs without sample preparation. In recent studies, the authors described the possible application of PTR-ToF-MS coupled with an auto-sampler and tailored data analysis tools for the automatic real-time monitoring of the mVOCs. The authors studied the mVOCs associated with the fermentative performances of yeast and lactic acid bacteria

(LAB), the main microbes involved in food fermentations. All these features, which allow rapid and accurate screening, are likely to be important for characterizing typical fermented foods and for developing new strategies in the standardization/ enhancement of microbial contribution to unique sensory qualities; both aspects are of a huge importance in the design of preservation and innovation paths in the field of traditional fermented foods.

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Chapter 1

BIOACTIVE COMPOUNDS IN FERMENTED DAIRY FOODS

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ABSTRACT

Bioactive compounds formed *in situ* or added during manufacture of fermented dairy foods, such as galacto-oligosaccharides (GOS), caseinophophopeptides (CPP) and conjugated linoleic acid (CLA) have shown to exert various biological activities affecting digestive, cardiovascular, immune and nervous systems. In particular, the prebiotic capacity of GOS, anticariogenic activity of CPP and anti-carcinogenic and anti-adipogenic effects of CLA, are some of the most important.

GOS, non-digestible carbohydrates comprised of galactose and glucose, are produced by the activity of β-galactosidases enzymes on lactose. In turn, CPP are derived from caseins by the action of proteolytic enzymes (trypsin, quimotrypsin), and CLA is synthesized by microbial activity from its precursor fatty acid (mainly linoleic acid).

This chapter presents an overview the current knowledge on technological approaches for manufacturing of fermented dairy foods enriched in GOS, CPP and CLA, as well as aspects concerning nutrition and health.

Keywords: functional foods, galacto-oligosaccharides, caseinophophopeptides, conjugated linoleic acid, beneficial effects, technological strategies

INTRODUCTION

In recent years, there has been a growing trend in the consumption of functional foods. Functional foods are those that when consumed regularly produce a specific beneficial health

effect beyond their basic nutritional properties. In this regard, recent studies have demonstrated the presence of a wide range of bioactive molecules or components in milk and fermented dairy products. The term 'bioactive component' refers to chemical compounds that are present naturally in foods or formed and/or formulated during processing (Shah 2000, Korhonen 2009, Park 2009). The dairy industry has an important role in the development of this food category; as dairy products that stimulate the immune system, reduce elevated blood pressure, inhibit the development of cancer, among others, are sold in some countries (Playne 2003, Korhonen 2009). In particular, the sector of fermented dairy foods is one of the most dynamic at industrial level, since it constantly develop new products to meet growing consumer demand towards natural, fresh and healthy foods.

There is a wide diversity of bioactive molecules, belonging to the families of carbohydrates, proteins and lipids, among which it may be mentioned galacto-oligosaccharides, caseinphosphopeptides and conjugated linoleic acid, respectively. GOS, non-digestible carbohydrates comprised of galactose and glucose, are produced by the activity of β-galactosidases enzymes on lactose. In turn, CPP are derived from caseins by the action of proteolytic enzymes (trypsin, quimotrypsin), and CLA is synthesized by microbial activity from its precursor fatty acid (mainly linoleic acid).

The addition of these components in the formulation of fermented foods is well documented. On the other hand, some studies also indicate that they can be generated *in situ* during manufacture, which constitutes a potential alternative to their use as additives. However, the development of functional foods holds many technological changes.

In the formulation of some fermented foods, different ingredients (skimmed milk powder, caseinates, concentrates or whey protein isolates, among others), could be included (Isleten and Karagul-Yuceer 2006, Peng et al. 2009). They provide different amounts of casein, whey protein, lactose, fat and minerals, some of which act as substrates of biochemical reactions for GOS, CPP and CLA production. In addition, the microbiota present in the food matrix together with other ingredients such as prebiotics, could contribute to the formation of these compounds.

This chapter reviews the current knowledge about claimed beneficial health effects of GOS, CPP and CLA and the technological aspects related to the development of fermented dairy foods enriched in these bioactive compounds.

GALACTO-OLIGOSACCHARIDES

Definitions and Characteristics of GOS

GOS are non-digestible carbohydrates; consist of a chain of galactose units usually with a terminal glucose unit. They are synthesized from lactose by the action of β-galactosidase enzyme (EC 3.2.1.23) in a transgalactosylation reaction that occurs simultaneously with the hydrolysis (Gosling et al. 2009, Park and Oh 2010). The mechanism of these reactions can be described as follows. The β-galactosidase is coupled to a molecule of lactose. A glucosyl moiety is released and the enzyme and galactosyl moiety form a covalent bond. At this point, hydrolysis or transgalactosylation can occur depending on the available galactosyl acceptor. The hydrolysis takes place if the galactosyl acceptor is water and so a galactose molecule is

released. In turn, if the acceptor is a saccharide (glucose, galactose, lactose or GOS), a transgalactosylation reaction occur and the result leads to a heterogeneous and complex mixture of GOS (Sangwan et al. 2011). These compounds have different degree of polymerization (from 2 to 10 monomer units), type of glycosidic bond between units (β-1-4, β-1-6) and molecular weight (Mahoney 2003, Gosling et al. 2010, Park and Oh 2010). The amount and nature of GOS synthesized is highly influenced by several factors such as enzyme origin (isolated from yeast, fungi or bacteria and then purified or obtained from a crude cell extract of microorganisms) and their state (in soluble form or immobilized on a support), concentration of substrate, and the conditions under which the reaction carried out (pH, temperature and time) (Boon et al. 2000, Martínez-Villaluenga et al. 2008a, Neri et al. 2009b, Park and Oh 2010, Gosling et al. 2010, Palai et al. 2012). Different authors have worked on identifying the individual structure of GOS, which requires the use of sophisticated analytical techniques, since prebiotic property depends on the linkage type and polymerization degree (Coulier et al. 2009, Gosling et al. 2010).

Initial investigations were focused on theoretical aspects of the reaction tending to elucidate the mechanism and kinetics of the process (Mahoney 1998). Then, interest was focused on the optimization of parameters for GOS production in order to increase the yield intended to be used as an additive in the food industry. The use of GOS as an additive was encouraged by knowledge of their beneficial effects on human health (mainly prebiotic activity) (Sako et al. 1999, Playne 2003, Splechtna et al. 2006, Mussatto and Mancilha 2007).

Commercial GOS preparations used are composed of a mixture of several types of GOS in liquid or powder form (Playne 2003, Mlichová and Rosenberg 2006).

On the other hand, some studies indicate that GOS can be generated in situ in food matrix under certain conditions. In particular, these bioactive compounds can be synthesized in fermented dairy products during processing (Playne 2003).

Health Benefits

GOS are an important group of bioactive compounds; its main function is the prebiotic capacity. In fact, they resist to salivary, pancreatic and intestinal enzymes and the stomach acid medium. They are fermented by the beneficial microflora of the large intestine inhibiting the growth of pathogenic and putrefactive bacteria and thereby contribute to reduce of toxic metabolites, prevention of diarrhea, constipation relief and improvement of lactose tolerance (Caselato de Sousa et al. 2011). Also, metabolism of GOS leads to the production of short chain fatty acids which show beneficial effects including increase in calcium and magnesium absorption, control of serum lipid and cholesterol level, reduction of cancer risk, among others (Cashman 2003, Sangwan et al. 2011, Whisner et al. 2013).

Prebiotic activity is measured mainly on the basis of increased numbers of intestinal bifidobacteria or lactobacilli upon consumption. It is important to know the type and composition of GOS as their metabolism by these bacteria is different. In fact, generally, lactobacilli ferment di- or trisaccharides whereas bifidobacteria employ complex polysaccharides as carbon source, although this activity is strain-dependent (Gänzle 2011, Sims et al. 2014).

Uses As Additive

Industrial production of GOS has been carried out mainly for the development of infant formulas, in which GOS are incorporated as bifidogenic factor to simulate human milk. Breast milk naturally contains a high concentration of carbohydrates, especially oligosaccharides (at least 20 different classes), which constitute the third most abundant component. Conversely, milk from cow and other ruminants are poor in these compounds, besides have a simpler oligosaccharides profile (Vandenplas 2002, Playne 2003, Pandya and Haenlein 2010). Recently, it have been reported the importance of GOS in food intended for elderly people in order to promote the health, due to that gastrointestinal problems are among the most common pathologies affecting this group of population (Surakka et al. 2009, Montilla et al. 2015).

These compounds have other properties that are useful in the food industry: their stability in acidic medium and at high temperature; ability to reduce the freezing point of foods; high moisture retaining capacity preventing excessive drying; high viscosity; excellent taste quality and low sweetness and calorie value (Splechtna et al. 2006, Torres et al. 2010). Because of these properties, the use of GOS has increased in a wide variety of other foods such as bakery products and confectionary, ice cream, beverages, chewing gum and jams. In addition, livestock feed and pet food industries, poultry, pig and aquaculture and cosmetic and pharmaceutical industries, can also take advantage of physicochemical and physiological properties of GOS (Torres et al. 2010, Sangwan et al. 2011).

GOS Synthesis

Several studies have been performed in order to study different variables on GOS synthesis; in particular, we discussed the origin of the enyme and if it is free or immobilized, type and concentration of substrate, GOS yield and composition of GOS mixture obtained and reaction conditions. Martinez-Villaluenga et al. (2008a) reported the synthesis of di- and tri-saccharides from lactose solution using a free commercial enzyme preparation (Lactozym) from *Kluyveromyces lactis*. The profile of GOS mixture was different depending on the conditions of pH and temperature/time employed. Cardelle-Cobas et al. (2009) reported different GOS profiles using β-galactosidases from *K. lactis* (Lactozym) and *Aspergillus aculeatus* (Pectinex) on lactose. Allolactose was the di-saccharide more abundant produced with Lactozym, while the tri-saccharide 4'-galactosyl-lactose was originated with Pectinex; the tri-saccharide 6'-galactosyl-lactose was formed with both enzymes. Neri et al. (2009a and 2009b) found that the initial lactose concentration in reaction mixture affected the GOS yield, using β-galactosidases from *A. oryzae*. No significative difference was observed for the free and immobilized enzymes. Temperature and pH showed to have a minimal effect on GOS production. The reaction rates for GOS formation increased with increasing temperature from 30ºC to 60ºC, but levels of GOS production was almost unchanged. The maximal values ranged from 26.9% to 64.1%, starting from an initial lactose concentration from 5 to 50 g/100 mL, respectively. Production of tri-saccharides predominated over that of tetra-saccharides. Recently, Palai et al. (2012) obtained a GOS mix composed mainly by tri-saccharides followed by tetra- and penta-saccharides employing β-galactosidase from *Bacillus circulans* (Biolacta FN5) on lactose. They also found a competitive inhibition of glucose on

transgalactosilation reaction. Meanwhile, Padilla et al. (2012) evaluated transgalactosylase activity of crude cell extracts from *Kluyveromyces* strains isolated from cheeses. The yield and types of GOS obtained were different for all strains studied. The yields ranged from 25 to 42 g/100 g. 6′-galactosyl-lactose was the main tri-saccharide formed followed by 6-galactobiose and 3′- and 4′-galactosyl-lactose. In studies conducted by our research group, GOS synthesis and lactose hydrolysis by commercial β-galactosidase enzyme from *K. lactis* (YNL-2, GODO) on lactose solution were evaluated (Vénica et al. 2015). The GOS formation was favored with increasing of initial lactose concentration and enzyme doses, while the hydrolysis dominated at lower levels of lactose. The maximum GOS content obtained was 13.1 g/100 mL starting from 20 g/100 mL of lactose.

Studies carried out on more complex matrices are discussed below. Golowczyc et al. (2013) employed whey permeate suspension (approx. 32 g lactosa/100 g) and β-galactosidase from *A. oryzae*; the reaction was performed at 37°C, pH 4.5 and 120 min. The maximum GOS concentration obtained was 27 g/100 g for a lactose conversion near to 57%. Song et al. (2013) examined the conditions for GOS synthesis using reconstituted cheese whey and a lactase from *Lactobacillus paracasei* YSM0308. The final GOS concentration was 19.41 g/100 mL for the following conditions: 30 g/100 g of whey powder, pH 6.5-7.0, 30°C and 4 h. Fischer and Kleinschmidt (2015) studied GOS synthesis of two commercial lactases from *A. oryzae* and *K. lactis* on sweet and acid wheys and lactose solution. The *A. oryzae* enzyme gave a GOS yield (including non-lactose disaccharides, trisaccharides and oligosaccharides of higher polymerization degree) of approximately 11% starting from 38 g/L of lactose for the three matrices under study. Thus, it is presumed that the components of whey did not influence the transgalactosilase activity. However, *K. lactis* enzyme showed a GOS yield different depends on type and concentration of whey. GOS yield was 10.7% in lactose solution and acid whey whereas it was of 4.3% in sweet whey, using 38 g/L of initial lactose. They observed that the inhibitory effect of sweet whey decreased with increasing initial lactose concentration, which resulted in even higher yields than in lactose solution. Corzo-Martínez et al. (2013) reported the maximum formation of GOS with degrees of polymerization from 2 to 4 (disaccharides 16%, trisaccharides 21% and tetrasaccharides 3%) after 5 h of reaction at pH 6.5 and 50ºC with 300 g/kg of total carbohydrates using whey permeate (previously isomerized and composed mainly by lactose and lactulose) and β-galactosidase from *B. circulans*.

In the case of employing milk as reaction medium, the GOS yields reported were minor in comparison to the results mentioned previously. Kwak and Jeon (1986) found that the GOS concentration depend on the enzyme (from *K. lactis* and *Candida pseudotropicalis*) level and temperature/time of reaction; the maximum values (0.37 g/100 g) were lower than those obtained with lactose solution. Then, GOS were almost entirely hydrolyzed as the reaction proceeded. Gosling et al. (2009) applied a thermal treatment to commercial enzyme (Biolacta FN5) prior to milk addition, which led to a higher concentration of GOS (2.78 g/100 mL). This fact was attributed to selective inactivation of enzymes with greater hydrolytic capacity, preserving the enzymes with transgalactosilase activity. Ruiz-Matute et al. (2012) analyzed the GOS content in commercial lactose-free milks and evaluated the formation of GOS during preparation of UHT milk hydrolyzed with Lactozym. GOS were detected in all commercial samples ranging from 0.09 to 0.43 mg/100 mL. During preparation of hydrolyzed UHT milk, the GOS reached a maximum about 1 g/100 mL and then they gradually decrease by half. On the other hand, the higher GOS values (0.70 g/100 mL) were obtained employing lactase from

K. lactis in comparison to those levels obtained with *B. circulans*, and *A. oryzae* (Rodriguez-Colinas et al. 2014).

To sum up, the results suggest that a variety of processing conditions influence the GOS formation and the composition of mixture obtained.

Generation *In Situ* during Manufacture of Fermented Milk

In relation to the presence and formation of GOS in fermented dairy products, the information available is limited (Table 1). Early results were reported in the 80s. Toba et al. (1982 and 1983) determined the content of GOS in commercial yogurts and in yogurts during manufacture. In both cases, GOS values obtained were low (less than 0.2%). The presence of GOS was attributed to an increase of extracellular or intracellular β-galactosidases released during autolysis of yogurt cultures. Then, Toba et al. (1986) observed an enhancement of GOS production when exogenous β-galactosidases from *A. oryzae* (Galantase and Sumylact L) were added simultaneously with starter. Maximum GOS levels were detected at two hours of fermentation (1.06-1.23%) and then the values diminished. In fact, GOS in yogurts made with higher enzyme doses were hydrolyzed mostly towards the end of manufacture, whereas the maximum GOS content was reduced approximately by half in yogurts prepared with lower enzyme doses. Subsequently, Lamoureux et al. (2002) found GOS values of approximately 0.27 g/100 g in traditional yogurts, which increased up to 0.71 g/100 g when probiotic bacteria were included in the formulation. The technological strategy employed was to add an incubation step (1.5 h/50ºC) with bifidobacteria strains (*Bifidobacterium animalis*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum*) prior to yogurt manufacture. GOS production mainly occurred during this period. *B. infantis* had the greatest ability to synthesize GOS (approximately 0.7%). No significant differences in GOS concentration was observed through 28 days of storage. Similarly, Yadav et al. (2007) reported different GOS values for fermented milks made with mixed dahi cultures, *Lactococcus lactis*, *L. acidophilus* and *L. casei*; the highest content was found in products with *L. acidophilus.* No changes occurred during storage, indicating that GOS were not hydrolyzed in this period. On other hand, Martinez-Villaluenga et al. (2008b) analyzed commercial fermented milks and found considerable variation in the concentration and composition of GOS among samples. Classical yogurts showed values of approximately 0.24 g/100 g and yogurts containing probiotic bacteria presented values between 0.3 and 0.6 g/100 g. This study revealed that GOS content remained unchanged during storage, suggesting that hydrolysis of these compounds does not occur. Recently, Martins et al. (2011 and 2014) prepared yogurts using classical starter culture and probiotics (*B. animalis* and *L. acidophilus*) and the addition of a mix of β-galactosidases from *K. lactis* and *A. niger.* They evaluated the effect of initial lactose concentration, enzyme doses and moment of enzyme addition (with a lag period in relation to the beginning of fermentation). The condition established to maximize GOS content (0.49 g/100 mL) was to add the maximum enzyme dose assayed 90 minutes after the start of fermentation. Yogurts without enzyme addition showed low GOS values (0.04 g/100 mL), suggesting that the starter cultures was not able to produce them.

Table 1. GOS in fermented milk products

Fermented milk product	Origin of β-galactosidase added	Total GOS	Oligosaccharide identified	References
Yogurts		30-90 mg/100 g	6-O-β-D-galactopyranosyl-D-glucose (Allolactose) 6-O-β-D-galactopyranosyl-D-galactose	Toba et al. (1982)
Yogurts		30-200 mg/100 g	Oligosaccharides not specified (other than lactose).	Toba et al. (1983)
Yogurts		30-170 mg/100 g	Disaccharides Trisaccharides Oligosaccharides not specified (other than lactose).	Toba et al. (1986)
Yogurts	*A. oryzae*	280-670 mg/100 g		
Yogurts		260-270 mg/100 g	Trisacharides	Lamoureux et al. (2002)
Yogurts with *B. animalis*		460-580 mg/100 g		
Yogurts with *B. bifidum*		480-520 mg/100 g		
Yogurts with *B. breve*		500-660 mg/100 g		
Yogurts with *B. infantis*		480-710 mg/100 g		
Yogurts with *B. longum*		480-520 mg/100 g		
Fermented milk with mixed dahi cultures		900-940 mg/100 g	Trisaccharides Tetrasaccharides Pentasaccharides	Yadav et al. (2007)
Fermented milk with *Lc. Lactis*		780-840 mg/100 g		
Fermented milk with *L. acidophilus*		1040-1170 mg/100 g		
Fermented milk with *L. casei*		1360-1420 mg/100 g		
Yogurts		223-249 mg/100 g	D-Galp-β(1-3)-D-Gal (3-galactobiose) D-Galp-β(1-6)-Lac (6´-galactosyl-lactose) D-Galp-β(1-3)-D-Glc (3-galactosyl-glucose) D-Galp-β(1-3)-Lac (3´-galactosyl-lactose) Other unidentified	Martinez-Villaluenga et al. (2008b)
Yogurts with bifidobacteria		357-585 mg/100 g		
Ready-to-drink yogurts with *L. casei*		292-435 mg/100 g		
Yogurts with *B. animalis* and *L. acidophilus*	*K. lactis* and *A. niger*	270-420 mg/100 mL	Oligosaccharides not specified (other than lactose).	Martins et al. (2011)
Yogurts with *B. animalis* and *L. acidophilus*		40 mg/100 mL	Oligosaccharides not specified (other than lactose).	Martins et al. (2014)
Yogurts with *B. animalis* and *L. acidophilus*	*K. lactis* and *A. niger*	90-490 mg/100 mL		
Yogurts	*K. lactis*	340-610 mg/100 g	Trisaccharides	Vénica et al. (2015)
Yogurts with *L. acidophilus*	*K. lactis*	380-660 mg/100 g		

Likewise, our research group studied the effect of a commercial β-galactosidase and *L. acidophilus* La-5 on GOS formation during the manufacture and storage of drinkable and stirred yogurts (Vénica et al. 2015). The enzyme and starter were added together, while *L. acidophilus* was incorporated at the end of manufacture. The presence of GOS was already evident at 45 min of fermentation. Mean concentrations were 0.36 and 0.62 g/100 g for fresh

drinkable and stirred yogurts, respectively. No changes in GOS levels were observed through storage, indicating that they were stable in the products. Probiotic culture was not able to produce GOS in the conditions studied.

On the other hand, to the best of our knowledge, there are no data reported on cheese enriched in GOS. Preliminary studies carried out in our institute show the possibility to obtain cheeses with GOS. However, the conditions of manufacture have to be studied in detail for obtaining chesses with suitable characteristics.

To sum up, the results show the great potential of fermented dairy products to be carriers of GOS by *in situ* generation during processing. In particular, the data published on GOS formation during manufacture of fermented milks and their stability during storage is not abundant and exhibits much variability. This fact can be attributed, as mentioned above, to that many factors affect the synthesis of these compounds such as β-galactosidase enzyme source and concentration, type and counts of microorganisms, concentration of substrate (lactose), composition of food matrix, conditions of fermentation and storage, and time/temperature of hydrolysis/transgalactosylation.

CASEINOPHOSPHOPEPTIDES

Definition and Characteristics of CPP

The term phosphopeptide was likely used by first time by Mellander in 1950 to describe phosphorylated peptides derived from calcium-sensitive caseins (α_{s1}, α_{s2} and β caseins), which enhanced vitamin D independent bone calcification in rachitic infants (FitzGerald 1998). These peptides are inactive fragments within the sequence of precursor protein, but they can exert specific biological activities after its release *in vivo* during the passage through the gastrointestinal tract. In addition, they are also produced *in vitro* by the action of specific enzymes or *in situ* during food processing (Korhonen 2009). The primary structures of α_{s1}, α_{s2} and β caseins are characterized by the presence of phosphoryl residues, which are present as monoesters of serine. These phosphoseryl residues occur mainly in clusters that involve two, three or four residues, and in general are next to two glutamic acid residues. Thus, most CPP contain the cluster sequence or acidic motif: SerP-SerP-SerP-Glu-Glu (FitzGerald 1998, Cross et al. 2005). These or related sequences occur in the following fractions of caseins: α_{s1}-CN (f66-70), α_{s2}-CN (f8-12), α_{s2}-CN (f56-60), α_{s2}-CN (f129-133) and β-CN (f15-19) (Meisel and FitzGerald 2003). This domain of high negative charge gives to CPP the ability to enhance bivalent mineral solubility, which is relevant to several biological functions in the organism (Nongonierma and FitzGerald 2012).

Health Benefits

Multi-functional bioactive effects have been attributed to CPP. Among the specific physiological functions that can exert these peptides, this review has been focused on their more recognized activities such as antiocariogenicity, antioxidant and mineral binding capacity. Nevertheless, it has been also demonstrated that CPP can exert cytomodulatory

effects, which comprise antiproliferative activities towards cancer cells as well as immunomodulatory effects on immunocompetent cells (Meisel and FitzGerald 2003). More recent studies have attributed new beneficial properties to CPP such as potential inhibitors of gastric secretion (Guilloteau et al. 2009) and modulator of bone cell activity, probably sustained by their ability as calcium carrier (Donida et al. 2009, Cosentino et al. 2010).

Anticariogenic Activity

The consumption of milk, milk concentrates, yogurt and cheese has been associated with a lower prevalence of dental caries in humans. Studies on anticariogenic properties of dairy products have been conducted through a number of *in vitro*, *in situ* and *in vivo* models and epidemiological studies (Silva et al. 1987, Vacca Smith and Bowen 2000, Ferrazano et al. 2008, Tanaka et al. 2010). Several milk components such as caseins, peptides (glycomacropeptide, CPP; proteose-peptone fraction), calcium, phosphorous, etc. seem to provide protection against dental caries by different mechanisms, which are not fully elucidated (Silva et al. 1987, Grenby et al. 2001, Warner et al. 2001, Andrews et al. 2006, Ferrazano et al. 2008, Herod 2009).

Prevention of dental caries by milk-derived peptides is a complex physical and chemical sequence of events including bacterial inhibition, competitive exclusion to enamel binding sites, improved buffering capacity in the pellicle surrounding teeth, reduction of enamel demineralization, and increasing of enamel remineralization (Aimutis 2004).

Among CPP, only those containing the sequence Ser(P)-Ser(P)-Ser(P)-Glu-Glu have demonstrated to have anticariogenic properties. The particular sequence of aminoacids and the conformational specificity of peptides are required for developing a full anticariogenic activity. At this respect, it has been observed that non-phosphorylated casein peptides had no anticariogenic effects and synthetic peptides having phosphorylated residues only exert a partial anticariogenic activity (Reynolds 1995, Aimutis 2004). The calcium binding and the calcium phosphate stabilization by the peptides is influenced by their net charge, length and sequence (Cross et al. 2005). Among peptides having anticariogenic activity, α_{s1}-CN(f59-79)5P, β-CN(f1/2-25)4P, α_{s2}-CN(f46-70)4P and α_{S2}-CN(f1/2-21)4P were reported (FitzGerald 1998, Reynolds 1998, Cross et al. 2005).

CPP have the distinctive ability to form soluble complexes with amorphous calcium phosphate (ACP); these peptides stabilize calcium phosphate in solution through their multiple phosphoserine residues and substantially increase the level of calcium in dental plaque, but without allowing growth to the critical size required for nucleation and precipitation of calcium phosphate. Calcium and phosphate are essential components of enamel and dentine and they form highly insoluble complexes, but in the presence of CPP remain soluble and biologically available. Thus, the mechanism of anticariogenicity for the CPP-ACP more accepted is that they localize ACP in dental plaque which buffers the ionic activities of free calcium and phosphate, helping to maintain a state of supersaturation with respect to tooth enamel. This depresses demineralization and enhances remineralization processes (Reynolds 1995, 2008). This fact has been demonstrated in several studies that include rat caries model (Reynolds 1995), *in situ* caries model (Reynolds 1987, 1998) and *in vitro* desmineralization/remineralization assays (Reynolds 1997, 1998; Grenby et al. 2001, Rahiotis and Vougiouklakis 2007, Kanekanian et al. 2008, Setareh Nejad et al. 2009).

The fact that some antibiotics are peptides has also suggested that CPP might have antibiotic activity. However, some authors considered as very unlikely a simple antibiotic

mechanism of these compounds (Andrews et al. 2006). On the other hand, there is contradictory evidence that CPP-ACP nanocomplexes influence bacterial adhesion to the dental hard tissues and reduce biofilm formation both in *in vitro* and *in situ* studies (Schüpbach et al. 1996, Rose 2000, Rahiotis et al. 2008, Grychtol et al. 2014). Finally, recent studies have postulated that the CPP could have a more passive role, by acting as protective coat over the tooth surface (Andrews et al. 2006, Kanekanian et al. 2008).

Antioxidant Activity

Among the beneficial effects of CPP, its antioxidant activity is the least known (Pihlanto 2006). Antioxidant compounds present in foods contribute to the maintenance of antioxidant defense systems of living systems (Aloğlu and Öner 2011). It has been recognized that reactive oxygen species (ROS) ($O_2^{\cdot-}$, OH, H_2O_2, etc.) and free radicals produced during the late stage of the oxidative reaction plays a key role in a great number of degenerative and age specific diseases (Pihlanto 2006, Virtanen et al. 2007). In addition, lipid peroxidation can generate deteriorations in food quality such as off-flavor development and shortening of shelf-life (Diaz and Decker 2004, Elias et al. 2008). Antioxidant agents can develop this activity in different ways: by preventing the formation of free radicals, by scavenging free radicals and ROS or by chelating transitions metal ions which act as pro-oxidants (Aloğlu and Öner 2011).

There are several studies that demonstrate the ability of milk caseins to inhibit lipid oxidation in foods. Quenching of free radicals by oxidation of amino acid residues and chelating metals by those domains that contain phosphorylated serine residues were proposed as possible explanations (Pihlanto 2006). However, the release of peptides through hydrolytic process seems to be the most promising approach to form proteinaceous antioxidants since peptides have significantly higher antioxidant activity than intact proteins (Elias et al. 2008).

The production of antioxidant peptides in fermented dairy products such as milk, yogurts and cheeses, which contain different lactic acid bacteria (LAB), indicate that the development of radical scavenging activity is a strain-specific characteristics, and radical scavengers are related to proteolysis (Kudoh et al. 2001, Hernández-Ledesma et al. 2005, Silva et al. 2006, Virtanen et al. 2007, Gupta et al. 2009, Farvin et al. 2010a, 2010b; Aloğlu and Öner 2011). Antioxidant peptides can be also produced by action of exogenous proteases on casein and whey proteins (Suetsuna et al. 2000, Peña-Ramos and Xiong 2001, Rival et al. 2001, López-Expósito et al. 2007, Bayram et al. 2008, Gómez-Ruiz et al. 2008, Peng et al. 2009). These hydrolysates possess great potential for use as natural antioxidants in food products (Peña-Ramos and Xiong 2001). Neither the structure-activity relationship nor the mechanism of inhibiting lipid oxidation by the enzymatic hydrolysates is fully understood (Peña-Ramos and Xiong 2001, Philanto 2006). The antioxidant activity of the peptides has been attributed to peptide molecular weight, aminoacid composition, structure, configuration and hydrophobicity (Peng et al. 2009, Virtanen et al. 2007). The prevalence of certain residues of aminoacids in the peptides such as histidine, valine, leucine, phenylalanine, tryptophan, tyrosine, methionine, lysine, etc. is related to the found activities (Peña-Ramos and Xiong 2001, Virtanen et al. 2007). In addition to the presence of the appropriate aminoacids, the specific positioning in the sequence plays an important role in the antioxidant activity of the peptides (Sarmadi and Ismail 2010). Unfortunately, the specific aminoacid sequence responsible of antioxidant activity has been identified only in a few research works. In these cases, none of the peptides contained phosphoseryl residues (López-Expósito et al. 2007,

Farvin et al. 2010b). Thus, the peptide fractions were not dominated by CPP. However, CPP preparations, derived from spray-dried whole tryptic digest containing 40% α_{s1}-CN (43-79) and 36% β-CN (1-25), demonstrated to prevent oxidation reactions through different primary or secondary antioxidant mechanisms that specifically involve direct free radical scavenging and sequestering of potential metal prooxidants (Kitts 2005). A similar study revealed that tryptic digest of bovine milk casein containing CPP were effective inhibitors of lipid oxidation (Chiu and Kitts 2004). Besides, CPP have been shown to be good antioxidants in foods (Díaz and Decker 2004, Sakanaka et al. 2005, Rossini et al. 2009) and in different systems models such as liposome (Díaz and Decker 2004) and oil-in-water emulsions (Díaz et al. 2003). Kim et al. (2007) and Rossini et al. (2009) reported that CPP prepared from sodium caseinate using proteolytic enzymes exhibited antioxidant activity. This activity was dependent on the pH values of the supernatants obtained from hydrolysates.

Enhanced Bioavailability of Minerals

The excellent bioavailability of calcium from dairy products has been attributed, at least partially, to the presence of CPP in the small intestine (FitzGerald 1998). As previously mentioned, these peptides are formed *in vivo* by normal digestion of caseins and, because they are relatively resistant to further enzymatic degradation, accumulate in the distal ileum (Adamson and Reynolds 1996, Phelan et al. 2009). CPP may form soluble organophosphate salts with minerals such as calcium, iron and zinc at intestinal pH, modulating their bioavailability, and therefore, they may act as mineral solubilizers and/or carriers. The binding sites for minerals involve Ser-bound phosphate groups, as well as the free carboxyl groups of Glu; the hydrophobic tail protects this complex from further interactions, and hence prevents the precipitation of calcium ions as calcium phosphate or phytate (Ferraretto et al. 2003). This suggested the possibility that CPP may enhance the soluble calcium amount in the intestinal lumen, thereby increasing the mineral availability for absorption in the small intestine (Cross et al. 2005). However, considerable controversy exists as to whether the CPP can improve dietary calcium absorption (FitzGerald 1998). *In vitro* assays performed in human intestinal tumor HT-29 cells, which are used as a study model for intestinal absorption, rat ileum sacs or ligated segments, as well as studies conducted on whole animals, have suggested a positive role exerted by CPP on intestinal calcium absorption and bioavailability (Lee et al. 1983, Sato et al. 1986, Erba et al. 2002, Ferraretto et al. 2003, Cosentino et al. 2010). These experiments, however, were not able to clarify the mechanism by which CPP induced calcium uptake, particularly to distinguish whether passive diffusion or active transport was affected (Ferraretto et al. 2003). Conversely, *in vivo* studies in animals revealed that there is no evidence that CPP can have a significant effect on the calcium absorption (Pointillart and Guéguen 1989, Brommage et al. 1991, Bennett et al. 2000). Such discrepancies have been attributed, in part, to differences in the methodologies used to measure extrinsic and intrinsic absorption of Ca (FitzGerald 1998). Besides, other factor that contribute to the explanation of the controversial results about the role of CPP on mineral bioavailability *in viv*o is the origin of CPP (αs- versus β-caseins), which display specific effects (Bouhallab et al. 2002).

In spite of a great number of studies have been focussed on calcium bioavailability, some investigations have attempted to elucidate the role of CPP on iron and zinc absorption. In this sense, the results on Fe metabolism suggest that the bioavailability of CPP bound iron was

improved in young deficient rats (Aït-Oukhatar et al. 1997) and in vascularised duodenal rat loop model (Aït-Oukhatar et al. 2002). The influence of type of phosphopeptide (Bouhallab et al. 2002), the mechanisms of absorption of CPP bound iron (Pérès et al. 1999) and the role of alkaline phosphatase (Ani-Kibangou et al. 2005) in the absorption of this complex, have been also studied. On the other hand, the contribution of CPP to the enhancement of zinc bioavailability has been recently reviewed (Miquel and Farré 2007).

Production of CPP

Different approaches for CPP isolation and industrial production have been developed in order to use these compounds as additives in different products because their functional properties. CPP can be produced by hydrolysis from precursor milk caseins using the following agents: a. Digestive enzymes, b. Proteolytic starter cultures, c. Enzymes derived from microorganisms or plants (Phelan et al. 2009).

Most of the commercially available CPP are usually prepared from sodium caseinate or whole casein using pancreatic endoproteinases, single or combined, such as trypsin, chymotrypsin and pancreatin. The tryptic hydrolysis is the most effective procedure used to yield these bioactive peptides (Qi et al. 2003). In addition, other proteinases, such as alcalase, papain and pepsin, have been also assayed for CPP production. (Adamson and Reynolds 1996, McDonagh and FitzGerald 1998). The type of enzyme, degree of hydrolysis, ratio of enzyme to substrate (E:S) as well as the method used for isolation and purification to obtain CPP, determine the composition of mixtures (Adamson and Reynolds 1995, 1996, 1997; McDonagh and FitzGerald 1998, Ellegård et al. 1999). For example, it has been reported that CPP released from tryptic digestion are β-CN(f1-25)4P and α_{s1}-CN(f59-79)5P with smaller quantities of α_{s2}-CN(f46-70)4P and α_{S2}-CN(f1-21)4P (Cross et al. 2005), whereas the main CPP released by pancreatin were β-CN(f7-24)4P, α_{s1}-CN(f61-78)5P and α_{s1}-CN(f59-78)5P (Adamson and Reynolds 1995). On the other hand, the use of alcalase produces truncated peptides in comparison to those released by tripsin (Adamson and Reynolds 1996). Finally, it is important to consider that the quality of CPP produced and isolated is highly dependent on temperatures used during this process; CPP are sensitive to heat because high temperatures induce dephosphorylation (Meisel and FitzGerald 2003).

The casein hydrolysate obtained is then adjusted to pH 4.6 and insoluble non-peptide material is separated by centrifugation. Phosphopeptides, which remain soluble at this pH, are then aggregated using mineral salts, mainly salts of calcium, and after that they can be precipitated with a hydrophilic solvent such as ethanol or can be separated by ultrafiltration (Reynolds et al. 1994). In addition, chromatographic methods using ion exchange resins have been proposed for the enrichment of CPP from aggregates precipitated (Adamson and Reynolds 1995, FitzGerald 1998, Meisel 1998). Mixtures of CPP are commercially available as spray-dried powders. The yield of CPP is low; overall, the percentages vary from 6% to 20% (w/w) of the original protein (McDonagh and FitzGerald 1998, Ellegård et al. 1999, Kim et al. 2007).

Uses As Additive

CPP were firstly incorporated in non-food matrices as a complex with ACP. The major contributions on production, identification and characterization of CPP-ACP were generated from studies carried out by Reynolds, which resulted in several patents and development of products for dental care (Reynolds 1991, 1993, 2002, 2005, among other patents). Patents were licensed exclusively to Recaldent Pty Ltd, who manufactures and markets CPP-ACP under the RECALDENT™ brand around the world. This product has been incorporated in chewing gum without sugar from brand Adams (Trident White™, Trident Xtracare™) and in several tooth products such as toothpastes and mouthrinses (Prospec MI Paste™, GC Tooth Mouse™) (Table 2).

A very large number of studies have been carried out to assess the efficacy of synthetic CPP-ACP nanocomplexes incorporated in sugar-free chewing gum, lozenges, mouth rinses, dental filling material, among others, to act as antiocariogenic agents using *in vitro* and mainly in human *in situ* experiments (Reynolds 1995, 1998, 2008; Shen et al. 2001, Cai et al. 2003, Morgan et al. 2008). Similarly, the addition of CPP-ACP to sugar confections resulted in a significant regression of the enamel subsurface lesions, and this effect was greater than that obtained with the sugar-free confection (Walker et al. 2010). In relation to dairy products, increased remineralization of tooth enamel by milk containing added CPP-ACP has been observed. This re-mineralizing effect was dose-dependent; levels from 0.2% to 0.5% CPP-ACP producing the higher increase in mineral content relative to the control milk (Walker et al. 2006, 2009).

CPP have been extensively used as resource for mineral supplementation. Currently, several multinational companies, particularly in Europe and Japan, commercialize products with CPP aimed at enhancing the bioavailability of mineral in functional foods (FitzGerald 1998, Korhonen 2009, Phelan et al. 2009).

Table 2. Commercial products with added CPP

Product name	Manufacturer	Type of product	Additive	Commercial product
Recaldent™	Recaldent Pty Ltd, US	Chewing gum	CPP-ACP	Trident; Recaldent
Recaldent™	Recaldent Pty Ltd; US	Toothpaste	CPP-ACP	GC Tooth Mousse; Prospect MI Paste
Capolac	Arla Food, Denmark	Hydrolyzate ingredient	CPP	
CE90CPP	DMV, Netherlands	Ingredient	CPP 20%	
Tekkotsu Inryou	Suntory, Japan	Soft drink	CPP	
Kotsu Kotsu calcium	Asahi, Japan	Soft drink	CPP	

CPP in Dairy Products

The natural presence of CPP in milk, yogurt and cheese is related to the action of native enzymes of milk and microbial enzymes from starters.

In milk, peptides occur naturally by the proteolytic activity of plasmin and, possibly, active cathepsins B and D. Although only a few sequences have been established, CPP have been reported as the most abundant among them (Baum et al. 2013, Dallas et al. 2014). In fermented milks, CPP appear during manufacture and, in the case of cheeses, the major number of peptides are produced during ripening. The sequences of CPP identified in cheeses suggest that they are probably produced throughout caseinolysis as a result of the plasmin activity followed by the action of milk-endogenous or microbial enzymes (Dupas et al. 2009). In this sense, many dairy starter cultures are highly proteolytic, and so, the release of different bioactive peptides from milk proteins by the action of these cultures is possible (Korhonen 2009, Pihlanto 2013). These peptides, once liberated, can influence the biochemical activities of the microbial communities; however, this role has probably been underestimated in dairy processing (Smacchi and Gobbetti 2000).

Considerable effort has been expended on the isolation and identification of peptides from cheeses. A large number of multiphosphorilated, diphosphorylated and monophosphorylated peptides derived from α-s1, α-s2 and β-caseins have been identified in the water-extract fraction of different cheese varieties such as Cheddar (Singh et al. 1997), Grana Padano (Ferranti et al. 1997, Sforza et al. 2004), Ragusano (Gagnarie et al. 2011), Parmigiano Reggiano (Addeo et al. 1994, Lund and Ardö 2004, Sforza et al. 2004), Herrgard (Lund and Ardö 2004, Ardö et al. 2007), Comté, (Roudot-Algaron et al. 1994), Emmental (Gagnaire et al. 2001) and Feta (Michaelidou et al. 1998), among others.

Although several enzymatic activities are common to many cheese varieties, the peptide composition at different ages is characteristic of the cheese type (Ardö 2007). Ripening conditions, type of starters and technology affect bioactive peptides synthesis. For example, a large number of small phosphopeptides derived from caseins were found in considerably higher levels in semi-hard cheeses than extra-hard cheeses (Lund and Ardö 2004). Certain fragments from β-caseins seems to be characteristic for cheese varieties made with pasteurized milk and mesophilic starter using medium-high temperatures and long holding times (Ardö et al. 2007). On the other hand, the level of bioactive peptides formed naturally in cheese depends on the balance between their formation and the degradation exerted by the proteolytic enzymes involved during ripening (Smacchi and Gobbetti 2000). Lactic acid bacteria have a complex enzymatic system including cell bound proteinases and intracellular peptidases. These enzymes release different oligopeptides, which are further hydrolyzed to peptides and aminoacids. Some attempts have been made to correlate the production of peptides with the proteolytic enzymes involved during cheese manufacture (Gagnaire et al. 2001). In fact, the sequences of phosphorylated peptides identified in different studies indicate that they are likely released by action of plasmin, cathepsin D, aminopeptidase, carboxypeptidase and other peptidases from starters. As a result of further proteolysis, bioactive peptides can be hydrolyzed to inactive fragments. It has also been observed that several peptides were totally or partially dephosphorylated, thus suggesting the action of phosphatases on phosphorylated caseins and their fragment during cheese ripening (Dupas et al. 2009). As can be seen, the formation of bioactive peptides is a dynamic process, and so, the type of bioactivity appears to be dependent on the stage of ripening of the cheese (Phelan et al. 2009). However, there is evidence that after synthesis, some CPP can accumulate during ripening of cheeses (Ardö et al. 2007). The greater resistance of CPP to further hydrolysis has been attributed to the absence of proteolytic enzymes with specificity for the phosphoserine

(Ellegård et al. 1999) or to the particular sequence of aminoacids containing at least three closely located phosphoserine residues (Ferranti et al. 1997).

In fermented milks, the higher level of CPP, compared with that in milk, has been also attributed to the proteolytic activity of starter cultures (Chianese et al. 2003, Ferrazano et al. 2008). The type of starter is one of the main factors that influence the synthesis of bioactive peptides; for example, proteolysis by *L. helveticus* is related to the production of antihypertensive peptides (Smacchi and Gobbetti 2000). In relation to this topic, most studies have been focused on the production of peptides in probiotic yogurts manufactured with the addition of *L. acidophilus* and *Bifidobacterium* spp. and several prebiotics. The results showed that these bio-yogurts contained a great number of peptides exhibiting a wide variety of biological activities (Ferrazzano et al. 2008, Pinto et al. 2012). On the other hand, Lorenzen and Meisel (2005) proposed a hydrolysis of milk with trypsin previous to manufacture, as a strategy for increasing CPP in yogurt. Two peptides identified as β-CN(f1-25) and α_{s1}-CN(f43-79) were the major peaks released during enzyme treatment. This technological approach had a negative effect on texture as the gel strength was lower and the syneresis was higher than traditional yogurts. The sensory attributes were also affected. Overall, the typical attributes of yogurts were less pronounced in those yogurts from trypsin-treated milks. In addition, other possible disadvantage of tryptic hydrolysis is the appearance of undesirable flavors due to the formation of bitter peptides, which depends on the enzyme and the hydrolysis conditions used (Brule et al. 1994). However, the bitter taste may be masked by the flavor compounds formed naturally in the product or even the starter could hydrolyze these peptides. In another study, Lorenzen (2004) evaluated the production of CPP in yogurt through a pre-treatment of milk with plasmin, reporting similar defects in texture than those above mentioned. In relation to this aspect, the bitter taste is masked by the incorporation of sucrose in CPP enriched yogurts by the milk treatment with trypsin (data not published obtained in our institute). Finally, it has been demonstrated that the type of the powder ingredients that are added in order to increase the total milk solids, can influence the production of peptides in milk fermented by *L. helveticus* (Leclerc et al. 2002).

To date, few data there are available in literature concerning the CPP levels in traditional dairy products. Kawahara et al. (2005) reported 171 mg of CPP per g protein in plain yogurt and 139 mg of CPP per g protein in Camembert cheeses. On the other hand, CPP level in Beaufort cheese was estimated in 73 mg per g of protein (Dupas et al. 2009). The higher level of CPP in plain yogurt than in Camembert cheese was attributed to the alteration in micelle structure of caseins. Consequently, the release of peptides from caseins can be favored or by contrast, formed peptides can be hydrolyzed.

CONJUGATED LINOLEIC ACID

Definitions and Characteristics

CLA is a collective term used to describe a heterogeneous mixture of positional and geometric isomers of octadecadienoic acid or linoleic acid (c9,c12-$C_{18:2}$) in which double bonds are conjugated (cis-, trans- or mixed configurations). Depending on the position and geometry of the double bonds, 54 CLA isomers are possible but only about 28 isomers of

CLA have been identified so far (Delmonte et al. 2004, Park 2009). In the last three decades, CLA have gained considerable attention since numerous potential beneficial health effects have been reported. The primary research focus has performed using commercial mixture containing the two major isoforms, c9,t11-$C_{18:2}$ (rumenic acid) and t10,c12-$C_{18:2}$ (Yu et al. 2003), accounting for more than 80% CLA intake in the diet, as naturally occur in foods derived from animals. Whigham et al. (2007) reveal that an intake of about 2-3 g per day for 6 to 12 months by an adult would impart optimum biological effects, which would be long lasting too. Recent evidence suggests that both major isomers may have myriad effects in different biological systems. However, some of physiological effects reported by CLA appear to be the result of multiple interactions between the two biologically active isomers, they even in some cases appear to act in opposition (Bhattacharya et al. 2006, Park 2009).

Health Benefits

Biological and biochemical roles attributed to CLA have been discussed in detail in several reviews (Pariza et al. 2000, Wahle et al. 2004, Bhattacharya et al. 2006, Nagpal et al. 2007, Park 2009, Abbas et al. 2014). The main activities include anticancer, body fat reduction, prevention of cardiovascular diseases through the reduction of atherosclerosis lesions and levels of cholesterol and triacylglycerides, anti-inflammatory and antioxidant. The mechanisms of action underlying are not fully established. While the activities were evaluated in different models of cell cultures and animal studies (Pariza et al. 1999, Belury 2002, Larsen et al. 2003, Kim et al. 2004, Parodi 2004, Hur et al. 2007, Whigham et al. 2007, Benjamin and Spener 2009, Bassaganya-Riera and Hontecillas 2010), few studies examined the effects of CLA or its individual isomers in human health. Most of them have not reflected the dramatic findings obtained in *in vitro* and *in vivo* studies, which makes it difficult to pin-point whether CLA offer a 100% safe ingredient functional food. Bhattacharya et al. (2006), Whigham et al. 2007, Park (2009), Benjamin and Spener (2009) are some of the reviews that summarize the results of clinical studies. After analyzing the results, authors suggested that further short- and long-term human studies and in different age-group subjects need to be undertaken in the near future to elucidate CLA's effects.

In relation to *anticancer effect*, the protective role towards gastrointestinal and colon, breast, skim and prostate cancers has been studied extensively both *in vivo* and *in vitro* models. CLA action on the every stage of cancer development including initiation, promotion, progression and metastasis are reported (Park 2009). However, results obtained in animal experiments and in tumor cell lines on effects of individual CLA isomers over several tumor types are divergent. It is important to take into account that different mechanisms (alteration of lipid peroxidation, tissue fatty acid composition, eicosanoid metabolism, cell proliferation, among others) are associated to each tumor type and different stages of tumor progression, as well as the method used for tumor induction in *in vivo* studies (Kelley et al. 2007). The first hints of anticancer effect appeared in the early 90 by the finding by Ha et al. (1987), which found an inhibition in the initiation of epidermal tumor in CLA-treated mice. Since this important initial observation a large number of apparent health benefits have been ascribed to CLA and numerous studies have been carried out about this topic. Ip et al. (1999) demonstrated that rat fed diets containing butter enriched with c9,t11-CLA and purified c9,t11-CLA developed fewer number of mammary tumors. Ip et al. (2002) found similar

anticancer efficacies of the c9,t11- and t10,c12-CLA through the evaluation of the reduction in premalignant lesions and carcinomas in the mammary gland of rats. Chen et al. (2003) confirmed that CLA shows inhibition on forestomach neoplasia in mice which is possibly related with the purity of isomers used in the assay. However, the authors mentioned the inhibitory mechanisms of CLA on carcinogenesis are complicated. Contrary, Rajakangas et al. (2003) suggested that t10,c12-CLA may act as a growth promoter in small intestine carcinogenesis, as the size of adenomas were greater in small intestine of mice fed with a diet containing this isomer compared with the control mice.

One of the most interesting aspects of CLA that has drawn much attention and that was first reported in 1995 by Park and coauthors, is their ability to *reduce body fat* while enhancing lean body mass. Park et al. (1999) observed that t10,c12-CLA is more effective than c9,t11-CLA in changing the body composition in a mice feeding study. In addition, t10,c12-CLA in comparison to c9,t11-CLA produced the suppression hepatic triglycerides secretion in cell culture (Lin et al. 2001) and decreased the expression of stearoyl-CoA desaturase in cultured adipocytes (Pariza et al. 2000). Park and Pariza (2007) suggested multiple mechanisms (reduction of lipid accumulation of adipose tissues, increase of the fatty acid β-oxidation in skeletal muscle, increase of energy expenditure, inhibition of enzyme involved in fatty acid metabolism and lipogenesis, decrease of adipocyte size) to explain this effect.

As regards the *immune and inflammatory responses*, they are attributed to the reduction of pro-inflammatory eicosanoids associated to inhibition of arachidonic acid biosynthesis and the increase of anti-inflammatory cytokines (Changhua et al. 2005), and reduction of antigen-induced cytokine production in immune cells antigen (Park et al. 2009).

Microbial Production of CLA

The fact that CLA is formed in the bovine rumen by the action of anaerobic bacteria *Butyrivibrio fibrisolvens* from polyunsaturated fatty acids (PUFA), mainly linoleic and linolenic acids, by isomerisation and biohydrogenation, and also in mammary glands by enzymatic desaturation of vaccenic acid (t11-C18:1) (Parodi 2004, Khanal and Dhiman 2004, Sieber et al. 2004) has led to the speculation that also other microorganisms may be able to form this metabolite. In fact, it have been demonstrated that several cultures such as LAB (lactobacilli, lactococci, streptococci), propionibacteria and bifidobacteria, many of them with probiotic role, are able to produce CLA from linoleic acid in a special growth medium or milk. Several reviews covering the CLA production by microorganisms have been published over the last decade (Sieber et al. 2004, Ogawa et al. 2005, Nagpal et al. 2007, Andrade et al. 2012).

Linoleate isomerase (LAI) enzyme is responsible of CLA synthesis, which is bond to the cell membrane of microorganisms. In particular, Lin et al. (2003) and Lin (2006) demonstrated the presence of LAI activity in crude enzyme extracts of *L. acidophilus* (CCRC 14079) and *L. delbrueckii* ssp. *bulgaricus* (CCRC14009) and the feasibility of CLA production from linoleic acid (LA). According to Lin (2006), yields of c9,t11- and t8,c10-CLA were larger than t10,c12-CLA and other isomers produced from enzyme extracts of *L. acidophilus* (CCRC 14079). Some differences in the distributions of isomers were detected for different enzyme extract levels of *L. acidophilus* (CCRC 14079) and for different doses of

LA (Lin et al. 2003). According to Kepler and Tove (1967), the optimum pH for the purified LAI enzyme from *Bu. fibrisolvens* was 7.0-7.2, although the pH range for the activity was broad (5.5-8.5).

The mechanism proposed previously by Jiang et al. (1998), in which the microorganisms perform LA isomerization as a detoxification process because of the toxic nature of unsaturated long-chain fatty acids, was confirmed by Gorissen et al. (2011). The pathway of LA isomerisation to CLA was investigated using *L. acidophilus* AKU 1137 as a representative strain by Ogawa et al. (2001). They suggested that hydroxy fatty acids are intermediates compounds of CLA biosynthesis, which consists of at least two successive reactions, i.e., the hydration of LA to 10-hydroxy-12-C18:1 and the dehydrating isomerization of the hydroxy fatty acids to CLA. Free form of LA only acts as a substrate for CLA production by LAB, and the ester and triacylglycerol of LA did not.

Among nineteen different strains commonly used as dairy starter cultures (lactobacilli, lactococci, streptococci and propionibacteria) tested by Jiang et al. (1998) for their ability to produce CLA from free LA in MRS broth, only two strains were found to be capable of converting LA to extracellular CLA. Kim and Liu (2002) screened several *Lactobacillus* and *Lactococcus* strains for CLA producing ability in MRS and whole milk, using sunflower oil at different levels (0.1-1.0 g/L) as substrate. *L. lactis* IO-1 showed the highest CLA production in milk, but its growth was completely inhibited at a dose higher than 0.5 g/L of LA in MRS. Alonso et al. (2003) found that *L. acidophilus* and *L. casei* were able to produce mainly c9,t11-CLA and t10,c12-CLA at a lesser extent, in MRS supplemented with LA (0.05-0.5 mg/mL); maximum CLA production was observed at 24 h of incubation with 0.02% LA and no significant increases in CLA levels were found after 24 h. The concentrations of different isomers produced in skim milk supplemented with the same concentration of LA were similar to those formed in MRS broth. Kishino et al. (2002) investigated the CLA productivity of the washed cells (resting cells) of *L. plantarum*, *L. rhamnosus*, *L. acidophilus*, *L. paracasei* and *Propionibacterium shermanii* to avoid the inhibitory effect of LA on cell growth. Strains produced c9,t11- and t9,t11-CLA, together with two hydroxy fatty acids. The authors revealed interesting points: the washed cells of LAB exhibiting high levels of CLA production were obtained by cultivation in medium containing a small amount of LA; the CLA production was observed under anaerobic conditions for avoiding the interfering oxidative metabolism of LA and the LA should be pretreated with a detergent or albumin to facilitate the dispersion in the reaction mixture and the availability for the cells. The antibacterial effect of LA on the growth is strain-dependant and also depends on cell density and growth phase (Jiang et al. 1998, Rainio et al. 2002, Ando et al. 2004).

Macouzet et al. (2009) found a readily incorporation of LA into biomass from washed cells of *L. acidophilus* La-5 at 72 h and the conversion of LA to CLA in the cells represented approx. 30%, but no noticeable accumulation of CLA was detected in the medium or in the biomass from active growing cells after 24 h. Aerobic incubation seemed to stimulate the production of c9, t11-CLA, but restriction of oxygen resulted in a preferential accumulation of t,t isomers. Lee et al. (2003) reported the ability of *L. reuteri* to form CLA after 24 h of incubation in MRS containing LA (0.9 g/L); CLA was mainly located in the extracellular space of the cells. Oh et al. (2003) found two CLA-producing *Bifidobacterium* strains from faecal origin; c9,11t-CLA production was paralleled by an increase in cell biomass for both bacteria cultivated in MRS with 0.05% LA and was maximal at 30 h. At higher concentration of 0.05% LA, growth was strongly inhibited. The majority of CLA was in the culture

supernatant, i.e., in the extracellular phase. Meanwhile, different strains of bifidobacteria were incubated in MRS containing L-cysteine hydrochloride and LA (550 ug/mL). Considerable interspecies variation was found; only *B. breve*, *B. dentium* and *B. lactis* produced high amounts of c9,t11-CLA in the cell supernatant fluids, whereas the other bifidobacteria studied did not convert LA to CLA at any significant level. The formation of CLA ceased when the cultured entered stationary phase (Coakley et al. 2003). On the contrary, Kishino et al. (2002) reported that CLA produced was accumulated as intracellular or cell-associated lipids in the free form. They suggested that the cells themselves could be used as a source of CLA after a simple centrifugation step.

The ability of 36 *Bifidobacterium* strains to convert LA to CLA was evaluated by Gorissen et al. (2010). Four strains of *B. breve* (LMG 11040, LMG 11084, LMG 11613 and LMG 13194), *B. bifidum* LMG 10645 and *B. pseudolongum* subsp. *pseudolongum* LMG 11595 produced mainly c9,11t-CLA and to a lesser extent t9,t11-CLA. The addition of LA (0.50 mg/mL) after 7 h of growth resulted in less growth inhibition than when adding LA immediately after inoculation. The same research group investigated the differences in production kinetics of CLA isomers of these six strains in MRS supplemented with LA (0.50 mg/mL); LA was added to the fermentation when the bacterial culture reached an optimal density in order to minimize its inhibitory effect on the growth. Formation of c9,11t-CLA occurred during the logarithmic to early stationary growth phase of cultures, but in the stationary phase or death phase, it was observed a decrease of c9,11t-CLA and an increase of t9,t11-CLA. Some strains displayed a high conversion of LA despite poor growth, whereas other strains grew well but displayed lower conversion (Gorissen et al. 2012a).

Villar-Tajadura et al. (2014) identified three strains of *B. breve* (ZL12-28, 29M2 and M7-70), among eight bifidobacteria strains assayed, by their ability to c9,t11-CLA production in MRS and skim milk medium supplemented with LA (500 ug/mL). Rodríguez-Alcala et al. (2011) selected CLA-producing strains belonging to the genera *Bifidobacterium*, *Lactobacillus* and *Lactococcus* after incubation in skim milk with free LA. Van Nieuwenhove et al. (2007a), in a first stage, chose seven dairy bacteria (*L. casei* CRL431 and CRL87, *L. rhamnosus* C14, *L. acidophilus* CRL730 and Q42, *B. bifidum* CRL1399, and *S. thermophillus* CRL728), among eight strains studied, by their capacity to form CLA (percentages of conversion ranged 17-36%) after 24 h of incubation in MRS broth containing LA (200 ug/mL). Then, the four strains (*L. rhamnosus* C14, *S. thermophillus* CRL728, *B. bifidum* CRL1399, *L. casei* CRL431) with the highest LA conversion were inoculated into buffalo milk. LA isomerization and CLA production were initiated after the first 4-7 h of incubation and then continued to rise as fermentation progressed. The highest CLA production was observed near stationary phase. Milk inoculated with *L. rhamnosus* C14 showed the highest CLA level; on the contrary, milk with *B. bifidum* CRL1399 had the lowest content of CLA.

On the other hand, the pH and temperature of incubation produce important effects on growth and fatty acid profiles of microorganisms. In fact, the pH of medium affects the enzyme activity and the microorganism adaptation to environmental conditions. The influence of temperature is related to the fluidity and the composition of the cell membrane and the isomerase activity, affecting finally the growth and fatty acid profile (Gorissen et al. 2011, Soto 2013). In fact, Gorissen et al. (2011) revealed the highest CLA production by *L. sakei* at 30°C and pH 6.2, but LA was not converted to CLA at 30°C (pH 5.5) and 37°C (pH 6.2). The authors suggested that the ability of lactobacilli to isomerize LA to CLA at low temperature is associated with changes in the cell membrane composition. The best results

obtained by Soto (2013) for CLA production by *L. plantarum* were in a medium at pH 6.5 supplemented with grape seed oil and incubated at 37°C; CLA was observed only inside of culture. Rainio et al. (2002) described the kinetics of isomerization by *P. freudenreichii* ssp. *shermanii* in pH-controlled batch and fed-batch fermentations in whey permeate medium formulated from whey permeate, yeast extract, tryptone and supplemented with LA (600-2000 ug/mL). In batch fermentation, the rate of CLA formation was highest during the exponential phase of growth. High amounts of CLA (80-87% conversion) were accumulated at the end of fermentation employing LA level up to 2000 mg/mL. In fed-batch fermentations, in which LA was added into the culture after exponential growth, a rapid but short conversion period occurred. Thus, both actively growing and non-growing cultures were able to effectively form CLA. However, the production rates per cell were significantly higher in growing cultures.

Several authors evaluated the effect of different additives added to growth medium on production of CLA isomers. In fact, the individual addition of lactose, sucrose, fructose and sodium chloride to skim milk medium produced a decrease in c9,t11-CLA after 24 h of incubation for *L. acidophilus*, *L. bulgaricus*, *Lc. Lactis* and *S. thermophilus*, except for *Lc. cremoris* (Lin 2000); the use of yeast extract and glucose significantly increased the cell growth and CLA production by *L. plantarum* (Khosravi et al. 2015); the supplementation with hemin solution reduced the CLA concentration, but an increase of CLA content was observed when the sunflower oil was included; the addition of L-serine, glucose, $AgNO_3$ and NaCl to the reaction mixture produced a selective production of c9,t11-CLA (Ogawa et al. 2005).

The source of LA substrate is other factor that has been studied. Among the five probiotic strains (*L. acidophilus*, *L. reuteri*, *L. plantarum*, *L. buchneri* and *L. brevis*) studied by Hosseini et al. (2015), washed cells of the late log phase of *L. plantarum* ATCC 8014 showed a considerable productivity of the two main CLA isomers from sunflower oil. It was verified the conversion from triacilglycerides to free LA by bacterial lipase. In fact, when oils are used as LA sources, bacteria must have the ability to produce lipases and esterases to release the fatty acids from the triacilglycerides, representing an extra reaction step (Holland et al. 2005). Xu et al. (2004) demonstrated the synthesis capacity of CLA by *Proponibacterium* strains in model systems containing hydrolyzed soy oil (1%) emulsified in milk, but it did not occur in model systems of unhydrolyzed soy oil (1%) emulsified in milk. Abd El Salam et al. (2010) analyzed the CLA formation by strains of *Lactobacillus*, *Lactococcus*, *Enterococcus*, leuconostoc and propionibacteria during incubation for 48 h in a skim milk medium containing lipolyzed sesame oil (0.2-1.0%); *Lc. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* subsp. *mesenteroides* gave the maximum CLA production. Ando et al. (2004) obtained high yield of CLA, mainly t9,t11-CLA and in a minor extent c9,t11-CLA, from castor oil by washed cells of *L. plantarum.* Castor oil is rich in ricinoleic acid (10-hydroxy-12-octadecaenoic acid) as a form of triacylglycerol; so for it to be accessible for bacteria, castor oil was treated previously with a lipase. Puniya et al. (2009) stated that *L. acidophilus* NCDC-14 and *L. casei* NCDC-19 had similar ability to CLA production using low level of sunflower oil (0.25%) in a medium prepared with skim milk and glucose. The highest CLA level was obtained by *L. casei* NCDC-19 with the higher amount of sunflower oil (1.0%) added to milk medium. Rodríguez-Alcala et al. (2011) selected five strains of the genera *Bifidobacterium*, *Lactobacillus* and *Lactococcus* for their ability to produce CLA after incubation in skim milk with free LA and safflower oil. Homologous values of bioconversion

of LA were found for cultures incubated with both substrates. Contrary, Macouzet et al. (2009) did not observe differences on the CLA content produced by *L. acidophilus* La-5 in a medium supplemented with milk fat.

The results show that CLA-producing strains can be found. However, the discrepancies found among different research groups suggest that the CLA productivity in the *in vitro* assays and the ratio between the isomers are influenced by numerous factors such as the intrinsic characteristics of the microorganism (genera and specie), cell number and cell growth, LA concentration, type/source of substrate (free acid or oil), pH and time/temperature of incubation and presence of oxygen, composition of growth medium. Therefore, the factors that control the yield of CLA need to be examined in detail.

Presence of CLA in Fermented Dairy Food

CLA is a natural component of animal foods (meat and milk), as mentioned above.

The levels of CLA naturally occurring in milk and dairy products are relatively low and highly variable (0.6-9.0 mg/g of fat). This fact as well as the presence of other isomers in addition to the majority aforementioned, show the influence of many factors in the biosynthesis, such as animal species, breed and animal´s diet, the age of animals, production system, environmental and geographic conditions, etc. (Parodi 2004, Collomb et al. 2006, Van Nieuwenhove et al. 2007b, 2009; Serafeimidou et al. 2012, Gómez-Cortés et al. 2013, Abbas et al. 2014). In addition, food processing conditions also contribute to the noted differences (Sieber et al. 2004, Akalin et al. 2007, Oliveira et al. 2009, Korhonen 2010). For this reason, various alternatives have been development aimed at increasing the CLA content in milk and dairy foods. In some countries, liquid milk, powdered milk, fermented milk, yogurt and cheese enriched in CLA are marketed (Rodríguez-Alcalá and Fontech 2007, Prandini et al. 2007, Cicognini et al. 2014). Considerable research efforts have been made through the herd nutritional practice to enhance the CLA content in raw milk and eventually in dairy products (Chilliard et al. 2001, Schroeder et al. 2003, Addis et al. 2005, Collomb et al. 2006, dos Santos et al. 2012, Gómez-Cortés et al. 2013, Mohan et al. 2013).

On the other hand, the known fact that several strains of bacteria possess the ability to synthesize CLA *in vitro* in the presence of precursor substrate, as was indicated above, raised the possibility for increasing the production of CLA *in situ* during manufacture of fermented dairy foods (Lin 2003, Sieber et al. 2004, Ogawa et al. 2005). In recent years, variety of information has been published about this topic. An interesting review of Bisig et al. (2007) suggests that the addition of LA may have a negative influence on the flavor of dairy products and the amount added of LA needs to be well controlled as the starter cultures are sensitive to it and too high amount reduces the conversion to CLA.

Xu et al. (2006) studied the effect of inoculation concentration of *L. rhamnosus* and yogurt starter cultures, added individually or in co-cultured, on the production of CLA using hydrolyzed soy oil as the lipid source in the manufacture of fermented milks. They found the higher CLA contents in fermented milks made with mix of cultures. Inoculation levels had no significant effect on CLA level and texture of products, but affected acidity and flavor. This research group also found increased levels of c9,t11- and t10, c12-CLA after 14 days of storage of fermented milks prepared with the combination of yogurt starter and propionibacteria. The organoleptic attributes of these products were comparable to the

fermented milks prepared without propionibacteria (Xu et al. 2005). Lin (2003) also observed an increase in CLA synthesis in yogurts made with the inclusion of *L. acidophilus* and LA (0.1%), and it was not evidenced a decrease in the overall acceptability of the products. In Ras cheeses made with the incorporation of *L. casei* and *L. acidophilus* and with the addition of sesame oil and hydrolyzed sesame oil, higher levels of rumenic acid and traces of t10,c12-CLA were detected after 90 days of ripening (Abd El-Salam et al. 2011). By contrast, Gorissen et al. (2012b) did not found increased levels of c9,t11-CLA in fermented milks by bifidobacteria and *L. sakei* and with the addition of vegetable oils, even having identified these strains as CLA producers in *in vitro* assays. Likewise, Perotti et al. (2014) reported that no increase in the CLA content was found in sheep cheeses prepared with a mix of *L. acidophilus* La-5 and *B. animalis* ssp. *lactis* Bb12. Among the reasons suggested by authors to justify this fact, they mentioned the inadequate amount of LA produced by lipolysis during cheese ripening or adverse environmental conditions to produce CLA by the strain cultures present in the matrix.

In recent years, the incorporation of prebiotic substances in the formulation of functional foods has received considerable attention. In addition to the known benefits of prebiotics, their addition may also affect the synthesis of CLA in dairy matrix. Akalin et al. (2007) found high CLA levels in yogurts made with *B. animalis* Bb12 and *L. acidophilus* La-5 and containing fructo-oligosaccharides (FOS); 9c,11t-CLA remained unchanged while 10t,12c-CLA decreased during cold storage. Similarly, a synergistic effect between the fruit fibers (composed by pectins and FOS) and *L. acidophilus* L10 and three strains of *B. animalis* ssp. *lactis* on CLA level of yogurts was reported by Espíritu Santo et al. (2012). Meanwhile, according to Oliveira et al. (2009) the largest amounts of CLA were found in fermented milks by a mix of *S. thermophilus* - *L. acidophilus* and supplemented with maltodextrin.

In the study of Rodrigues et al. (2011), c9,t11-, t10,c12- and t9,t12-CLA were detected upon 15 d of ripening of cheeses inoculated with *B. lactis* B94, *L. casei* 01 and *L. acidophilus* La-5, being c9,t11-CLA the predominant isomer produced by the three bacteria at stake. In addition, it was included FOS:inulin in the formulation of chesses; however, the mix of prebiotics did not favor the CLA formation. Similar results were found by de Lima Alves et al. (2011); they did not observe significant changes in the CLA content of cream cheeses prepared with the inclusion of *B. animalis* Bb12 and *L. acidophilus* La-5 and inulin. According to Manzo et al. (2015), *L. acidophilus* La-5 and *B. animalis* Bb12 in association with *S. thermophilus* and *L. bulgaricus* did not able to produce isomers of linoleic acid during milk fermentation; even if prebiotic (GOS) was added.

To sum up, some bacteria are able to modify the fatty acid profile and produce fatty acids with potential health properties during the fermentation process as a result of their growth and metabolism. Results reported suggest that the levels of CLA in fermented dairy food may possibly be increased by the addition of substances (prebiotics) that improve the metabolic activity of microbiota, whenever linoleic acid required for the CLA synthesis is present in the medium. The success of this strategy and the level of CLA achieved in the dairy matrix depend on the strain or combination of strains employed and the inoculum level, the concentration added of LA (free acid or oil), the characteristics of food matrix, and the processing and storage/ripening conditions of food. In addition, special attention on the effect of these changes on acidification kinetics, aroma compounds synthesis and final product quality may be required, in order to develop the new products.

Uses As Additive

Other approach to increase the human dietary intake of CLA isomers is to consume CLA-fortified dairy products, which are obtained by the addition of CLA (in free form or esterified as triacylglycerols) during food manufacture. Most studies on food fortification with nutritionally important fatty acids are focused in ω-3 (eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA) fatty acids. For that, different components such as lecithin have been used to get a better dispersion and stability of fatty acids in food matrix. Encapsulation with different materials (gelatin and sorbitol) was also tested (Sanguansri and Augustin 2007, Tamjidi et al. 2012). In relation to fortification of food with CLA (from an oil rich in CLA or synthetic CLA), little data are published. Campbell et al. (2003) evaluated the impact of "high-CLA" oil addition on the quality of fluid milk. Pasteurization caused a decrease in the CLA content and significant losses of the major isomers were observed after 3 weeks of refrigerated storage. Besides, high CLA milks had lower acceptability compared to normal milks. Rodríguez-Alcalá and Fontecha (2007) reported the CLA contents of CLA-fortified dairy products (milk, milk powder, fermented milk, yogurt, cheese) available in the Spanish market, and they analyzed the CLA evolution during processing and storage. The total CLA content of different products (supplemented with a synthetic oil rich in CLA) varied considerably depending on the presence of milk fat in the products. c9,t11- and t10,c12-CLA were the predominant fatty acids present in all foods at similar levels. They were unaffected by the yogurt and cheese processing, while a loss of total CLA was detected in cheeses after 10 weeks of storage. Recently, microencapsulation has been proposed as the tool to protect CLA and avoid defects in the sensory quality of food. Several studies have evaluated different components for the preparation of the microcapsules; besides, microcapsules have been characterized from the morphological point of view and the stability of CLA has also been analyzed (Park et al. 2002, Jimenez et al. 2004, 2006; Choi et al. 2010). In relation to the incorporation of these structures into dairy products, Jimenez et al. (2008) studied the impact on the sensory characteristics of milk, yogurt and butter. In this study, CLA microcapsules were prepared using whey protein concentrate as wall material. Consumer acceptability was higher for butter samples compared with milk and yogurt samples. The authors suggested that the effect of supplementation on acceptability is dependent on the food matrix and the amount of CLA to be added is determined by the detection threshold. Among other encapsulation systems, liposomes technology is one of the most interesting, and is widely used in pharmaceuticals and food. These structures are vesicles that form spontaneously by the self-assembly of phospholipids in aqueous solutions. They consist of phospholipids' bilayers containing aqueous core, which allows the encapsulation of substances both fat-soluble and water-soluble. Liposomes have been successfully used for drug administration (Al-Meshal et al. 1998, Zhang et al. 2005) due to its versatility, low toxicity, biocompatibility and biodegradability. Furthermore, it is known that liposomal structures promote solubility and bioavailability of fat soluble components, and that 1000 nm vesicles or smaller can be absorbed in the intestine (Um et al. 2003, Sekhon 2010). Due to the ability of these structures to encapsulate a variety of compounds, application in the food industry is promising. In particular, the use of liposomes in dairy foods has become an area of widespread interest, along with other nanotechnologies (Banville et al. 2000, Kheadr et al. 2002, da Silva Malheiros et al. 2012). So far, to our knowledge, there is no information published about the

fortification of yogurt and cheese with CLA encapsulated in liposomes. Research on ω-3 fatty acids and CLA loaded liposomes is being in progress in our team work (Vélez et al. 2015).

CONCLUSION

The current global interest in developing functional foods provides a timely opportunity to obtain fermented dairy products enriched in bioactive compounds derived from milk.

In this chapter, we have presented an overview of the claimed health promoting effect of GOS, CPP and CLA and the technological conditions used in order to obtain yogurts and cheeses enriched in these compounds. In particular, the aspects related to the use of GOS, CPP and CLA as functional additives and their *in situ* formation during manufacture of products are discussed.

The scientific interest of this topic is demonstrated through the large number of research papers available up to date. However, it is important to emphasize that there are only few food products commercially available. The results published are widely variable and in some cases are controversial. In relation to health benefits, further research is needed particularly in humans to fully substantiate the role of GOS, CPP and CLA. The success of the technological approaches outlined for developing high-GOS, -CPP and -CLA cheeses or yogurts depend on multiple factors: environmental conditions of the food matrix, substrate concentrations, concentration and type of enzymes, genera and species of the microbiota present, conditions of processing and storage/ripening, stability of the active component. Finally, it is important to consider the impact of these strategies on the final quality of the products and the consumer acceptability, which is vital for developing new functional foods.

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Chapter 2

PROBIOTICS IN FERMENTED FOODS

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ABSTRACT

Probiotic application in food product development is one of the major industries worldwide. The whole concept of probiotic is not new and probiotics in the form of fermented foods have been consumed by humans for thousands of years. Originally, probiotic delivery was mainly associated with fermented dairy foods such as yoghurt and, even today fermented dairy foods play a significant role in delivering probiotics to humans. Probiotic delivery has also moved progressively towards the non-dairy fermented foods including fruits, vegetables, cereals and meat and, even as nutraceuticals in the form of capsules. Despite the mode of delivery and the type of carrier food, fermented probiotic foods have been considered to promote the health and well-being of consumers. Inclusion of probiotics with known health features into the food matrices before the fermentation process is one of the most common practices in manufacturing fermented probiotic foods. Other than the nutritional and health benefits, probiotics may also positively influence the sensory characteristics such as texture, aroma and taste of the final product and may responsible for extending the shelf life of the fermented product. This chapter focuses on discussing the role of probiotics applications in

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developing fermented foods, quality characteristics of both dairy and non-dairy fermented foods and their therapeutic effects in human nutrition and health.

1. INTRODUCTION

During the last few years, functional fermented food research has moved progressively towards the development of dietary supplementation, including the concept of probiotics. Probiotics are live microorganisms which when administered in adequate amounts confer health benefit on the host, by improving its intestinal microbial balance (Fuller 1989; FAO 2002). The whole concept of probiotics is not new, and in fact they have been consumed by humans in the form of fermented foods, for many years. The therapeutic properties of probiotics have also been long known with scientist in early ages being reported that fermented milk could cure some digestive problems (Cross et al. 2001; Kopp-Hoolihan 2001; Ranadheera et al. 2010).

Although the mechanisms of health promoting properties of probiotics are still not fully understood, these health improving properties may be related to pathogen interference, exclusion or antagonism, immune-modulation, anticarcinogenic and antimutagenic activities, alleviation of lactose intolerance symptoms, reduction in serum cholesterol levels, reduction in blood pressure, prevention and decreasing incidence and duration of diarrhea, prevention of bacterial vaginosis and urinary tract infection, maintenance of mucosal integrity, and improved periodontal health (Franz et al. 2014). In order to deliver these health benefits to the host, the carrier food should contain an adequate amount of live probiotic bacteria (at least 10^6-10^7 cfu/mL or g) at the time of consumption (Ranadheera et al. 2010; Sidira et al. 2014).

Bacteria primarily belong to genera *Lactobacillus* and *Bifidobacterium* are mainly consider as probiotics with GRAS (generally-recognized-as-safe) status, however, some other bacteria and yeast species have also considered as probiotics (Table 1).

Table 1. Microorganisms considered as probiotics

Category and genus	Species
Bacteria	
Lactobacillus	*Lb. acidophilus, Lb. amylovorus, Lb. brevis, Lb. casei, Lb. curvatus, Lb. crispatus, Lb. delbrueckii subsp. bulgaricus, Lb. fermentum, Lb. helveticus, Lb. gasseri, Lb. johnsonii, Lb. reuteri, Lb. rhamnosus, Lb. salivarius, Lb. paracasei, Lb. plantarum*
Bifidobacterium	*B. adolescentis, B. animalis, B. bifidum, B. lactis, B. breve, B. infantis, B. longum, B. thermophilum, B. essensis, B. laterosporus*
Streptococcus	*S. cremoris, S. diacetylactis, S. intermedius, S. salivarius*
Propionibacterium	*P. freudenreichii, P. freudenreichii subsp. shermanii, P. jensenii*
Enterococcus	*E. faecalis, E. faecium*
Lactococcus	*L. lactis subsp. cremoris, L. lactis subsp. lactis*

Category and genus	Species
Other bacteria	*Pediococcus acidilactici, Leuconostoc mesenteroides, Bacillus cereus, Clostridium butyricum, Escherichia coli* Nissle 1917
Yeast	*Kluyveromyces lactis, Saccharomyces boulardii, Saccharomyces cerevisiae*

Adapted and modified from Parvez et al. (2006), Prado et al. (2008); Lew and Liong (2013); Ranadheera et al. (2014b); Ranadheera et al. (2014c).

There are some concerns regarding the safety of some of these genera such as *Enterococcus* since they can be pathogenic causing illness in the host. Although some therapeutic effects of certain starter culture microorganisms such as *Streptococcus thermophiles* and *Lactobacillus delbrueckii* spp *bulgaricus* have been reported, it is still debatable to consider those microorganisms as "probiotics" due to their failure in meeting the minimum selection criteria to be considered as probiotics (Table 2) (Meydani and Ha 2000; Guarner et al. 2005). Especially the gastrointestinal survival and colonization ability of these starter cultures are poor compared to probiotic bacteria (Harnett et al. 2011).

Table 2. Important criteria for the selection of probiotics

Criteria	Property
Safety criteria	Origin Pathogenicity and infectivity Virulence factors-toxicity, metabolic activity and intrinsic properties , i.e., antibiotic resistance
Technological criteria	High viability retention during processing and storage Good sensory properties Ability to produce at large-scale Genetically stable strains Phage resistance Large scale production
Functional criteria	Tolerance to gastric acid and juices Bile tolerance Adhesion to mucosal surface Validated and documented health effects
Desirable physiological criteria	Immunomodulation Antagonistic activity towards gastrointestinal pathogens Antimutagenic and anticarcinogenic properties

Adapted and modified from Saarela et al. (2006); Vasiljevic and Shah (2008); Ranadheera et al. (2014c).

Probiotic can be utilized to produce fermented as well as non-fermented food products (Figure 1). Along with the starter culture microorganisms, probiotics have widely been used as a co-culture or adjunct culture in the food fermentation process. Nevertheless, only probiotics alone (without any starter cultures) can also be used to produce fermented food products (Ranadheera et al. 2014a).

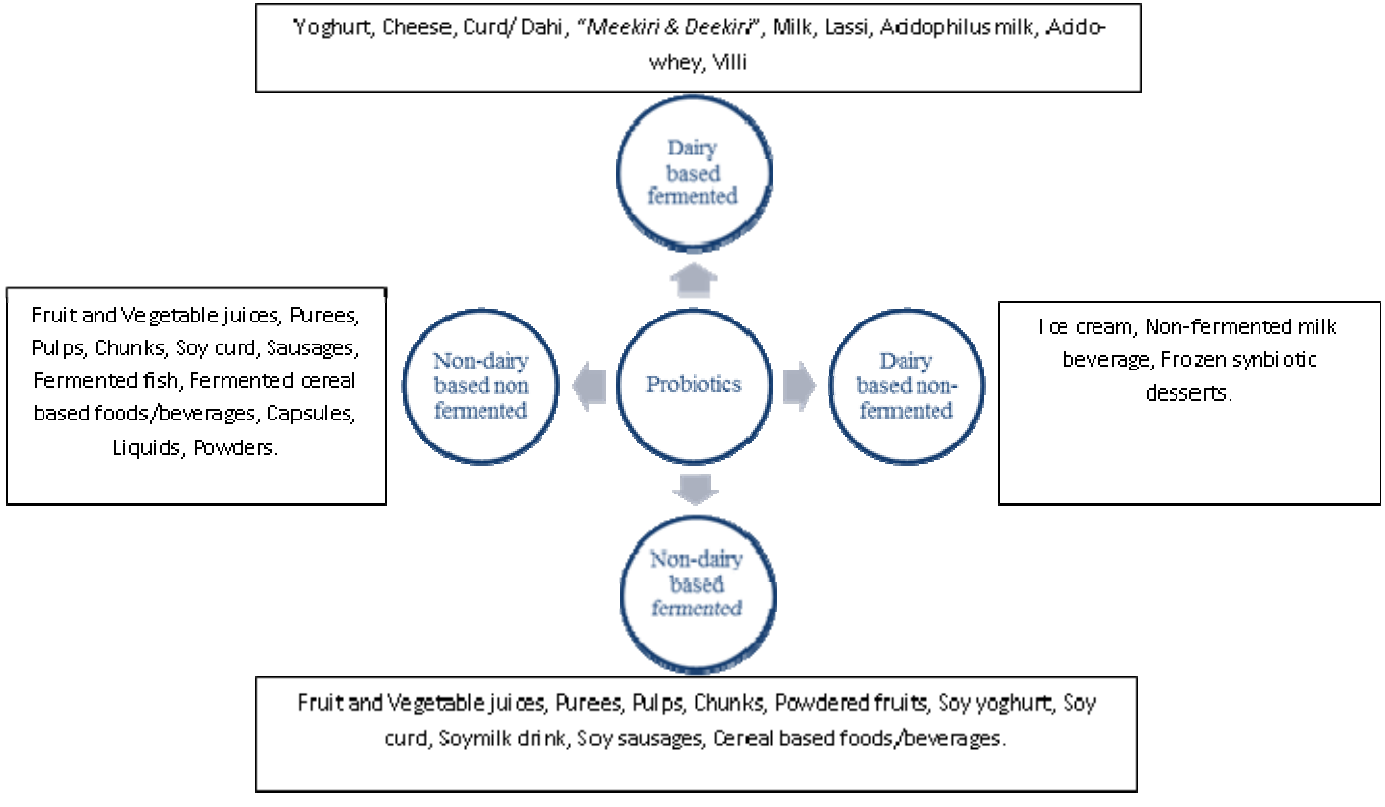

Figure 1. Classification and types of probiotic foods (Adapted and modified from Kumar et al, 2015).

While developing fermented foods, selection of suitable food matrices and appropriate probiotic/probiotic combinations is a vital factor, because efficiency of the fermentation process, viability retention of probiotics during food processing, storage and gastrointestinal delivery, organoleptic properties of the product and the consumer acceptability could be influenced by the appropriate food matrix-probiotic combination. Mattialla-Sandholm et al. (2002) described that the probiotic viability in the food matrix depends on many factors such as pH, storage temperature, oxygen levels, presence of competing microorganisms, and inhibitors. Thus, the strains chosen as probiotics should have multifunctional characteristics (Holzapfel 2002), hence they can ferment foods and survive in the product, while maintaining probiotic properties in the food matrix as well as in the human gastrointestinal tract.

Interactions between probiotics and starter organisms play a major role in the production of probiotic fermented products. According to Heller (2001), the strength of the interactions between added probiotics and both the medium in the food and the starter organisms depends on; whether they are added during fermentation or after; maintenance of the cold chain during processing and storage; physiological state of the added probiotics. Further, it is important that the formulation maintains the activity and viability of the probiotic for extended period of time. Their physical and genetic stability should be maintained during storage of the product, and all of their properties are essential for expressing their health benefits after consumption (Heller 2001).

2. PROBIOTICS IN FERMENTED DAIRY PRODUCTS

2.1. Milk As a Food Matrix for Probiotic Growth

Most of the probiotic bacteria grow slowly and some of them do not grow at all in milk due to their poor proteolytic activity, which limit their possible application in fermented dairy products (Yonezawa et al. 2010; Prasanna et al. 2014). This is due to poor availability of some nutrients such as amino acids which are essential for growth of probiotic bacteria in milk. Therefore, sometimes probiotic fermented dairy products do not contain sufficient number of probiotic bacteria at the consumption (10^6-10^7 cfu/mL or g). However, number of viable cell counts in a probiotic fermented dairy product is very important to exert the claimed health benefits. To overcome the problem of low growth and viability, many researchers have successfully used many ingredients to stimulate the growth and activity of probiotic bacteria in milk, for example various sugars (glucose and galactose), protein sources (yeast extract, liver extract, peptones, and corn steep liquor), and different vitamins (Gomes et al. 1998; Sodini et al. 2005; Zhao et al. 2006). However, due to poor *organoleptic* properties of some of the sources mentioned above, milk-derived compounds have been evaluated as additives, such as whey protein concentrate, whey protein isolate, and casein hydrolysate; studying mainly the effect of such compounds on the growth of probiotic bacteria in milk either singly or as part of starter a mixed starter culture (Oliveira et al. 2001; Prasanna et al. 2014; Zhang et al. 2015).

2.2. Applications of Probiotics in Fermented Dairy Products

Fermented dairy products are produced by using lactic acid bacteria which convert lactose of milk into lactic acid. Human beings have been using fermented dairy products as a part of their diets for thousands of years and originally fermented milk products were processed in Middle Eastern countries around 100-150 BC. It was started with using the natural cultures found in unpasteurized milk, fermentation vessels, and tools (Shiby and Mishra 2013). In the global context, there are various fermented dairy products which are produced by multinational companies and small scale enterprises under various trade names. However, the concept of probiotic has been discussing under various levels for more than a century and probiotic dairy foods are considered as the fastest growing functional foods around the world. Probiotic dairy products represent one third of the market share of functional foods (Raeisi et al. 2013). Furthermore, the intervention of the United Nations, World Health Organization and some Europe countries in mid 2000s increased the awareness of probiotics among people around the world (Reid 2015). Fermented probiotic dairy products have been increasingly popular among people around the world due to the claimed health benefits with the consumption of these products. In addition, consumption of fermented probiotic dairy products has been proposed as a natural mechanism to restore or to enhance gut health of human (Del Bono et al. 2015). Therefore, there is a continuous development and introduction of new dairy based fermented probiotic products to cater the demand of the market (Demers-Mathieu et al. 2015).

Many species and strains of bacteria have been evaluated and described to have probiotic properties which are suitable for application in fermented dairy products.

Table 3. Overview of probiotic bacteria used in fermented dairy products

Species	Product/s	Reference/s
Lactobacillus species		
Lb. plantarum	Yoghurt; Greek set-type yoghurt	Brinques and Ayub, (2011)
Lb. acidophilus	Yoghurt; Dahi; Fruit Yoghurt; Fermented milks; Synbiotic fermented milk	Donkor et al. (2007); Yadav et al. (2007); Kailasapathy et al. (2008); Sendra et al. (2008); Oliveira et al. (2009)
Lb. paracasei	Greek set-type yoghurt	Maragkoudakis et al. (2011)
Lb. casei	Dahi; Yoghurt; Fermented milk	Yadav et al. (2007); Donkor et al. (2007); Sendra et al. (2008)
Lb. johnsonii	Fermented milk	Yamano et al. (2006)
Lb. helveticus	Fermented milk; Yoghurt	Vinderola et al. (2007); PAVUNC et al. (2011)
Lb. reuteri	Yoghurt	Hekmat et al. (2010); Anukam et al. (2008)
Lb. rhamnosus	Yoghurt; Synbiotic fermented milk	Anukam et al. (2008); Oliveira et al. (2009); Hekmat et al. (2010)
Bifidobacterium species		
B. longum	Stirred yoghurt; Yoghurt	Adhikari et al. (2003); Al-Sheraji et al. (2012); Prasanna et al. (2013)

Species	Product/s	Reference/s
B. lactis	Yoghurt; Fruit yoghurt; Synbiotic fermented milk	Kailasapathy et al. (2008); Oliveira et al. (2009); Ejtahed et al. (2011)
B. bifidum	Fermented milk; Carbonated fermented milk	Vinderola et al. (2000b); Sendra et al. (2008)
B. animalis	Strawberry flavored yogurt	Cruz et al. (2010)
B. adolescentis	Yoghurt	Shihata and Shah, (2000); Vinderola et al. (2000a)
B. breve	Yoghurt	Picot and Lacroix (2004)
B. infantis	Yoghurt	Shihata and Shah (2000); Prasanna et al. (2013)
Other lactic acid bacteria		
Enterococcus faecalis	Yoghurt	Chang et al. (2011)
Enterococcus faecium	Yoghurt	Crittenden et al. (2003)
Lactococcus lactis	Fermented milk	Tillisch et al. (2013)
Leuconostoc mesenteroides	Fermented whey	Virtanen et al. (2007)
Non lactic acid bacteria		
Propionibacterium freudenreichii	Yoghurt	Saxelin et al. (2010)
Propionibacterium jensenii	Yoghurt	Ranadheera et al. (2012); Ranadheera et al. (2014a)

Table 3 shows an overview of probiotic bacteria which have been used in different fermented dairy products. The majority of probiotic bacteria used to manufacture probiotic fermented dairy products also belongs to genera *Lactobacillus* and *Bifidobacterium* (Ranadheera et al. 2012). In addition, there are few other species of lactic acid bacteria and non-lactic acid bacteria which are considered to be as suitable probiotic organisms for dairy products. Furthermore, some species of yeasts have been shown to have probiotic properties.

Table 4. Types of fermentation used to produce different fermented dairy products

Fermentation type	Type of microorganism involved	Products
Lactic Fermentation	Mesophilic type	Cultured buttermilk
	Thermophilic type	Yoghurt, Bulgarian buttermilk, Dahi, Labneh
	Therapeutic	Acidophilus milk, Yakult
Yeast-lactic Fermentation	Yeast and lactic acid bacteria	Kefir, Koumiss, Acidophilus yeast milk
Mould-lactic Fermentation	Mould and lactic acid bacteria	Villi

Adopted and modified from Robinson and Tamime (2006).

2.3. Fermented Dairy Products with Probiotic Microorganisms

There are many types of probiotic fermented milk around the world market produced under various brand names. Major physicochemical properties of probiotic fermented milk products vary basically on the type of probiotic microorganism, type of milk and use of other

starter cultures in the product. In addition, fermented probiotic milk products vary in their textures ranging from liquid drinks such as acidophilus milk, kefir and koumiss to semi-solid/ropy or firm products such as yoghurt, dahi, leben and villi. A classification of some fermented dairy based products based on fermentation type is given in Table 4.

2.3.1. Fermented Liquid Probiotic Dairy Products

Fermented liquid milk is produced by using lactic acid or alcoholic fermentation of milk. Specific character of these products is the physical nature which they exist in the form of liquid at room temperature (Clark et al. 2007). Few common liquid fermented probiotic dairy products are discussed below.

2.3.1.1. Acidophilus Milk

During acidophilus milk production, low fat sterilized milk is fermented by using pure culture of *Lb. acidophilus*. Incubation is carried out for around 18 to 24 hours until acidity reaches 1% and then product is packed. Some people are reluctant to consume acidophilus milk due to its characteristics sour taste. Therefore, sometimes sweet acidophilus milk is produced by adding small amount of sugar to the final product which is preferred by consumers (Clark et al. 2007). Acidophilus milk is claimed to have probiotic properties (Hui 2006) and some other probiotics and lactic acid bacteria have been incorporated to the product to enhance the quality and health benefits of the product. These probiotic bacteria include strains of *Bifidobacterium* spp. and *Lactobacillus casei* (Baek and Lee 2009; Chandan 2013).

2.3.1.2. Cultured Buttermilk

Cultured buttermilk is a unique product manufactured from commercial buttermilk which is obtained as a byproduct from churning sweet cream into butter. Commercial butter milk is also called as sweet buttermilk (Sodini et al. 2006). Skim milk is commonly used to produce buttermilk at commercial scale. The manufacturing of cultured buttermilk is done by using low fat milk. Pasteurized milk is subjected for lactic acid fermentation followed by quick refrigeration of the final product. The fermentation of milk is carried out using lactococci (*Lac. lactis* subsp. *cremoris*, *Lac. lactis* subsp. *lactis*, and *Lac. lactis* subsp. *lactis* biovar. *diacetylactis*) and aroma-producing bacteria leuconostocs (*Leuconostoc mesenteroides* subsp. *cremoris*). These bacteria have different roles in the manufacturing process. *Lac. lactis* subsp. *lactis* biovar. *diacetylactis* and *Leu. mesenteroides* subsp. *cremoris* are responsible for the development of characteristics aroma of cultured buttermilk. Diacetyl and acetaldehyde are the main aromatic compounds produced by these bacteria. Cultured buttermilk has been used in bakery industry and cooking. In addition, different probiotic bacteria have been successfully used in different probiotic buttermilk. These include *Lb. rhamnosus* GG, *Lb. rhamnosus* 271, *Lb. reuteri*, *Lb. casei* 431, *Lb. acidophilus*, and *Bifidobacterium* spp. (Tamime 2005; Baek and Lee 2009).

2.3.1.3. Probiotic Based Whey Drinks

Cheese production yields a large quantity of main byproduct which is called as whey. The characteristics and composition of whey vary on the type of the cheese and the quality of the milk (Özer and Kirmaci 2010). Normally whey is used in its powder form in many food

preparations which is a costly application. Therefore, production of whey based beverages can be helpful to use whey in its liquid form. Selection of suitable strains of probiotic bacteria for production of whey based beverage is important to have the unique flavor and texture of the final product. Most of whey based probiotic drinks are produced by fermentation of liquid whey concentrates or fermentation of milk enriched with whey protein concentrate and whey protein isolates. Many studies have reported the production of whey based probiotic drinks with acceptable sensory and other physicochemical parameters. *Lb. acidophilus* LA-5, *Lb. casei* LBC-81, *B. bifidum* Bb–12, *Lb. casei* Lc-01, *Lb. rhamnosus,* and *B. animalis* subsp. *lactis* are common probiotic bacteria which have been used in production of whey based probiotic beverages (Drgalic et al. 2005; Almeida et al. 2008; Pescuma et al. 2010; Lollo et al. 2013).

2.3.1.4. Kefir

Kefir is considered as an ancient milk beverage originated in many centuries ago in the Caucasian mountains (Garrote et al. 1998). It is produced with fermentation of milk with lactic acid bacteria, acetic acid bacteria and several genera of yeasts. These microbes are confined into a matrix called as kefir grains which are used to add into milk as starter culture. Milk obtained from various species (cow, goat, sheep, camel, and buffalo) can be used to manufacture kefir. According to Tamime, (2005) kefir grains contain bacteria such as *Lac. lactis* subsp. *cremoris*, *Lac. lactis* subsp. *lactis*, *Lb. kefir*, *Lb. kefiranofaciens*, *Lb. brevis*, *Lb. acidophilus*, *Leuconostoc* spp., and *Acetobacter* spp. In addition, yeast plays a major role in kefir fermentation because of the production of ethanol and carbon dioxide. In general, kefir grains have lactose fermenting yeast (*Kluyveromyces lactis*, *Kluyveromyces marxianus* and *Torula kefir*), as well as non-lactose fermenting yeasts (*Saccharomyces cerevisiae*). The fermentation process of kefir leads to production of compounds such as lactic acid, acetaldehyde, acetoin, diacetyl, ethanol, and CO_2 (Irigoyen et al. 2005). It is fizzy and slightly alcoholic. Kefir consumption has been suggested to have several health-promoting properties such as antimicrobial, antitumoral, immunological, and hypocholesterolemic effects (Farnworth 2005; Romanin et al. 2010). In addition, kefir is considered as a natural probiotic food which has a high interest among scientists around the world. Consumption of kefir has been increased in western countries including Austria, Latvia, Turkey, and the USA as an alternative probiotic product for conventional probiotic products such as yoghurt, cheese, and ice cream (Cogulu et al. 2010). Probiotic properties of some bacteria isolated from kefir have been reported. These include *Lb. kefir*, *Lb. plantarum* (Golowczyc et al. 2010; Huang et al. 2013) and *Lb. rhamnosus* (You et al. 2005).

2.3.2. Semi Solid/Ropy Probiotic Fermented Products

2.3.2.1. Probiotic Yoghurt

Yogurt is a product obtained by fermentation of milk using a mixed starter culture, typically of *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*.

These bacteria are responsible to convert lactose to lactic acid which decreases pH of the medium leading for development of a delicate gel. However, these bacterial species are not able to survive passage through the digestive tract of human (Lourens-Hattingh and Viljoen

2001). Nevertheless, yoghurt is considered as a popular carrier for probiotic bacteria (Ranadheera et al. 2010).

Different strains of probiotics are incorporated as cell suspensions or as freeze dried form depending on type of yoghurt. Many types of probiotic yoghurt have been reported such as plain (Aryana et al. 2007; Ranadheera et al. 2007), stirred (Ramasubramanian et al. 2008; Cruz et al. 2012), carbonated, flavored (Cruz et al. 2010; Gonzalez et al. 2011), fruit (Kaur et al. 2009; Cakmakci et al. 2012), low fat (Penna and Barbosa-Cánovas, 2007; Ramchandran and Shah 2010) and fat free (Antunes et al. 2005; Aryana et al. 2007). In addition, according to Vedamuthu (2006) a probiotic yoghurt could be produced using only *Lb. acidophilus* or *Lb. acidophilus* and *Bifidobacterium* spp. (known as AB culture) or *Lb. acidophilus*, *Bifidobacterium* spp. and *Lb. casei* (known as ABC culture). Furthermore, *Lb. reuteri*, *Lb. casei, Lb. rhamnosus GG*, *Lb. gasseri* and *Lb. johnsonii LA1* have been shown to be effective as adjunct cultures in probiotic yoghurt manufacturing process (Chandan 2013).

2.3.2.2. Viili

Viili is a fermented product which is mainly produced in Finland. It has a mild acidic taste and it is characterized with ropy thick texture. It is produced by fermentation of milk using mesophilic cultures such as *Lac. lactis* subsp. *cremoris*, *Lac*. *lactis* subsp. *lactis* biovar. *diacetylactis* and *Leu. mesenteroides* subsp. *cremoris*, and a mould (*Geotrichum candidum*).

The incubation is carried out in retail cups since fat rises to the surface during the incubation where the mould grows to produce the musty aroma. The characteristic thick texture is due to complex carbohydrate produced by microorganisms used in the fermentation process (Chandan 2013). In addition, *Lb. rhamnosus* GG and *B. lactis* BB12 have been reported to use as probiotic organisms in production of viili (Tamime 2005; Ruas-Madiedo et al. 2006).

2.3.2.3. Curd/Dahi

Dahi is produced by fermentation of cow or water buffalo milk with lactic acid bacteria. Dahi made of buffalo milk is also known as curd or "*meekiri*/*deekiri"* in Sri Lanka (Jayamanne and Adams 2004). Curd is an acidic flavor fermented dairy product with a texture which is similar to that of yoghurt.

In South Asian countries such as India and Sri Lanka, it is mainly produced at home level or by small scale dairy producers. Use of a good quality starter culture is essential to produce a good quality final product. In the production process, pasteurized or boiled milk is inoculated with the curd/dahi from the previous day as the starter. Incubation is normally carried out in a warm place for overnight.

Usually the mixed starter culture contains *S. thermophilus, Lb. delbrueckii* subsp. *bulgaricus, Lac. lactis* subsp. *lactis, Lac. lactis* subsp. *cremoris, Lac. lactis* subsp. *lactis* biovar. *diacetylactis*, *Lb. helveticus, Lb. casei* and *Lb. acidophilus* (Chandan 2013). Dahi can be considered as a suitable carrier for probiotic bacteria (Jain et al. 2008). *Lb. acidophilus* (Yadav et al. 2007; Rajpal and Kansal, 2009; Kaushal and Kansal 2012), *Lb. casei* (Yadav et al. 2007), *B. bifidum* (Rajpal and Kansal, 2009; Kaushal and Kansal 2012) and *Lac. plantarum* (Mohania 2013) have been shown to be effective as probiotic bacteria in dahi production.

2.4. Effect of Probiotic Bacteria on Quality Parameters of Fermented Dairy Products

Even though probiotic bacterial strains are already used in fermented dairy products, they have some inferior behavioral characteristics compared with traditional lactic acid bacteria used in fermented dairy products, which limit their applications in products. More specifically, they exhibit weaker growth in cow milk and require long fermentation times, anaerobic conditions, and low redox potential for their growth (Gomes and Malcata 1999; Janer et al. 2004; Prasanna et al. 2012b). Hence, poor acidifying performance of probiotic bacteria has a direct effect on textural properties of fermented milk products. It has been shown poor rheological properties of probiotic fermented milk products. The rheological properties of fermented dairy products are important at industrial level since they are considered as quality parameters of finished products (Damin et al. 2008; Akalin et al. 2012). Selection of suitable strains of probiotic bacteria to be incorporated into dairy products has been used to overcome the above problems. These include strains that can acidify milk quickly, strains that grow with traditional starter cultures and strains that do not produce unpleasant textures during fermentation process (Martínez and Gómez 2007; Prasanna et al. 2012a).

Probiotics used in dairy products should not create bad sensory properties which may affect the consumer preference. Product appraisal could be conducted to identify specific sensory attributes which are important parameters prior to the introduction of new dairy based probiotic yoghurt to the market (Granato et al. 2010). Specially, from a product development point of view, bifidobacteria which do not produce much acetic acid are more appropriate for fermented milk applications, as high concentrations of acetic acid lead to an undesirable vinegar flavor in fermented dairy foods (Prasanna et al. 2012b). However, many studies concluded that probiotic products are highly accepted by consumers. In one study, probiotic yoghurts manufactured using *L. rhamnosus* GR-1 and *L. reuteri* RC-14 were shown to have good sensory attributes which were accepted by consumers (Hekmat and Reid 2006). Similarly, Atunes et al. (2005) did not observe changes in sensory properties of yoghurt with addition of probiotic bacteria (*L. acidophilus* and *B. longum*). However, some researchers observed changes of sensory attributes of dairy products with probiotic addition (Uysal-Pala et al. 2006; Allgeyer et al. 2010).

3. PROBIOTICS IN NON-DAIRY FERMENTED FOODS

Drying and salting are believed to be the common fermentation practices among the oldest methods of food preservation (Swain et al. 2014). However, lactic acid fermentation has become the widespread fermentation method used to maintain and improve the nutritional and sensory features of food commodities (Karovicova and Kohajdová 2003; Acar et al. 2006; Gobbetti et al. 2013). Food fermentation is practiced to prevent foods from microbial spoilage and development of food toxins, improve flavor and texture and increase nutritional value of the foods (Steinkraus 1988; Rolle and Satin 2002). However, purposes of food fermentation vary depending on the geographical areas in the world where method and the purpose of fermentation depends on various traditions and cultural preferences related to those areas. Daily consumption of probiotic food products is essential to get long-term

beneficial effects for humans since the live bacteria present in fermented products are quickly eliminated with the feces without colonizing or residing in the human gastrointestinal tract (St-Onge et al. 2000).

Many studies have reported that the best carrier for probiotics is dairy fermented products (Rivera-Espinoza et al. 2010). However, allergic reactions occur in sensitive individuals and lactose intolerance (Prado et al. 2008) and growing vegetarianism (Martinsa et al. 2013) affect negatively on consumption of fermented dairy products as the sole matrices of delivering probiotic bacteria to the human gastrointestinal tract. Moreover, in some developing countries, cultural aspects and economic reasons limit the use of dairy fermented products as carriers for probiotic bacteria (Prado et al. 2008). Furthermore, dairy products as carriers for probiotic bacteria may not be appropriate for some regions in some parts of the world such as African countries, especially in rural settings where there are lack of infrastructure facilities and other problems with maintaining a cold chain (Franz et al. 2014). In this scenario, people cannot rely totally on dairy fermented products for probiotics. Therefore, it is worthwhile to create other delivery vehicles for probiotics including desserts, confectionary and soy products as well as meats and fermented vegetables (Franz et al. 2014). In fact the trend toward using probiotics in different food systems rather than using dairy products has increased over the decades (Ranadheera et al. 2010). The level of consumer awareness of different types of probiotics has improved significantly over the years which improved the research efforts into the development of alternative carriers (Granato et al. 2010). Thus, to cater to the increasing demand for probiotic products, food industry succeeded in promoting the consumption of non-dairy fermented foods which have completely different sensory and physiochemical attributes compared to dairy fermented foods.

3.1. Traditional Probiotic Non-Dairy Fermented Foods

As mentioned earlier, fermentation process is one of the oldest forms of food preservation in the world. Food fermentation still plays a major role in combating food spoilage and food borne diseases in countries like Africa (Franz et al. 2014). Deferent species of yeast, lactic acid bacteria and fungi have been used for fermentation of fermented non-dairy probiotic products (Blandino et al. 2003). Therefore, traditional fermented foods are considered as plentiful sources of microorganisms and some of them show probiotic characteristics (Rivera-Espinoza et al. 2010). Traditional fermented foods are generally fermented by lactic acid bacteria such as *Lb. acidophilus, Lb. plantarum, Lb. pentosus, Lb. brevis, Lb. fermentum, Lb. casei, Leuconostoc mesenteroides, Leu. fallax, Weissella confusa, W. koreenis, W. cibaria,* and *Pediococcus pentosaceus,* which are considered as probiotics (Rivera-Espinoza et al. 2010; Swain et al. 2014). There is a wide array of traditional non-dairy foods developed around the world. Table 5 illustrates examples for traditional fermented non-dairy foods with possible probiotic organisms. Many of them are non-alcoholic beverages, manufactured with cereals as the main raw material (Franz et al. 2014). Although some of the traditional fermented foods do not contain probiotics, information provided by traditional fermented foods and scientific research could help to develop new fermented probiotic products for the food industry as these traditional foods can be used as matrix for new product development, where traditional starter cultures could be replaced by probiotic cultures.

Table 5. Commonly available traditional probiotic enriched non-dairy fermented foods

Raw material source	Raw material	Name	Responsible organism for fermentation	Country of origin/Place where the product is popular	Reference/s
Cereals/ Legumes	Rice and Lentils	Adai	Lactic acid bacteria	India	Farnworth (2005)
	Maize	Atole	Lactic acid bacteria	Mexican, Guatemalan, and El Salvador	Escamilla-Hurtado et al. (1993)
	Pearl millet	Ben-saalga	Lactic acid bacteria	Burkina Faso/Africa	Tou et al. (2006)
	Cereals	Boza	*Lb. plantarum, Lb. brevis, Lb. rhamnosus, Lb. fermentum, Lb. mesenteroides* subsp. *dextranium*	Bulgaria, Albania, Turkey, and Romania	Hancioglu and Karapinar (1997), Moncheva et al. (2003); Botes et al. (2007); Todorov et al. (2008)
	Sorghum	Bushera	*Lb. plantarum, Lb. paracasei* subsp. *paracasei, Lb. fermentum, Lb. brevis* and *Lb. delbrueckii* subsp. *delbrueckii, S. thermophilus*	Western highlands of Uganda	Muyanja et al. (2003)
	Rice batter and lentils	Dosa	*Lb. plantarum, Leu. mesenteroides, Lb. fermentum, S. cerevisiae*	India	Soni et al. (1986)
	Cereal, legumes	Idli	*Leu. mesenteroides,* Lactic acid bacteria, Yeast	India	Agrawal et al. (2000); Aidoo et al. (2006)
	Maize	Ilambazilokubilisa	Lactic acid bacteria	Zimbabwe	Farnworth (2005)
	Wheat, soybeans	Kecap	Lactic acid bacteria	Indonesia	Roling et al. (1999)
	Maize	Kenkey	*Lb. casei, Lb. lactis, L. plantarum, Lb. brevis, Lb. acidophilus, Lb. fermentum, Lb. casei,* Yeast	Ghana	Olsen et al. (1995); Olasupo et al. (1997)
	Cereal and milk	Kishk	Lactic acid bacteria	Turkey and Iran	Tamime and McNulty (1999)
	Sorghum	Kisra	*Lactobacillus sp., Lb. brevis*	Ethiopia	Mohammed et al. (1991)
	Millet	Koko	*Lb. fermentum, Lb. salivarius*	African savanna areas	Lei and Jacobsen (2004)
	Maize	Mahewu	*Lb. brevis*	East Africa	McMaster et al. (2005)
	Maize	Mawe	*Lb. fermentum, Lb. brevis, Lb. salivarius, S. cerevisiae*	South Africa	Hounhouigan et al. (1999)
	Maize	Ogi	*Lb. plantarum, Lb. fermentum, Leu. mesenteroides, Sacch. cerevisiae*	Nigeria, Benin	Odunfa and Adeyele (1985); Adeyemi (1993); Ijabadeniyi (2007); Omemu et al. (2007)

Table 5. (Continued)

Raw material source	Raw material	Name	Responsible organism for fermentation	Country of origin/Place where the product is popular	Reference/s
	Flour, water	Sourdough	*Lb. reuteri, S.cerevisiae*	Egypt	Gobbetti et al. (2007); Chae et al. (2011)
	Parboiled wheat meal and yogurt	Tarhana	*Streptococcus thermophilus, Lb. bulgaricus, Lb. plantarum*	Turkey	Blandino et al. (2003); Patel et al. (2004)
	Black or rye bread	Kvass	*Lactobacillus* sp.	Russia	Dlusskaya et al. (2008)
	Kaffir maize	Kaffir beer	*Lactobacillus* spp	South Africa	Otles and Cagindi (2003); Farnworth (2006)
	Soybeans	Miso	*Aspergillus oryzae, Zygosaccharomyces* sp*., Pediococcus sp.*	Japan	Nichols (2007); Fujisawa et al. (2006)
	With cocoa and cornmeal	Pozol	*Leu. mesenteroides, Lb. plantarum, Lb. confusus, Lb. lactis and Lactococcus raffinolactis*	Southeastern Mexico	Escalante et al. (2001)
	Soybean	Tempeh	Lactic acid bacteria, *Lb. plantarum*	Indonesia	Ashenafi and Busse (1991); Feng et al. (2005)
	Maize, Finger millet	Togwa	Lactic acid bacteria	East Africa	Mugula et al. (2003a, b)
	Maize, sorghum cassava, finger millet	Uji	Lactic acid bacteria	Sudan	Onyango et al. (2003, 2004)
Fruit and Vegetables	Cabbage	Kimchi	*Lb. plantarum, Lb. curvatus, Lb. brevis, Lb. sake, Leu. mesenteroides*	South Korea	Chin et al. (2006); Lee et al. (2006)
	Green cabbage	Sauerkraut	*Lb. plantarum, Leu. mesenteroides*	Germany	Harris et al. (1992); Lu et al. (2003)
Fish/ Meat	Fish	Som-fug	Lactic acid bacteria	Thailand	Riebroy et al. (2007)
	Fish	Ngari	*Lactococcus lactis* subsp. *cremoris, Lactococcus plantarum, Enterococcus faecium, Lb. fructosus, Lb. amylophilus, Lb. coryniformis* subsp. *torquens, Lb. plantarum*	India	Thapa et al. (2004)

Raw material source	Raw material	Name	Responsible organism for fermentation	Country of origin/Place where the product is popular	Reference/s
	Fish	Tepa	Lactic acid bacteria	Alaska	Chilton et al. (2015)
	Herring	Surströmming	*Haloanaerobium praevalens, Haloanaerobium alcaliphilum*	Sweden	Grześkowiak et al. (2009)
	Meat	Fermented sausage	*Lactobacillus* sp., *Pediococcus* sp., *Micrococcus* sp.	Greece and Italy	Papamanoli et al. (2003)
Other	Cassava	Agbelima	*Lb. plantarum, Lb. brevis, Lb. fermentum, Leu. mesenteroides*	Ghana	Amoa-Awua (1996)
	Black, green, white, pekoe, oolong, or Darjeeling tea, sugar	Kombucha	*Gluconacetobacter, Zygosaccharomyces*	Russia and China	Kozyrovska et al. (2012)
	Agave plant sap	Pulque	Lactic acid bacteria, *Zymomonas mobilis*	Mexico	Riveros-Mckay et al. (2014)

Adapted and modified from: Granato et al. (2010); Rivera-Espinoza et al. (2010); Franz et al. (2014); Chilton et al. (2015).

3.2. Recently Developed Probiotic Non-Dairy Fermented Food Products

East Asia is the region where non-dairy fermented foods have been developed using various raw materials such as cereals, soybeans, fruits, vegetables, and fish (Rhee et al. 2011). They are not commonly found in many countries (Granato et al. 2010).

Fruit and vegetables have become the most prevalent among these raw materials and they are ideal substrates for microbial fermentation. In addition, their textural properties could be changed to suit for microbial fermentation (Betoret et al. 2003; Luckow and Delahunty, 2004; Champagne et al. 2005; Sheehan et al. 2007). Availability of certain specific nutrients such as vitamins, minerals, together with acidic nature of fruits and vegetables provide conducible medium for fermentation by lactic acid bacteria (Swain et al. 2014). Furthermore, some research studies have been shown that cereals are also suitable substrates for the growth of some probiotic bacteria (Mårtenson et al. 2002; Angelov et al. 2006; Trachoo et al. 2006; Kedia et al. 2007). However, other several raw materials including soy bean, different cereals, meat and fish have been extensively investigated to determine the suitability of substrates to produce non-dairy fermented foods with probiotic microorganisms (Rivera-Espinoza, et al. 2010; Martins et al. 2013). Table 6 summarizes the non-dairy based probiotic products developed recently in the world.

Table 6. Recently developed non-dairy probiotic products

Category	Product	Reference
Fruit and vegetable based	Many dried fruits	Betoret et al. (2003)
	Fermented banana pulp	Tsen et al. (2004)
	Blackcurrant juice	Luckow and Delahunty (2004)
	Tomato-based drink	Yoon et al. (2004)
	Beets-based drink	Yoon et al. (2005)
	Vegetable-based drinks	Rakin et al. (2007)
	Onion	Roberts and Kidd (2005)
	Grape and passion fruit juices	Saarela et al. (2006)
	Cabbage juice	Yoon et al. (2006)
	Cranberry, pineapple, and orange juices	Sheehan et al. (2007)
	Ginger juice	Chen et al. (2009)
	Green coconut water	Prado et al. (2008)
	Carrot juice	Nazzaro et al. (2008)
	Fermented banana	Tsen et al. (2009)
	Peanut milk	Kabeir et al. (2009)
	Noni juice	Wang et al. (2009)
	Probiotic banana puree	Tsen et al. (2009)
	Minimally processed apple	Rößle et al. (2010);
	Pomegranate juice	Alegre et al. (2011)
	Pear juice	Mousavi et al. (2011)
	Plum juice	Ankolekar et al. (2012)
	Olive	Sheela and Suganya (2012)
	Cocoa	Hurtado et al. (2012)
		Possemiers et al. (2010)
Soy based	Stirred yogurt-like drinks	Beasley et al. (2003)
	Fermented soymilk drink	Donkor et al. (2007)

Category	Product	Reference
	Defatted soy flour Soybean bar	Chen et al. (2011) Chen and Mustapha (2012)
Cereal based	Rice-based yogurt	Boonyaratanakornkit and Wongkhalaung (2000)
	Malt based	Rozada-Sánchez et al. (2008)
	Oat-based drink	Angelov et al. (2006)
	Oat-based products	Martensson et al. (2002)
	Yosa (oat-bran pudding)	Blandino et al. (2003)
	Mahewu (fermented maize beverage)	McMaste et al. (2005)
	Wheat, rye, millet, maize	Blandino et al. (2003)
Probiotic beverages	Malt-based drink	Kedia et al. (2007)
	Maize, sorghum, and millet malt fermented probiotic beverages	Blandino et al. (2003)
	Millet or sorghum flour fermented probiotic beverage	Muianja et al. (2003)
	Starch-saccharified probiotic drink	Kitabatake et al. (2003)
	Barley, tomato pulp and whey powder L. acidophilus Food mixture	Jood et al. (2012)
Other	Probiotic cassava-flour product	Molin (2001)
	Meat products Cashew juice	Krockel (2006) Vergara et al. (2010); Pereira et al. (2011)

Adapted and modified from Granato et al. (2010) and Martins et al. (2013).

3.2.1. Fruits and Vegetables Based Probiotic Products

Taste profiles of the fruits and vegetables based products are preferred by all age groups which is a positive factor for marketing aspects of the product compared to other non-dairy sources. Thus, there is a genuine interest among the researchers for the development of fruit or vegetables based probiotic food products. Occasionally there may be problems with off flavors and odors related to fermented fruit and vegetable products. However, various strategies could be used to mask the occasional unpleasant odors in such products. Luckow et al. (2006) observed noticeable off-flavors caused by probiotics in orange juice and were able to mask the off-flavors by adding10% (v/v) of tropical fruit juices (a mixture of pineapple, mango and passion fruit juices). Typically, fruit and vegetables create an acidic media for the growth of microorganisms and some microorganisms show sensitivities towards extreme pH conditions of the substrate (Vinderola and Reinheimer 2003). In general, the optimum pH for the growth of probiotic bacteria is between 4.5 and 6.4 and the growth is ceased when the pH reaches to a level of 3.6–4.0 (Shah 2007). However, research studies have been conducted to minimize the negative effect of pH (in fruit juices) on probiotic viability and Kailasapathy (2002) reported that such negative effects could be minimized by providing a physical barrier (such as encapsulation) for probiotic bacteria by using compounds such as alginate, agar, k-carrageenan, and locust bean gum. In addition, it has been reported that fruits provide a protection towards probiotics during processing (Betoret et al. 2003; Kourkoutas et al. 2005). Some fruit juices stimulate the growth of probiotics since these are considered as rich sources of simple sugars, mainly glucose and fructose, and minerals (Kailasapathy 2002; Ranadheera

et al. 2014b). Martins et al. (2013) reported that the expansion of the dairy industry leads to the introduction of new products called 'hybrid dairy products,' made by combining the dairy and fruits as pieces, pulp, juice or puree and these products are responsible for more than half of the market segment of the dairy industry, where these can act as both pro and prebiotics (synbiotics). According to Rivera-Espinoza (2010), *Lb. plantarum* and *Leu. mesenteroides* are the most common bacteria in natural vegetable lactic acid fermentation in addition to *Lb. paracasei/casei, Lb. delbrueckii* and *Lb. brevis* (Shah 2001).

3.2.2. Cereal Based Probiotic Products

Cereals are the staple foods in Asian and African countries which are considered to be one of the most important sources of proteins, carbohydrates, vitamins, minerals, and fiber. However, the organoleptic qualities of cereals and their products are sometimes inferior in comparison with milk, milk products, and fruit or vegetables due to the coarse nature of the grains. Thus, many methods have been adopted to improve the nutritional quality and sensory properties of cereals such as genetic improvement, amino acid supplementation, processing technologies which include cooking, sprouting, milling and fermentation (Blandino et al. 2003).

Table 7. Genera of lactic acid bacteria involved in cereal fermentations and most common strains responsible for giving probiotic effects

Genera of lactic acid bacteria	Most common strains responsible for giving probiotic effects
Lactobacillus	*Lb. fermentum, Lb. bulgaricus, Lb. johnsonii, Lb. crispatus, Lb. salivarius, Lb. bifidus, Lb. rhamnosus or GG, Lb. acidophilus, Lb. reuteri, Lb. plantarum, Lb. helveticus, Lb. casei subsp. rhamnosus, Lb. gallinarum, Lb. brevis, Lb. delbrueckii subsp. bulgaricus, Lb. gasseri, Lb. cellobiosus , Lb. vitulinus, Lb. collinoides, Lb. cremoris, Lb. ruminis, Lb. dextranicum, Lb. lactis, Lb. thamnosus, Lb. lactis biover oliacetylactis, L. casei*
Streptococcus	*S. faecalis*, *S. diacetilactis, S. salivarius subsp. thermophilus, S. cremoris, S. faecium, S. lactis S. equinus*
Pediococcus	*P. pentosaceus, P. acidilactici, P. halophilus*
Lactococcus	*Lac. lactis* subssp. *lactis* and *cremoris*
Leuconostoc	*Leu. mesenteroides* subsp. *dextranium* *L. paramesenteroides or lactis*
Bifidobacterium	*B. adolescentis B. bifidum, B. brevis B. longum, B. animalis B. infantis, B.thermophilum, B. breve* *B. lactis*
Enterococcus	*E. faecium, E. faecalis*
Sporolactobacillus	*S. inulinus*
Lactosphaera	
Oenococcus	
Vagococcus	
Aerococcus	
Weissella	

Adapted and modified from Blandino et al. (2003).

Fermentation is one of the best methods to improve the nutritional quality and sensory properties of cereal based products (Mattila-Sandholm et al. 2002). Fermentation of cereals is

carried out by different types of microorganisms, including bacteria *(Leuconostoc, Lactobacillus, Streptococcus, Pediococcus, Micrococcus* and *Bacillus)*, fungi (*Aspergillus, Paecilomyces, Cladosporium, Fusarium, Penicillium* and *Trichothecium*) and yeasts *(Saccharomyces)* (Steinkraus 1998). However, lactic acid bacteria are the predominant type of microorganism responsible for safe and quality cereal based fermented foods (Blandino et al. 2003) (Table 7).

In addition, cereals are considered as one of the best sources for the growth of lactic acid bacteria since cereals can provide wide array of nutrients such as fermentable carbohydrates, amino acids, vitamins, nucleic acids and minerals required by them (Gomes and Malcata 1999). Furthermore, non-digestible carbohydrates present in cereals can act as prebiotics which can stimulate the growth of probiotics.

In fact, cereals are the most abundant raw material used in the traditional fermented foods in the world and recently many research studies have been conducted to demonstrate that cereals are suitable substrates for the growth of probiotic bacteria (Table 6). Table 7 provides information of lactic acid bacteria involved in cereal fermentation and the strains that are commonly added to improve the probiotic effects.

3.2.3. Soy Based Probiotic Products

Soybean and its byproducts have received attention as an excellent source of proteins since soy proteins contain a rich amino acid profile compared to other plant protein sources. Moreover, soy is a source of soluble fiber, magnesium, phosphorus, vitamins K, riboflavin, thiamine, and folic acid (Granato et al. 2010). Also studies have disclosed that fermented soy products provide much more health benefits than non-fermented soy products (Esaki et al. 1994).

According to Granato et al. (2010), soy is considered as a good substrate for functional foods and soybean has been used to prepare fermented products such as Kecap, Kisra, Miso, and Tempeh (Table 6). Soybean contains oligosaccharides such as raffinose and stachyose that are sources of carbon for the growth of various *Lactobacillus* species, such as *Lb. acidophilus* and *Lb. delbruecki* subsp. *bulgaricus,* as well as *Bifidobacterium* species (Scalabrini et al. 1998). Therefore, soy is a good culture medium for inoculation and growth of probiotic strains (Wang et al. 2003). Granato et al. (2010) further stated that the main probiotic bacteria studied for growth in soy beverages are *Lb. acidophilus, Lb. fermentum, Lb. rhamnosus, Lb. lactis* R0187 and bifidobacteria. Farnworth et al. (2007) reported about successful growth of probiotic bacteria and bifidobacteria in soy yogurt and predominant bacterial species in the product were *Lb. johnsonii* NCC533 (La-1) and *Lb rhamnosus* ATCC 53103 (GG). Furthermore, a number of new products incorporating probiotics have been researched such as stirred yogurt-like drinks (Saris et al. 2003), fermented soymilk drink (Donkor et al. 2007), defatted soy flour (Chen et al. 2011), and soybean bar (Chen and Mustapha 2012).

The health promoting effects of soy based probiotics have also been reported. Recently an *in vitro* study conducted by Wagar et al. (2009) suggested the health promoting effects of soy probiotic products as they have immunomodulatory properties. Additionally, combination of soy with either a probiotic or a prebiotic resulted in lowering total cholesterol and low-density lipoprotein (LDL) (Larkin et al. 2007).

3.3. Beneficial Effects of Non-Dairy Probiotic Based Fermented Products

The concept of 'functional foods' became very popular since the last decade and trend towards healthy foods have become increased. The demand for foods which contain ingredients that optimize beneficial properties such as probiotics, prebiotics, vitamins and minerals has increased drastically over the years (Franz et al. 2014). Probiotic fermented non-dairy based foods are also claimed to have health benefits, such as improved intestinal immune system, displaced enteric pathogens, provide antimutagens and antioxidants, reduction in serum cholesterol levels, reduction in blood pressure, prevention and decreasing incidence and duration of diarrhea, prevention of bacterial vaginosis and urinary tract infection, maintenance of mucosal integrity, and improved periodontal health (Perdigón et al. 2001; Rivera-Espinoza et al. 2010; Franz et al. 2014). In addition, there are some other benefits that can be gained through adding probiotic bacteria into different products such as food preservation, promotes better digestion, improving the flavor and palatability and preventing unfavorable softening of the vegetables (Rhee et al. 2011).

4. CHALLENGES AND FUTURE DIRECTIONS FOR DEVELOPING FERMENTED PROBIOTIC PRODUCTS

At present, there is a genuine interest among researchers and the industry for the development of probiotic based functional fermented foods, due to higher demand from the health conscious consumers. Development of new non-dairy fermented probiotic food products seems to be increasingly challenging, as it has to fulfill the consumer's expectancy (Shah 2007), because the dairy fermented probiotic products are well established in the market as carriers for probiotics. The commercial success of both dairy and non-dairy probiotic products ultimately depends on taste, appearance, price, and health claim of the product. Besides, developing a new dairy-free probiotic food is an expensive process since it requires carrying out comprehensive research activities (Granato et al. 2010).

Maintaining the survival of the probiotic bacteria in sufficient numbers in the food matrices during the storage is a challenge. In addition, probiotic bacteria should be able to survive until they reach in the colon. Although they can survive in the food matrices, the conditions in the small intestine may not be appropriate for their survival. However, to increase the survival of probiotics in different food matrices and human gut, different techniques have been adopted such as (1) addition of 0.01% baker's yeast in to the food matrix (Kailasapathy 2002); (2) addition of raffinose, stachyose, fructo-, isomalto- and galacto-oligosaccharides like substances in to the food matrix (Mitsuoka 1992); (3) use of a concentrated deep-frozen or freeze-dried culture (Costello 1993) and (4) microencapsulation and coating of cultures (Rao, et al. 1989; Kailasapathy 2002). In order to improve the probiotic survival, the use of these techniques in developing fermented probiotic food products may be useful. In addition more research is needed to improve the organoleptic properties of probiotic enriched fermented food products.

CONCLUSION

Fermented food products can be produced with or without probiotic microorganisms. However, most of the starter cultures used in producing fermented foods possess probiotic properties up to some extent. Both dairy and non-dairy food matrices have been used for the production of probiotic fermented food products for thousands of years. Nevertheless, the application of probiotic cultures in fermented food products is a challenge. Proper understanding of probiotics, starter culture microorganisms and the carrier food matrices is important in developing fermented probiotic food products, because these factors can influence the viability of probiotics during processing, storage and gastrointestinal transit, flavor and texture of the food product and the potential health benefits after consumption.

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Chapter 3

FERMENTED PLANT PRODUCTS: LACTIC ACID BACTERIA AND THEIR HEALTH BENEFITS

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ABSTRACT

Numerous fermented foods (fermented milk, fish, meat, vegetables, and plant products) are consumed around the world. These food products are prepared by the fermentation of various raw materials. Lactic acid bacteria (LAB) from the genera *Lactobacillus (Lb), Lactococcus (Lc), Leuconostoc (Ln), Pediococcus (P)* and *Weissella (W)* are the most important bacteria in desirable food fermentations. These bacteria are involved in the flavor, aroma compounds, γ-aminobutyric acid (GABA), and antimicrobial substances formation during fermentation. Several fermented fruit and vegetable products that arise from lactic acid fermentation are important for the nutritional requirements of a large proportion of the world's population. Diversity and probiotic potentials of LAB, *Lb. plantarum*, *Lb. paraplantarum*, *Lb. pentosus*, *Lb. sakei*, *Lb. casei*, *Lb. fermentum*, *Lb. paracasei*, *Lb. brevis*, *Lb. buchneri*, *Lb. parabuchneri*, *Lb. pantheris*, *Lb. harbinensis*, *Lb. kimchi*, *Lb. fallax*, *Lc.lactis*, *Ln. mesenteroides*, *Ln. pseudomesenteroides*, *P. pentosaceus*, *P. acidilactici*, *W. confusa*, *W. koreenis* and *W. cibaria* isolated from plant products have been reported. LAB produce bacteriocins with strong antibacterial activity to *Listeria*, *Bacillus* and *Staphylococcus*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, and *Carnobacterium*. LAB, *Lb. brevis*, *Lb. plantarum*, *Lc. lactis* subsp. *lactis*, *En. casseliflavus*, and *Streptococcus thermophilus* are found to produce GABA. Traditions and economic factors that limit the use of dairy fermented products in some developing countries promote the idea of reduction of milk components as vehicles for probiotic agents. Recently, the development of non-dairy probiotic products, including fruit, vegetables, and cereals, has been widely studied. Lactic acid fermentation contributes to improvement in the nutritive quality of vegetable

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juices. Beverages such as fruit and vegetable juices would be the food category where the healthy probiotic bacteria will make their mark. Fruit and vegetable juices and cereals, the potential substrate for the production of probiotic beverages by *Lb. plantarum*, *Lb. casei*, and *Lb. delbrueckii*, *Lb. rhamnosus* and *Lb. acidophilus*, are evaluated for their ability to survive during storage. These beverages could be a good vehicle for delivery of probiotic LAB to consumers, and have the potential to become a commercial product. Consumption of these probiotic products and pickles that contain GABA for reducing blood pressure has been associated with good health and longevity. In addition, acetic acid bacteria play role in vinegar fermentation. Antioxidant, antimicrobial, mineral, volatile, physicochemical, and microbiological characteristics of traditional vinegars have been characterized. Vinegar ingestion favorably influences biomarkers for heart disease, cancer, and diabetes. Traditionally fermented plant products not only have served as food supplements but have also had numerous health benefits attributed to them.

1. INTRODUCTION

Fermentation is one of the oldest processing techniques for extending the shelf life of perishable food. It is a slow decomposition process of organic substances induced by microorganisms or enzymes that essentially convert carbohydrates to alcohols or organic acids (Battcock & Azam-Ali, 2001). Lactic acid fermentation (LA fermentation) plays an important role, which has been widely studied for many years, while acetic acid fermentation by acetic acid bacteria causes the oxidation of alcohol to acetic acid (Stamer et al., 1971; Battcock & Azam-Ali, 2001; Swain et al., 2014). Fermentation has many benefits, providing food security, improved nutrition, and better social wellbeing for millions of people in marginalized and vulnerable sections of societies around the world (Battcock & Azam-Ali, 2001). Fermented foods can be produced with inexpensive ingredients and simple techniques, and make a significant contribution to the human diet, especially in rural households and village communities worldwide (Hui & Evranuz, 2012). LA fermentation of vegetables and fruits is a common practice to maintain and improve the nutritional and sensory features of food commodities. The availability of certain specific nutrients, such as vitamins and minerals, and the acidic nature of vegetables and fruits provide a conducible medium for fermentation by LAB (Swain et al., 2014). A large number of probiotic potential LAB – *Lactobacillus* (*Lb.*) *plantarum*, *Lb. paraplantarum*, *Lb. pentosus*, *Lb. sakei*,*Lb. casei*, *Lb. fermentum*, *Lb. paracasei*, *Lb. brevis*, *Lb. buchneri*, *Lb. parabuchneri*, *Lb. pantheris*, *Lb. harbinensis*, *Lb. kimchi*, *Lb. fallax*, *Lc.lactis*, *Ln. mesenteroides*, *Ln. pseudomesenteroides*, *P. pentosaceus*, *P. acidilactici*, *Weissella confusa*, *W. koreenis*, and *W. cibaria*– have been isolated from plant products (Swain et al., 2014).

As noted above, fermented plant products, including fermented vegetables and fruits, can bring many benefits to people in developing countries (Battcock & Azam-Ali, 2001). The intake of a specific dose of vegetables and fruits in the daily diet prevents chronic pathologies, such as hypertension, coronary heart problems, and lowers the risk of strokes, and has been recommended by World Health Organization (WHO) and Food and Agriculture Organization (FAO). Consumers tend to prefer food and beverages which are fresh, highly nutritional, health promoting and ready to eat or ready to drink (Endrizzi et al., 2009). The probiotic LAB in products of vegetable origin and the characteristics that enable the use of these food matrices as potential carriers of probiotic bacteria have been applied (Martins et

al., 2013). The consumption of these probiotic products and pickles that contain GABA for reducing blood pressure has been associated with good health and longevity. In addition, acetic acid bacteria are important in the production of vinegar (acetic acid) from fruit juices and alcohols. Antioxidant, antimicrobial, mineral, volatile, physicochemical, and microbiological characteristics of traditional vinegars have been characterized. Vinegar ingestion favorably influences biomarkers for heart disease, cancer and diabetes. This chapter outlines the current research prospects regarding fermented plant products, lactic acid bacteria, potential probiotics, and vinegar, and their health benefits.

2. WORLD FERMENTED PLANT PRODUCTS AND LAB

The fermented foods consumed around the world include fermented milk, fish, meat, soybean, vegetables, bread and porridges, and alcoholic beverages. LAB and yeasts play important roles in many fermented products. In America and Mexico, LAB and *Candida* spp. are found in acid-fermented starch, *pozol*, and the cereal-based fermented product *atole* (Lee, 1997; Blandino et al., 2003). In Europe, various LAB, such as *Lb. sanfranciscensis, Lb. plantarum, Lb. acidophilus, Lb. fermentum, Lb. coryniformis, Lb. pentosus, Lb. rhamnosus, Lb. mali, Lb. brevis, Lb. casei, Lb. curvatus, Lb. paracasei , Lb. delbrueckii , Lb. pontis, Lb. brevis, Lb. acetotolerans, Lb. vaccinostercus, P. pentosaceus, P. acidilactici, En. casseliflavus, En. italicus, W. paramesenteroides, W. cibaria, Ln. paramesenteroides, Ln. mesenteroides, Ln. citreum,* and *Lc. Lactis*, and yeasts, such as *Saccharomyces (S. uvarum, S. cerevisiae), Pichia fermentans, C. humilis,* and *Candida* spp., are distributed in many kinds of plant products, as described below. The most well-known product is the pickled vegetable *sauerkraut*. There are olives and table olives in Spain, and in Italy, the acid-leavened sourdough bread (Viander et al., 2003; Wang et al.,2010; Yang et al., 2010; Argyri et al., 2013; Nychas et al., 2002; De Bellis et al., 2010; Lattanzi et al., 2013). *Shalgam* or *salgam* (lactic acid fermentation of black carrot, turnip, rock-salt, sourdough, bulgur flour and drinkable water (Tanguler & Erten, 2012)) and *hardaliye* (a grape-based, non-alcoholic traditional beverage (Arici & Coskun, 2001)) are produced in Turkey, and in Turkey and Bulgaria there are cereal-based fermented products such as *Boza* (barley, oats, rye, millet, maize, wheat or rice).

In the Indian sub-continent there are many kinds of pickled vegetables, and strains of *Lb. plantarum, Lb. brevis, Lb. fermentum, Lb. fallax, Lb. lactis, P. pentosaceus, P. acidilactici, Pediococcus* spp., and *Lactobacillus* spp. are distributed in gundruk, sinki, Inziangsang, and soidon, produced in India, with gundruk and sinki also found in Nepal and Bhutan (Steinkraus 1997; Dahal et al., 2005; Tamang et al., 2008; Tamang, 2009). *Saccharomyces cerevisiae* and lactic acid bacteria such as *Pediococcus*, *Streptococcus* and *Leuconostoc* are distributed in cereal-based fermented products such as nan, adai, anarshe and vada, produced in India and also consumed in Pakistan, Afghanistan, and Iran (Blandino et al., 2003). The strains of *Lb. plantarum* and *P. pentosaceus* are found in khalpi, produced in Nepal (Tamang, 2009; Dahal et al., 2005). Acid-leavened breads Idli, dosa, and dhokla are produced in India and Sri Lanka (Mukherjee et al., 1965) and *Ln. mesenteroides* and *S. faecalis* strains are found, while in the Sri Lankan Hoppers (*appa*) yeast and lactic acid bacteria are involved (Lee, 1997).

In East Asia, *Lb. pentosus*, *Lb. plantarum*, *Lb. brevis*, *Lb. lactis*, *Lb. fermentum*, *Lb. lactis*, *Ln. mesenteroides*, *W. paramesenteroides*, *W. minor*, *W. cibaria*, and *En. faecalis* strains are distributed in pickled vegetables, in China in paocai (Yan et al., 2008; Feng et al., 2012) and in China and Taiwan in yan-taozih (Chen et al., 2013b). *Lb. plantarum*, *Lb. curvatus*, *Lb. sakei*, *Lb. fermentum*, *Lb. brevis*, *Lb. maltaromicus*, *Lb. bavaricus*, *Ln. mesenteroides*, *Ln. citreum*, and*W. confusa* strains are found in kimchi in Korea (Lee, 2001). *Lb. curvatus*, *Lb. plantarum*, *Lb. brevis*, *P. pentosaceus*, and *Bacillus coagulans* are found in nozawana-zuke (Kawahara & Otani, 2006) and sunki in Japan (Battcock & Azam-Ali, 2001). *Lb. casei*, *Lb. cellobiosus*, *Lb. fermentum*, and *Ln. mesenteroides* are found in the acid-fermented mungbean starch noodle produced in China, Thailand, Korea, and Japan (Lee, 2001); *Lb. sakei*, *Lb. pobuzihii*, *Lb. plantarum*, *P. pentosaceus*, *Tetragenococcus halophilus*, *Lc. Lactis*, *W. cibaria*, *W. hellenica*, *W. paramesenteroides*, *W. minor*, *Ln. mesenteroides*, *Ln. lactis*, *En. sulfurous*, and *En. casseliflavus* strains are found in jiang-gua, pobuzihi, suan-tsai, yan-jiang, yan-dong-gua, and yan-tsai-shin (Chang et al., 2011; Chen et al., 2006; 2010; 2012; 2013a; 2013c; Lan et al., 2009) produced in Taiwan. In addition, in alcoholic beverages, *S. cerevisiae*, *Lb. casei*, *Lb. cellobiosus*, *Lb. fermentum*, *Lb. pentosus*, *Lb. plantarum*, *Lb. sakei*, *P. pentosaceus*, and *P. acidilactici* strains are found in takju and omegisool in Korea (Lee, 1997; Oh & Jung, 2015). *Aspergillus oryzae*, *Torulopsis etchellsii*, *Zygosaccharomyces rouxi*, and LAB strains are found in cereal-based fermented products hamanatto, miso and shoyu (soy sauce), and LAB in soybean milk (Blandino et al., 2003) produced in Japan.

In Africa, *Lb. fermentum*, *Lb. brevis*, *Lb. curvatus*, *Lb. buchneri*, *Lb. plantarum*, *Ln. mesenteroides*, *Lb. cellobiosus*, *Lb. salivarius*, *P. acidilactici*, *P. pentosaceus*, *Lb. reuteri*, *W. confusa*, *Acetobacter*, moulds, *S. cerevisiae*, *S. chavelieri*, *Candida*, *C. mycoderma* strains are found in cereal-based fermented products, including mawé in Benin (Hounhouigan et al., 1993; 1994), ogi in Benin and Nigeria (Odunfa & Adeyele, 1985; Nago et al., 1998; Omemu, 2011; Sanni et al., 2013), burukutu in Nigeria, Benin and Ghana, kwunu-zaki in Nigeria (Blandino et al., 2003), uji in Kenya, Uganda and Tanganyika (Mbugua, 1985; Blandino et al., 2003), kenkey, banku and koko (cereal: pearl millet) in Ghana (Olsen et al., 1995; Blandino et al., 2003; Marsch et al., 2014), ben-saalga in Burkina Faso (Omar et al., 2006; Songré-Ouattara et al., 2008), hussuwa, hulumur, kisra and nasha in Sudan (Lee, 1997; Yousif et al., 2010; Blandino et al., 2003), mahewu in South Africa, enjera in Ethiopia, bouza in Egypt (Lee, 1997), and bushera (sorghum, millet flour) in Uganda. Lactic acid bacteria, yeasts and moulds are found in ilambazi lokubilisa, mutwiwa and tobwa produced in Zimbabwe (Blandino et al., 2003). In addition, *Lb. plantarum*, *Lb. fermentum*, *P. pentosaceus*, *Ln. fallax*, and *W. confusa* strains are found in acid fermented starch, fufu in Nigeria (Sanni et al., 2013), gari in Benin (Kostinek et al., 2007), and lafun in West Africa (Padonou et al., 2009). *Lb. plantarum*, *Lb. lactis*, *Lb. helveticus*, *Lb. salivarius*, *Lb. casei*, *Lb. brevis*, *Lb. buchneri*, *P. damnosus*, *S. cerevisiae*, *Candida krusei*, *S. chevalieri*, *S. elegans*, *Bacillus subtilis*, *Aspergillus niger*, *A. flavus*, and *Mucor rouxii* strains are found in alcoholic beverages such as bussa in Kenya (Lee, 1997), Busaa in Nigeria and Ghana (Blandino et al., 2003), seketeh in Nigeria, and sorghum beer in South Africa (Blandino et al., 2003). In South East Asia, *Lb. plantarum*, *Lb. pentosus*, *Ln. mesenteroides*, *Lb. mali*, *Lb. fermentum*, *Lb. brevis*, *Lb. paracasei*, *Lb. pantheris*, *P. pentosaceus*, *P. acidilactici*, *Lb. mesenteroides*, *Lb. confusa*, and *Lb. curvatus* strains are found in pickled vegetables such aspak-gard-dong, pak-sian-dong, and pak-koom-dong produced in Thailand (Tanasupawat & Komagata, 2001), dua

muoi, ca muoi, and dhamuoi in Vietnam (Steinkraus, 1997; Nguyen et al., 2013), burong mustala in the Philippines (Rhee et al., 2011), sayur asin in Indonesia (Puspito & Fleet, 1985), and tempoyak in Malaysia (Leisner et al., 2001). In addition, *Ln. mesenteroides* and *S. faecalis* strains are found in acid-leavened bread, puto, in the Philippines (Lee, 2001); *Lb. plantarum*, *Lb. fermentum*, and *P. acidilactici* strains are found in acid-fermented noodle, ka-nom-jeen, in Thailand (Tanasupawat & Komagata, 2001), acid-fermented starch and LAB in me in Vietnam, *Saccharomyces*, *Mucor*, *Rhizopus*, *Aspergillus*, *Leuconostoc spp.*, and *Lb. plantarum* in the alcoholic beverage tapuy in the Philippines (Lee, 1997), and *Aspergillus oryzae*, *Lactobacillus sp.*, *Hansenula sp.*, *Saccharomyces sp.* strains in the cereal-based fermented product kecap in Indonesia (Blandino et al., 2003).

3. POTENTIAL HEALTH BENEFITS OF LAB

3.1. Probiotics

Probiotic foods contain live microbes or components of microbial cells that have a beneficial effect on the health and wellbeing of the consumer host (Salminen et al., 1999). The concept of ingesting live microorganisms for the purpose of improving the intestinal health and general wellbeing can be traced to the beginning of the 20th century (Metchnikoff, 1907; O'Sullivan, 2001). Probiotic microorganisms have been included in various types of food products, including dairy foods, food supplements, and dietary supplements, and in particular in fermented milk. Fermented milk products containing viable probiotic bacteria have been used by humans primarily as a prophylactic, and their use has been extended for treatment of intestinal infections. Some researchers have suggested the use of probiotics to prevent and treat diarrhea induced by *Salmonella* or *Shigella* (Alm, 1983; Zychowiez et al., 1974). A recent formal definition of probiotics, recommended by a working group of the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO), defines probiotics as live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002). Lactic acid bacterial strains are the major representatives of probiotics; they are generally recognized as being harmless (GRAS) organisms that are safe to consume, and they have a long history of use in food (Bredholt et al., 2001). Lactobacilli and bifidobacteria possess antimicrobial properties, and are the most frequently used probiotics (Gorbach, 2002). *Bifidobacterium bifidum*, *Lb. paracasei*, and *Lb. rhamnosus* strains have been used as probiotic starter cultures (De Bellis et al., 2010).

Probiotic strains are isolated from numerous sources such as dairy products or gut and the other is vegetable-derived probiotics (VDP) which are isolated from fermented vegetable food such as pickles or a soy-sauce (Okada et al., 2011). There has been a great deal of research on potential probiotics (Table 1) in fermented plant products, including, for example, *W. koreensis* FKI21 from fermented *koozh*, which demonstrates cholesterol-reducing potential (Anandharaj et al., 2015).

Table 1. Probiotic LAB from fermented plant products

Probiotic LAB	Product Name	Activity	References
W. koreensis FKI21 and *Lb. crispatus* GI9	Gherkins and koozh	Cholesterol reduction, antimicrobial activity against *Ps. aeruginosa* MTCC 2642; *E. coli* MTCC 1089; *K. pneumoniae* MTCC 7028; *B. subtilus* MTCC 8561; *S. aureus* MTCC 7443; *C. albicans* BS3	Anandharaj et al., (2015)
Lb. plantarum subsp. *plantarum* SW03 and *P. acidilactici* SW05	*Omegisool* (fermented millet alcoholic beverage)	Acid and bile tolerance, high levels of antioxidant activity, strong adhesion to HT-29 cell	Oh and Jung (2015)
Ln. mesenteroides; *Ln. pseudomesenteroides*; *Lb.casei*; *Lb.buchneri* and *Lb. plantarum* strains	*Gilaburu* [Turkish fermented European cranberrybush (*Viburnum opulus* L.) fruit drink]	Acid and bile tolerance, high hydrophobicity properties, inhibit growth of some pathogenic bacteria (*B. cereus*, *E. coli*, *L. monocytogenes*, *S. typhimurium*, *S. aureus*, *Y. enterocolitica*)	Sagdic et al., (2014)
Lb.pentosus E281, E97, E104, E108); *Lb.plantarum* B282, E10, E69 and *Lb.paracasei* subsp. *paracasei* (E93, E94)	Fermented olives	Acid and bile tolerance, higher adherence properties to Caco-2 cells than the well-known probiotic strains (*Lb.rhamnosus* GG and *Lb.casei* Shirota)	Argyri et al., (2013)
Lactobacillus strain s193	Funazushi (Sushi; salted and fermented with rice and a crusian carp)	Anti-inflammatory effect on DSS-colitis	Okada et al., (2011)
P. pentosaceus strain MP12 and *Lb. plantarum strain* LAP6	Pickled cabbage	Adhere and survive on the mouse intestinal epithelium, antagonistic activity on *Salmonella* spp.	Peres et al., (2012)
Lb. plantarum C06and *Lb. acidophilus* C11	Pickled cabbage	*In vitro* performance in terms of duodenum cell adhesion, tolerance to gut biotic stress associated with exposure to gastric juice and bile salts, antibacterial activity against *B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli* and *Sal. enterica*) pathogens, β-galactosidase activity	Peres et al., (2012)
Lb. pentosus strains	Fermented green olive brines	High autoaggregation ability, low hydrophobicity properties, and lower survival to gastric than to pancreatic digestion	Bautista-Gallego et al., (2013)
Lb. plantarum strains	Raw fruits and vegetables	High resistance to simulated gastric and intestinal fluids, stimulate 27 immune-mediators, strong adhesion to Caco-2 cell	Vitali et al., (2012)
Lb. plantarum; *Lb. brevis*; *Lb. curvatus*; *P. pentosaceus; P. acidilactici*; *Leuconostoc* spp.; *En. durans*	Ethnic fermented vegetables and tender bamboo shoots	Adherence to the mucus secreting HT29 MTX cells, high hydrophobicity (70%), antagonistic activity on *L. innocua* DSM 20649, *B. cereus* CCM 2010, *S. aureus* S1, *St. mutans* DSM 6178, *K. pneumonia* subsp. *pneumonia* BFE 147, *Enterobacter cloacae* BFE 282, *Enterobacter agglomerans* BFE 154, *Ps. aeruginosa* BFE 162	Tamang et al., (2009)
Lactobacillus spp.; *Leuconostoc* spp.; *Pediococcus* spp.	Manzanilla Aloreña green table olives	Tolerance to low pH and bile salts, production of bile salt hydrolase, inhibit growth of *S. aureus*, *L. monocytogenes*, *En. faecalis*, *B. cereus*, *St. mutans* and *Salmonella*	Abriouel et al., (2012)

A mixture of *Lc. lactis* BFE920 and *Lb. plantarum* FGL0001 may serve as an immunostimulating feed additive useful for disease protection and innate immunity and

disease resistance in olive flounder (*Paralichthys olivaceus*) in the fish farming industry (Beck et al., 2015). *Lb. pentosus* SW02, *Lb. plantarum* subsp. *plantarum* SW03, *Lb. sakei* subsp. *sakei* SW04, *Lb. plantarum* subsp. *plantarum* SW06, *Lb. plantarum* subsp. *plantarum* SW07, *P. pentosaceus* SW01 and SW05 isolated from *Omegisool*, a traditionally fermented millet alcoholic beverage in Korea, survived exposure to pH 2 for 3 h and 0.3% bile salts for 24 h or 48 h. They exhibited strong abilities to adhere to HT-29 cells. The isolates also demonstrated DPPH radical scavenging activity in the range of 22.0-56.3%. They have the potential use as probiotic preparations for the fermentation industry (Oh & Jung, 2015).

The probiotic potentials of LAB strains isolated from fermented gilaburu (*Viburnum opulus*) juice, a traditional Turkish fermented European cranberrybush (*Viburnum opulus* L.) fruit drink, were able to grow at pH 2.5. *Lb. casei* (G20a), and *Lb. plantarum* (G19e) showed the highest cell hydrophobicity degrees and antibacterial activity to *L. monocytogenes* and *B. cereus*, but not *E. coli*or *S. aureus* (Sagdic et al., 2014).

Lb. pentosus, *Lb. plantarum* and *Lb. paracasei* subsp. *paracasei* strains from fermented olives were found to possess desirable *in vitro* probiotic properties similar to or even better than the reference probiotic strains *Lb. casei* Shirota and *Lb. rhamnosus* GG. These strains are good candidates for further investigation, both with *in vivo* studies to elucidate their potential health benefits, and in olive fermentation processes to assess their technological performance as novel probiotic starters (Argyri et al., 2013). The *Lb. pentosus* strain from naturally-fermented Aloreña green table olives has potential probiotic traits, such as inhibition of human pathogenic bacteria, survival at low pH (1.5), and bile-salt tolerance (3%) (Abriouel et al., 2011). *Lb. pentosus*, *P. parvulus*, and *Ln. pseudomesenteroides* strains from Aloreña green table olives exhibited useful antimicrobial substances active against pathogenic bacteria such as *L. monocytogenes*, *B. cereus*, *S. aureus*, *St. mutans* and *Sal. enterica*, utilization of raffinose and stachyose, and production of bile salt hydrolase and phytase. On the basis of data obtained, selected strains with potential traits were tested for their survival at low pH and their tolerance to bile salts, and the survival capacity demonstrated by some of the analyzed strains was sufficient to encourage further study on their potential as probiotics (Abriouel et al., 2012). *Lb. pentosus* strains from spontaneously fermented green olive brines showed promising potential probiotic characteristics, such as high autoaggregation ability, low hydrophobicity properties, and lower survival to gastric than to pancreatic digestion, which are even better than probiotic reference strains. Due to the autochthonous origin of the strains, their use as starter cultures may contribute to improving natural fermentation and the nutritional characteristics of table olives (Bautista-Gallego et al., 2013). Hence, a probiotic potential is expected to greatly increase the already important nutritional value of table olives originally arising from their being a source of fiber, organic acids, vitamins, and minerals. Development of probiotic olive products may indeed convey a favorable economic impact, especially in light of the fact that such products originate in less developed regions (Peres et al., 2012).

The autochthonous lactic acid bacteria from raw fruit and vegetables have functional features that enable them to be considered novel probiotic candidates. *Lb. plantarum* strains are found to maintain high cell densities under simulated gastric and intestinal conditions. All strains stimulated all immune-mediators by peripheral blood mononuclear cells (PBMC). Only a few strains increased the synthesis of cytokines with anti-inflammatory activity. Four *Lb. plantarum* strains were defined as strongly adhesive strains (more than 40 bacteria adhering to one Caco-2 cell), and two as adhesive strains. Five strains grew and acidified

chemically defined medium with fructooligosaccharides (FOS) as the only carbon source. All strains inhibited enterohemorrhagic *E. coli* K12 and *B. megaterium* F6 isolated from human sources (Vitali et al., 2012). *Lactobacillus* strain s193 isolated from Japanese "Funazushi" (Japanese old-style sushi; salted and fermented with rice and a crusian carp) could suppress mRNA levels of proinflammatory cytokines and the number of non-probiotic bacteria such as *E.coli* on DSS colitis. *Lactobacillus* strain s193 have anti-inflammatory effect on DSS-colitis, not only by the suppression of proinflammatory cytokines and the modulation of intestinal bacterial flora, but also by reductions of neuropeptides such as substance P and its receptors (Okada et al., 2011). *Lb. plantarum* IB2 (BFE 948) isolated from inziangsang, a fermented leafy vegetable product from the Himalayas, produced a bacteriocin against *S. aureus* S1. Some strains of *Lb. plantarum* showed more than 70% hydrophobicity. Adherence to the mucus secreting HT29 MTX cells was also shown by seven strains, indicating their probiotic nature (Tamang et al., 2009). The synbiotic effects of fermented rice bran, containing dietary fiber that has potency as a prebiotic, fermented by probiotic in the colon to produce lactic acid and short chain fatty acid (SCFA) using *Lb. plantarum* B2 and *Lb. casei* in Wistar rats (*Rattus norvegicus*), were investigated. The newly isolated *Lb. plantarum* B2 in conformity with rice bran showed higher characteristics in comparison to the commercial *Lb. casei*. Therefore, the fermented rice bran using *Lb. plantarum* B2 has potential as symbiotic product (Zubaidah et al., 2012).

3.2. Antimicrobial Substances of LAB

A large number of bacteriocin-producing lactic acid bacteria, including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Weissella*, and *Enterococcus* strains have been isolated from a variety of foods. These antimicrobial substancesare proteinaceous compounds that have been used extensively in food preservation (Gao et al., 2015).*Lb. plantarum* C19 isolated from fermented cucumber produced plantaricin C19, which exerted a bacteriostatic action on sensitive cells of *Listeria grayi* IP 6818 in BHI broth (Atrih et al., 2001). *Lactococcus* sp. GM005 isolated from Miso-paste was found to produce a bacteriocin with strong antibacterial activity. GM005 bacteriocin exhibits a bactericidal activity against *Lb. sakei* JCM1157^{T} (Onda et al., 2003). *En. mundtii* strain ST15, isolated from soy beans, produced a 3944 Da bacteriocin that inhibited the growth of *Lb. sakei*, *En.faecalis*, *B, cereus*, *Propionibacterium* sp., *C. tyrobutyricum*, *A. baumannii*, *K. pneumoniae*, *Ps. aeruginosa*, *S. aureus*, *St. pneumonia*, and *St. caprinus*. The mode of activity is bactericidal. Bacteriocin ST15 differs from other broad-spectrum bacteriocins described for *Enterococcus* spp. by being active against Gram-negative bacteria (De Kwaadsteniet et al., 2005). *Lb. plantarum* strains ST194BZ, ST414BZ, and ST664BZ, *Lb. pentosus* ST712BZ, *Lb. rhamnosus* strains ST461BZ and ST462BZ, and *Lb. paracasei* strains ST242BZ and ST284BZ, isolated from *boza*, produced bacteriocins active against *Lb casei*, *E. coli*, *Ps. Aeruginosa*, and *En. faecalis*. Based on tricine-SDS-PAGE, the bacteriocins ranged from 2.8 to 14.0 kDa in size (Todorov & Dicks, 2006). *Leuconostoc* spp. and *Lb. plantarum* corresponded to *Weissella* spp., and *Lc. lactis* isolated from fresh vegetables and fruit showed the ability to inhibit the growth of foodborne human pathogens (*E. coli*, *L. monocytogenes*, *Ps. aeruginosa*, *S. typhimurium*, and *S. aureus*). This supports the potential use of lactic acid bacteria as bioprotective agents against foodborne human pathogens in ready-to-eat fresh fruit and vegetable products (Trias

et al., 2008). The use of LAB from minimally processed fruits and vegetables as biocontrol agents –for example, applying *Lb. plantarum* strains CIT3 and V7B3 to apples and lettuce, respectively – increased both safety and shelf-life. The combining of the selected strains with natural antimicrobials produced a further increase in the shelf-life of these products without detrimental effects on their organoleptic qualities (Siroli et al., 2015). An antimicrobial substance (nonbacteriocin) produced by *W. paramesenteroides* DFR-8, isolated from cucumber (*Cucumis sativus*), a non-proteinaceous antimicrobial molecule with molecular mass ~2.5 kDa, is thermostable up to 121°C at pH ~4.0. It is insensitive to proteolytic enzymes, lipase, amylase, and catalase, and shows a broad spectrum of activity toward food-borne/spoilage pathogens including Gram-negative organisms. The broad inhibitory spectrum and heat stability of the antimicrobial substance advocates its application as a food-biopreservative (Pal & Ramana, 2009).*P. pentosaceus* strain ST44AM, isolated from marula, produces a 6.5 kDa class IIa bacteriocin, active against lactic acid bacteria, *E. coli, Ps. aeruginosa*, *K. pneumoniae*, *L. innocua*, *L. ivanovii* subsp. *ivanovii* and *L. monocytogenes.* The mode of activity against *L. ivanovii* susbp. *ivanovii* ATCC19119 and *En. faecium* HKLHS is bactericidal. Bacteriocin ST44AM may be a derivative of pediocin PA-1 (Todorov & Dicks, 2009). *Lb. sakei* strain C2 isolated from traditional Chinese fermented cabbage produced a bacteriocin which strongly inhibited *S. aureus* ATCC 63589 and *E. coli* ATCC 25922. This bacteriocin had a molecular weight of 5.5 kDa like sakacin C2 which exhibited a wide range of antimicrobial activity, strong heat stability (15 min at 121°C) and pH stability (pH 3.0-8.0). Sakacin C2 was sensitive to protease but insensitive to lipase, a-amylase and b-amylase. These characteristics suggested that sakacin C2 was a novel Class II bacteriocin with a broad inhibitory spectrum and might broaden the application range of bacteriocins from LAB in the food industry (Gao, et al., 2010). *Lc. lactis* subsp. *lactis* KT2W2L and *En. faecalis* KT2W2G, TS9S17 and TS9S19 isolated from mangrove forests (soil, water, leaf, twig and fruit) in southern Thailand were screened for bacteriocin production. Only four strains that produced bacteriocin-like inhibitory substance (BLIS) in MRS broth, named KT2W2G, KT2W2L, TS9S17, and TS9S19, showed an inhibition zone against *Lb. sakei* subsp. *sakei* JCM 1157, *L. monocytogenes* DMST 17303, and *Brochothrix thermosphacta* DSM 20171. These BLISs showed a wide range of antibacterial activity against similar bacterial strains, food-spoilage and food-borne pathogens, but were inactive against the Gram-negative bacteria tested (Hwanhlem et al., 2014). *Lc. garvieae* LG34 isolated from traditional Chinese fermented cucumber produced Garviecin LG34, a novel Class IIa bacteriocin consisting of 46 amino acid residues. Garviecin LG34 exhibited inhibitory activity not only against Gram-positive bacteria but also Gram-negative bacteria. Garviecin LG34 showed resistance to heat and low pH, and was sensitive to proteolytic enzymes but not to lipase or amylase. This bacteriocin had the potential to be used as food biopreservative (Gao et al., 2015).

3.3. γ-Aminobutyric acid (GABA) and Antioxidants of LAB

Lb. brevis, Lb. plantarum, *Lc. lactis* subsp. *lactis*, *En.casseliflavus*, and *S. thermophilus* isolated from *kimchi* were found to produce γ-aminobutyric acid (GABA) in the culture filtrate. *Lb. brevis* strains produced 40-50 mM GABA from 59 mM monosodium glutamate

per liter of culture medium (Ayakawai et al., 1997). *Lb. brevis* IFO-12005 showed good growth in rice shochu distillery lees (kome shochu kasu) and converted free glutamic acid (10.50 mM) in shochu kasu to GABA within 2 d of stationary culture at 30°C (Yokoyama et al., 2002). *Lb. brevis* OPK-3, isolated from *kimchi*, produced 84.292 mg/L/h of GABA productivity (Park & Oh, 2007a). *Lb. brevis* BJ-20, isolated from salt-fermented Jot-gal (cod gut), possessed the highest GABA-producing ability in MRS broth with 1% monosodium glutamate (MSG). A sea tangle solution was fermented over 5 days to produce GABA using *Lb. brevis* BJ20. The fermented solution exhibited strong antioxidant activities, such as DPPH scavenging, superoxide scavenging and xanthine oxidase inhibition (Lee et al., 2010).Antioxidants are oxidizing agents which protect the oxidation of cellular oxidizable substrates by scavenging free radicals and reactive oxygen species (ROS) (Lee et al., 2010). There are antioxidants from natural sources are more desirable, and lactobacilli possess antioxidative activity and are able to decrease the risk of accumulation of ROS and degrade the superoxide anion and hydrogen peroxide (Das & Goyal, 2015).

Lc. lactis subsp. *lactis* strain B, isolated from kimchi, showed the highest GABA-producing ability (3.68 g/L) in MRS broth with 1% monosodium glutamate (MSG). The maximum GABA yield of *Lc. lactis* B was 6.41 g/L when the mixing ratio of brown rice juice, germinated soybean juice, and enzymolyzed skim milk was 33:58:9 (v:v:v) (Lu et al., 2008). Fermented soya milk (GABA soya yogurt) produced with starter *Lb. brevis* OPY-1 isolated from kimchi and substrate had the GABA concentration of 424.67 μg/g DW. *Lb. brevis* OPY-1 and germinated soybean possessed a prospect to be applied in dairy and other health products with high nutritive values and functional properties (Park & Oh, 2007b). Black raspberry juice was fermented to produce GABA using *Lb. brevis* GABA 100 at different temperatures and pHs for 15 days. It can be GABA-enriched using lactic acid bacteria (Kim et al., 2009). A new type of pickle (nukazuke) in Japan, which contained GABA and angiotensin converting enzyme inhibitory peptides, was reported to reduce blood pressure of rats (Oda et al., 2015). A probiotic *Lb. plantarum* DM5 isolated from fermented beverage Marcha of Sikkim displayed antioxidant properties and produced bioactive GABA, a major inhibitory neurotransmitter in the mammalian brain. *Lb. plantarum* DM5 has the potential to protect the oxidative damage mediated by the ROS and can act as an antioxidative probiotic (Das & Goyal, 2015).

4. PLANT-BASED PROBIOTIC PRODUCTS FOR HEALTH

Consumption of functional probiotic foods has increased over recent decades, with increasing consumer and researcher awareness of their health-promoting effects. This has prompted an interest in the development of novel functional food formulations (Peres et al., 2012). Food additives such as probiotics and prebiotics may exert positive effects on the composition of gut microbiota and are the subject of intensive research (Prado et al., 2008). Among probiotic microorganisms, *Lactobacillus* strains are the most commonly used by the food industry. Fermented dairy products are generally good food matrices for probiotics, but the consumption of these products is limited due to an increase in vegetarianism and the large number of individuals who are lactose intolerant or on cholesterol-restricted diets (Peres et al., 2012). Despite fermented dairy products still remaining the most common vectors for the

delivery of probiotics to humans, other food matrices such as fruits and vegetables offer a promising performance as sources and carriers of probiotic strains (Peres et al., 2012). Vegetables are rich sources of the biologically active compounds which have beneficial effects in the prevention of some diseases and certain types of cancer (Rakin et al., 2007). Thus, the development of non-dairy probiotic products, including food matrices based on fruit, vegetables, and cereals, has been widely studied (Table 2) (Martins et al., 2013).

Table 2. Plant-based probiotic products

Products	Probiotic or LAB strains used	References
Beet juice	*Lb. acidophilus*, *Lb. casei*, *Lb. delbrueckii*, *Lb. plantarum*	Yoon et al., (2005)
Cabbage juice	*Lb. plantarum* C3, *Lb. casei* A4, *Lb. delbrueckii* D7	Yoon et al., (2006)
Beetroot and carrot juice	*Lb. acidophilus* NCDO1748	Rakin et al., (2007)
Fruit juice (pineapple, apple, orange, pear and/or grape, passion fruit, lemon), purees (peach, strawberry, mango and kiwi),	*Lb. acidophilus* LB2, LB3 and LB45, *Lb. brevis* LB6, *Lb. rhamnosus* LB11 and LB24, *Lb. fermentum* LB32, *Lb. plantarum* LB42 and *Lb. reuteri* LB38.	Champagne and Gardner (2008)
Fermented red and yellow peppers	*Lb. plantarum* PE21, *Lb. curvatus* PE4 and *W. confusa* PE36	Di Cagno et al., (2009a)
Tomato juices	*Lb. plantarum* POM8, *W. cibaria/confusa* POM11, *P. pentosaceus* POM10, *Lb. plantarum* POM1, POM27, POM35, POM43, *Lb. brevis* POM2, *En. faecium/faecalis* POM3, *Lactobacillus* sp. POM44, *Lb. plantarum* LP54	Di Cagno et al., (2009b)
Minimally processed pineapple	*Lb. plantarum* 1OR12 *and Lb. rossiae* 2MR10	Di Cagno et al., (2010)
Arbequina naturally green olives	*Lb. pentosus* and *Lb. plantarum*, and *C. diddensiae*	Hurtado et al., (2010); Aponte et al., (2012)
Cashew apple juice	*Lb. casei* NRRL B-442	Pereira et al., (2011)
Sweet cherry puree	*P. pentosaceus* SWE5 and *Lb. plantarum* FP3	Di Cagno et al., (2011)
Pear juice	*Lb. acidophilus*	Ankolekar et al., (2012)
Fermented chestnut purees	*Lb. rhamnosus* VT1, RBM526, and RBT739; *Lb. casei Lbc491 and Lbc496;Lb. rhamnosus GG*	Blaiotta et al., (2012)
Vegetable yogurt-like beverages [Cereal (rice, barley, emmer and oat) and soy flours and concentrated red grape must]	*Lb. plantarum 6E* and *M6*	Coda et al., (2012)
Sonicated pineapple juice	*Lb. casei* NRRL B442	Costa et al., (2013)
Peanut-soy milk	*P. acidilactici* and *S. cerevisiae*	Santos et al., (2014)
Litchi juice	*Lb. casei*	Zheng et al., (2014)
Fermented soy milk beverage with added apple juice	*Lb. acidophilus* LA-5	İçier et al., (2015)
Non-dairy probiotic drink (sprouted wheat, barley, pearl millet and green gram separately with oat, stabilizer and sugar with soymilk)	*Lb. acidophilus* NCDC14	Mridula and Sharma (2015)
Clarified apple juice	*Lb. paracasei* ssp. *paracasei*	Pimentel et al., (2015)
Lactobacilli-fermented cereal beverages (oat, barley and malt)	*Lb. acidophilus* NCIMB 8821, *Lb. plantarum* NCIMB 8826, *Lb. reuteri* NCIMB 11951	Salmerón et al., (2015)

At present, some non-dairy probiotic beverages are being commercialized, and beverages such as fruit and vegetable juices would be the next food category where the healthy probiotic bacteria will make their mark (Prado et al., 2008).

LA fermentation represents the easiest and most suitable way for increasing the daily consumption of fresh-like vegetables and fruits. LAB make up a small part of the autochthonous microbiota of vegetables and fruits. The diversity of the microbiota markedly depends on the intrinsic and extrinsic parameters of the plant matrix. Notwithstanding the reliable value of spontaneous fermentation to stabilize and preserve raw vegetables and fruits, a number of factors support the use of selected starters. Two main options may be pursued for the controlled lactic acid fermentation of vegetables and fruits: the use of commercial/allochthonous starters, and the use of autochthonous starters (Di Cagno et al., 2013). *Lb. acidophilus*, *Lb. casei*, *Lb. delbrueckii*, and *Lb. plantarum* strains were used for the production of probiotic beet. All the lactic cultures were found capable of rapidly utilizing beet juice for cell synthesis and lactic acid production. The viable cell counts of these lactic acid bacteria except for *Lb. acidophilus* in the fermented beet juice still remained at 10^6-10^8 CFU/ml after 4 weeks of cold storage at 4°C (Yoon et al., 2005).The viability of *Lb. plantarum* C3, *Lb. casei* A4, and *Lb. delbrueckii* D7 grew well on cabbage as a raw material for production of probiotic cabbage juice, reaching nearly 10 x10^8 CFU/mL after 48 h of fermentation at 30°C. *Lb. casei* strain did not survive the low pH and high acidity conditions in fermented cabbage juice and lost cell viability completely after 2 weeks of cold storage at 4°C. Fermented cabbage juice could serve as a healthy beverage for vegetarians and lactose-allergic consumers (Yoon et al., 2006).

Lb. rhamnosus strains are more stable to survive in a commercial fruit drink stored at 4°C, pH 4.2, for up to 80 days than *Lb. acidophilus*, but viability was still mostly strain-dependent (Champagne & Gardner, 2008). Strains of *Lb. plantarum*, *W. cibaria*/*confusa*, *Lb. brevis*, *P. pentosaceous*, *Lactobacillus* sp., and *En .faecium*/*faecalis* fermented in unstarted tomato juice (TJ) and TJ fermented with the autochthonous strain showed marked decreases of ascorbic acid (ASC), glutathione (GSH), and total antioxidant activity (TAA) during storage. On the contrary, several TJs fermented with autochthonous strains, especially with *Lb. plantarum* strains, maintained elevated values of ASC, GSH, and TAA (Di Cagno et al., 2009b). The fermented cashew apple juice with *Lb. casei* NRRL B-442 is a good and healthy alternative functional food containing probiotics. Cashew apple juice proved to be as efficient as dairy products for *Lb. casei* growth. Viable cell counts were higher than 8.00 Log CFU/mL throughout the storage period (42 days) (Pereira et al., 2011). Fermenting pear juice with *Lb. acidophilus* has been shown to have potential for use in diet designs for managing type 2 diabetes due to the enhanced inhibitory bioactivity of the juice using relevant *in vitro* enzyme assay models. It may be used as a novel method for delivering probiotic lactic acid bacteria in lactose intolerant people in a fermented fruit-based system with multiple potential health benefits (Ankolekar et al., 2012).

Lb. casei NRRL B442 using the sonicated pineapple juice as substrate for producing a probiotic beverage at 31°C and pH 5.8,after 42 days of storage under refrigeration (4°C) exhibited the viability as 6.03 Log CFU/mL in the non-sweetened sample and 4.77 Log CFU/mL in the sweetened sample. Sonicated pineapple juice was a suitable substrate for *Lb. casei* NRRL B442 cultivation and for the development of an alternative non-dairy probiotic beverage (Costa et al., 2013). The use of litchi juice treated by high hydrostatic pressure

(HHP) as substrates for producing a probiotic beverage by *Lb. casei*has been reported. Comparison of quality attributes and product stability of fermented heat- and HHP-treated litchi juice by *Lb. casei* showed that both viability counts of *Lb. casei* were more 8.0 log CFU/mL in heat- and HHP-treated litchi juice after 4 weeks of storage at 4°C. This study is relevant to fermentation of litchi juice by probiotic *Lb. casei* (Zheng et al., 2014). Oligofructose was stable to storage in apple juice and could be used as a sugar substitute. Oligofructose added products had similar physicochemical characteristics and acceptability to products with sucrose, and enhanced the probiotic *Lb. paracasei* ssp. *paracasei* survival during storage (Pimentel et al., 2015a). It was possible to develop a synbiotic apple juice that showed a similar sensory profile and acceptance to that of the sucrose-added juice by adding *Lb. paracasei* as a probiotic culture and oligofructose as a sugar substitute and prebiotic (Pimentel et al., 2015b). *Lb.rhamnosus* and *Lb. casei* strains as promising probiotic candidates are able to grow and survive in chestnut puree at a population level higher than 8 log10 CFU/mL along 40 days of storage at 4°C for the production of a new food, lactose-free and with reduced fat content (Blaiotta et al., 2012). The probiotic strain, *Lb. paracasei* IMPC2.1, successfully colonized the olive surface dominating the natural LAB population and decreasing the pH of brines to ≤ 5.0 after 30 days until the end of fermentation. The human strain *Lb. paracasei* IMPC2.1 can be considered an example of a strain used in the dual role of starter and probiotic culture (De Bellis et al., 2010).

Cereal (rice, barley, emmer, and oat), soy flours and concentrated red grape have been used for making vegetable yogurt-like beverages (VYLB). Two selected strains of *Lb. plantarum* have been used for lactic acid fermentation, according to a process which included the flour gelatinization. All VLYB had values of pH lower than 4.0, and both selected starters remained viable at ca. 8.4 log CFU/g throughout storage. Beverages made with a mixture of rice and barley or emmer flours seemed to possess the best combination of textural, nutritional, and sensory properties (Coda et al., 2012). Cereals have been extensively investigated to develop new probiotic foods (Rivera-Espinoza & Gallardo-Navarro, 2010). Shalgam juice, hardaliye, and boza are the most well-known traditional Turkish fermented non-alcoholic beverages which include vegetables, fruits, and cereals. *Lb. plantarum*, *Lb. brevis* and *Lb. paracasei* subsp. *paracasei* strains in shalgam fermentation and *Lb. paracasei* subsp. *paracasei* and *Lb. casei* subsp. *pseudoplantarum* strains in hardaliye fermentation are predominant. Boza is prepared by using a mixture of maize, wheat, and rice (or flours thereof) and water. Generally, pre-produced boza or sourdough/yoghurt are used as starter cultures, rich in *Lactobacillus* spp. and yeasts (Altay et al., 2013).

The fermented soy milk beverage with added apple juice was produced by using *Lb. acidophilus*. The beverage was found to be a pseudoplastic fluid with a shear thinning nature. *Lb. acidophilus* had good growth and viability in the beverage with or without apple juice. *Lb. acidophilus* counts were in the range of 8.73-9.11 log cfu/g after storage at 4°C for 21 days. This beverage could be a good vehicle for delivering probiotic microorganisms to consumers, and has potential to become a commercial product (İçier et al., 2015). The physicochemical characteristics and acceptance of lactobacilli-fermented cereal beverages individually inoculated with human derived *Lb. acidophilus* NCIMB 8821, *Lb. plantarum* NCIMB 8826, and *Lb. reuteri* NCIMB 11951 were evaluated after 10 h of incubation at 3 °C. The beverage formulated with *Lb. plantarum* and malt substrate exhibited greater acceptance, and contained the highest concentration of acetaldehyde (Salmerón et al., 2015). Non-dairy probiotic drink (PD) was developed utilizing sprouted wheat, barley, pearl millet and green

gram separately with oat, stabilizer and sugar using *Lb. acidophilus* NCDC14; with soymilk and distilled water as liquid portion. Probiotic count ranged from 9.10 to 11.06, 10.36 to 11.17, 10.36 to 11.51 and 10.36 to 11.32 log cfu/mL in wheat, barley, pearl millet, and green gram-based PD samples, respectively, which increased with increasing level of grain flour (Mridula & Sharma, 2015).

5. ACETIC ACID (VINEGAR) AND HEALTH BENEFITS

Acetic acid bacteria (AAB) are distributed in various natural sources, such as alcoholic juices, hard cider, wine or beer, fruits, flowers, and fermented rice products. While they play a positive role in the production of certain foods and beverages, they can also spoil other foods and beverages, such as wine, beer, soft drinks, and fruits (Sengun & Karabiyikli, 2011; Tanasupawat et al., 2011). In food fermentations, *Acetobacter* strains are important in the production of vinegar (acetic acid) from fruit juices and alcohols by causing the oxidation of alcohol to acetic acid (Östman et al., 2005). Vinegar had been used to fight infections and other acute conditions since the time of Hippocrates, but recent research suggests that vinegar ingestion favorably influences biomarkers for heart disease, cancer, and diabetes (Johnston, 2006). Rice vinegar has been shown to have an antimicrobial effect on the survival of *E. coli* O157:H7 in inoculated lettuce (10^4 and $10^7 CFUg^{-1}$). The treatment of inoculated lettuce ($10^7 CFUg^{-1}$) with commercial vinegar containing 5% acetic acid (pH 3.0) for 5 min would reduce 3 logs population at 25°C (Chang & Fang, 2007). The maximum observed log reduction of *L. monocytogenes* was 2.15 ± 0.04 for balsamic vinegar, 50% (v/v), 1.18±0.06 for white wine vinegar, 50% (v/v) and 1.13 ± 0.06 for acetic acid, 50% (v/v). Washing with water only reduces 0.05 ± 0.04 log CFU/mL of *L. monocytogenes* numbers. Balsamic vinegar washings may be a promising method for reducing other foodborne pathogens present in produce or other foods, both in the home and in retail environments (Ramos et al., 2014).

The antioxidant activities of grape juice and wine vinegar have been attributed to their different phenolic contents and compositions and other non-phenolic antioxidants present in the samples, and are good dietary sources of antioxidants (Dávalos et al., 2005). Melanoidins, the brown polymers formed through Maillard reaction during the vinegar process, are one of the major high-molecular-weight fractions of the vinegar production process which may have health promotion activity (Xu et al., 2007). Daily acetic acid ingestion, in the form of vinegar, dill pickle, or commercial vinegar pill, has been shown to influence hemoglobin A1c in diabetic patients. Regular vinegar use modestly improved glycemic control (Johnston et al., 2009). Although the exact mechanism of vinegar action is not known, several possibilities have been proposed, including suppression of disaccharidase activity, delayed gastric emptying, enhanced glucose uptake in the periphery and conversion to glycogen, and increased satiety (Salbe et al., 2009). The traditional balsamic vinegar TBV melanoidins may have a role in oxidative damage prevention. Fe2+-chelating and heme-binding activities, as well as the mechanisms of antioxidant activity of TBV Melanoidins, have also been compared with coffee, barley coffee, and dark beer melanoidins (Verzelloni et al., 2010). Studies on the effect of apple vinegar and balsamic vinegar on hydatid cyst showed that vinegar has scolicidal activity, and that its activity is related to the concentration and exposure time (Hajihossein et al., 2015). Aqueous extract of nipa palm vinegar (NPV) possesses

antihyperglycemic activities comparable to metformin, while ethyl acetate extract precipitated significant antioxidant effects attributable to its high phenolic content. The antioxidant compounds of NPV do not contribute much toward the overall observed antidiabetic effect (Yusoff et al., 2015). A beverage made of wine vinegar and grape juice (Budo-no-megumi™) has recently been developed for people wishing to obtain an effective amount of both polyphenols and vinegar for their health. This new beverage may be useful for people who are worried about palpitation and/or hypertension (Sugiyama, 2003). The addition of vinegar or peanut products to a high-glycemic load meal significantly reduced postprandial glycemia (Johnston & Buller, 2005).

The total phenols index (TPI) and antioxidant activity values of persimmon vinegars were always higher than those obtained from white- and red-wine vinegars, indicating that persimmon vinegar is a competitive product in the market (Ubeda et al., 2011). From twenty traditional home-made vinegars in Turkey, the most abundant compounds in some were a-terpineol and ethyl acetate, with phenethyl alcohol another common compound detected in the vinegars. Traditional Turkish vinegars exhibited very distinct properties, their antioxidant and antimicrobial properties independent from the raw materials used (Ozturk et al., 2015). In vinegar fermentation with *Pinus morrisonicola* Hayata powder (50 mg/mL), the cultured saccharified broth was produced at day 1, and wine with an ethanol content of 8.23% was produced at day 12. After 11 days of fermentation, vinegar with an acidity of 5.21 g/100 mL was obtained. The alcoholic and vinegar products were developed to provide beneficial health effects (Chen et al., 2011). Black vinegar (BV) contains abundant essential and hydrophobic amino acids and polyphenolic contents, especially catechins and chlorogenic acid. The component profiles of BV contributed to the lipid lowering and antioxidant effects on HFCD-fed hamsters (Chou et al., 2015).

Recent studies on animals have suggested that vinegar consumption may confer an antiobesity effect through the activation of the AMP-activated protein kinase (AMPK) signaling pathway. Pomegranate vinegar (PV) beverage tended to suppress downstream gene expression, such as that of sterol regulatory element binding protein-1c and acetyl coenzyme carboxylase, in adipose tissue. PV is an excellent AMP-activated protein kinase (AMPK) activator, and may exert beneficial effects on adiposity (Park et al., 2014). Tomato vinegar (TV) prevented obesity by suppressing visceral fat and lipid accumulation in adipocyte and obese rats, and TV can be used as an anti-obesity therapeutic agent or functional food (Lee et al., 2013). Pear vinegar (PV) may act as a new functional food for inflammatory bowel disease patients (Wakuda et al., 2013). A simple addition of vinegar to meals has antiglycemic effects in adults at risk of type 2 diabetes, possibly related to carbohydrate maldigestion (Johnston et al., 2013).

In conclusion, the nutritional quality of fermented fruits and vegetables can be enhanced by fermentation. Lactic acid fermentation increases their shelf-life, enhances nutritive value and flavors, and reduces toxicity. Fermented fruits and vegetables can be used as a potential source of probiotics as they harbor several lactic acid bacteria. Probiotic LAB strains improve digestive functions, enhance the immune system, reduce the risk of colorectal cancer, and control serum cholesterol levels. LAB play a defining role in the preservation and microbial safety of fermented foods. The development of non-dairy probiotic products, including fruit and vegetable juices and cereals, contributes to improving the nutritive quality the products. Consumption of these products, as well as pickles that contain GABA, has been associated

with good health. In addition, ingestion of traditional vinegars has a positive influence on health due to their antioxidant and antimicrobial characteristics.

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Chapter 4

HEALTHY PROBIOTIC: SURVIVAL IN VARIETY OF PRODUCTS AND SENSORY PROPERTIES

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ABSTRACT

Recently, probiotic products were developed and rapidly increase in the functional food market. Commercial probiotic products include primarily dairy products (fermented milks, yoghurts, ice cream, cheeses), non-dairy products (beverages, breakfast cereals, fermented meats, dry-foods) and dietary supplements. The application of probiotic bacteria in foods for promoting health benefits is based on the concept that the maintenance of a healthy gut microflora provides protection against gastrointestinal disorders including infections and inflammatory syndromes of the bowel. The viable cell concentration of the probiotic bacteria in a food product or nutraceutical formulation should be as high as possible (at least 10^6-10^7 CFU per gram of product at the time of consumption) because a significant number of bacterial cells die during storage and passage through the stomach and the small intestine. Therefore, identifying the factors influencing probiotic survival in food and developing ways to enhance probiotic survival during storage is an important area of research with considerable impact for the food industry.

It is possible to make probiotic cells more robust to external conditions. From an industrial perspective, the application of encapsulation has helped to increase the incorporation of probiotics in various foods. However, in general, before such a product reaches the market organoleptic assessment of the product needs to be carried out to

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ensure consumer acceptability. The sensory quality of the product is a challenge for probiotic-containing products.

1. INTRODUCTION

Foods that have been fortified with ingredients that can impart health benefits are referred to as functional foods. Due to their aim being the promotion of health and well-being that is more than just basic nutrition, functional foods have received a lot of attention as consumers have become more aware of the effect of their diet on their health. This type of product contains ingredients that are functional, which might be fatty acids, phenolic, bioactive peptides, prebiotic carbohydrates and probiotic microorganism, etc. An estimate has been made that of the complete functional food market, probiotic foods account for 60 – 70% (Soccol et. al. 2012, Tripathi and Giri 2014).

Japan is the place that probiotics originated from and the market has reached maturity with only moderate growth, while in Europe, particularly for products related to gastrointestinal health, the market is large and accounts for 45% of the total market. The 2013 value for the global probiotic market was 58,700 million USD with an estimate of 96,046 million USD by 2020, by Global Industry Analysis (GIA). Some of the main companies that produce probiotics are Probi AB, BioGaia AB, Chr. Hansen A/S, Danisco, Nestlé S.A., Yakult Honsha Co. Ltd. and Lifeway Foods Inc. The global probiotic market covers applications in dietary supplement, food and beverages and animal feed.

A definition of probiotics is "Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO 2002). The use of probiotic bacteria in food acts to promote health via the maintenance of the gut microflora so that it is healthy and can provide protection against inflammation of the bowel and other gastrointestinal disorders and infections (Sheehan et al. 2007). The two most commonly used and widely investigated genera for beneficial effects are *Lactobacillus* and *Bifidobacterium* (Islam et al. 2010).

Probiotics are delivered in two main formats, either as a dried nutraceutical product or incorporated in various foods, such as fermented dairy products.

The viable cell concentration of the probiotic bacteria in a food product or nutraceutical formulation should be as high as possible because a significant number of bacterial cells die during storage and passage through the stomach and the small intestine (Champagne et al. 2005, Gueimonde et al. 2004, Shah 2000, Vinderola et al. 2000).

2. DAIRY AND NON-DAIRY PRODUCTS

Dairy-based probiotic products constitute the majority of commercial products. In the production of many products, such as fermented milks (Alamprese et al. 2002) and other dairy products, such as yoghurt (Kailasapathy 2006), ice cream (Homayouni et al. 2008), cheese (Fritzen-Freire et al. 2010), as well as baby foods, breakfast cereals, confectionary products, oat-based desserts, soya-based products, fermented fruit juices, vegetables, cereals

and fermented meats (Champagne et al. 2005; Tripathi and Giri 2014),or mixture thereof are being utilized for delivering these beneficial microorganisms.

The development of non-dairy probiotic products is a challenge to the food industry in its effort to utilize the abundant natural resources by producing high quality functional products and are also ideal choice for consumers who are interested in low cholesterol foods, or suffer from lactose intolerance (Prado et al. 2008, Granato et al. 2010).

There have been many studies about the creation of non-dairy products. For example, about:

- *Fermented almond milk*. This uses the probiotic bacteria *Lactobacillus reuteri* and *Streptococcus thermophiles*, which had survival rates of 51% after vitro digestion. This was possibly due to the presence of inulin, which could enhance the beneficial health effects of the product, and so add value (Bernat et al. 2014).
- *Peanut-soy milk*. This had six different lactic acid bacteria (LAB) being inoculated, and during fermentation they reached concentrations of 8.3 log CFU/ml. A pH of 4.3 required a time of 12 hours for it to be reached. Once fermentation was finished, a test was performed to show that the ethanol content was 0.03% (v/v) or less, thereby conforming that the product was non-alcoholic (Santos et al. 2014).
- *Fermented oat milk*. In this the selected formulation allowed the starters *L. reuteri* and *S. thermophiles*to survive at levels greater than10^7CFU/ mL. Therefore, this is a functional food that could be maintained for the full period of the 28 day test. The finished product also contained beta-glucans that had a good effect on the viscosity (Bernatet al. 2015).
- *Raspberry powder.* The raspberry powder was obtained by spray drying raspberry juice and then *Lactobacillus rhamnosus NRRL B-4495* and *Lactobacillus acidophilus NRRL B-442* encapsulated it with the additive maltodextrin (Anekella and Orsat 2014).
- *Fruit powders*. The powders of cranberry, blackcurrant, strawberry and pomegranate were mixed with *Lactobacillus plantarum* that had been freeze dried.
  The water activity was linked to the survivability. For cranberry there as a 1-1.5 log increase in the cell survival due to the presence of inulin and gum arabic. It was shown that powdered instant juice was an excellent carrier of probiotic cells, and so represents a viable alternative to fruit juices that are highly acidic (Nualkaekul et al. 2012b).

3. DIETARY SUPPLEMENTS

When compared to the market for dairy based probiotics, the market for dietary supplement based probiotics is more extensive. There are a wide range of different product formats and contents, for example, tablets, capsules, powders and liquids. If the probiotic is in a dried form that has been prepared and stored correctly it is possible for it to still be alive when reaching the intestine. This type of product contains a wide range of genera and spices, which include *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Bacillus* and *Enterococcus* (Table 1).

Table 1. Examples of commercial probiotic supplements

Probiotic	Company	CFU (bn)	Species
Kyo-Dophilus	Kyolic	1.5	*L. gasseri, B. bifidum, B. longum*
Life Start 2	Natren	2	*B. infantis*
L. Acidophilus	Natural Healthy Concepts	150	*L. acidophilus*
D.L.ULTRA-DOPHILUS	Douglas Laboratories	4	*L acidophilus*
Daily Probiotic	Ganaden Sustenex	2	*Bacillus coagulans*
Probiotic Acidophilus	Good 'N Natural	6	*L acidophilus, B bifidum, L salivarius, L bulgaricus*
Super 10 Probiotic Complex	GNC	10	*L salivarius, L rhamnosus, B bifidum, B longum, B infantis, L acidophilus, B lactis*
Complete Probiotics	Dr. Mercola	70	*L. casei, L. plantarum, L. salivarius, L. rhamnosus, L. brevis, B. lactis, B. longum, B. bifidum, S. thermophilus*
Propolis Plus	Essential Formulas	21.6	*B. breve, B. infantis, B. longum, Enterococcus faecalis, L. acidophilus, L. brevis, L. bulgaricus, L casei, L. fermentum, L. helveticus, L. plantarum, Streptococcus thermophilus*
Nexabiotic	Bioprosper Labs	30	*Saccharomyces boulardi, S. thermophilus, L. fermentum, L. acidophilus, L. brevis, L. bulgaricus, L .casei, L. helveticus, L. paracasei, L. pantarum, L. rhamnosus, L. salivarius, L. lactis, Bacillus coagulans, B. bifidum, B. breve, B. infantis, B. lactis, B. longum, Pediococcus acidilacti*

4. HEALTH BENEFITS OF PROBIOTICS

When consuming probiotics, a range of positive effects have been identified with much work determining the possible rolls of probiotics in enhancing responses to lactose intolerance (Lye et al. 2009), lowering cholesterol level (Ooi and Liong 2010), stimulation of immune system (Pozo-Rubio et al. 2011), treatment of irritable bowel syndrome (Rogers, and Mousa 2012), prevention of intestinal and vaginal infections (Zuccotti et al. 2008), inhibition of pathogen colonization (Dhanani et al. 2011), prevention of food allergies (Khani et al. 2012), treatment of constipation (Riezzo et al. 2012) and prevention of diarrhoea due to pathogenic bacteria and viruses (Malaguarnera et al. 2012). However, it is important to

consider that for the cases identified above, the evidence related to the beneficial impact of the probiotics varies significantly. The gut microflora being modulated or the gut barrier function being altered as well as the immune system being regulated have all be suggested as possible explanations for the beneficial effects. Pathogenic bacteria can be inhibited by the probiotics as the can production of antimicrobial compounds, for example, carbon dioxide, hydrogen peroxide, acetic or lactic acid and a range of bacteriocins (Fooks et al., 1999; Ozdogan et al. 2012), or via competition for the gut wall adhesion sites or nutrients that the pathogenic bacteria require (Tuohy et al. 2003, Liong 2011).

5. DOSAGE AND ADMINISTRATION OF PROBIOTICS

For human consumption, there should still be at least 10^6 -10^7 CFU per gram of probiotic product when the expiration date is reached for them to be effective (Ouwehand et al. 1999, Homayouni et al. 2008; Ding and Shah 2008, Rauch and Lynch 2012). These numbers have been suggested because during passage through the stomach and the small intestine upon ingestion, a significant number of bacterial cells die (Shah 2000). To enable products to be effectively commercialised it is vital that the probiotics are able to survive the processing and storage.

It was shown by Astashkina et al., (2014), the level of CFUs shown on the commercial products Imunele, Dannon and Pomogayka that contained live cultures of the genera *Lactobacillus* and *Bifidobacterium* corresponded to the levels of 10^7 CFU/mL published on the label. In pasteurized products the survival of the test strains was not better than 10%.

6. FACTORS INFLUENCING PROBIOTIC SURVIVAL

In food products the viability of probiotics is mainly due to the species/strain used (Kailasapathy et al. 2008), probiotic production process (Saarela et al. 2009), composition of the food product (Shah 2000), storage temperature and duration (Saarela, et al. 2006a), oxygen levels (particularly for *Bifidobacterium* species) (Shah, 2000) and container type (Champagne et al. 2008). These aspects will be discussed in more detail in the followings sections.

6.1. Genera / Species / Strains

The first stage in the development of a probiotic food product is to select a suitable strain. Each probiotic type has significantly different technical properties and levels of robustness (Alamprese et al. 2002, Champagne and Gardner 2008). Generally, *Lactobacillus* species are more robust than those of *Bifidobacterium* (Shah 2000, Annan et al. 2008, Nualkaekul et al. 2011a, Nualkaekul et al. 2011b); however, the majority of work on different levels of robustness between species has been conducted in *Lactobacilli*. In cheese products it has been shown that *L. casei* and *L. rhamnosus* survive better than *L. acidophilus* at low temperatures (Champagne et al. 2005). While in fruit juice, good survival was shown by *L. rhamnosus* for

80 days at 4°C, but the lowest stability was shown by two strains of *L. acidophilus* (Champagne and Gardner 2008). In addition, Sadaghdar et al. (2012) showed that *L. casei*, out of several *Lactobacillus* strains, showed the greatest survival while being stored at 5°C for 21 days in flavored fermented milk products and the lowest survival was recorded in *L. acidophilus*.

6.2. Probiotic Production Process

A fermentation process is conducted in which bioreactors are used to grow cells that are harvested before being re-suspended in a medium that is a cryo-protectant before being freeze dried to produce the probiotics. Very little research has been undertaken to study this process, but still it is believed that both upstream and downstream processing will have effects on the probiotic while it is being added to the food products and then stored. The parameters that are most likely to affect the fermentation are the pH, composition of media, time for growth and gas atmosphere as they have the potential to influence the cell physiology and therefore the stability of the cells (Saarela et al. 2009). It was reported by Palmfeldt and Hahn-Hagerdal (2000) that the viability of *L. reuteri* during the freeze drying was increased when a pH of 5 was used as opposed to a pH of 6. Cell growth at pH 5 for 2.5 hours in the stationary phase gave a survival rate of 80%, which was the highest. In addition, the same authors also determined that if the cells were starved they were protected from different stresses. Further work determined that stationary phase cells rather than exponential phase cells had the best tolerances to stressful conditions (Kim et al. 2001, Saarela et al. 2009). Despite no working being done, it is possible to predict that cells that are more technologically robust would be able to survive the storage process better.

It is possible to add either fresh, dried or frozen probiotic concentrated cultures to food products (Saarela et al. 2006a). Due to them being easier to handle, freeze dried cells are generally preferred to frozen cells when making dry bacterial powders (Saarela et al. 2006a). Only minimal work has been published about the effect that the dying process has on the probiotics' survival in the food product (Gardiner et al. 2000). Only a single study was found related to this, in which Saarela et al. (2006b) showed that when added to apple juice there was better survival of fresh *L. rhamnosus* cells mixed with oats (20% β-glucan in oat flour) than the freeze dried cells. It was suggested by the authors that the acidic product damaged the freeze dried cells as they could have been already injured during the freezing process. As there is a limited amount of information available on this aspect, more work is needed that will hopefully enhance the production techniques.

6.3. Composition of Food Products

The food product composition also affects the probiotic bacteria's viability via the pH; phenolic, protein, dietary fiber, sugar or organic acid contents; and water activity (Shah 2000, Champagne and Gardner 2008). Differences in these parameters are probably the cause of the different survival rates observed. For example, the survival in apple juice was lower than in chocolate-coated breakfast cereal for *L. rhamnosus* (Saarela et al. 2006b), while the survival

rate was also lower in fruit juice compared to milk for *B. animalis* subsp. *lactis* E-2010 (Bb-12) (Saarela et al. 2006a).

It appears that the most important factors related to probiotic survival are low pH values and the content of the organic acid (Champagne et al. 2005, Saarela et al. 2009). The intracellular pH is increased by acids as they dissociate once entering the cytoplasm and inhibit the metabolic processes, thereby damaging the bacterial cells. To combat this and maintain the correct intracellular pH the cells have to expend energy (Shabala et al. 2006, Saarela et al. 2009). The energy that is used to increase cell survival generally comes from fermentable sugars (glucose) (Dave and Shah 1998, Sandholm et al. 2002). When this was considered using a model system, it was shown that *Lactobacillus* strains had their short-term survival increased by the presence of fermentable sugars when the pH was about 2. However, as *L. rhamnosus* GG (Charalampopoulos et al. 2003) was able to survive better than *L. rhamnosus* E800 (Corcoran et al. 2005) the affect could be strain specific. When the nitrogen source was whey protein (often added to yoghurts) the storage survival was better, possibly due to peptides and amino acids being provided (Dave and Shah 1998, Shah 2000).

Fiber can be eaten but is not absorbed or digested in the small intestine, but rather it is fermented in the large intestine (Saarela et al. 2006b). The survival of probiotic cells during processing and storage has been increased due to them being physically be immobilized on the fiber (Saarela et al. 2006b, do Espirito Santo et al. 2012). When *B. animalis* subsp. *lactis* was added to yoghurt with oat β-glucan fiber, the survival after long term cold storage was enhanced (Vasiljevic et al. 2007). In addition, *L. rhamnosus* was protected during the storage of apple juice when oat flour with 20% β-glucan was added (Saarela et al. 2006b). Other work has suggested that *Lactobacilli* and *Bifidobacteria* were protected in the large intestine by prebiotic compounds (Vernazza et al. 2006), which are 'non-digestible food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract' (Gibson et al. 2004).

Examples of prebiotic compounds are fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), transgalacto-oligosaccharides (TOS), isomalto-oligosaccharides, xylo-oligosaccharides and soybean-oligosaccharides (Fooks and Gibson, 2002; Krasaekoopt et al. 2003). As an example, when low-fat ice-cream was stored for 90 days at -18^{o}C, is that the viability of *L. acidophilus* and *B. animalis* was improved by oligofructose (Akalin and Erisir 2008). Hernandez-Hernandez et al. (2012) stated that the growth and survival of *Lactobacillus* strains was improved in the gastrointestinal tract by GOS and lactulose. While frozen yogurt with 2% inulin caused the viability of *L. acidophilus* and *B. lactis* to significantly improve (Rezaei et al. 2014). Bedani et al., (2014) showed that the pulps of mango and guava did not affect the viability of *L. acidophilus* and *B. animalis* Bb-12 in soy yoghurt, but did result in a significantly decreased survival rate under a simulation of gastrointestinal stress.

Especially in dried formulations, for example, infant formulas, the water activity is another factor that is important for the stability (Mattila-Sandholm et al. 2002, Abe et al. 2009, Rokka and Rantamaki 2010).

It has been stated that if the water activity is greater than 0.25 then there will be a negative effect on the cell survival (Teixeira et al. 1995). When freeze dried *L. acidophilus* was stored at 20^{o}C for 10 weeks the survival decreased as the water activity was changed from 0.11 to 0.23 before reaching 0.43, which caused a three log CFU/g loss (Kurtmann et al. 2009).

6.4. Storage Temperature and Time

Products, such as yoghurt and fermented milk, as well as the majority of the other acidic probiotic products, are stored in refrigerators, and this improves the probiotics' viability (Krasaekoopt et al. 2003). Generally, as the storage temperature gets lower the probiotics' survival increases, and therefore, 4^{o}C is the most commonly used storage temperature (Saarela et al. 2006a, Champagne et al. 2008). The lower temperature causes the diffusion rate of acid into the cells to be lower, which prevents damage (Salminen and Wright 1998). In addition to the temperature, the storage time also influences the survival of the probiotics: a longer storage time results in a lower survival rate (Lankaputha et al. 1996, Cruz et al. 2007). The most important consideration is that the probiotic remains at a suitably high rate (>106 CFU/g or mL) throughout the period that the product will be stored for (four or five weeks for yoghurt or six weeks for fresh juice) as it is the organoleptic properties and safety requirements that dictate the applicable storage time.

6.5. Oxygen Levels

Oxygen has an effect on probiotic bacteria, but the specific level is strain dependent (Talwalkar and Kailasapathy 2003, Simpson et al. 2005, Champagne, et al. 2008). Generally, *Lactobacilli* species are less sensitive than *Bifidobacterium* ones (Talwalkar and Kallasapathy 2003, Burgain et al. 2011). It was shown by Champagne et al. (2008) that *L. rhamnosus* R0011 was not affected by oxygen in apple juice under aerobic or anaerobic conditions (open or closed bottles). Despite them being anaerobic, the sensitivity of *Bifidobacteria* to oxygen is strain dependent (Biavatiet al. 2000). Oxygen does not damage the cells, but it is partially reduced to water via metabolic processes, which can cause reactive oxygen species, such as superoxide anion radical (O2-), the hydroxyl radical (OH•) and hydrogen peroxide (H2O2), to be formed. The oxidizing potential of these intermediates is high and this causes the toxicity in the cells due to oxygen (Miyoshi, et al. 2003). Processing operations, for example, homogenization or blending, affect the dissolved oxygen in the fruit juice as well as the packaging and the ability of oxygen to diffuse through it, the headspace and size of the product. Some products, such as yoghurt, have antioxidants added sometimes to reduce the oxygen's effect and enhance the probiotic's survival as they scavenge any oxygen present. Adding ascorbic acid or L-cysteine are examples of this process (Shah 2000, Champagne et al. 2005).

6.6. Type of Container

Another factor that affects the cell survival is the container or packing material. To prevent cell death the oxygen levels should be as low as possible during the storage time (Cruz et al. 2007). A study by Dave and Shah (1997) investigated the effect of storage in either glass or high-density polyethylene containers on the probiotic bacteria in yoghurt for 35 days. As the glass container had significantly less dissolved oxygen, the survival rate was much better. Jayamanne and Adams (2004) also found that glass containers were the best when they looked at *Bifidobacterium* strains in fermented buffalo milk (meekiri) that was

stored for four days at 29°C, with plastic containers being the next best and clay pots the worst. The clay pots allowed the diffusion of oxygen, which resulted in the low number of cells.

7. MEASURING THE VIABILITY OF PROBIOTICS

Breeuwer and Abee (2000) define live cells as those that have an intact membrane, are able to perform DNA transcription and RNA translation, generate energy for maintenance of metabolism and biosynthesis of components, and are able to multiply. Methods such as plate counts, which are culture-based, are used to determine the viable cells. There are a range of species specific media available to enable the counting of cells, such as deMan Rogosa Sharpe (MRS) agar for *Lactobacilli*, M17 for *Lactococci* and Trypticase-Phytone-Yeast Extract (TPY) for *Bifidobacteria* (Shah 2000, Rosaria et al. 2007). Despite this, there are still problems with the counting of viable cells, for example, when they are dormant or sub-lethally injured, no colonies will be formed by *Biffidobacterium* strains (Lahtinen et al. 2007). Under these conditions, the microorganisms do not replicate when exposed to conventional culture conditions but still have metabolic activity and are alive, and they are generally referred to as viable but non-culturable (VBNC) (Breeuwer and Abee 2000, Lahtinen 2007). The plate count method also has other disadvantages, such as long incubation times required (up to three days), some bacterial strains do not have any information about suitable conditions, neighboring cells can inhibit each other that leads to the viable cells being underestimated and the growth media's composition could affect the growth of the cells (Breeuwer and Abee 2000, Bunthof and Abee 2002, Gunasekera et al. 2000, Lahtinen 2007).

Fluorescent probes can be used instead of the plate cell count method. These probes can be used with flow cytometry or fluorescence microscopy for the enumeration of cells that are viable, damaged, metabolically active or dead (Gunasekera et al. 2000, Maukonen et al. 2006). This can be achieved due to the different states showing different cell physiologies. Probes, such as for pump activity, enzyme activity, membrane potential and membrane integrity, have been used to determine the cell's physiology (Breeuwer and Abee 2000, Joux and Lebaron 2000). The dye exclusion based LIVE/DEAD® BacLight TM assay (Invitrogen 2004) method is the most common way of determining the membrane integrity of cells. In this a mixture of the dyes, SYTO 9 and propidium iodine (PI), are used. Live cells are stained by the SYTO 9 green-fluorescent nucleic acid dye as it can diffuse passively through the intact cytoplasmic bacterial membranes. In contrast, only bacteria with damaged membranes are stained with the red-fluorescent nucleic acid stain PI. While dead cells show a red staining color as both stains can pass the damaged cell membranes (Invitrogen 2004, Lahtinen 2007). Assay kits for use with either a flow cytometer or fluorescence microscope have been used by many workers to determine the number of probiotic microorganisms in food products, such as fermented and non-fermented (Lahtinen et al. 2007), non-dairy drinks and pharmaceutical products (Maukonen et al. 2006).

The cationic Rhodamine123 (Rh123) and carbocyanines as well as the anionic bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC4(3)] dyes have been used to measure the cells' membrane potentials. To achieve this, the voltage between the outside and inside of the cell is determined (Joux and Lebaron 2000). Green fluorescence is emitted when Rh123,

DiOC6(3) and DiBAC4(3) are excited at 488 nm. Amor et al. (2002) used flow cytometry to determine the membrane integrity and potential as well as the intracellular enzymatic activity of *B. lactis* and *B. adolescentis* when in the presence of large amounts of bile salts with the use of [DiBAC4(3)].

It is possible to use the level of enzyme activity as a marker to determine the bacterial cells' metabolic activity. To achieve this, the nonpolar carboxyfluorescein diacetate (cFDA) can diffuse through cell membranes so it can be used as a probe. The cells are then stained green by the fluorescent anion carboxyfluorescein (cF) that is released by the hydrolyzed cFDA being esterased (Lahtinen 2007). Sunny-Roberts et al. (2007) showed the intracellular enzymatic activities of *L. rhamnosus* GG using flow cytometry to show the osmotic stress (high sugar concentrations) via cFDA.

A common disadvantage when using a fluorescent method with a microscopy is that often the colors are not clear, which makes the counting subjective and operator dependent. Then there are also general problems with all fluorescent techniques as they are often labor intensive, have low fluorescence signals, the cells need a pre-treatment step and, sometimes, as influenced by the dye, can be expensive.

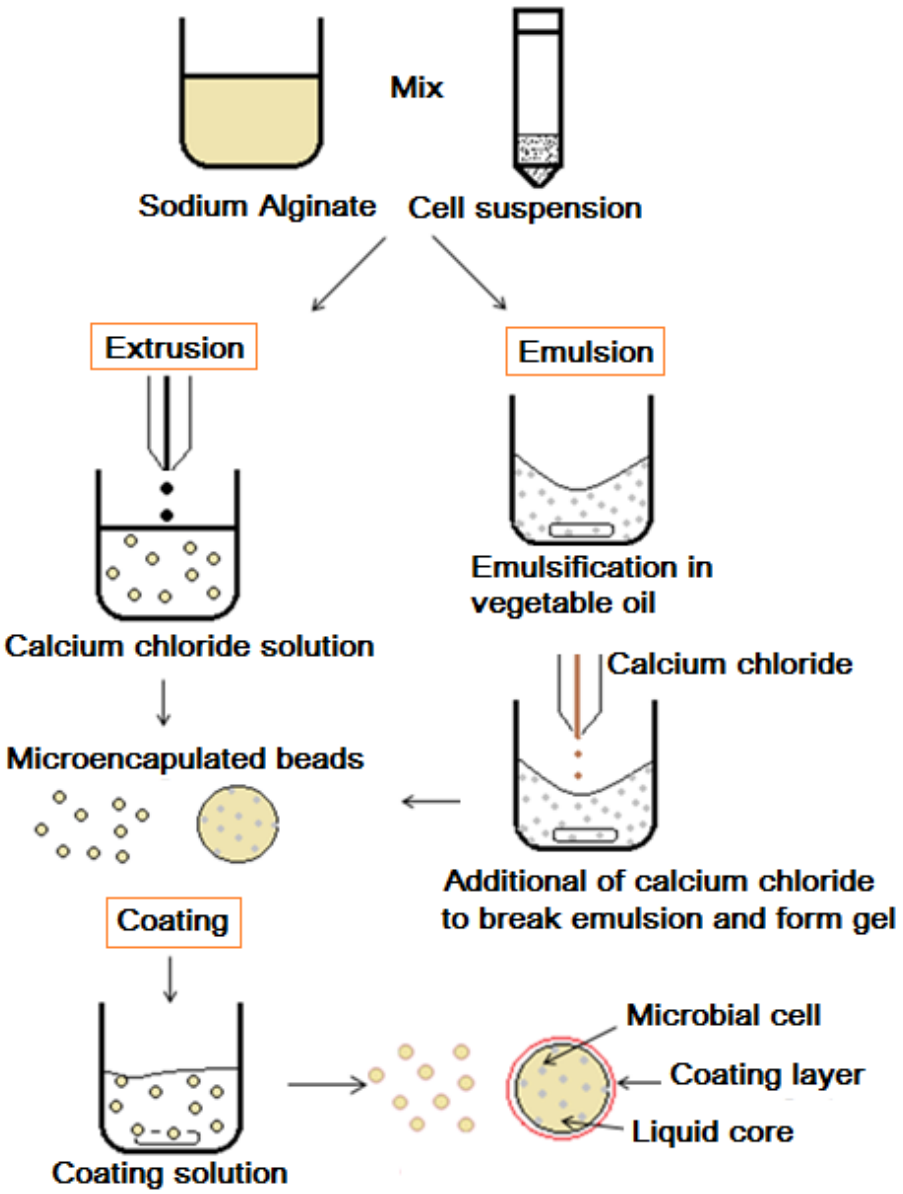
8. ENCAPSULATION METHODS FOR IMPROVING PROBIOTIC VIABILITY

During encapsulation cells are covered by an encapsulating polymer that enhances the cells' survival due to preventing injury or loss (Krasaekoopt et al. 2003, Islam et al. 2010). Probiotics can be protected from dangerous environments, for example, mild heat treatment when being processed or high acid levels in the product (Mortazavian et al. 2007). This means that the cells are less likely to be injured or killed during the processing or storage (Ding and Shah 2007). Due to their properties of being low cost, biocompatible, bioavailable, easy to handle and non-toxic, alginates are commonly used to encapsulate microbial samples (Islam et al. 2010) to enable drug delivery and tissue engineering.

Pectin, a natural polymer, has recently been used for drug delivery in the pharmaceutical industry after having been widely used in the food and beverage industries as a colloidal stabiliser, gelling agent and thickening agent. In addition, it can be used to encapsulate probiotic bacteria in food products.There is a range of methods that can be used to immobilize or encapsulate probiotic cells, such as spray coating, co-extrusion, emulsion, extrusion or spray drying (Kailasapathy 2002, Krasaekoopt et al. 2003, Anal and Singh 2007, Umer et al. 2011). These methods generate beads that have various properties of size, shape and texture (Burgain et al. 2011). The emulsion and extrusion methods are the most regularly used (Krasaekoopt et al. 2003).

8.1. Extrusion

A polymer, such as sodium alginate solution, is used in the extrusion process, into which a cell suspension is added and then a needle is used to extrude it into a calcium chloride solution. This process is presented in Figure 1.



Adapted from Krasaekoopt et al. 2003, Cook et al. 2011.

Figure 1. Flow chart of encapsulation of bacteria using extrusion and emulsion methods and subsequent coating.

This process has several benefits, such as simple operation, production of beads with uniform sizes and shapes, minimal cell injuries and high viability of the probiotic cells. Unfortunately, the process cannot be easily scaled up as the beads form quite slowly (Martinsen et al. 1989, Krasaekoopt et al. 2003), while when moving to the industrial scale it is more likely that several systems will be employed at once (scale out) rather than just one very large system. The gel is formed from alginate and calcium chloride with concentrations

of 0.6-3% and 0.05-1.5 M, respectively (Krasaekoopt et al. 2003, Rokka and Rantamaki 2010).

The formed beads usually have diameters of 2-3 mm (Krasaekoopt et al. 2003), with the size being influenced by the sodium alginate solution's viscosity, the needle's diameter and the distance separating the syringe and calcium chloride solution (Smidsrod and Skjak-Braek 1990, Anal and Singh 2007).

A study by Lee and Heo (2000) showed that when using sodium alginate at 2, 3 or 4% to encapsulate *B. longum* the survival when exposed to simulated gastric juices and bile salts was affected by the bead size and alginate concentration.

Sun and Griffiths (2000) studied *B. infantis'* survival in pasteurized yoghurt that was stored for five weeks in a refrigerator and which was encapsulated in gellan-xanthan beads (0.75% gellan and 1% xanthan gum, diameter ~ 3 mm) and exposed to simulated gastric juice with pH values of 2.5, 2.0 or 1.5. The encapsulation resulted in significantly better cell survival, for example, a survival rate that was eight log better at pH 2.5.

8.2. Emulsion

For this process, there is a large volume of a continuous phase, such as an oil (soybean, sunflower, canola or corn) with an emulsifier added (tween 80), and the cell suspension and sodium alginate are added to it. Then a water-in-oil emulsion is formed by stirring (Figure 1). After this calcium chloride is added, which causes the water soluble polymer to become insoluble and gel particles to form. Smaller beads will be formed when the water phase has smaller particles (Krasaekoopt et al. 2003, Mortazavian et al. 2007).

To form the emulsion the polymers should be water soluble and able to form a gel via ionotropic gelation, with possible examples being cellulose acetate phthalate, locust bean gum, k-carrageenan (Rao et al. 1989), chitosan (Groboillot et al. 1993), gelatin (Hyndman et al. 1993) and alginate (Sheu et al. 1993, Ding and Shah 2008). The beads that are produced can be in the range of 25 μm and 2 mm (Mortazavian et al. 2007). This method has the disadvantages of producing beads with a large range of shapes and sizes as well as being expensive. As there is oil remaining in the beads it is possible that they cannot be used for low-fat food products (Kailasapathy 2002, Gbassi and Vandamme 2012).

It was reported by Ding and Shah (2008) for orange and apple juices that were stored at 4^{o}C for six weeks that the cell survival of *L. rhamnosus, B. longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei* and *B. lactis* were significantly improved. A further study in yoghurt stored at 4^{o}C for seven weeks showed that when calcium-induced alginate-starch microencapsulation was used *L. acidophilus* and *B. lactis* survival was increased by two and one log times compared to the free cells (Kailasapathy 2006).

A range of probiotic strains had their stability investigated under high acid and bile conditions by Ding and Shah (2009a). Encapsulation was preformed using alginate and various types of gum (locust, guar, carrageenan or xanthan) with the strains of *L. rhamnosus, B. longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei, B. lactis* and *B. bifidum.* The particle size was reduced with a microfluidizer (100 bar, 22^{o}C and 34 cycles) to mix the emulsion before the calcium chloride was added. Better survival (>5 log CFU/mL) was achieved after exposure to acid conditions (pH 2) for two hours when the encapsulation was performed with carrageean or xanthan gum as well as alginate.

Table 2. Comparison of extrusion and emulsion methods

	Extrusion	Emulsion
Technological feasibility	Difficult to scale up	Easy to scale up
Cost	Low	High
Simplicity	High	Low
Survival of microorganism	80-95%	80-95%
Size of bead	2-3mm	25 μm-2mm
Shape and size	Uniform	Non-uniform

From Krasaekoopt et al. 2003, Burgain et al. 2011.

Lower survival rates of about 3.8 and 4.8 log CFU/mL were obtained for the guar and locust bean gums, respectively. However, all encapsulated cells had better survival rates that the free cells. Very few comparisons have been made of the extrusion and emulsion methods.

Jayalalitha et al. (2011) showed that when *Lactobacillus* and *Bifidobacterium* cells were encapsulated using the two methods, those produced from extrusion had better survival in yoghurt for 21 days.

This could be due to bigger beads being produced by the extrusion method that offer greater protection than smaller beads (Lee and Heo 2000). Table 2 summarizes the advantages and disadvantages of the two methods.

9. COATING OF BEADS

Extra protection can be added to the beads by coating them with polymers, which can prevent the encapsulated cells being exposed to oxygen or low pH (Rokka and Rantamaki 2010). The coating of the beads can also result in their mechanical strength increasing (Smidsrod and Skjak-Braek 1990). These changes are important as the beads' textures could influence the survival of the cells as well as the products' organoleptic properties. The coating process usually entails mixing the beads in a coating solution (Figure 1) (Cook et al. 2011). Alternatively, a spray coating method can be used, in which the beads are sprayed with a liquid coating material that forms a hard layer at the surface. Spray coating has the advantages of being easy to scale up and the ability to apply several layers of coating (Burgain et al. 2011).

Material such as gelatin, gum, starch, cellulose acetate phthalate (CAP), diethylaminoethyl-dextran (DEAE-extran), whey proteins, poly-1-lysine or chitosan can be used for the coatings (Gbassi and Vandamme 2012). The specific choice relates to the interaction with the encapsulating polymer. The properties of stability, impermeability, flexibility and strength should be provided by a coating material (Umer et al. 2011). The majority of the work related to coating beads has been related to chitosan (Krasaekoopt et al. 2004, Chavarri et al. 2010, Rokka and Rantamaki 2010). This is due to chitosan having good film-forming ability, being low cost, nontoxic, biodegradable, biocompatible and it being a cationic polymer. Chitosan is a polycation as when below a pH of 6.5 the amine residues are protonated (Cook et al. 2011). Chitosan can form polyelectrolyte complexes as its cationic character means it can react with polyanions, for example, alginate (Peniche et al. 2003). This

means that under acidic conditions probiotic cells will be protected well by alginate coated beads.

It was shown by Nualkaekul et al. (2012) that there was a significant influence of using multi-layer coatings (double vs single vs none) on alginate beads in pomegranate juice. The double coating (alginate-chitosan-alginate-chitosan) was thicker than the single coat, which meant it was harder for the organic acids to penetrate the beads.

Poly-1-lysine (PLL) has also been used to coat beads encapsulated in alginate (Cui et al. 2000, Krasaekoopt et al. 2004, Ding and Shah 2009b). A semi-permeable membrane is formed when a complex develops between the poly-amino acid and the alginate (Krasaekoopt et al. 2004). Ding and Shah (2009b) showed that the viability of *Lactobacillus* and *Bifidobacterium* species was increased after exposure for two hours to pH 2 by one log CFU/mL (compared to uncoated) when poly-1-lysine and palm oil were used as the coating of the alginate beads.

It was reported by Nualkaekul et al. (2013) that alginate and pectin could be used for probiotic encapsulation, as they were stable when stored at a cold temperature and low pH. They could be used with a range of fruit juices, such as cranberry and pomegranate, and that there was moderate cell survival for four weeks of storage (>10^4 CFU/mL), while six weeks of storage was possible with the pomegranate juice (~ 10^4-10^6 CFU/mL). There is significant potential to use multi-layer chitosan or gelatine coatings for probiotic encapsulation to improve the survival in acidic fruit juices. The polymers had a strong ionic interaction in gelatin coated beads as shown by their good survival and stability in juices with low pH values.

However, it is important to use polymers that do not come from animals so that they can be used in vegetarian food. They could be obtained from plant or microbial sources and should be cationic; in addition, they should have a low production cost, be non-toxic, edible and food-grade, while also giving high protection levels. For example, *Bifidobacteria* cells have been protected in fruit juice when a poly-glutamic acid (PGA) polymer produced by *Bacillus subtilis* was used. This also enhanced the cell's survival when they moved through the gastrointestinal tract (GIT) and its harsh conditions (Bhat et al., 2015).

10. SENSORY PROPERTIES

The sensory quality of products containing probiotics is a challenge. In fermented products containing free cells, they often have a sour flavor, depending on the specific food product and probiotic used. This however does not always affect the sensory acceptability, and this is especially true for dairy products. For example, for cheese that was stored at 4^{o}C for 28 days there was a positive sensory evaluation when the strains CECT5713 and PS2 of *Lactobacillus salivarius* were added (Cardenas et al. 2014). Then, when *Lactobacillusparacasei* ssp. *paracasei* was added to low-fat yogurt, the sensory profile and acceptance were not altered (Pimentel et al. 2013). However, Luckow et al. (2006) showed that customers may be dissatisfied with probiotics in fruit juice as they could cause off flavors, but that the off flavors could be hidden by adding other flavors or tropical fruit juice.

Table 3. Effect of coating polymers on survival of probiotic cells

Strain	Encapsulation polymer	Coating polymer	Test conditions	Cell survival result	Reference
*L. acidophilus*547, *B. bifidum*ATCC 1994, and *L. casei*01	2% (w/v) sodium alginate	0.4% (w/v) chitosan, 0.17% (w/v) sodium alginate, and 0.05% (w/v) poly-l-lysine	Simulated gastric juice (pH 1.55) and 0.6% bile salt solution	Coated beads improved cell survival up to 1-3 logs compared with uncoated beads	Krasaekoopt et al. (2004)
B. adolescentis 15703T	13% (w/v) gelatin	1% (w/v) alginate	Simulated gastric solution (pH 2.0, 2 h), and intestinal solution (pH 7.4, 4 h)	Coated beads improved cell survival up to 1 log compared with uncoated beads	Annan et al. (2008)
L. plantarum 299v, *L. plantarum*800 and *L. plantarum*CIP A159	2% (w/v) alginate	2% (w/v) of whey protein	Simulated gastric solution (pH 1.8) and Simulated intestinal solution (pH 6.5)	Coated beads improved cell survival ~ 4-5 log compared with uncoated beads	Gbassi et al. (2009)
*L. acidophilus*PTCC1643 *and L. rhamnosus* PTCC1637	1% (w/v) sodium alginate	0.5% (w/v) sodium alginate	Simulated gastric juice (pH 1.5, 2 h),	Coated beads improved cell survival up to 1-2 logs compared with uncoated beads	Mokarram et al. (2009)
B. breve NCIMB 8807	2%(w/v) alginate	0.4% (w/v) chitosan	Simulated gastric solution (pH 2) 1 and 2 h	Coated beads improved ~ 2 log (wet beads) and ~ 1 log (dry beads) in 1 h compared with uncoated beads	Cook et al. (2011)
L. rhamnosus CRL 1505	Pectin (PE) and pectin–whey protein (PE–WP).	8% (w/v) of whey protein	Simulated gastric solution (pH 1.2 and pH 2)	The $MC_{PE/WP}$ beads were more stable than the $MC_{PE-WP/WP}$ beads	Gerezet al. (2012)

However, despite this being an important topic, very little work has been done on evaluating the acceptance of juices containing probiotics, in particular when they are in the form of encapsulated beads. As encapsulation has been used more widely, it has increased the level of probiotics that are incorporated in different types of food. For example, a process by Barry Callebaut has been established that enables encapsulated probiotic bacteria to be added into chocolate. Probiotic cells that have been encapsulated have been added to chocolate tablets and bars, nutrient bars and yoghurt-covered raisins by the Institute Rosell-Lallemand. An orange juice that can be stored for 10 weeks and that contains encapsulated probiotic bacteria was developed by Chr Hansen. It is however important to ensure the beads are the correct size as otherwise they influence the sensory properties of the product. Krasaekoopt and Kitsawad (2010) reported that when the probiotic beads were 100-200 μm sized 80% of the consumers approved of the product, and those that did not like the products stated that the beads remained in their mouths or got lodged in the throat. The swallow ability scores were ~ 2 and ~ 7 for fruit juices with and without beads, respectively. When using the extrusion technique the beads of alginate were about 2 – 4 mm, which was much greater than the 100 to 200 μm target. The size of the beads can be masked or combatted by making the product thicker (by adding thickening agents) or making it into a smoothie formulation, with the other option being making the beads smaller. Much smaller beads, between 20 μm and 200 μm, can be made with the emulsion technique, but the size is less uniform. Alternatively, vibrational nozzles can be used with the extrusion technique to get smaller beads. It is also necessary to study the beads textural characteristics and how the hardness/softness relates to the finished products' overall properties. It has been shown that a rough, gritty and unpleasant feeling was produced when there was a large concentration of hard big particles in products that had low viscosity (Heidebach et al. 2012). Therefore, to develop a process that decreases the size and increases the softness of the beds is very important to make sure that consumers accept the products. While it has been shown that in Feta cheese, the aroma-related compounds were improved by the immobilization of cells (Dimitrellou et al. 2014).

As the number of coating layers increased, the texture of the beads decreased. The texture of the beads is most likely due to the concentration of the sequestering agent (malate or citrate, for example) and, particularly in fruit juices, monovalent ions as well. As far as the organoleptic perspective is concerned, these aspects are very important as they are related to the characteristics of the beads and product during storage and its acceptability to the consumer (Nualkaekul et al. 2013).

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Chapter 5

FUNCTIONAL DAIRY FOODS: PHENOLIC PHYTOCHEMICALS PROVIDE BENEFICIAL EFFECTS OF ANTIOXIDANTS IN YOGURT

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ABSTRACT

Several studies have shown that free radicals extant in the human organism cause oxidative damage to different molecules such as lipids, proteins and nucleic acids and thus are involved in the beginning stage of some degenerative diseases. Biological antioxidants are substances which are able to delay or inhibit the oxidative damage of different biomolecules associated with several diseases including cancer, liver disease, aging, inflammation, diabetes, hypertension, Parkinson's disease and atherosclerosis. Plants present a good source of nutrients and antioxidants compounds. One of the important global market trends is searching for unique food ingredients and flavours with enhanced health properties. Yogurt is slow lactic fermentation of lactose from milk by thermophilic lactic acid bacteria, and is one of the most consumed fermented foods in many countries.

It is widely recognized as functional food because of the nutritional properties, being a good vehicle to deliver probiotics to consumers. Incorporation of plant materials in yogurt during fermentation is an effective strategy to increase antioxidant intake and therapeutic value that may help to reduce the risk of developing chronic disease. The aim of the present study is to review the effects of supplementation of yogurt with appropriate plant materials for developing novel functional yogurt with antioxidant properties.

Keywords: functional foods, yogurt, phenolic phytochemicals, antioxidant activity

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1. INTRODUCTION

Oxidation is important to living organisms for biological processes (Unal and Akalın, 2006). Free radicals and reactive oxygen species including the superoxide radical, hydroxyl radical, hydrogen peroxide, and the peroxide radical are physiological metabolites produce in the living systems. The excessive amount of free radicals can damage cellular macromolecules (Unal and Akalın, 2006). This damage has been strongly associated with a wide variety of chronic diseases including atherosclerosis, arthritis, diabetes, and cancer (Sarmadi and Ismail, 2010). It is also recognized that lipid oxidation happening in food products causes decline in food quality, i.e., formation of off flavor, undesirable taste and shortening of shelf life. Synthetic antioxidants have been used as antioxidants in food but because of their carcinogenicity effects become less desirable (Brash and Havre, 2002; Rahimi et al., 2005). There is very increasing interest to find natural antioxidants from food to protect the human body from the free radicals and delay the development of many chronic diseases as well as inhibit lipid oxidative rancidity in foods (Liu et al., 2005; Kadri et al., 2011). Natural antioxidants from plant origins such as rosemarinic acid, catechin, tocopherols, ascorbate and phenolic compounds have been widely used food manufacture. However, natural antioxidants have extended to include peptides and protein hydrolysates from fermented dairy food (López-Expósito et al., 2007; Contreras et al., 2009; Srinivas and Prakash, 2010; Kamau et al., 2010; Nagpal et al., 2011).

Yogurt is slow lactic fermentation of lactose from milk by thermophilic lactic acid bacteria. It is traditionally considered as functional food because of its therapeutic and nutritional properties (rich in potassium, calcium, protein and vitamins).

Lactic acid bacteria (LAB) used as starters are mainly responsible for the liberation of bioactive peptides with antioxidative effect during milk fermentation (Pihlanto, 2006). There are growing interests in applying plant materials as functional food ingredients since they are rich source of bioactive compounds with antioxidants proprieties. The aim of the present study is to review the effects of supplementation of yogurt with appropriate plant materials for developing novel functional yogurt with antioxidant activities.

2. WHAT ARE FUNCTIONAL FOODS?

Foods are functional when they provide additional properties other than nutritive values. However, added physiologic benefits to foods are now being examined intensively which may either a state of well-being and health and/or to the reduction of the risk of a disease. Functional foods have no universally accepted definition. The concept was first developed in Japan in the 1980s when the Ministry of Health and Welfare introduced a controlling system to approve certain foods with recognized health benefits in hopes of developing the health of the nation's aging population (Yamada et al., 2008). These foods are also called "Foods for Specified Health Use" (FOSHU, Yamada et al., 2008). Functional foods may also be defined as "any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (Hasler, 2002).

The International Life Sciences Institute defines them as further refinement i.e., "foods that, by virtue of the presence of physiologically-active components, provide a health benefit

beyond basic nutrition" emphasise the importance of therapeutical values inherent in functional foods. Functional foods must not be consumed as medicine but rather as foods that are "whole, fortified, enriched or enhanced" but more importantly, states that such foods must be consumed as "part of a varied diet on a regular basis, at effective levels" for consumers to reap their potential health benefits" (American Dietetic Association, 1999). Nutraceuticals is another term commonly used with functional foods. This term created in 1991 by the Foundation for Innovation in Medicine refers to nearly any bioactive component that provides a health benefit. A food can be assumed to be functional if it follows one of the next criteria (Ramchandran and Shah, 2009):

a) It comprises a food component (being nutrient or not) which have positive effects targeted one or a limited number of function(s) in the body.
b) It has physiological or psychological properties further than the traditional nutritional effect.

The component that makes the food "functional" can be 'either an essential macronutrient with particular physiological properties or an essential micronutrient for body needs on a daily basis. In addition, some of food components may not recorded as essential, such as some oligosaccharides, or they have no nutritive value, such as live microorganisms or plant chemicals' (Nakakuki, 2002). The main types of functional foods are indicated in Table 1.

3. FUNCTIONAL DAIRY PRODUCTS

Dairy products are established as healthy natural products and they form one of the four major food groups (the other three being protein, fruits and vegetables and grains) that make up a balanced diet (Ramchandran and Shah, 2009). Regular consumption of certain dairy products has beneficial effects in the prevention of disease (Bozanic et al., 2001) because they contain a number of active compounds with putative roles in both nutrition and health protection (Table 2).

Table 1. Different type of functional foods

Type	Description	Some examples
Fortified products	Increasing the content of existing nutrients	Grain products fortified with folic acid, fruit juices fortified with additional vitamin C
Enriched products	Adding new nutrients or components not usually present in a certain food	Fruit juices enriched with calcium, foods with probiotics and prebiotics
Altered products	Replace existing components with beneficial components	Low-fat foods with fat replacers
Enhanced commodities	Changes in the raw commodities that have altered nutrient composition	High lysine corn, carotenoid containing potatoes, lycopene enhanced tomatoes

Source: Spence, 2006.

Table 2. Dairy components and ingredients in functional foods and their health claims

Component	Sources	Claim areas
Minerals	Calcium Casein peptides	Optimum growth and development, dental health, osteoporosis
Fatty acids	Conjugated linoleic acid (CLA)	Heart disease, cancer prevention, weight control
Prebiotics/ carbohydrates	Galactooligosaccharides Lactulose Lactose	Digestion, pathogen prevention, lactose intolerance, immunity and gut flora balance
Probiotics	Lactic acid bacteria Bifidobacteria	Immunity, heart disease, digestion, vitamin production, remission of inflammatory bowel disease, antitumor activity, alleviation of diarrhea and prevention of allergy
Proteins/peptides	Whey proteins, caseins, lactoferrin, immunoglobulins, glycoproteins, specific peptides	Growth, antibacterial activity, dental health, immunomodulation and hypertension regulation (angiotensin inhibitors)

Source: Shortt et al., 2004.

4. YOGURT AS A FUNCTIONAL FOOD

The most common functional dairy products are those containing probiotic bacteria, quite frequently enriched with prebiotics, such as yogurt (Saxelin et al., 2003). Yogurt is fermented milk obtained by lactic acid bacteria fermentation of milk and is a popular product throughout the world. The highest production and consumption of yogurt are recorded in countries in the Mediterranean, South Asia and central Europe which surround the possible origin of yogurt i.e., in the Middle East (Lore et al., 2005; Rahman et al., 2009; Shori, 2013a and b).

Yogurt is recognized as a healthy food due to the beneficial action of its protein and its rich contents of potassium, calcium, protein and B vitamins (Table 3).

Yogurt is formed during the slow fermentation of milk lactose by the thermophilic lactic acid bacteria *S. thermophilus* and *L. delbrueckii ssp. Bulgaricus*. However, these bacteria are not indigenous to humans and cannot colonize the intestine to promote human health.

Thus probiotics, mainly *Lactobacillus acidophilus* and *Bifidobacterium* spp. are added to improve the fermentation process for production probiotic yogurt (Donkor et al., 2006) and offer many advantages for the consumer. *S. thermophilus* and *L. delbrueckii ssp. Bulgaricus* are required to convert milk to yogurt whereas *L. acidophilus* and *Bifidobacterium* are added to increase the functional and health-promoting properties.

5. PHENOLIC PHYTOCHEMICALS

Phenolic compounds are widely present in fruits, vegetables and spices. These compounds may have potent antioxidants activity by applying antioxidative action as terminators of free radicals and chelating metals that have ability for catalyzing lipid peroxidation.

Table 3. The nutritional value of 100g yogurt

Constituents	Yogurt (low fat and plain)	Constituents	Yogurt (low fat and plain)	Constituents	Yogurt (low fat and plain)
Energy Value (kJ)	220.0				
Major Constituents (g)	Major Constituents (mg)			Vitamins	
Protein	5.00			A (IU)	70-130
Fat	1.00	Orotic Acid	4.00	Thiamine (µg)	37-50
Lactose	5.00	Fumaric Acid	8.00	Riboflavin (µg)	220-260
Galactose	1.50	Succinic Acid	19.00	Pyridoxine (µg)	40-54
Lactic Acid	1.00	Benzoid Acid	7.00	Cyanobalamine (µg)	0.1-0.35
Citric Acid	0.30	Cholesterol	7.00	Ascorbic Acid (µg)	0.1-0.1
Potassium	0.24	Urea	0.02	Tocopherol (µg)	30
Calcium	0.18	Glucose	30.00	Folic Acid (µg)	4
Phosphorus	0.14	5'-UMP	0.50	Nicotinic Acid (µg)	120-130
Chloride	0.18	3'+ 5'-GMP	0.40	Panthothenic Acid (µg)	380
Sodium	0.08	5'-AMP	0.10	Biotin (µg)	1.2-4.0
Bacterial mass	0.15	NAD	0.60	Cholinc (µg)	0.6

Source: Cmckinley, 2005.

They may act by donating a hydrogen atom to radicals which results in the formation relatively stable phenoxy radical intermediates making it more difficult for a new chain reaction to initiate (Sroska and Cisowski, 2003; Ranilla et al., 2010). The efficiency of phenolic compounds may be associated with factors such as number of hydroxyl groups and the site of binding as well as the mutual position of hydroxyls in the aromatic ring (Sroska and Cisowski, 2003). Thus diets rich in vegetables, fruits and spices have been associated with a lowered incidence of degenerative diseases, diabetes (Brash and Havre, 2002) and hypertension diseases (Kris-Etherton et al., 2002). Some phenolic substances may have applications in controlling pathogens in foods (Mandavia et al., 2000; Anson et al., 2009) and crops (Mandavia et al., 2000) as well as possessing anti-inflammatory effects (Trouillas et al., 2003). Phenolics compounds occur primarily in conjugated form with one or more sugar residues linked to hydroxyl groups as well as with other compounds such as carboxylic, organic acids, amines and lipids (Sroska and Cisowski, 2003). Enzyme hydrolysis of these phenolic glycosides appears to be a usefull way to improve the number of free phenolics with nutraceutical and pharmacological properties (Zheng and Shetty, 2000). Examples of classification of dietary polyphenols with their pharmacological properties are given in Figure 1.

6. NATURAL FOODS ANTIOXIDANTS AND THEIR HEALTH BENEFITS

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are synthetic antioxidants which have been used as antioxidants in food because of its excellent solubility

in food, heat stable and providing extended shelf life. There are limitations on applying of these compounds in food because of their carcinogenicity effects (Brash and Havre, 2002; Rahimi et al., 2005). There are other options to replace synthetic antioxidants by natural and safe sources of food antioxidant (Psaltopoulou et al., 2011) which include vegetables, fruits and plants in general. Plants are increasingly used for the manufacture of raw ingredients or preparations including phytochemicals with major antioxidant activities and therapeutic properties (Exarchou et al., 2002). Crude extracts rich in phenolics such as fruits, herbs, vegetables and cereals are widely used in the food manufacturing and processing since they increase the quality and nutritional value of food by delaying oxidative degradation of lipids. In fact many plants spices and herbs have protective effect related to the presence of antioxidant and antimicrobial compounds in their cell walls (Srinivasan, 2005; Wilson and Adams, 2007; Ranilla et al., 2010). The role of food antioxidants in the maintenance of health and reduction of risks developing cancer, high blood pressure, diabetes and other diseases is increasingly apparent as reflected in the increase in consumer's preference for functional foods with specific health properties (Anonymous, 2002). Antioxidants compounds are known to delay or prevent the oxidation of lipids or other compounds by inhibiting the beginning or proliferation of oxidative chain reactions (Sroska and Cisowski, 2003). Thus the addition of antioxidants to food products specifically to lipids and lipid-containing foods can improve the food shelf life. The antioxidative effect is generally caused by phenolic components such as flavonoids (Chan et al., 2012), phenolic acids and phenolic diterpenes (Chan et al., 2012) which can absorb and neutralize free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Psaltopoulou et al., 2011; Chan et al., 2012).

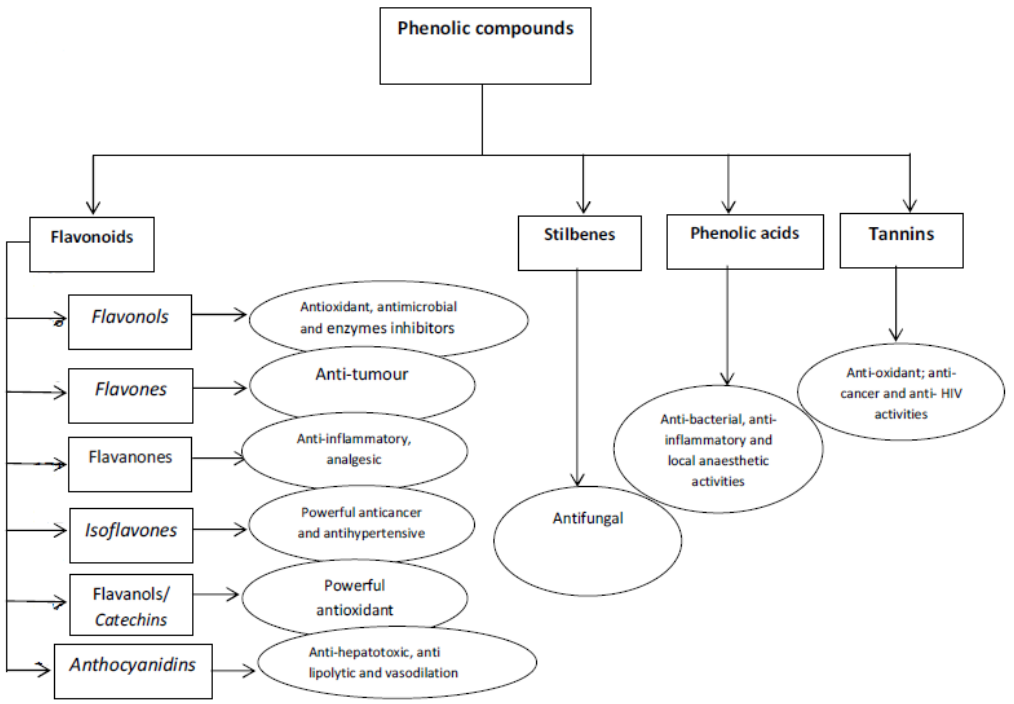

Figure 1. Classification of dietary polyphenols with their pharmacological properties.

A lot of these phytochemicals have significant antioxidant activity such as effective neutralizing effects on free radicals that can control high blood pressure and diabetes (Hunter and Fletcher, 2002; Anonymous, 2002). The exposure of living organisms to reactive oxygen species (ROS) is unavoidable in aerobic life since the generation of ATP from molecular oxygen demands electrons. ROS fall into two groups i.e., those that contain unpaired electrons (O_2^-, OH^-) or those that have the ability to extract electrons from other molecules (H_2O_2, HOCl). These species may damage biomolecules directly or initiate chain reactions in which ROS are passed from one molecule to another resulting in extensive damage to cell structures such as membranes and proteins.

Breakdown or deficiency of these defenses against ROS can lead to damage which has been strongly associated with a wide variety of chronic diseases including Alzheimers, autoimmune disease, cancer, cardiovascular disease, diabetes, multiple sclerosis and arthritis (Trouillas et al., 2003; Rahimi et al., 2005; Shetty et al., 2008; Ranilla et al., 2010). In contrast, levels of ROS must not become too low given their important roles in the immune system. Therefore, there is a need for constant monitoring and regulation of the redox potential of the blood.

7. ANTIOXIDANTS OF YOGURT

Milk proteins are a rich source of bioactive peptides. Milk and its fermented products have health benefits beyond basic nutrition, which can have a protective effect on some age diseases caused by inappropriate diet such as cardiovascular diseases, diabetes type II and obesity. These peptides are inactive in the initial protein of the milk (casein and albumins). The fermentation of the milk with LAB results in releasing a vast number of bioactive peptides and free amino acids with antioxidative activity (Pena-Ramos et al., 2001). The potential antioxidant activities of the peptides have been observed as chelation of transition metals and scavenging free radicals (Karadag et al. 2009). The concentration of these antioxidants in yogurt is related with the nutrition of the dairy cattle (specific amino acid sequence of the milk protein variants) and milk heat treatment.

Galleher et al., (2005) reported that heat treatment (95°C for 15 min) undergone by the milk for the production of yogurt causes denaturation of proteins exposed initially buried reactive sites with antioxidant capacity. Yogurt is rich in antioxidants with specific reaction. Since many components in yogurt have a certain antioxidant capacity, the exact function of each antioxidant in milk cannot be assumed. The measurement of the total antioxidant capacity is a useful method for determining the amount of the antioxidant role of each component of the yogurt (Lindmark and Akesson, 2000).

Antioxidant vitamins in yogurt, for example vitamin E and carotenoids are located in the membranes of the fat globules in milk and can inhibit auto oxidation of the milk fat. Vitamin C is playing an important role in antioxidant activity by a complex interaction with the iron and is used as the electron donor in the conversion of the tocopheroxyl radical back to antioxidant active vitamin E (Lindmark and Akesson, 2000). Moreover, lactoferrin is a protein with molecular mass of 80 kDa found in milk whey. It has a great contribution in chelating iron ions, thus preventing the oxidation of fatty acids and releasing peroxy radicals (Bihel and Birlouez-Aragon, 1998).

Moreover, the antioxidant activity is strongly influenced by strain-specific characteristics of LAB. Kim et al., (2005) indictaed that yogurt LAB such as *L casei* 01, *L. acidophilus* LA100, *L. rhamnosus* GG 744, *L. acidophilus* LA5 and *L. bulgaricus* LB 207 have a good antioxidant effect on inhibition of lipid peroxidation. *L. bulgaricus* LB207 showed the strongest antioxidant activity with 81.3% whereas *L. rahamnosus* GG744, L. casei 01, *L. acidophilus* LA100 and *L. acidophilus* LA5 showed high antioxidant activity, lipid peroxidation inhibition of 72.87%, 68.17% , 65.32% and 38.27% respectively.

8. EFFECTS OF PLANTS PHENOLIC COMPOUNDS BASED YOGURT ON ANTIOXIDANTS

Plant food antioxidants rich in vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been known as potential to reduce disease risk. Phenolic compounds have great oxygen radical scavenger activity because of the low electron-transfer potential, thus can scavenge reactive oxygen intermediates leading to the prevention of further oxidative reactions (Ainsworth and Gillespie, 2007). Most of the antioxidant compounds belong to different classes of antioxidant activity such as gallates, have strong antioxidant activity while others such as the mono-phenols are weak antioxidants (Zainoldin and Baba, 2009). Fermented food product like fruit yogurt is being available in many countries and it has significant nutritional and therapeutic value i.e., antioxidant activity, bioactive compounds or edible fibers, minerals and vitamins (Alzamara and Akin, 2015).

Recently, there has been an increasing interest in the devolvement of yogurt with health effects by using natural food additives and incorporation of antioxidant-promoting substances (Zainoldin and Baba, 2009; Alzamara and Akin, 2015). Yogurt additive such as plant materials have beneficial effects attributed to the strong antioxidant activity of the plant phenolic compounds. Several plants have used to enrich yogurt during fermentation process (Table 4) which might possess strong antioxidant properties that could protect the body from damage caused by free radical-induced oxidative stress. According to McCue and Shetty (2005), phenolic antioxidants did not affected by yogurt LAB during fermentation in presence of kefir culture. In addition, microbial cells have a number of antioxidant defence mechanisms to remove or inactivate ROS to protect biological system (Korpela et al., 1997; Lin and Yen, 1999 aandb; Amanatidou et al., 2001). Some LAB possessed significant antioxidant activity, allowing the preservation of phenolic compounds from oxidation during yogurt fermentation (Kachouri and Hamdi, 2006).

Oxidation of LDL plays an important factor in the initiation and progression of diseases (Kojima et al., 1998). Most phenolic phytochemicals that have positive effect on health are believed to be functioning by countering the effects of reactive oxygen species (ROS) generated during cellular energy metabolism (Ranilla et al., 2010). Therefore, the enriched yogurt with plants antioxidants may serve as the basis for assessment of the preventative role of plant-yogurt against free radical-mediated lipid peroxidation, and thus is able to enhance the therapeutic effect of the functional food proposed. The oxidation of lipids in foods is responsible for the formation of off-flavours and undesirable chemical compounds which may be detrimental to health.

Table 4. Supplementation of yogurt with appropriate plant materials for developing novel functional yogurt with antioxidant properties

Type of yogurt	Additive	Extracts ratios	starter culture	DPPH radical scavenging activity	ABTS radical scavenging	Ferric reducing antioxidant power	Anti radical power	ferrousion chelating	Total phenolic content	References
NM	Artichoke extracts (edible part) Artichoke extracts (not edible part) Strawberry fruits (ripe) strawberry fruits (unripe) cherries	NM	NM	60 mg TE/100g 47 mg TE/100g 109 mg TE/100g 70 mg TE/100g 20 mg TE/100g	ND	50 mg TE/100g 46 mg TE/100g 42 mg TE/100g 39 mg TE/100g 37 mg TE/100g	ND	ND	49 mg GEA/100g 34 mg GEA/100g 28 mg GEA/100g 23 mg GEA/100g 25 mg GEA/100g	(Cossu et al., 2009)
Set yogurt	Red dragon fruits extracts White dragon fruits extracts	30%	*Mixture of S. thermohilus, L. acidophilus, L. bulgaricus, L. casei, L. rhamnosus, B. bifidum, B. infantis, and B. longum* (10%)	45.74% 39.96%	ND	ND	ND	ND	49.61 mg GAE/ml 64.43 mg GAE/ml	(Zainoldin and Baba, 2009)
Set yogurt	Roselle syrup	10%	Yogurt starter culture (0.132 g) + Lactobacillus casei (0.6 g)	7%	ND	ND	ND	ND		(Lawin and Kongbangkerd, 2010)
Set yogurt	Cinnamon extracts	10%	*Mixture of S. thermohilus, L. acidophilus, L. bulgaricus, L. casei, L. rhamnosus, B. bifidum, B. infantis, and B. longum* (10%)	35.3 ± 1.0%	ND	ND	ND	ND	39.55 µg GAE/g	(Shori and Baba, 2011)
Set yogurt	Peppermint Dill	10%	*Mixture of S. thermohilus, L. acidophilus, L. bulgaricus, L. casei, L. rhamnosus, B. bifidum, B. infantis, and B. longum* (10%)	50% 57%	ND	ND	ND	ND	26.6 ± 3.8 mg GEA/ml 35.2 ± 2.4 mg GEA/ml	(Amirdivani and Baba, 2011)

Table 4. (Continued)

Type of yogurt	Additive	Extracts ratios	starter culture	DPPH radical scavenging activity	ABTS radical scavenging	Ferric reducing antioxidant power	Anti radical power	ferrousion chelating	Total phenolic content	References
Set yogurt	Basil	10%		35%	ND	ND	ND	ND	20.1 ± 1.4 mg GEA/ml	(Amirdivani and Baba, 2011)
Set yogurt	Skim milk powder whey protein concentrate sodium calcium caseinate	4% 4% 4%	Yoghurt starter culture (0.4%)	88% 94% 87%	ND	ND	ND	19.07 ± 5.05% 30.5 3 ± 9.01% 23.46 ± 14.28%		(Unal and Akalın, 2012)
NM	Hickory-black soybean	12%	Yogurt starter culture (6%)	42.85 g/L (IC_{50})	ND	ND	ND	16.17 g/L (IC_{50})	0.62 ± 0.08 mM GAE	(Ye et al., 2013)
NM	Black carrot jams Pumpkin jams Strawberry jams	1.5%	Yogurt starter culture (2%) and Bifidobacterium lactis B6-12 (2%)	36.34% 31.69% 40.12%	ND	ND	ND	ND	22.92 mg TAE/100g 27.44 mg TAE/100g 28.48 mg TAE/100g	(Abou El Samh et al., 2013)
NM	Blackberry pieces Cherry Mango Peach pieces pulp and pineapple pieces	10% 10.2% 10% 13% 11.6%	NM	43.61% 36.07% 42.47% 37.22% 44.21%	ND	ND	ND	ND	2.69 ± 0.09 mg GEA/g 3.49 ± 0.03 mg GEA/g 1.07 ± 0.04 mg GEA/g 1.42 ± 0.02 mg GEA/g 6.90 ± 0.31 mg GEA/g	(Pereira et al., 2013)
NM	Rehydrated plums and plums puree Raspberry pieces	10% 9.4%	NM	82.01% 57.03%	ND	ND	ND	ND	2.18 ± 0.00 mg GEA/g 2.38 ± 0.04 mg GEA/g	(Pereira et al., 2013)

Type of yogurt	Additive	Extracts ratios	starter culture	DPPH radical scavenging activity	ABTS radical scavenging	Ferric reducing antioxidant power	Anti radical power	ferrousion chelating	Total phenolic content	References
NM	Strawberry pieces, kiwi pulp and pieces	6%, 5.6%	NM	45.45%	ND	ND	ND	ND	4.12 ± 0.16 mg GEA/g	(Pereira et al., 2013)
NM	Wine grape pomace	3%	Yoghurt starter culture + Lactobacillus acidophilus and Bifidobacterium lactis	936 mg AAE/kg	ND	ND	ND	ND	1338 mg GAE/kg	(Tseng and Zhao, 2013)
Set yogurt	Soybean extracts	10%	*Mixture of S. thermohilus, L. acidophilus, L. bulgaricus, L. casei, L. rhamnosus, B. bifidum, B. infantis, and B. longum* (10%)	61.76 ± 3.3%	ND	ND	ND	ND	34.33 µg GAE/g	(Shori, 2013a)
Set yogurt	Chickpea extracts	10%	*Mixture of S. thermohilus, L. acidophilus, L. bulgaricus, L. casei, L. rhamnosus, B. bifidum, B. infantis, and B. longum* (10%)	37%	ND	ND	ND	ND	38.0 ± 0.1 µg GAE/g	(Shori, 2013b)
Set yogurt	Neem extracts	10%	*Mixture of S. thermohilus, L. acidophilus, L. bulgaricus, L. casei, L. rhamnosus, B. bifidum, B. infantis, and B. longum* (10%)	30.1 ± 5.1%	ND	ND	ND	ND	59 µg GAE/g	(Shori and Baba, 2013)
Stirred yogurt	Pomegranate peel extracts	35%	Traditional starter culture (3%)	43.81 ± 2.54%	12.22 ± 1.89%	ND	ND	ND	7.61 ± 1.00 mg GAE/g	(El-Said et al., 2014)
Set yogurt	Carrot Broccoli Pumpkin red sweet pepper extracts	10%	commercial YC-180 DVS yogurt culture (2%)	ND	ND	1.733 mmol Fe2+/L 2.029 mmol Fe2+/L 1.793 mmol Fe2+/L 1.939 mmol Fe2+/L	0.236 µmol TE/g 0.336 µmol TE/g 0.196 µmol TE/g 0.310 µmol TE/g	ND	ND	(Najgebauer-Lejko et al., 2014)

Table 4. (Continued)

Type of yogurt	Additive	Extracts ratios	starter culture	DPPH radical scavenging activity	ABTS radical scavenging	Ferric reducing antioxidant power	Anti radical power	ferrousion chelating	Total phenolic content	References
Set yogurt	-	-	Yogurt starter culture Yogurt starter culture + *Lactobacillus casei* (0.01% w/v) Yogurt starter culture + *Lactobacillus acidophilus* (0.01% w/v) Yogurt starter culture + *Bifidobacterium bufidus* (0.01% w/v)	52.44% 56.51% 63.99% 54.93%	ND	ND	ND	ND	ND	(Gjorgievski et al., 2014)
NM	Dried grape pomace	5%	Yogurt starter culture + Bifidobacterium lactis (2%)	84.72%	ND	ND	ND	ND	190 mg GAE/100g	(Mohamed et al., 2014)
yogurt-like beverage	oat flakes	5%	Lactobacillus plantarum LP09	15.7 ± 0.4%	ND	ND	ND	ND	ND	(Luana et al., 2014)
NM	encapsulated cardamom encapsulated nutmeg cinnamon	0.5%	Yoghurt starter culture + Lactobacillus acidophilus strain 5(LA5) (0.02%)	80% 70% 69%	ND	ND	ND	ND	ND	(Illupapalayam et al., 2014)
NM	encapsulated cardamom encapsulated nutmeg cinnamon	0.5%	Yoghurt starter culture + Bifidobacterium animalis ssp. Lactis (Bb12) (0.02%)	59% 71% 59%	ND	ND	ND	ND	ND	(Illupapalayam et al., 2014)
Set yogurt	Garlic extracts	10%	*Mixture of S. thermohilus, L. acidophilus, L. bulgaricus, L. casei, L. rhamnosus, B. bifidum, B. infantis, and B. longum* (10%)	37.9 ± 0.8%	ND	ND	ND	ND	39.55 µg GAE/g	(Shori and Baba, 2014)

Type of yogurt	Additive	Extracts ratios	starter culture	DPPH radical scavenging activity	ABTS radical scavenging	Ferric reducing antioxidant power	Anti radical power	ferrousion chelating	Total phenolic content	References
Set yogurt	Lycium barbarum extracts	10%	*Mixture of S. thermohilus, L. acidophilus, L. bulgaricus, L. casei, L. rhamnosus, B. bifidum, B. infantis, and B. longum* (10%)	35.5%	ND	ND	ND	ND	38.7 ± 1.7 µg GAE/g	(Baba et al., 2014)
NM	wild Thyme extracts	1.5%	Yogurt starter culture	70%	ND	ND	ND	ND	ND	(Nikjooy and Hashemi, 2015)
NM	Rose hip marmalade	15%	Yogurt starter culture	62.34%	14.62 ± 0.10 Mm TE/g	ND	ND	ND	0.47 ± 0.10 mg GAE/ml	(Alzamara and Akin, 2015)

NM: Not mention; ND: Not detected; TAE: Tannic acid equivalent phenolics; AAE: Ascorbic acid equivalents, TE: Trolox equivalent.

Antioxidants are used by the food industry to delay the oxidation process (Berset, 1994). Therefore, the presence of plants antioxidants may give rise to the possibility of prolonged shelf life of yogurt.

CONCLUSION

Plant-based yogurt has potentials in the development of an effective dietary strategy for antioxidant activity and will result in the increase of novel functional dairy products. However, several factors need to be taken into consideration prior to make the manufacture of the plant-based yogurt a success. The variations in the phenolic content and phenolic profile in plants could limit their success as therapeutic agents regardless of their many beneficial properties. For instance, plants which originate from different heterozygous seeds are phenotypically variable causing substantial phytochemical inconsistency, which translates into unreliable clinical effects as well as inconsistent health benefits. Other factors, such as cultivar, maturity, processing, and storage may also influence the plant phenolics content. There are few reports on the antioxidant action of plant-based yogurt on the gastrointestinal digestion, thus further in vivo experiments are needed.

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Chapter 6

MONITORING OF MICROBIAL VOLATILE ORGANIC COMPOUNDS IN TRADITIONAL FERMENTED FOODS: THE IMPORTANCE OF TAILORED APPROACHES TO OPTIMIZE VOC CONTRIBUTION TO CONSUMER ACCEPTANCE

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ABSTRACT

The bio-preservation of perishable edible raw materials through fermentations represents the first biotechnological application and one of the first forms of food processing in human history. Nowadays, this millenary tradition declined in many geographical contexts by reason of differences in raw materials, environmental conditions and in traditional knowledge. Flavour is one of the factors mainly characterizing the degree of typicality of fermented products. Many volatile organic compounds (VOCs) responsible for flavour (and off-flavour) perception are of microbial

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origin. Hence, microbial volatile organic compounds (mVOCs), by influencing the quality of food, affect consumer preference on fermented food. The huge number of geographical, compositional, microbiological and technological variables highlights the important need for tailored technologies to improve consumer acceptance of traditional fermented foods. Direct-Injection Mass Spectrometric (DIMS) technologies, associate time resolution with high sensitivity and robustness, thus offering interesting insights in the field. In particular, Proton Transfer Reaction Time of Flight Mass Spectrometry (PTR-ToF-MS) is an analytical tool for the detection and quantification of very small quantities of VOCs with the possible simultaneous real-time monitoring of these VOCs without sample preparation. In recent studies, we described the possible application of PTR-ToF-MS coupled with an auto-sampler and tailored data analysis tools for the automatic real-time monitoring of the mVOCs. We studied the mVOCs associated with the fermentative performances of yeast and lactic acid bacteria (LAB), the main microbes involved in food fermentations. All these features, which allow rapid and accurate screening, are likely to be important for characterizing typical fermented foods and for developing new strategies in the standardization/ enhancement of microbial contribution to unique sensory qualities; both aspects are of a huge importance in the design of preservation and innovation paths in the field of traditional fermented foods.

INTRODUCTION

Food fermentation is one of the first forms of food preservation (and processing) in human history (Hutkins 2006). The bio-preservation/processing of perishable/edible raw materials through fermentations is also known as the 'oldest biotechnology' (Leroy and De Vuyst 2004). Without knowing the existence of microscopic forms of life, our ancestors used the metabolic activities associated with 'virtuous' microorganism development to transform, for example, milk into cheese and must into wine (Steinkraus 2002). This historic, cultural, and transdisciplinary significance explains well the relevance of fermented foods in the important sector of traditional, typical, and artisanal foods and beverages (Capozzi et al. 2012a, Capozzi et al. 2012b). For instance, without considering wines, fermented products represent more than 80% of the EU Geographical Indications (GIs; "GI is a sign used on goods that have a specific geographical origin and possess qualities, reputation, or characteristics that are essentially attributable to that place of origin" (WIPO 2015)) (37% cheeses, 20% beer, 16% meat products, 4% fruits and vegetables, 4% bakery products, biscuits, confectionery (Capozzi and Spano 2011)). The result is a field of food market where most products, even when produced on an industrial scale (e.g., yoghurt production (Arena et al. 2015)) are a traditional production developed by millenary human cultures. Nowadays, we find this millenary tradition on the shelves of the food industry and declined in many geographical contexts by reason of the differences in raw materials, environmental conditions and in traditional knowledge, leading to surprising breadth of different fermented typical products. Considering microbial resources associated with these manufactures, traditionally, food fermentations rely on naturally selected microbial consortia; however, large scale production usually standardizes safety and quality of the final products by means of the 'starter cultures' regiment (Ross et al. 2002), where starter culture can be defined as "a microbial preparation of large numbers of cells of at least one microbial species to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process" (Leroy and De Vuyst 2004).

FOOD FERMENTATIONS AND THE IMPORTANCE OF MICROBIAL VOLATILE ORGANIC COMPOUNDS (MVOCS)

Encompassing the exploitation of micro-organisms and/or microbial enzymes which led to desirable biochemical changes and substantial variation to the food matrices, five are the main desired activities of microorganisms in fermented foods: *i)* enhancing the human dietary by means of improved/new flavours, aromas and textures, *ii)* increasing the shelf life and microbiological safety (lactic acid, alcoholic, acetic acid, alkaline fermentations and high salt fermentations), *iii)* enriching foods in vitamins, protein, essential amino acids and essential fatty acids, *iv)* reducing the toxicity of the substrate, *v)* decreasing cooking times and fuel requirements (Caplice and Fitzgerald 1999, Steinkraus 2002, Capozzi et al. 2012c, Capozzi et al. 2012d, Russo et al. 2014). Besides, fermentations might lead to more digestible foods and improve the functional content of final products (Caplice and Fitzgerald 1999, Arena et al. 2014). The preservation and the improvement of aroma, flavour and texture connected with microbial resources are key drivers of consumers' acceptance and choice (Borresen et al. 2012). Microbial associated volatile organic compounds (mVOCs) provide the molecular basis of aroma/flavour perception. In fact, many microbial metabolites are volatile organic compounds (VOCs), chemicals generally defined as organic compounds characterized by low water solubility, whose vapour pressure is at least 0.01 kPa at 20°C (Pagans et al. 2006). In pursuance thereof characteristics, this peculiar class of metabolites is usually able to diffuse across the membranes of microbial cells and be released into the food matrix. In 1921, Zoller and Clark (1921) described for the first time the production of volatiles by bacteria (formic and butyric acid). Schulz and Dickschat (2007), studying the chemistry of a panel of bacterial strains grown on defined artificial media, demonstrated the high heterogeneity of contribution in terms of bacterial volatiles (75 fatty acid derivatives, 50 aromatic compounds, 74 nitrogen-containing compounds, 30 sulfur compounds, 96 terpenoids, and 18 halogenated, selenium, tellurium, or other metalloid compounds), while Dickschat et al. (2005) and Kai et al. (2007) demonstrated the importance of species/strains contribution (e.g., Kai et al. (2007) reported up to 60 compounds per strain). The correlations between flavour properties and microbial VOCs (mVOCs) provide the basis for making qualified decisions in producing quality fermented foods, a subject of relevant economic and social importance. For example, it is noteworthy to consider that *a)* only GIs agro-food market size is approximately $50 billion (Capozzi and Spano 2011) and that *b)* fermented foods and beverages represent a relevant part of human diet, accounting for approximately one-third of the global food intake (Campbell-Platt 1994) (in fact, traditional fermented foods are diffused staple food for most developing countries and also the crucial healthy foodstuff for developed countries (Yang et al. 2010).

MICROBIAL VOCS RELEASE IN TRADITIONAL FERMENTED FOOD AND BEVERAGES: A MULTIFACETED PROBLEM

Contrary to other areas of standardization and innovation of sensory quality in the food industry, the optimization of mVOCs in traditional fermented foods is a broad and heterogeneous sector by reason of the many geographical, compositional, microbiological and

technological variables which have been reported in many recent review papers: traditional Indian fermented foods (Satish Kumar et al. 2013), fermented fruits and vegetables of Asia (Swain et al. 2014), African fermented foods (Franz et al. 2014), cereal based functional food of Indian subcontinent (Das et al. 2012), traditional healthful fermented products of Japan (Murooka and Yamshita 2008), traditional fermented plant foods and beverages in Eastern Europe (Sõukand et al. 2015), traditional fermented foods and beverages of Turkey (Kabak and Dobson 2011), cereal fermentations in Africa and Asia (Nout 2009). In each geographical region, fermented foods are produced from different raw materials (justifying important differences addressable to chemical composition): cereal products, dairy products, fish products, fruit and vegetable products, legumes, and meat products (Campbell-Platt 1987). In each of these categories, and for each geographical region, we might encounter hundreds of different productions. See, for instance, the quote attributed to Charles De Gaulle "How can you govern a country that makes five hundred different cheeses?" (Rudduck et al. 1994). Further differences are induced by fermentation activities: fermentations producing textured vegetable (e.g., Indonesian tempe and ontjom), high salt sauce and paste fermentations (e.g., Chinese soy sauce and Japanese miso), lactic acid fermentations (e.g., Russian kefir and sauerkraut), alcoholic fermentations (e.g., Egyptian bouza and Ethiopian tej), acetic acid fermentations (e.g., wine vinegars in the West and coconut water vinegar in the Philippines), alkaline fermentations (e.g., Nigerian dawadawa and Indian kenima) (according to the classification reported by Steinkraus (2002), with minor modifications). For each kind of fermentation, the biochemical changes are associated to a complex microbiota, in which several species might have protechnological properties. Table 1 provides a non-exhaustive list of protechnological yeasts and bacteria (Capozzi et al. 2011, Tristezza et al. 2013, Garofalo et al. 2015a) involved in wine fermentations (excluding spoilage microbes that produce undesired volatiles; e.g., *Brettanomyces bruxellensis* (Di Toro et al. 2014).

Finally, it is worth to stress the intraspecific diversity (Bisson et al. 2012) and the related fundamental strain-dependent protechnological characters. Given the huge number of interconnected variables there is a clear need for innovative analytical approaches that can manage the complexity of this sector which is relevant for both the economy and the nutrition and, in particular, for analytical techniques suitable for on-line monitoring without destroying the analyzed sample (e.g., in dry-cured or dry-fermented food as Turkish sausages (Kaban 2013)).

Table 1. Non-exhaustive list of microbial species protechnological interest involved in wine fermentations

Microbial species	Wine-making phase	References
Saccharomyces cerevisiae	Alcoholic fermentation	Legras et al. 2007
Hanseniaspora uvarum	Alcoholic fermentation	Moreira et al. 2011, Garofalo et al. 2015b
Torulaspora delbrueckii	Alcoholic fermentation	Bely et al. 2008
Candida zemplinina	Alcoholic fermentation	Masneuf-Pomarede et al. 2015
Candida guillermondii	Alcoholic fermentation	Moreira et al. 2011, Garofalo et al. 2015b
Oenococcus oeni	Malolactic fermentation	Campbell-Sills et al. 2015
Lactobacillus plantarum	Malolactic fermentation	Capozzi et al. 2012d
Pediococcus damnosus	Malolactic fermentation	Juega et al. 2014

THE NEEDS FOR SUITABLE APPROACHES FOR VOCS MONITORING: THE ADVANTAGES OF DIRECT-INJECTION MASS SPECTROMETRY (DIMS)

The recent and fast growing development of direct-injection mass spectrometric (DIMS) technologies for VOCs analysis opens new opportunities for the rapid monitoring and quantification of volatile organic compounds (VOCs). This class of techniques embraces different approaches including MS-e-noses, atmospheric-pressure chemical ionization mass spectrometry (APCI-MS), secondary electrospray ionization mass spectrometry (SESI-MS), selected ion-flow-tube mass spectrometry (SIFT-MS), and proton-transfer-reaction mass spectrometry (PTR-MS) (Biasioli et al. 2011, Bean et al. 2015). This panel of instrumental analytical systems can nowadays combine considerable mass and time resolution with high sensitivity and robustness (Biasioli et al. 2011).

Each approach has peculiar strengths and weaknesses, as a function *i)* of the instrumental design, *ii)* of ionization conditions, and *iii)* of the approach of analysis (for reviews, refer to Biasioli et al. (2011), Berchtold et al. (2014)).

In general, the option of switching between different precursor ions, together with the enhanced design of time-of-flight-based instruments, allows the effective detection of most VOCs of interest by DIMS (Biasioli et al. 2011). Several recent uses of the DIMS technologies for the rapid monitoring and quantification of VOCs in fermented foods testify the wide range of application of the existing analytical methods (Table 2).

DIMS-based MS-e-noses, simulating the behaviour of human olfaction, provide a digital fingerprint of the analysed product, with good prospect for the large sample sets screening (Ballabio et al. 2006, Biasioli et al. 2011), but with possible disadvantages such as high sensitivity to moisture, poor linearity, and poor reproducibility. APCI, performing ionization at atmospheric pressure, lessens any loss of volatiles addressable to an inefficient transport of neutral molecules into the vacuum, considered to be robust, sensitive, and reproducible in flavor-release applications (Biasioli et al. 2011, Berchtold et al. 2014). While APCI ionization is quite complex due to the presence of many possible ionization agents, SIFT-MS and PTR-MS allow an improved control of precursor-ion generation and hence of the ionization process. The SIFT technique, focusing on control of the ionization process, is very effective in the investigation of ion-molecules reactions (Lourenço and Turner 2014).

On the other hand, PTR-MS focuses on sensitivity and, thanks to the recent implementation of high-resolution mass analysers, on chemical information. The further coupling with automated sampling systems and tailored data analysis tools constitutes a complete set-up which is suitable for both the identification of very small quantities of VOCs and the real-time monitoring of VOCs without sample preparation (Yener et al. 2014, Romano et al. 2014, Romano et al. 2015).

Recently, this strategy has been employed for the study of processes of outmost interest for the industry of food fermentations, such as lactic acid fermentation and alcoholic fermentation, allowing, in particular, the real-time monitoring of VOCs associated with the fermentative performances of different starter cultures of the principal protechnological microbes involved in food fermentations: *Saccharomyces cerevisiae* and lactic acid bacteria, in bread (Makhoul et al. 2014) and yogurt (Benozzi et al. 2015).

Table 2. Exemplificative list of scientific studies applying Direct-Injection Mass Spectrometric (DIMS) technologies to monitor VOC content in fermented food/ beverage matrices

Fermented foods and beverages matrices	Fermentation monitored (Y/N)	DIMS technologies	References
Cheese	N	APCI-MS	Taylor et al. 2000
Cheese	N	MS-eNOSE	Pérès et al. 2002
Yogurt	N	PTR-MS	Mei et al. 2004
Fermented Whey	Y (LAF)	PTR-MS	Gallardo-Escamilla et al. 2005
Yogurt	N	APCI-MS	Saint-Eve et al. 2006
Fermented whey	N	PTR-MS	Gallardo-Escamilla et al. 2007
Cheese	N	PTR-MS	Boscaini et al. 2008
Cheese	N	PTR-MS	Bovolenta et al. 2008
Cheese	N	MS-eNOSE	Botre et al. 2009
Cheese	N	PTR-ToF-MS	Fabris et al. 2010
Yogurt	Y (LAF)	PTR-ToF-MS	Soukoulis et al. 2010
Wine	N	MS-eNOSE	Vera et al. 2010
Dry Fermented Sausage	N	SIFT-MS	Olivares et al. 2011
Cheese	N	PTR-ToF-MS	Galle et al. 2011
Yogurt	N	PTR-ToF-MS	Soukoulis et al. 2011
Cheese	N	SIFT-MS	Langford et al. 2012
Yogurt	Y (LAF)	PTR-ToF-MS	Tsevdou et al. 2013
Cheese	N	SIFT-MS	Taylor et al. 2013
Bread	Y (AF)	PTR-ToF-MS	Makhoul et al. 2014
Cheese	N	SIFT-MS	Castada et al. 2014
Wine	N	PTR-ToF-MS	Romano et al. 2014
Bread	Y (AF)	PTR-ToF-MS	Makhoul et al. 2015
Cheese	N	PTR-ToF-MS	Bergamaschi et al. 2015
Cheese	N	SESI-MS	Bean et al. 2015
Yogurt	Y (LAF)	PTR-ToF-MS	Benozzi et al. 2015
Sourdough	Y (LAF, AF)	SIFT-MS	Van Kerrebroeck et al. 2015

Direct-injection mass spectrometric (DIMS); Proton Transfer Reaction-Mass Spectrometry (PTR-ToF-MS); Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS); Secondary Electrospray Ionization-Mass Spectrometry (SESI-MS), Atmospheric Pressure Chemical Ionization-Mass Spectrometry (APCI-MS); Lactic Acid Fermentation (LAF); Alcoholic fermentation (AF); Yes (Y); Not (N).

CONCLUSION

In this note, we propose that direct-injection mass spectrometric (DIMS) technologies, being a valid compromise between rapid/on-line and high analytical informational content, are interesting methodologies in order to develop strategies for the rapid monitoring and quantification of microbial volatile organic compounds in fermented foods.

The final aim is to conceive 'tailored' approaches to improve authenticity preservation and innovation management in the field of traditional fermented foods, a fascinating sector of food industry relevant for the economy and human nutrition.

In addition, direct high-resolution mass spectrometry techniques, if considered high-throughput and non-target applications in 'foodomics' analysis (Ibáñez et al. 2015), represent an intriguing opportunity for the formulation of new hypotheses in food science and nutrition, particularly in the light of the recent attention deserved to fermented foods as valuable models of microbial ecosystems (Wolfe and Dutton 2015).

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