Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Food Microbiology 86 (2003) 23–50
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Review

Spoilage yeasts in the wine industry

V. Loureiro*, M. Malfeito-Ferreira

Laboratório de Microbiologia, Departamento de Botânica e Engenharia Biológica, Instituto Superior de Agronomia, 1349-017 Lisbon, Portugal

Accepted 14 May 2003

Abstract

Yeasts play a central role in the spoilage of foods and beverages, mainly those with high acidity and reduced water activity (a_w). A few species are capable of spoiling foods produced according to good manufacturing practices (GMPs). These can survive and grow under stress conditions where other microorganisms are not competitive. However, many of the aspects determining yeast spoilage have yet to be clarified. This critical review uses the wine industry as a case study where serious microbiological problems are caused by yeasts. First, the limitations of the available tools to assess the presence of spoilage yeasts in foods are discussed. Next, yeasts and factors promoting their colonisation in grapes and wines are discussed from the ecological perspective, demonstrating that a deeper knowledge of vineyard and winery ecosystems is essential to establish the origin of wine spoilage yeasts, their routes of contamination, critical points of yeast infection, and of course, their control. Further, zymological indicators are discussed as important tools to assess the microbiological quality of wines, although they are rarely used by the wine industry.

The concepts of the susceptibility of wine to spoilage yeasts and wine stability are addressed based on scientific knowledge and industrial practices for monitoring yeast contamination. A discussion on acceptable levels of yeasts and microbiological criteria in the wine industry is supported by data obtained from wineries, wholesalers, and the scientific literature.

Finally, future directions for applied research are proposed, involving collaboration between scientists and industry to improve the quality of wine and methods for monitoring the presence of yeast.

Keywords: Yeast; Spoilage yeast; Wines; Contamination; Differential media; Zymological indicators; *Zygosaccharomyces bailii*; *Brettanomyces*/*Dekkera* spp.

1. Introduction

When we look at any subject on food microbiology published during the last 50 years, it appears that food spoilage caused by yeasts receives little attention, even in foods commonly spoiled by yeasts. Analysis of the works published on food spoilage by yeasts, starting from the classical review of Ingram (1958) to the book of Deak and Beuchat (1996) leads to the conclusion that little has changed in the knowledge of the biological processes and microbial interactions involved in food spoilage by yeasts. The following questions should still be asked. (i) What is spoilage yeast? (ii) Does the food industry have adequate information to be sufficiently aware of the microbiological problems of a food commodity? (iii) What are the sources of

* Corresponding author. Tel.: +351-21-3653448; fax: +351-21-3635031.
E-mail address: vloureiro@isa.utl.pt (V. Loureiro).

0168-1605/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0168-1605(03)00246-0

spoilage yeasts in the food industry? (iv) Does the food industry have the appropriate zymological indicators to assess the quality of foods and to establish fair commercial contracts with retailers and wholesalers?

2. Concept of "spoilage yeast"

In many cases, microbial spoilage is not easily defined, particularly in fermented foods and beverages, where the metabolites produced contribute to the flavour, aroma, and taste of the final products. In fact, for cultural or ethnic reasons, there is little difference between what is perceived as spoilage or beneficial activity (Fleet, 1992). An example of this can be found in the wine industry, where the production of 4-ethylphenol by *Brettanomyces*/*Dekkera* spp. in red wines is only regarded as spoilage when this secondary metabolite is present at levels higher than about 620 μg/l (Chatonnet et al., 1992, 1993). At less than 400 μg/l, it contributes favourably to the complexity of wine aroma by imparting aromatic notes of spices, leather, smoke, or game, appreciated by most consumers. Above 620 μg/l, the wines are clearly substandard for some consumers, but remain pleasant for others.

One of the most recent handbook of yeast taxonomy describes the characteristics of 761 species (Boekhout et al., 2002). Of these, about a quarter may be isolated from foods, but only a handful plays a significant role in food spoilage. Those that can survive in foods but are not able to grow and, for that reason, do not affect the sensory appeal of the food may be termed adventitious or innocent; those responsible for undesirable changes are called spoilage yeasts. However, for food technologists, the concept of spoilage yeast has, in general, a stricter sense. It applies only when a particular species is able to spoil foods which have been processed and packaged according to the standards of good manufacturing practices (GMPs) (Pitt and Hocking, 1985), in spite of the subjective character of these practices. If this is not achieved, many other adventitious yeast contaminants can develop in a product (Pitt and Hocking, 1985). This distinction is shown by the contamination species listed in order of frequency in Table 1, where widespread adventitious contaminants are not re-

Table 1
Contamination and spoilage yeast recovered from foods and beverages

Most frequent contaminants[a] (Deak and Beuchat, 1996)	Spoilage species (Pitt and Hocking, 1985)	Additional spoilage species (Tudor and Board, 1993)
Saccharomyces cerevisiae	*Brettanomyces intermedius*[b]	*Candida dattila*
Debaryomyces hansenii	*Candida holmii*	*Candida globosa*
Pichia anomala	*Candida krusei*	*Candida humicola*
Pichia membranifaciens	*Debaryomyces hansenii*	*Candida lactis-condensi*
Rhodotorula glutinis	*Kloeckera apiculata*	*Candida lipolytica*
Rhodotorula mucilaginosa	*Pichia membranifaciens*	*Candida parapsilosis*
Torulaspora delbrueckii	*Saccharomyces cerevisiae*	*Candida saké*
Kluyveromyces marxianus	*Schizosaccharomyces pombe*	*Candida versatilis*
Issatchenkia orientalis	*Zygosaccharomyces bailii*	*Candida zeylanoides*
Zygosaccharomyces bailii	*Zygosaccharomyces bisporus*	*Cryptococcus* spp.
Candida parapsilosis	*Zygosaccharomyces rouxii*	*Hansenula anomala*
Zygosaccharomyces rouxii		*Hansenula subpelliculosa*
Candida guilliermondii		*Kluyveromyces marxianus*
Candida albidus		*Pichia burtonii*
Candida tropicalis		*Pichia fermentans*
Saccharomyces exiguus		*Sporobolomyces roseus*
Pichia fermentans		*Torulaspora delbrueckii*
Trichosporon pullulans		*Trichosporon cutaneum*
Hanseniapora uvarum		*Trycosporum pullulans*
Candida zeylanoides		

[a] Species presented by decreasing level of occurrence.
[b] Anamorph of *D. bruxellensis*.

garded as spoilers (e.g., *Rhodotorula* spp.) and dangerous spoilage species are not necessarily frequent contaminants (e.g., *Brettanomyces bruxellensis*).

The observation that only a small group of about 10 species of yeast is responsible for food spoilage was made by Pitt and Hocking (1985). Tudor and Board (1993) added a second group composed of 18 species and 1 genus (Table 1). These "second-division" yeasts are associated with spoilage of foods, which also allow the growth of bacteria, mainly Gram-negative with simple nutritional requirements. In these foods, the factor(s) favouring the growth of yeasts over bacteria cannot be identified. These yeasts should be added to those listed by Pitt and Hocking (1985), which usually appear in foods preserved by extreme abiotic stress factors, mainly low water activity (a_w) and high acidity, that inhibit bacterial growth (Tudor and Board, 1993). These authors presented a table of contamination yeasts comprising 33 genera and 213 species (no attempt was made to determine the number of species according to the latest nomenclature), while Deak and Beuchat (1996) listed 35 genera and 109 species most frequently occurring in foods, without specifying their relative spoilage potential. Although the exact number of species important in food spoilage is debatable, there is no doubt they represent a small proportion of yeasts that can be found in foods.

In fermented alcoholic beverages, the concept of spoilage yeast has a more complex meaning than in nonfermented foods, where any yeast able to change food sensorial characteristics can be regarded as a "spoilage yeast." In fermented drinks or foods, yeast activity is essential during the fermenting process. Detrimental and beneficial activity must therefore be distinguished. In the wine industry where alcoholic fermentation occurs in the presence of many yeast species and bacteria (mainly lactic and acetic), it is very difficult to draw a line between beneficial fermenting activity and spoilage activity. For this reason, spoilage yeasts are rarely sought during wine fermentation, but during storage or aging and during the bottling process. However, many detrimental effects of yeasts occur before fermentation (e.g., ethyl acetate produced by *Pichia anomala* (Plata et al., 2003)) or the early stage of fermentation (e.g., acetate production by *Kloeckera apiculata*/*Hansaniaspora uvarum* (Romano et al., 1992)). Thus, monitoring of spoilage yeasts should include all phases of winemaking.

The concept of wine spoilage yeasts sensu stricto includes only those species able to affect wines that have been processed and packaged according to GMP. As an example of the abovementioned definitions, Deak and Beuchat (1996) listed 39 species as the most frequent wine-related yeast contaminant, but spoilage are much fewer. For instance, Kunkee and Bisson (1993) put them in the following groups: (i) fermenting strains (*Saccharomyces cerevisiae*), able to referment sweet bottled wines; (ii) *Zygosaccharomyces bailii*; (iii) film-forming yeasts (*Hansenula*, *Kloeckera*, *Pichia*, *Metchnikowia*, *Debaryomyces*); (iv) *Brettanomyces* spp. Similarly, Sponholz (1992) highlighted the problems due to ester production and film formation by species of *Hansenula*, *Candida* and *Pichia*, while referring to *Z. bailii* and *Brettanomyces* spp. as the most dangerous wine spoilage yeasts. The relevance of *Z. bailii* and *Brettanomyces* spp. was also stressed by Boulton et al. (1996) and Fugelsang (1997), who described their spoilage abilities in detail. *Schizosaccharomyces pombe* and *Saccharomycodes ludwigii*, although dangerous spoilers, are not regarded as common contaminants (Kunkee and Bisson, 1993; Fugelsang, 1997). Basically, the experience of the present authors confirms the statements above, and the species most frequently encountered are described in Section 5.1.3. We consider *D. bruxellensis*, *Z. bailii*, and *S. cerevisiae* as spoilage yeasts sensu stricto. However, this last species appears to be more dangerous than indicated by the abovementioned authors, as some strains isolated from dry white wines seem to be a more potential spoilage yeast than *Z. bailii* due to its sorbic acid and sulphite tolerance at high ethanol levels (Malfeito-Ferreira et al., 1989). Furthermore, strains of *S. cerevisiae* have frequently been associated with refermentation of bottled "dry" red wines due to the presence of residual sugars in high ethanol (>13% v/v) wines (unpublished observations).

2.1. The increasing importance of yeasts in food spoilage

The spoilage of foods and beverages by yeasts has gained an increasing importance in food industry (Thomas, 1993). The reasons for this include the use of modern technologies in food processing, the great variety of new formulations of foods and beverages, the tendency to reduce the use of preservatives, par-

ticularly those effective against yeasts (e.g., sulphur dioxide and benzoic acid), and less-severe processing (Fleet, 1999; Loureiro and Querol, 1999). The first handbook of spoilage yeasts (Deak and Beuchat, 1996) reflects increasing awareness of the problems caused by yeast spoilage in food.

The increasing importance of yeasts in food spoilage is well illustrated by the case of wine industry. Microbial spoilage of wines may also be due to the activity of lactic and acetic bacteria. In fact, most traditional wine ''diseases'' are bacterial in origin (Sponholz, 1992; Boulton et al., 1996; Ribéreau-Gayon et al., 2000). However, advances in wine technology and improvement in GMPs, e.g., equipment design, sanitation procedures, and use of preservatives, have led to the virtual extinction of these diseases, most of which have never been encountered by today's oenologists. On the contrary, yeasts are now the most feared cause contaminants leading to wine spoilage. The common spoilage effects are film formation in stored wines, cloudiness or haziness, sediments, and gas production in bottled wines, and off-odours and off-tastes at all stages of wine production.

Increasing quality demand by consumers also extended the range of spoilage problems or decreased the tolerance to aspects which were not formerly taken to be defects, most of which are due to yeast activity (e.g., slight haziness in bottled wines, phenolic tainted wines). For example, *Dekkera*/*Brettanomyces* spp. have been well known since the beginning of the 20th century (see references cited in Van der Walt and van der Kerken, 1958, 1961), but has only attracted the attention of wine technologists in the last decade. Peynaud and Domercq (1956) referred to the production of acetic acid and ''mousy'' off-odours in grape juice, but their main effect —off-flavours due to volatile phenols (4-ethylguaiacol and 4-ethylphenol)—was yet to be discovered. Tucknot et al. (1981) reported that these yeasts were the only species isolated from wines with mousy and other ill-defined off-odours. Heresztyn (1986) demonstrated the production of volatile phenols by *Brettanomyces* in grape juice, but lactic acid bacteria were thought to be also responsible for their production in wines (Cavin et al., 1993). Hock (1990) described the concern caused by these yeasts in Californian wines, but the problem was then related to obnoxious flavours and odours and not specifically to the production of 4-ethylphenol (Kunkee and Bisson, 1993). Thomas (1993) did not link *Brettanomyces* with the production of volatile phenols but with other ''classical'' spoilage activities like haze formation and acetic acid and tetrahydropyridine production. Sponholz (1992) mentioned the production of 4-ethylphenol by *Brettanomyces*, but did not relate it to phenolic taint. By that time Chatonnet et al. (1992, 1995, 1997) had demonstrated that the genera *Dekkera*/*Brettanomyces* are the sole agents of phenolic off-flavours in wines. The monographs of Boulton et al. (1996) and Fugelsang (1997) described thoroughly, for the first time, the characteristics of *Dekkera*/*Brettanomyces* spp., including the production of phenolic off-odours described as ''barnyard-like'' or ''horsey'' and the positive or negative responses by consumers. In addition, we have found other species capable of producing 4-ethylphenol, with variable efficiency in grapes, grape juice, insects, and cellar equipment (Rodrigues et al., 2001; Dias et al., in press). Among these, *Pichia guilliermondii* showed conversion rates of *p*-coumaric acid into 4-ethylphenol similar to *Dekkera bruxellensis* (Dias et al., in press), but apparently, it cannot grow in wines (unpublished observations). Therefore, *D. bruxellensis* is today considered to be the main cause of wine spoilage, especially of fashionable premium red wines matured in oak casks, where it can be responsible for serious economic losses.

3. Methods of assessing the presence of spoilage yeasts in food ecosystems: very limited and few improvements in last decades

There are several techniques used to show the presence of spoilage yeasts in foods. However, undoubtedly, the spread plate technique is still the most popular and will be described in more detail.

The microbiological analysis of a food sample may be compared to taking a photo of the sample, aiming to show the species and size of the yeast population. As in photography, the sharpness depends on the tools and techniques used, which are, for the plate technique (i) sampling, (ii) pretreatment techniques (maceration/blending of the sample, dilution, and enrichment), (iii) counting techniques (culture media, incubation conditions), and (iv) identification procedures.

Traditionally, in most studies of microbial ecology of foods, more attention is paid to the identification of

isolated strains than to the previous steps. This statement is well illustrated by reading the many papers using molecular methods mentioned in Section 3.3. The titles of most publications may give the idea that the aim is to study yeast dissemination or ecology, but in fact, the main concern seems to be the discussion of the identification or molecular typing methods used. This leads to many redundant papers that add little or nothing to results obtained before molecular typing was used. Similarly, the ecology of "spontaneous" wine fermentation is studied by a lot of these research teams, but true spontaneity is absent once grape juice is sulphited, as described in many papers. On other hand, in routine industrial analysis, attention is seldom given to strain identification, and previous steps are carefully performed according to standardised procedures. Such standardisation, however, does not mean that the techniques of sampling, pretreatment, and counting are adequate.

3.1. Pretreatment techniques

3.1.1. Maceration/blending of the food sample

This operation is much more important for solid or liquid foods with suspended solids than for clear liquids like wine because yeast cells may adhere with different intensity to solid surfaces. Scanning electron microscopy studies of the distribution of microbial cells on vegetable tissues, such as leaves (Beech and Davenport, 1970) and grapes (Belin, 1972), showed that yeasts are present in the form of microcolonies firmly adhering to the substrate. A more complicated situation is the entrapment of cells in the reticulate structures of certain foods, where they are immobilised and localised in high densities (Fleet, 1999). For the isolation of yeast and moulds, maceration/blending procedures may consist of manually shaking the sample, after grinding if necessary, with a known volume of diluent, mixing with a diluent in a blender, or pummelling with a diluent in a Stomacher® (peristaltic agitator). Diluents commonly used comprise distilled water, saline, phosphate buffer and the most common, 0.1 % (w/v) peptone water. Contact time ranges from less than 1 to several minutes (generally 5–10 min).

Based on the assumption that the separation of yeast cells from natural habitats requires much rougher treatment of samples, a series of ecological surveys were conducted on the yeast population of different fruits (Martini et al., 1980). The overall results clearly indicate that pre-isolation treatments based on vigorous shaking, percolation with an excess of water, and the sonication of samples allowed the recovery of a higher number of colony forming units and species. In addition, as Fleet (1999) stated, "the assumption that maceration is an ecologically sound prelude to microbiological analysis requires a more rigorous scrutiny, especially since it is already known that extracts of vegetables, herbs, and spices are toxic to some microorganisms."

3.1.2. Dilution

To many microbiologists sample dilution is a routine operation, perfectly defined and harmless to yeast cells. There is even the conviction that yeasts are more resistant to osmotic shock than bacteria, and so the diluent composition is not relevant. However, holding periods of 1 (Beuchat et al., 2002) or 2 h (Mian et al., 1997) have been reported to cause significant reductions in yeast populations, regardless of the type of diluent. These periods may seem long to a bench microbiologist but are common in industry.

Sterile peptone water (0.1% w/v) is the recommended diluent for preparing samples to be plated on general-purpose enumeration media (Samson et al., 1992). However, given the diversity of food intrinsic characteristics and yeast biodiversity, there is no ideal diluent. It is always necessary to consider the nature of the food and the species sought in order to select the diluent (Deak and Beuchat, 1996). Furthermore, it should not be forgotten that the most important objective is to recover spoilage yeasts. Although it is necessary to standardise this operation, it is recognised by the International Commission on Food Mycology that specific protocols are not yet available, either with respect to type of food or sample contact time. This makes it difficult to compare results from different laboratories. In addition, according to Fleet (1999), based on an international collaborative study under the auspices of the abovementioned Commission, it is possible to conclude that apart from diluent composition and timing between dilution and plating, other factors such as stage of cell life cycle, cell stress prior to dilution, degree of cell clumping and aggregation, shear forces during shaking, presence of contaminating metal ions, pH, and temperature could all have an impact on the survival of the yeast cells during

dilution. Contrary to what is generally believed, dilution may not be a harmless procedure for yeasts.

3.1.3. Enrichment

Enrichment cultures are commonly used in food bacteriology to detect pathogenic species and other minority species present in foods. For yeasts, it is not common to do so, and it is unclear if it brings any advantage. As mentioned before, the results of Martini et al. (1980) suggested that vigorous and disruptive sample treatments of natural substrates achieve better results than enrichment cultures. However, the same authors suggest the use of enrichment cultures to detect fermenting species. Accordingly, higher frequencies of detection of *S. cerevisiae* (van der Westhuizen et al., 2000a) and *S. cerevisiae* and *Saccharomyces paradoxus* (Redzepovic et al., 2002) in sound grapes may be attributed to an enrichment step using a fermentation broth. As most of spoilage yeasts are fermenting species that are present in foods and natural substrates at very low levels, it is conceivable that enrichment may improve their detection. In addition, the recovery of cells sublethally injured by heat, osmotic or acid shock may require use of resuscitation techniques (Fleet, 1992; Deak and Beuchat, 1996), which may be also designed to select the spoilage yeast (Thomas and Ackerman, 1988).

3.2. Counting techniques

3.2.1. Culture media

Isolation and enumeration media for foodborne yeasts are usually complex and nutritionally rich, containing sugar as energy source (e.g., glucose, fructose, sucrose), a digested protein as nitrogen source (e.g., peptone, tryptone, casitone), and a complex supplement (e.g., yeast extract, malt extract). Additionally, they can contain one or more antibiotics against bacteria (e.g., oxytetracycline, chloramphenicol), a compound to inhibit the most rapidly spreading moulds (e.g., rose bengal, dichloran, sodium propionate, or the antibiotic oligomycin), sometimes a pH indicator (e.g., bromocresol green, bromophenol blue). Many studies have concluded that these media generally recover yeasts better than the earlier media acidified with organic or inorganic acids to pH around 3.5 (Beuchat, 1993). Unfortunately, all of these media are specially designed to recover the maximum number of yeast cells present in foods instead of targeting only spoilage yeasts. This is a problem because most innocent yeasts are fast growers, which inhibit the growth of slow-growing yeasts, which include some of the most dangerous spoilage yeasts (e.g., *Zygosaccharomyces* spp. and *Dekkera* spp.). In conclusion, the usual culture media used in food mycology may be inappropriate to give a ‘‘real image’’ of the food ecosystem. Ideally, the best medium to enumerate yeast cells on foods should prevent the growth of all innocent yeast and promote the growth of all spoilage yeasts. As this is virtually impossible, other strategies must be used, as described below.

Several media have been developed by choosing formulations and incubation conditions favourable to particular groups, including psychrotrophic, acid-resistant, and xerotolerant (osmophilic) yeasts (Fleet, 1992; Deak and Beuchat, 1996). In brewing, it is important to discriminate between *S. cerevisiae* fermenting yeasts and ‘‘wild yeasts,’’ which may be undesirable *S. cerevisiae* strains, other *Saccharomyces* species, or non-*Saccharomyces* species. Many culture media have been developed with that goal (Lysine agar, Lin’s medium, Schwarz differential medium, copper sulphate medium, etc.) with different efficiencies (Deak and Beuchat, 1996). A recent report indicated that copper sulphate medium was the best medium to discriminate between wild yeasts (including wild *S. cerevisiae*) and fermenting yeasts in lager beers (Kühle and Jespersen, 1998). Molybdate agar supplemented with 0.125% propionate was found adequate to distinguish several yeast species isolated from tropical fruits, but it was not tested in other food commodities (Rale and Vakil, 1984).

In wines, ethanol (11.4% v/v) has been successfully used as a selective agent in a medium developed to detect spoilage yeast (Thomas and Ackerman, 1988). Rodriguez (1987) devised a scheme based on growth in several media to distinguish food spoilage yeasts, but the scheme seems not to be appropriate given that the most dangerous species, *Z. bailii*, gave results coincident with other species. Heard and Fleet (1986) used Lysine agar to detect non-*Saccharomyces* species in wine and preferred the generic medium malt extract agar (MEA) to count *S. cerevisiae* instead of ethanol sulphite agar (ESA) (containing 12% v/v ethanol and 150 mg/l total sulphite), which was developed by Kish et al. (1983) to select wine yeast

in the presence of excessive numbers of apiculate yeasts. Cadaverin lysine ethylamine nitrate (CLEN) agar aimed at non-*Saccharomyces* in beer is not appropriate for wines because it supports growth of *S. cerevisiae* wine strains (Fernández et al., 2000). Fugelsang (1997) described media developed for the detection of *Dekkera* spp. and *Z. bailii.* To the best of our knowledge, only one "specific" medium is commercially available to detect *Dekkera* spp., i.e., *Brettanomyces* specific medium (BSM, Millipore), containing cycloheximide and 20 g/l glucose. According to our experience, a culture medium with 20 g/l of glucose is not appropriate to detect *Brettanomyces* cells, because the sugar favours fast-growing species, e.g., *K. apiculata*, *Candida tropicalis*, and *P. guilliermondii*. It is not then a medium specific for *Brettanomyces*, but merely a medium for species resistant to cycloheximide. Confirming our expectations, practical results demonstrate the recovery on BSM of colonies of non-*Dekkera* species characterised by small spherical cells which may be confused with *Dekkera* by inexperienced users (Stender et al., 2001).

The classical developments of selective media rely on the introduction of stress factors leading to the selection of few or single species. This approach may leave undetected strains of the target species with lower resistance to such stress factors, or may detect highly resistant strains of species assumed to be sensitive. This situation may be irrelevant for a particular food, but may be a severe limitation for widespread use in the food industry. For instance, *Z. bailii* agar (ZBA) medium has been found effective for detecting *Z. bailii* in acidified ingredients, mainly due to the addition of acetic and sorbic acids (Erickson, 1993). However, when it was tested in other food commodities, it was found to be less efficient than other general media with added acetic acid (Hocking, 1996), particularly in acidic foods with reduced a_w or acidic beverages (Makdesi and Beuchat, 1996a,b).

Another approach exploits particular enzymatic features which are restricted to a single species, in combination or not, with stress factors. The medium of Chaskes and Tyndall (1975) may be regarded as the precursor of this type of medium. It allows a clear-cut identification of the pathogen yeast *Cryptococcus neoformans* by containing 3,4-dihydroxyphenylalanine (DOPA), which is converted to black pigments. Following this strategy, several media have been proposed to detect particular spoilage species (Table 2). The degrees of efficiency are variable. *Dekkera*/*Brettanomyces* differential medium (DBDM) selects *D. bruxellensis* and other species owing to the utilisation of ethanol as single carbon and energy source and resistance to cycloheximide and by the use of a pH indicator and *p*-coumaric acid, the precursor of 4-ethylphenol, which is easily detected by imparting a phenolic smell to the medium. This medium proved its high efficiency to recover *D. bruxellensis* from wine samples. However, its use in a wider range of samples (grapes, insects, grape juice) showed the presence of *P. guilliermondii*, which was for the first time recognised as a strong 4-ethylphenol producer (Dias et al., in press). To distinguish these two species, another feature must be considered: colonies of *D. bruxellensis* take at least 6–7 days to become visible, while those of *P. guilliermondii* appear within 2–3 days. This feature exemplifies another limitation of current enumeration protocols which usually consider that 48–72 h of incubation is enough to detect

Table 2
Culture media for the enumeration of particular foodborne yeasts

Medium	Target species	Differential characteristics	Target food	Reference
YLM	*Yarrowia lipolytica*	brown discoloration of the agar medium containing tyrosine	cheese	Carreira and Loureiro (1998)
KDM	*Kluyveromyces marxianus* and *Kluyveromyces lactis*	blue colonies indicating presence of β-galactosidase in the absence of lactose, agar colour changes from green to blue	dairy products	Valderrama et al. (1999)
ZDM	*Zygosaccharomyces bailii*	blue colonies growing on glucose and formic acid	wine	Schuller et al. (2000)
DBDM	*Dekkera*/*Brettanomyces* spp.	pin-point yellow to green colonies, phenolic odour, slow growth, agar colour changes from blue to yellow	wine, soft drinks	Rodrigues et al. (2001)

foodborne yeasts. This is not so, particularly for slow-growing wine spoilage species as *Z. bailii* or *D. bruxellensis*, requiring incubation periods as long as 14 days (Millet and Lonvaud-Funel, 2000; Rodrigues et al., 2001).

he use of these selective and differential media is not yet widespread in industry and, given the short time of application, their acceptance among scientists has yet to be ascertained. In the field of clinical microbiology, there are some media directed to the selective and differential recovery of pathogenic yeasts (Freydiere et al., 2001), but they do not seem to be suitable for the food industry.

3.2.2. Other techniques

Numbers of yeast may be determined by techniques other than the standard plate counts. They include, for instance, Petrifilm, Redigel, and SimPlate methods, which are reported to yield comparable results (Beuchat et al., 1998). These methods still depend on cultivation, but other techniques avoid this step, such as epifluorescence microscopy, bioluminescence, and flow cytometry as reviewed by De Boer and Beumer (1999) and Veal et al. (2000). Up to now, these techniques are not familiar to most wineries, although specific research has been published on the subject (Kuniyuki et al., 1984; Henschke and Thomas, 1988; Thomas and Ackerman, 1988; Bouix et al., 1999; Kopke et al., 2000).

A classical technique for estimating microbial loads in foods is the most probable number (MPN) (Harrigan, 1998). It is more common for solid foods, but it is also adequate for liquid samples with suspended solids such as fortified wines (Vaz-Oliveira et al., 1995). This technique was also essential to recover *D. bruxellensis* present in numbers less than 0.1% of total microbial population (Rodrigues et al., 2001). We believe that the MPN technique, using selective media, is especially useful for enumerating minority spoilage or fermenting yeast. It seems not to be used very often compared to membrane filtration and incubation of the filter on agar surfaces.

3.3. Typing and identification techniques

Classical identification is based on physiological, biochemical, or sexual characteristics and cannot be routinely used in the food industry. As a consequence, various miniaturised and simplified identification methods have been developed. However, they use the same approach as the classical identification methods, being time consuming, even when procedures are automated and computerised, and often result in false identifications (Deak and Beuchat, 1996). To overcome these difficulties, faster typing methods have been developed, based, among others, on analysis of total proteins, long-chain fatty acids, and isoenzymes (Fleet, 1992; Deak and Beuchat, 1996; Loureiro and Querol, 1999). Presently, physiological and biochemical tests are still being developed and used. The simplified identification method (SIM) updated and published by Deak and Beuchat (1996) has recently been applied to fruit juice isolates (Sancho et al., 2000), and Velásquez et al. (2001) presented a system to identify foodborne yeasts updating an initial 10-test kit for wine yeasts.

3.3.1. Nucleic acid-based typing

The amazing development of molecular biological techniques in the last 20 years justifies a closer look regarding their application at the industrial level. Recent progress in molecular biology has contributed to the development of powerful typing techniques, almost always following techniques primarily developed for bacterial identification. Techniques like restriction fragment length polymor-phism (RFLP) of mitochondrial DNA, chromosomal DNA electrophoresis, restriction enzyme analysis of polymerase chain reaction (PCR)-amplified ribosomal DNA, random amplified polymorphic DNA (RAPD) assay are now familiar to food microbiologists. For description of techniques and critical reviews of the bases of molecular taxonomy, see, for instance, Van der Vossen and Hofstra (1996), Loureiro and Querol (1999), and Querol et al. (in press). However, in spite of huge efforts in the development of these techniques, they have not yet reached the bench of industry microbiologists.

Table 3 summarises the most recent applications of molecular methods for yeast typing in wine industry. Molecular identification techniques rely mostly on rRNA gene sequences because of their taxonomic significance, but are not suitable for routine use in industry (Boekhout et al., 1994; Cai et al., 1996; Kurtzman and Fell, 1998). Therefore, references to

Table 3
Recent molecular biological techniques for typing wine-related yeasts

Technique	Target species	Biomass source	Reference	Observations
RFLP-mtDNA restriction analysis with several restriction enzymes	*S. cerevisiae* and *Saccharomyces* spp.	broth cultures or plate colonies	Guillamón et al. (1994), Epifanio et al. (1999), Gutiérrez et al. (1999), Esteve-Zarzoso et al. (2000), Comi et al. (2000), Torija et al. (2002)	based on Querol et al. (1992a,b)
RFLP-mtDNA restriction analysis with *Hinf*I and *Dde*I	*S. cerevisiae* and several wine related species	broth cultures in microcentrifuge tubes	López et al. (2001)	improvement of Querol et al. (1992a,b) technique; time: 25, 5 h
PCR-RFLP of the 5.8S, 18S or 28S ITS regions of the rRNA gene, followed by restriction with several enzymes	Several yeast species	plate colonies or broth cultures	Guillamón et al. (1998), Esteve-Zarzoso et al. (1999), Dlauchy et al. (1999), Fernández et al. (2000), Egli and Henick-Kling (2001), Granchi et al. (1999), Redzepovic et al. (2002)	based on White et al. (1990)
RFLP-mtDNA restriction analysis	*S. cerevisiae* and several wine species	plate colonies	Gutiérrez et al. (2001)	based on Querol et al. (1992a,b)
PCR-RFLP of 18S rRNA and ITS 1 region	several wine species			based on Esteve-Zarzoso et al. (1999)
RFLP-mtDNA restriction analysis; electrophoretic karyotyping by CHEF; PCR amplification of δ sequences	*Saccharomyces* spp. starters	plate colonies	Fernández-Espinar et al. (2001)	based on Querol et al. (1992a,b)
RFLP-mtDNA restriction analysis	*S. cerevisiae*	broth cultures	Constantí et al. (1998)	based on Querol et al. (1992a,b)
PCR-RFLP of the 5.8S-ITS region of the rRNA gene	several yeast species			based on White et al. (1990)
PCR fingerprinting with $(GAC)_5$ and $(GTG)_5$ primers and NTS region amplification and restriction with *Hae*III and *Msp*I	*S. cerevisiae* and *K. apiculata*	broth cultures	Caruso et al. (2002)	based on Baleiras-Couto et al. (1995, 1996)
Electrophoretic karyotyping by CHEF	several species	plate colonies	Schütz and Gafner (1993), Egli et al. (1998)	according to Schütz and Gafner (1993)
RFLP-PCR of rDNA	*Dekkera*/*Brettanomyces* spp.	broth cultures	Molina et al. (1992)	
Nested PCR of DNA	*Dekkera*/*Brettanomyces* spp.	plate colonies or broth cultures	Alguacil et al. (1998)	based on Ibeas et al. (1996)
RAPD-PCR of total DNA	several species	plate colonies	Quesada and Cenis (1995)	
	Dekkera/*Brettanomyces* spp.	plate colonies	Mitrakul et al. (1999)	
AFLP selective PCR amplification of restriction fragments of total DNA	several species	broth culture	Barros-Lopes et al. (1999)	
Staircase electrophoresis of low-molecular-weight RNA profiles	several species	not described	Velásquez et al. (2001)	
PCR amplification of DNA SSRs	*S. cerevisiae* starters	broth cultures	Techera et al. (2001)	
PNA FISH targetting rRNA	*D. bruxellensis*	plate colonies	Stender et al. (2001)	probes based on the sequence of D1–D2 region of 26S rDNA

(continued on next page)

Table 3 (*continued*)

Technique	Target species	Biomass source	Reference	Observations
PNA CISH targetting rRNA	*D. bruxellensis*	plate microcolonies	Connel et al. (2002)	based on Stender et al. (2001)
PNA FISH targetting rRNA	*D. bruxellensis*	plate colonies	Dias et al. (inpress)	based on Stender et al. (2001)
PCR-RFLP of the 5.8S-ITS region (ITS1 and ITS2) of the rRNA gene	several species			based on Esteve-Zarzoso et al. (1999)
PCR of introns in the mitochondrial gene COX 1	*S. cerevisiae* starters	direct analysis of grape juice	López et al. (2002)	
DGGE of PCR amplified 26S rRNA genes	several species	direct analysis of grape juice	Cocolin et al. (2000, 2001, 2002), Mills et al. (2002)	threshold: $>10^3$ cells/ml; sample volume: 100 ml; time: 1 day

taxonomically oriented, or other more applied but "older" works, may be found in the articles cited in Table 3.

Table 3 shows the predominance of two techniques based on RFLP-PCR analysis of the 5.8S-ITS region and on mitochondrial DNA restriction patterns.

Analysis by RFLP-PCR of the 5.8S-ITS region is mostly used to identify spoilage yeast, since the 5.8S rRNA gene carries greater interspecific differences than the 18S and 26S rRNA genes (Cai et al., 1996; Esteve-Zarzoso et al., 1999). Arias et al. (2002) increased the available database of 5.8S-ITS profiles to 132 yeast species (Esteve-Zarzoso et al., 1999) and identified correctly 98% of the isolates from orange juices. Heras-Vasquez et al. (2003) advised the use of sequence analysis of the ITS region until the database obtained by restriction analysis is completed. The database published by Esteve-Zarzoso et al. (1999) has been expanded to identify 300 yeast species, and it is available at the website http://motor.edinfo.es/iata (Querol et al., in press).

The utilisation of peptidonucleic acid (PNA) molecular probes appears to have a particularly promising future in the rapid identification of yeast species (Stender et al., 2002). The results take, after strain isolation, about 2 h to obtain, hybridisation is done on microscope slides, and positive results are checked by microscopy, which is easier to interpret than profiles of gel bands. We have successfully applied a PNA probe specific for *D. bruxellensis*, but another PNA probe directed to *Z. bailii* (Perry-O'Keefe et al., 2000) was found not to be specific, requiring further improvement (unpublished observations). A disadvantage is the necessity of a costly fluorescence microscope. However, in spite of its great potential, PNA probes do not seem to interest industry (Stender, personal communication). At present, molecular techniques seem to be more appropriate to central laboratories, certification institutions, or other support laboratories, which may provide epidemiological studies or occasional consultancy as industry demands.

4. Origin of spoilage yeasts in the wine industry

The wine production environment may be divided in two fundamental parts: the vineyard, which is a natural ecosystem, influenced by cultural practices, and the winery, which is the environment associated with grape fermentation, wine storage and aging, and bottling. A deep knowledge of these two ecosystems—vineyard and the winery—is essential to establish the origin of wine spoilage yeasts, their routes of contamination, critical points of yeast infection, and their control.

4.1. Vineyard

Ecological surveys performed in vineyards and on grape surfaces during ripening are relatively few when compared to those performed on grape musts and on their spontaneous fermentation. Moreover, the majority of them used less optimal sampling, pre-isolation techniques, enrichment methods (Martini et al., 1996), isolation culture media, and incubation times, leading to an insufficient knowledge of grape microbial ecology. In general terms, the available information about the presence of microbial communities in vineyards

and on grape surfaces may be summarised as follows.

(i) Mature sound grapes harbour microbial populations at levels of 10^3-10^5 CFU/g, consisting mostly of yeasts and various species of lactic and acetic bacteria (Fleet, 1999), and filamentous moulds; (ii) the sources of yeasts and yeast-like microorganisms include all the vine parts, as well as the soil, air, other plants, and animal vectors in the vineyard (Davenport, 1974); (iii) insects are the principal vectors for the transportation of yeasts (Mortimer and Polsinelli, 1999); (iv) yeast colonisation on grapes is influenced by the degree of ripeness of the bunch (Rosini et al., 1982); (v) the occurrence and growth of microorganisms on the skin of the grapes is affected by the rainfall, temperature, grape variety, and application of agrochemicals (Davenport, 1974; Van der Westhuizen et al., 2000b; Sabate et al., 2002); (vii) yeasts are mainly localised in areas of grape surface where some juice might escape and are embedded in a fruit secrete; outer surface of the berries is covered by a waxy layer, which affects the adherence of microbial cells and their ability to colonize the surface (Belin, 1972); (viii) oxidative basidiomycetous yeasts, without any enological interest—*Sporobolomyces*, *Cryptococcus*, *Rhodotorula*, and *Filobasidium*—are mostly prevalent in the vineyard environment (soil, bark, leaves, grapes), as well as *Aureobasidium pullulans*, which seems to be a normal inhabitant of grape skin (Davenport, 1976; Sabate et al., 2002); (ix) apiculate yeasts (*Hanseniaspora* and *Kloeckera* spp) and oxidative yeasts (mostly *Candida*, *Pichia*, and *Kluyveromyces* spp.) are predominant on ripe sound grapes (Davenport, 1976; Sabate et al., 2002); (x) the main wine yeast—*S. cerevisiae*—contrary to many early reports, is virtually absent from sound grapes, being present in one berry among 2016 tested (Vaughan-Martini and Martini, 1995), or about 1 in 1000 berries (Mortimer and Polsinelli, 1999).

Despite the abovementioned statements, there are still no definitive studies on how microorganisms contaminate and colonize the grape bunch. The controversy on the origin of *S. cerevisiae* (Martini, 1993; Vaughan-Martini and Martini, 1995; Török et al., 1996) is beyond the scope of this review, but illustrates the need for more work on the subject and highlights as well the need to improve appropriate sampling and recovery techniques. In fact, the dissemination of yeasts on the grape surface is quite variable (Van der Westhuizen et al., 2000a,b), the microbial ecology of damaged grapes is poorly studied, or even unknown, and grape rupture is associated with the increasing occurrence of fermentative species (Mortimer and Polsinelli, 1999). The abovementioned studies have been addressed to *S. cerevisiae*, but the fact that spoilage species are also fermentative suggests that the knowledge of their dissemination may greatly improve if more attention is given to the microbiology of damaged grapes. Furthermore, as mentioned earlier, selective media and long incubation periods are essential to recover spoilage yeasts from grapes, such as *S. pombe* or *Brettanomyces* spp. (Florenzano et al., 1977).

4.1.1. The microbiology of damaged and dried grapes

Damaged grapes may result from different causes: (i) increase of berry volume due to rapid rainwater absorption by the vines, especially when the bunches are rather tight and the berry skin is thin; (ii) other meteorological accidents like hail and heavy rain; (iii) attack by *Drosophila* spp., honey bees, wasps, moths and birds; (iv) attacks of phytopathogenic moulds (e.g., downy and powdery mildews, noble or grey rot).

Grapes infected with powdery mildew harbour much higher microbial loads of microorganisms (yeasts, lactic and acetic bacteria) and volatile compounds (ethanol, ethyl acetate and acetic acid) than sound grapes (Gadoury et al., 2002). Among yeasts, significant numbers of *Dekkera* and *Kloeckera* were detected, which are probably disseminated by insects attracted to the infected grapes by the volatiles given off by ripening berries (Gadoury et al., 2002). In another work, no particular yeast contaminant species were found with powdery mildew infected grapes by Stummer et al. (2002), but the resulting wine was scored higher in "yeasty" and "estery" aromas, probably resulting from unwanted fermentation microorganisms (Stummer et al., 2002).

There are several types of rot, although the most frequent is that caused by the mould *Botrytis cinerea*. In particular climatic conditions, with alternating wet and dry periods, *B. cinerea* induces controlled dehydration of the grapes, leading to the well-known noble rot, which is the base for the production of some of the most famous dessert wines of the world, like Tokay Aszú and Sauternes. In *B. cinerea*-infected grapes, the presence of *K. apiculata* and *Candida stellata* seems to be favoured when compared with yeast populations of

healthy grapes (Donèche, 1992). In one of the few studies on the subject, Mills et al. (2002) showed the presence of *Hanseniaspora osmophila* and a nonculturable fructophilic *Candida* population, besides the expected populations of the genera *Saccharomyces*, *Hanseniaspora*, *Pichia*, *Metschnikowia*, *Kluyveromyces*, and *Candida*. In addition, *B. cinerea* frequently produces grey rot, which severely damages wine quality and causes serious economic losses. Both rots—noble and grey—change the chemical composition of grape juice dramatically. The first is essentially characterised by a significant increase in sugar concentration as a rule higher than 300 g/l, a slight increase in pH usually about 0.3 pH units, production of gluconic acid, and a significant increase in acetic acid (Donèche, 1992). In grey rot, the sugar concentration is not usually increased, but there is significantly more glycerol, gluconic acid, and due to acetic bacteria, acetic acid. *B. cinerea* also produces various antibiotic substances in grapes (Donèche, 1992). In contrast to noble rot, grey rot yields rather unbalanced wines, with weak maturation ability and with a typical mould odour, which reduces their quality.

When grey rot is accompanied by growth of other moulds, like *Aspergillus niger*, *Penicillium* sp. and *Cladosporium* sp., contaminated grapes are frequently extremely bitter and with aromatic flavours, yielding wines with phenolic and iodine odours (Ribereau-Gayon, 1982, cited by Donèche, 1992). It is easy to conclude that besides a sudden increase in microbial load to about 10^6–10^8 CFU/g (Fleet, 1999), deep alterations in yeast microbiota might occur compared with sound grapes. Surprisingly, the studies on microbial ecology of grapes spoiled by *B. cinerea* are so scarce that it is not clear if poor wine quality is a result of changes in the chemical composition of the grape caused by the mould or change in the fermentative microflora or both. Although in a relatively recent review (Donèche, 1992) and in most recent oenology monographs (Boulton et al., 1996, Ribéreau-Gayon et al., 2000), there is no information on the alteration of grape microbial community by grey rot; it is plausible that a significant increase of fermentative yeasts, some of which are spoilage yeasts, moulds, lactic and acetic bacteria, occurs. Our empirical experience confirms the observation of Ribereau-Gayon (1982), describing the phenolic taint of wine made from rotten grapes, suggesting that growth of 4-ethylphenol-producing species like *Dekkera*/*Brettanomyces* spp. or *P. guilliermondii* is favoured. Winemaking experience in Portugal also indicates that in vintages with a high incidence of grey rot, the frequency of wines affected by 4-ethylphenol is higher (unpublished information).

Grapes can also be affected by another type of rot, generally known as "sour rot," where yeasts and acetic bacteria appear to have a dominating role and where moulds are hardly detected. Sour rot was reported for the first time in Italy (Bisiach et al., 1986) and is frequently initiated in the area near berry pedicel or at the level of skin damage. Grape sour rot is easily recognised by browning and desegregation of the internal tissues, detachment of the rotten berries from the pedicel, and a strong ethyl acetate smell (Bisiach et al., 1986, Guerzoni and Marchetti, 1987). Another component of the system is *Drosophila* flies, although their role in the process has not been studied in depth. The yeast species most frequently reported as actively proliferating in rotten berries are *H. uvarum* and its anamorph *K. apiculata*, *C. stellata*, *Metshnikowia pulcherrima*, *Candida krusei*, *Pichia membranifaciens*, *Saccharomycopsis vini*, *Saccharomycopsis crataegensis*, and *Candida steatolytica* (Bisiach et al., 1986; Guerzoni and Marchetti, 1987; Blancard et al., 1999). Occasionally, *Zygosaccharomyces* spp. can also be present in high densities, together with other spoilage yeast species, like *Brettanomyces* spp. (Guerzoni and Marchetti, 1987). The contribution of acetic bacteria to this disease seems to be well established, and several studies on rotten berries confirm the presence of levels of acetic acid as high as 40 g/l (Donèche, 1992), of ethyl acetate, and of *Gluconobacter* spp. and, less commonly, of *Acetobacter* spp. (Marchetti et al., 1984; Bisiach et al., 1986; Blancard et al., 1999; Gravot et al., 2001). However, in other studies, acetic bacteria were rarely or never recovered (Guerzoni and Marchetti, 1987), suggesting that acetic acid and ethyl acetate may result from the yeast activity. Once more, surprisingly, there are no studies that cast light on the microbial ecology of this type of sour rot. Besides, most studies were performed without using pre-isolation techniques, selective/differential media or long incubation periods that favour the recovery of slow-growing and minority yeasts. Thus, it is legitimate to think that for many of the most important wine spoilage species, e.g., *Dekkera*/*Brettanomyces* spp., the main entry to

the winery is grapes affected by sour rot, which cannot be eliminated during harvesting.

Another example of unbalanced grapes that are used in winemaking with some frequency are those affected by mealy bugs (*Pseudococcus* spp.) excreting honeydew that may not damage grape skin, but a high concentration of sugar is accumulated in the surface. In some regions of Mediterranean countries, including Portugal, this disease can affect more than 10–20% of the crop, so that their yeast population might have an impact on wine quality. As far as we are aware, the microbial ecology of grapes with honeydew has never been investigated, although the typical black colour due to the growth of filamentous fungi on the grape surface and the abundant presence of ants are well known. Bearing in mind that honeydew is essentially plant sap with high sugar content, it is conceivable that damaged grapes are a habitat favourable for yeast growth, especially osmophilic and osmotolerant species, such as *Zygosaccharomyces* spp.

Similar conditions could occur in dried undamaged grapes used in the production of certain table (e.g., Amarones) or dessert (e.g., Muscat) wines, where the initial sugar concentration may suffer relative increases of 30–40%. However, very limited valid information is available.

As a consequence of the above description, many gaps exist in the knowledge of grape microbial ecology, particularly concerning wine spoilage yeasts.

4.2. Winery

Essentially, the microorganisms in the winery come from the grapes and vectors, among which, *Drosophila* flies are likely to be the most important (Mortimer and Polsinelli, 1999). Conditions enabling colonisation of wines and contact surfaces depend on the stringency of GMP. The intrinsic properties of wine are of major importance in influencing the evolution of microbial communities.

Considering the winery environment, two sections are relevant: (i) winemaking and bulk wine storage and (ii) the bottling line:

4.2.1. Winemaking and wine storage

Studies of microbial ecology in cellars are relatively scarce compared with those of grapes and grape juice. However, all the results obtained seem to confirm that the yeast population of wineries is quite different from that of grapes, particularly due to the high proportion of *S. cerevisiae* (Martini, 1993; Vaughan-Martini and Martini, 1995). The association between the winery and this species is so close that these authors called it "the first domesticated microorganism" and claim that it is a result of yeast species evolution in this environment. Besides *S. cerevisiae*, other species frequently recovered from wine or grape juice contact surfaces—tank walls, crushers, presses, floor, winery walls, pipes, etc.—are *P. anomala*, *P. membranifaciens*, *Candida* spp., *Cryptococcus* spp., and more rarely *Rhodotorula* spp., *A. pullulans*, *Trichosporon cutaneum*, *Debaryomyces hansenii*, *K. apiculata*, *M. pulcherrima*, and *T. rosei* (Martini, 1993). Some of these species, in spite of being common contaminants, are obligate aerobes (e.g., *Rhodotorula* spp., *Cryptococcus* spp., *D. hansenii*, and *A. pullulans*) and therefore have little or no ability to grow in or spoil wines.

The species able to grow abundantly in wine, with fully aerobic or weakly fermentative metabolism (e.g., *P. membranifaciens*, *P. anomala*, and *Candida* spp.) are known for film formation on the surface of bulk wines in unfilled containers and with sulphite levels insufficient to prevent their growth. Given their oxidative metabolism and high growth rate, at winery temperature, they rapidly colonise surfaces contaminated with wine residues, being regarded as indicators of hygiene and of the stringency in avoiding wine contact with air. When measures are not taken to prevent their growth, they may affect wine and favour growth of acetic bacteria with much more serious consequences. However, adequate GMP, adequate levels of molecular sulphite, efficient wine protection from air contact with nitrogen, and low storage temperatures (8–12 °C) allow high control of these yeast (Sponholz, 1992). Occasionally, they can be tolerant to molecular sulphite levels (Warth, 1985). *P. anomala*, *M. pulcherrima* and *H. uvarum* (*K. apiculata*) are known for producing high levels of ethyl acetate and acetic acid before and during initial fermentation steps, leading to serious wine deterioration (Sponholz, 1992; Romano et al., 1992; Plata et al., 2003). It seems that ethyl acetate is not produced by *K. apiculata* and *Candida pulcherrima* during fermentation by *S. cerevisiae* (Zohre and Erten, 2002). Although these species are common winery contaminants, their activity is especially dangerous when associated with damaged

berries, which encourage their growth, leading to high initial populations at the beginning of fermentation. High juice settling temperatures with low protective levels of sulphite can also lead to massive growth, originating from contaminated grapes or poorly sanitised equipments (unpublished observations).

Surprisingly, the yeast species regarded as the most dangerous to wines, i.e., *Dekkera*/*Brettanomyces* spp., *Z. bailii*, and *S. ludwigii*, are seldom detected in yeast studies performed in wineries. Although classical studies by Van der Walt and van der Kerken (1958, 1961) on *Brettanomyces* spp., Rankine and Pilone (1973) and Minarik (1983) on *Z. bailii*, and Peynaud and Domercq (1955) on *S. ludwigii* have demonstrated that they may be winery contaminants, most results from literature suggest that their prevalence is low. In our opinion, the utilisation of inadequate culture media and short incubation periods favouring mould and fast-growing species might have contributed to the underestimation in wineries of these yeast. In support of this, Alguacil et al. (1998) showed the presence of *Dekkera*/*Brettanomyces* in grapes and at various sites of grape-crushing processing lines by using direct PCR techniques. Connel et al. (2002) also recovered *D. bruxellensis* from air samples of crush, tank, barrel, and bottling line areas using BSM medium (Millipore) followed by a filter-based chemiluminescent in situ hybridisation technique.

From the technological point of view, the main question is to know which factors and under which conditions they enable slow-growing yeasts like *Dekkera*/*Brettanomyces* spp. and *Zygosaccharomyces* spp. to become competitive, attain high contamination levels, and cause serious wine defects. Only when this information is available can it be possible to establish adequate control measures.

Van der Walt and van der Kerken (1961), using appropriate culture media and a method developed for the recovery of *Brettanomyces* species from materials heavily contaminated with others species, reported that these yeasts are common contaminants in the winery and its equipment. However, the authors did not recover these yeasts from husks, pomaces, or fresh grapes, suggesting that the infection of wines and musts by *Brettanomyces* species is due to contamination spreading from foci of infection within the winery. Much later, Chatonnet et al. (1992, 1993) were the first authors to identify oak barrels as an ecological niche for *Dekkera*/*Brettanomyces* spp., which become more dangerous with repeated use. This suggests that barrel sanitation and sulphite utilisation (sulphur burning in empty barrels) is not enough to eliminate *Dekkera*/*Brettanomyces* spp., which develop during the lifetime of the barrel. Laureano et al. (2003) reported that treatment with hot water and steam is not enough to eliminate yeasts and moulds entrapped in barrel staves. It is now generally accepted that control of *Dekkera*/*Brettanomyces* spp. cannot be achieved by efficient sanitation of all cellar equipment, but demands much more stringent microbiological control and judicious utilisation of sulphite or dimethyldicarbonate (DMDC). However, the primary source of these yeasts remains obscure. Ongoing research in our laboratory suggests that rotten grapes, *Drosophila* spp, particularly those frequenting piles of husks, lees and grape leftovers, and wine residues on equipment are foci where *Dekkera*/*Brettanomyces* spp. can be found by using an appropriate culture medium and MPN enumeration technique. It is then admissible that rotten grapes are the main entry source of these yeasts in wineries and that lees and husks' leftovers are important infection sources, frequently visited by *Drosophila* flies which carry them into the winery.

Yeasts of the genus *Zygosaccharomyces*, and particularly *Z. bailii*, are very rare in sound grapes and are not regarded as common winery contaminants. However, its presence is well known in wineries processing sweet or sparkling wines using concentrated or sulphited grape juice (Rankine and Pilone, 1973; Neradt, 1982; Minarik, 1983; Wium et al., 1990). A similar situation is observed in other food and beverage industries using processed raw materials such as fruit juices, concentrated juice, glucose syrups, flavouring compounds, and colouring agents (Dennis and Buhagiar, 1980; Thomas and Davenport, 1985; Esch, 1992; Sancho et al., 2000). The fact that these yeasts are extremely resistant to preservatives, particularly *Z. bailii* (Thomas and Davenport, 1985), *Zygosaccharomyces bisporus*, and *Zygosaccharomyces rouxii* (Esch, 1992), means that addition of high, but sublethal, doses increases their competitiveness and makes them seriously dangerous. Hence, it is regarded as a good manufacturing practice to add the preservative to sweet wines just prior to bottling and to limit the circulation of concentrated grape juice to specific pipes and pumps. This is frequently forgotten in most

wineries. It would also be highly desirable to have a strict microbiological control of concentrated grape juice, which is not common in wineries.

S. ludwigii is another yeast species that may cause serious problems in wines, although it is not regarded as a typical contaminant of winery microflora. Its remarkable tolerance to sulphite makes it a frequent isolate in wineries where high sulphite doses or sulphited grape juices are used. Thomas (1993) defined this species as the "winemaker's nightmare" because of the infection of bulk wine which is notoriously difficult to eradicate from a winery.

Although it is known that mummified fruits are natural habitats of *Z. bailii* (Davenport, 1980), that *Z. bisporus* can be isolated from fruit tree exudates (Thomas and Davenport, 1985), and that *S. ludwigii* is present in slime fluxes of *Quercus* spp. (Phaff and Starmer, 1980), the winery contamination routes and vectors for these yeasts are barely known, justifying further studies on this subject.

4.2.2. Bottling line

Wine bottling is a critical operation since, with the exception of hot bottling, it is the last contamination source before wine is released to the market. In most dry red wines, yeast contamination during bottling is not serious (Rankine and Pilone, 1973). However, for wines with residual sugar and for some dry white wines, it can be very serious, being responsible for a major part of the microbiological problems in bottled wines.

When sweet wines are processed, either with natural sweetener stabilised with sulphite and sorbate, or alternatively, with concentrated grape juice, the contaminating flora of the bottling line is usually dominated by species that are resistant to chemical preservatives and low a_w, namely, *Z. bailii*, *S. cerevisiae* and *S. ludwigii* (Rankine and Pilone, 1973; Minarik, 1983; Fleet, 1992). This situation leads to the conclusion that high sulphite levels (Delfini, 1988) and sorbate (Warth, 1985), when used in sublethal doses, play an essential role in the favouring highly resistant yeast. This is mainly observed in bottling lines. The same applies to the use of concentrated grape juice, a well-known source of *Zygosaccharomyces* spp. and other dangerous species (Rankine and Pilone, 1973; Thomas and Davenport, 1985; Wium et al., 1990).

Some authors have studied the critical points of bottling lines. The outlet side of the sterilising filter, the filler, in particular, the bell rubbers and rubber spacers, the corker, in particular, the bells/cork jaws and cork hopper, the bottle sterilizer, the bottle mouth, and the air inside the bottling room (Donnelly, 1977a,b; Neradt, 1982; Malfeito-Ferreira et al., 1997) are important critical points. Furthermore, according to our experience, the importance of each point is strongly dependent on suitably designed equipment. Packaging materials such as bottles, corks, and rip-caps are generally not significant contamination sources, because they are frequently infected by fungi, spore-forming bacteria, and adventitious yeasts, which do not survive in wine. However, they can be important sources of spoilage yeasts when wine is improperly stored for long periods in a humid and contaminated winery environment (unpublished results). However, we have observed cork contamination with dangerous species prior to winery entrance in two separate cases: one with *S. cerevisiae*, due to contamination of the silicone used in cork surface treatment, and the other with *S. ludwigii*, resulting from an inadequate cork routine treatment before cork packaging with sublethal sulphite doses (unpublished information).

Good quality of bottling equipment is also essential to prevent yeast growth. When oxygen is introduced in wines during bottling, it stimulates growth of *Z. bailii* (Malfeito-Ferreira et al., 1989). In recent years, new bottling equipment, revision of bottling line sanitation programs and overall plant hygiene standards, and the better implementation of HACCP systems in wineries have contributed to a significant improvement of the microbiological quality of wine bottling. However, these improvements have not sufficed to reduce the levels of preservatives used in sweet and dry white wines sterilized by filtration prior to bottling. In our opinion, this is due to technological and microbiological limitations. The former are related to the design of plant layout and human failures, mainly, the incorrect execution of sanitation programs and the result of cross-contamination. A clear example of this is steam disinfection of the filler, which, according to our experience, is a classical case of a frequently incorrect procedure. In fact, if after steam application sterile air is not injected into the filler during the cooling period, a negative pressure will be formed inside the filler, leading to ingress of air contaminated with potential

spoilage yeasts. The limitations of a microbial nature are concerned, once more, with the lack of efficient tools to examine and interpret the contamination of bottling lines on line.

5. Quality control and indicators of spoilage yeast in wines

In a well-implemented and designed HACCP system, the evaluation of the microbiological quality of foods is not limited to the retrospective analysis of the final product. It also includes the estimation of the microbiological quality of raw materials, ingredients, sanitation procedures, processing operations, as well as the product shelf life. In turn, modern international trade is evolving towards the evaluation of microbiological quality of foods and beverages according to standardised methods and analytical parameters—microbiological or chemical—accepted by all parties involved. In this context, microbiological indicators are essential tools, either to production control or to quality evaluation and food trade regulation.

5.1. Zymological indicators

In wines produced according to GMP, pathogens do not grow or survive. The presence of spoilage yeasts is thus the main microbiological concern. Concerning the role of spoilage yeast, wine can be grouped in two categories: one concerns wines with residual sugar, and the other concerns wines without residual sugar, or fortified wines in which fermentative yeasts are incapable of alcoholic fermentation. In the first, yeast occurrence should be considered as both hazardous for food safety, i.e., exploding bottles, and detrimental to the final product, whereas in the second, yeast should be considered as a hazard to quality of the final product only. Even taking into account that refermentation is a rare event in sweet bottled wine (Deak and Reichart, 1986), one could assume that the most appropriate microbiological indicator for the evaluation of the quality of sweet wines is screening for "fermentative yeasts." Surprisingly, this is not the case. The evaluation, as a rule, is done through general yeast plate counts using a general culture medium. This enumeration of "total" viable yeasts (broadly known as "yeasts and moulds"), like the indicator "total viable count" used in food bacteriology, provides very limited information, which is clearly insufficient from the wine quality point of view. Unfortunately, most wine companies, and food industries in general, accept "what is currently done" (Mossel and Struijk, 1992), instead of choosing target organisms on the basis of their spoilage potential.

Other specific indicators can be used to evaluate the presence or activity of spoilage yeasts in wineries. These indicators may be divided into three categories, which may or may be not used together, as described below.

5.1.1. Indicators based on selective and differential culture media

Yeast enumeration on selective and indicative media can be used as indicators. For instance, "acid-resistant yeasts," enumerated on ZBA (Erickson, 1993) or tryptone glucose yeast extract agar (TGYA) (Makdesi and Beuchat, 1996a,b), and "xerotolerant yeasts," enumerated on dicloran 18% glycerol agar (DG18) (Deak and Beuchat, 1996), can be useful to assess the quality of sweet wines as well as concentrated and sulphited grape juices, although incubation times should be larger than 10 days. In these products, the utilisation of *Zygosaccharomyces* differential medium (ZDM) (Schuller et al., 2000) can be used to enumerate *Z. bailii* and *Z. bisporus.* This medium also enables the monitoring of these yeasts in bottling lines, particularly when they are used to bottle wines sweetened with grape juice concentrate. Lysine agar can be used to detect non-*Saccharomyces* species (Heard and Fleet, 1986), which may be regarded as a hygiene indicator under certain conditions. Another important indicator for assessing the quality of red wines, particularly those aged in oak barrels, is the 4-ethylphenol-producing yeasts obtained by growth on DBDM medium (Rodrigues et al., 2001). In this medium, positive responses for *Dekkera*/*Brettanomyces* spp. are obtained after 1 week of incubation. However, for other 4-ethylphenol producers that are unable to grow in wine but able to grow in grape juice, e.g., *P. guilliermondii*, colonies appear after 2–3 days (Dias et al., in press).

As already mentioned, growth in general purpose culture broth may be used to detect the presence of "fermentative yeasts" in sweet bottled wines. Unfortunately, ESA medium (Kish et al., 1983) was not

appropriate to detect *S. cerevisiae* (Heard and Fleet, 1986). Alternatively, a comparison between counts in general purpose medium and lysine medium (Heard and Fleet, 1986), or a general purpose medium with 4 ppm of cycloheximide, may be used to estimate the population of *Saccharomyces* spp. in wines.

5.1.2. Chemical and organoleptic indicators

An alternative approach to time-consuming yeast indicators based on microbiological analysis is to examine food samples for chemical or sensorial evidence of microbial activity. However, only very few metabolites have been accepted as a means of assessing the degree of yeast spoilage in foods. Ethanol and acetoin levels provide reliable indexes of the quality of the fruit on arrival at the factory and of hygiene in the processing plant, respectively (Mossel et al., 1995). Analysis of carbon dioxide in the head-space of sealed culture vials has been proposed for rapid enumeration of fermentative yeasts in food, using a selective medium and gas-chromatographic analysis (Guerzoni et al., 1985). In turn, 4-ethylphenol can be used as a sensorial or chemical marker to spot wines infected by *Dekkera*/*Brettanomyces* spp. (Boulton et al., 1996). As a sensorial indicator, the 4-ethylphenol is used when its concentration in wine is higher than the detection threshold—which is dependent on the type of wine and grape variety (Laureano, personal communication). Analysis by gas chromatography allows more efficient control of the 4-ethylphenol concentration. The execution of two analyses separated in time by 1 or 2 weeks indicates whether *Dekkera*/*Brettanomyces* spp. are active, enabling the oenologist to take measures to stop the process. These include sterilising by filtration, sulphite addition, or flash pasteurisation. We consider that tests for 4-ethylphenol should be used routinely for all red wines in which there is risk of *Dekkera*/*Brettanomyces* spp. being present. These include wine matured in oak barrels or produced with poor sanitary quality grapes.

Ethyl acetate can also be used as chemical marker to evaluate the spoilage activity of yeasts, e.g., *P. anomala*, particularly during pre-fermentative maceration and white juice settling. However, it is of limited interest because the production of ethyl acetate is very fast (Plata et al., 2003), and by the time the result is obtained, the grape juice can already have deteriorated.

5.1.3. Indicators based on biomarkers

Another approach is based on the long-chain fatty acid composition of contaminating yeasts (Malfeito-Ferreira et al., 1989, 1997; Augustyn et al., 1992; Sancho et al., 2000). The rationale of this approach has been given elsewhere (Loureiro and Querol, 1999; Loureiro, 2000). Briefly, it is possible to separate the yeasts in into three broad groups with different technological significance based on the presence or absence of polyunsaturated C18 fatty acids (Table 4). Most potential spoilage yeasts are located in Group II (with C18:2 and without C18:3). Group I yeasts (without C18:2 and without C18:3) may also be serious spoilage organisms in some conditions. Group III yeasts (with C18:2 and with C18:3) are considered less serious and regarded as indicators of poor GMP. The separation into three groups may be followed by a discrimination using multivariate statistical analysis, which allocates strains to distinct clusters of spoilage species. Nucleic acid-based methods should be used after a first screening by fatty acid profiling, but only to confirm or to provide further intraspecific information on the probable identities given by the fatty acid technique.

This approach has been used for detection of *Z. bailii* in wine bottling facilities (Malfeito-Ferreira et al., 1997) and fruit concentrates (Sancho et al., 2000) and *D. bruxellensis* in wines (Dias et al., in press). However, its widespread use in industry is strongly limited by the lack of readily available databases and by the difficulty of interpreting fatty acid profiles under industrial conditions.

5.2. Acceptable levels of yeasts in wines

The Office International de la Vigne et du Vin (OIV), a regulatory authority that includes most wine-producing countries, do not define maximum levels of microbial contamination in wine. The single condition is that bottled wine should be clear, that is, the microbial load should be less than 10^4–10^5 CFU/ml (in white wines) for microorganisms producing powdery sediments, or less than 10^2–10^3 CFU/ml, for microorganisms producing flocculent sediments. As far as we are aware, the only legal limit for yeasts in wines is imposed in Norway, being of 10 cells/ml of "total" microorganisms present in bulk wines (Anon., 1999).

Table 4
Contamination wine yeast species and their significance (according to Malfeito-Ferreira et al., 1989, 1997; Wium et al., 1990; Rodrigues et al., 2001 and unpublished data)

Indicator[a]	Species	Occurrence	Significance
Group I	*S. cerevisiae*	bottled dry wines	spoilage by sediment or cloudiness formation
		bottled sweet wines	spoilage by refermentation
		corks	contamination of silicone film
	S. ludwigii	bottled wines	spoilage by sediment or cloudiness formation
		corks	contamination
	K. apiculata	grape juices	spoilage by ethyl acetate production
Group II	*Z. bailii*	bottled wines	spoilage by sediment or cloudiness formation
		winery equipment	contamination
	Z. rouxii	grape juice concentrate	contamination
	T. delbrueckii	desulphited grape juice and storage tanks	contamination
	D. bruxellensis	bulk, barrel matured, and bottled wines	spoilage by 4-ethylphenol production
		sparkling wine	spoilage by cloudiness formation
Group III	*P. membranifaciens*	bottled wines	spoilage by sediment formation, contamination
		winery equipment	contamination
	P. anomala	winery equipment	contamination
	L. elongisporus	winery equipment	contamination
	Rhodotorula spp.	winery equipment	contamination
	Trichosporon spp.	winery equipment	contamination

[a] Grouping according to long-chain fatty acid composition of contaminating yeast in wines and their significance. Groups defined according to long-chain fatty acid composition: group I, without C18:2 and C18:3 acids; group II, with C18:2 and without C18:3 acids; group III, with C18:2 and C18:3 acids.

Wine vulnerability to yeast growth seems to be independent of the type of wine (white, red, rosé, dry, sweet, etc.) (Deak and Reichart, 1986). However, it has been established that yeast growth potential is much higher in sweet than in dry wines. In dry wines, yeast growth is due to the aerobic assimilation of other wine constituents, e.g., ethanol, organic acids, glycerol, and it is limited by the concentration of dissolved oxygen (Malfeito-Ferreira et al., 1989, 2001). Wine colour is also relevant because in a red wine, the observation of suspended cells is much more difficult than in rosé or white wines. Therefore, it is in most cases understandable that acceptable levels of yeast counts are more stringent for sweet than for dry wines and much more stringent for white than for red wines. However, this general statement may not be valid after the emergence of *Dekkera*/*Brettanomyces* as the agents of organoleptic alteration of dry wines—particularly red—due to their ability to produce 4-ethylphenol in amounts higher than the preference threshold of 620 μg/l (Chatonnet et al., 1992, 1993). The significance of this problem may be illustrated by the proportion of wines with levels of volatile phenols higher than the preference threshold shown in Table 5. Thus, off-flavour production in dry red wines has become, in our opinion,

Table 5
Incidence of volatile phenols in red wines

Country/region	Samples	>426 ppb[a] (%)	>620 ppb[b] (%)	Reference
Italy/Piemonte	47	49	19	Di Stefano (1985)
France/Bordeaux	–[c]	36	28	Chatonnet et al. (1992)
Australia	61	59	46	Pollnitz et al. (2000)
Portugal	366	42	27	Rodrigues et al. (2001) and unpublished data

[a] Preference threshold of 4-ethylphenol + 4-ethylguaiacol (10:1), according to Chatonnet et al. (1992).
[b] Preference threshold of 4-ethylphenol, according to Chatonnet et al. (1992).
[c] Not mentioned.

a spoilage hazard, at least as important as refermentation of sweet white wines. Unfortunately, the industry awoke too late to this problem. *Dekkera*/*Brettanomyces* are probably the biggest microbiological problem of modern oenology, causing large economic losses in the wine sector worldwide (Boulton et al., 1996; Fugelsang, 1997, unpublished data). It is from this viewpoint that the important theme of limits and criteria for yeast in the wine industry should be faced.

It is relevant to comment that OIV legislation concerning chemical preservatives (sulphite and sorbic acid) recognises implicitly different degrees of wine vulnerability, as maximum levels authorised in wines for total sulphite are 150 mg/l in red wine, with less than 4 g/l of reducing sugars, 200 mg/l for white and rosé wines, with less than 4 g/l of reducing sugars, 300 mg/l for white and rosé wines, with more than 4 g/l of reducing sugars, and 400 mg/l for certain special sweet white wines (e.g., Sauternes, Trockenbeerenauslese) (Anon., 1998).

5.2.1. Prediction of wine yeast spoilage

The establishment of acceptable levels of microorganisms in the final product is a concern common to many food industries. The aim of the wine producer is to comply with levels that are attainable under industrial conditions and ensure product stability during its shelf life.

Scientific and technical literature on acceptable levels and spoilage prediction of foods and beverages caused by yeasts is surprisingly scarce, revealing the low priority given to it by food microbiologists and technologists. Davenport (1986) stated that one viable cell of *Z. bailli* per any package unit of wine may cause spoilage. Deak and Reichart (1986) stated that a few cells per bottle of *Z. bailii* may cause loss of stability, and so no definite correlation was found between yeast population and time of stability (Deak and Reichart, 1986). The Workshop on Standardization of Methods for the Mycological Examination of Foods stated that the presence of *Z. bailii* at any level in wines is unacceptable (King et al., 1986). Accordingly, Thomas (1993) found that in the case of strong wine spoilers, such as *Z. bailii* and *S. cerevisiae*, and highly vulnerable wines, one cell may be enough to spoil the wine (Thomas, 1993).

For prediction of stability, the work of Delle, made in Odessa, at the beginning of the 20th century (cited by Amerine and Kunkee, 1965) stated the biological stability of dessert wine is reached when the sum (Delle units) of the sugar content (% by weight) and six times the ethanol content (% by weight) is at least 78. Amerine and Kunkee (1965) also tested the applicability of Delle's equation to the Californian musts fortified at various steps of fermentation and concluded that Delle units for wine stability depended upon the stage of fermentation at which wine was fortified and on the variety of must and wine yeast strain. Higher Delle units were required for stability when the fortification was made in the late stages of fermentation. Whiteley (1979) inoculated 16 sweet and dry table wines with Delle units ranging from 47.52 to 82.70, with different levels of *Z. bailli*, *S. cerevisiae*, and *S. ludwigii* per bottle. Spoilage was assessed by visual observation of growth after 8 weeks. This author concluded that the only wine that was microbiologically stable had more than 80 Delle units. More recently, Kalathenos (1995) studied the effectiveness of different levels of organic acids and ethanol in controlling the growth of *Z. bailli*, *S. cerevisiae*, *S. pombe*, *B. bruxellensis*, and *P. anomala* strains. His results served as a basis for the establishment of a commercial model related with wine stability—Food Micromodel, version 2 (Anon., 1996)—where the amount of free sulphur dioxide for wine preservation is predicted as a function of ethanol concentration and pH. Unfortunately, as in many other food industries, most predictive models commercially available are seldom used by industry that still uses, as a rule, the classical determination of the misleading "total viable counts." It remains to be seen if this attitude is due to model deficiency or to the resistance of industry to changing routines.

5.2.2. Acceptable levels of yeasts in wine industry and wholesalers

In a survey made by Andrews (1992) covering several Australian wineries, the acceptable levels of yeasts listed were relatively low. A similar inquiry was performed by us for this critical review and sent to wine companies and wholesalers all over the world. Despite finding the same difficulties as Andrews (1992) in getting data from the industry, a number of answers were received, providing a reasonable illustration of practical microbiological control procedures. The industry tends to be on the safe side, and so, very low levels of contamination are reported as acceptable,

Table 6
Bottled wine microbial specifications used by wineries and wholesalers

Winery[a]	Acceptable levels	Observations
1	<1:100 ml total counts	wines with residual sugar >6.0 g/l
2	<100/ml yeast	fast consumption wines
	<2/ml yeast	red wines
	<10:100 ml yeast	white wines
3	<1/ml yeast	with residual sugar
	<1/ml total counts	bag-in-box wines
4	<30:750 ml total counts	white and rosé dry wines
	0:750 ml total counts	white sweet wines
5	<25:200 ml total counts	white wines <7.0 g/l residual sugar
	<5:200 ml total counts	white wines ≥7.0 g/l residual sugar
	<25:200 ml total counts	red wines 3.0–6.9 g/l residual sugar
	<5:200 ml total counts	red wines >7.0 g/l residual sugar
6	<2:250 ml yeast	wines >5.0 g/l residual sugar
7	<100:100 ml total counts	dry white
	<1000:100 ml total counts	common dry red
	no limit	premium dry red and sparkling wines
8	<75:100 ml total counts	all wines
9	<10:100 ml yeast	all wines
10	0–5:100 ml yeast	red and white dry wines, fortified wines
	<1:100 ml yeast	sweet wines (sugar 15–20 g/l)
11	0:500 ml yeast	sweet wines (sugar 15–20 g/l)
	<15:500 ml yeast	dry white wines
12	<1:500 ml yeast	sugar <2 g/l, or sugar >2 g/l and free SO_2 >40 mg/l
	0:500 ml yeast	sugar >2 g/l and free SO_2 <40 mg/l
Wholesaler	**Unacceptable levels**	
1	>100:100 ml total counts	table wines
	>200:100 ml total counts	fortified wines
2	>100:100 ml yeast	dry red wine; analysis in WLN broth, incubation at 32 °C for 72 h, 1 sample/2 h
3	>100:100 ml yeast	dry white wines
	>1000:100 ml yeast	dry red wines
4	>0:100 ml yeast	all wines; sampling at the beginning, middle, and end of bottling day
5	>200:100 ml yeast	fortified wines
6	>200:100 ml yeast	fortified wines

Table 7
Procedures used by wineries when microbial specifications are not met

Winery	Procedures
3, 5, 8, 10	hold for a period of time and retest
4, 11	hold for enough time to meet specifications or to reprocess the affected product
6	a second set of bottles is analysed the following day to confirm yeast presence
7	hold and repeat; if still over limits, hold until values are lower than 10% of the specification

frequently less than 1 cell/100 ml especially in sweet wines (Table 6). As a rule, numbers of contaminant flora are obtained after growth on general media, and so results do not reflect the spoilage flora. The most frequently used medium is WLN (Wallerstein Laboratories Nutrient), which may provide some distinction between yeast species based on colony morphology (Pallman et al., 2001; Techera et al., 2001). Winery 6 uses this approach to differentiate yeasts based on colony morphology (on WLN, *Saccharomyces* produces cream to yellowish brown or green colonies; *Pichia* or *Candida* colonies are cream or white and *Rhodotorula* colonies are pink) and cell morphology by microscopy observation. One winery uses BSM (Millipore), while others use external services to detect *Brettanomyces* spp. Incubation conditions are 25–28 °C for 48–72 h.

When yeast levels are higher than acceptable, most wineries hold the product for long enough to meet specifications or to reprocess the affected product (Table 7). This procedure gives an indication of the contaminant flora because if counts increase, the wine is likely to be contaminated with spoiling yeasts. Most wineries that monitor final product also monitor the efficiency of sanitation, the integrity of membrane filters, and the levels of sulphite and sorbate.

The sampling criteria used by all the wine companies in our inquiry are based on sample collection during the bottling process without an obvious statistical justification. The main objective of sampling is to

Note to Table 6:
[a] Companies answering to the inquiry, prepared for this review, were from the following countries: Australia (wineries 1, 5 and 6), Hungary (winery 12), Portugal (wineries 7, 8, 9, 10 and 11, and wholesalers 5 and 6), Spain (wineries 2 and 4) and UK (winery 3, wholesalers 1, 2, 3 and 4).

identify the occurrence of microbiological problems (e.g., filter rupture) during bottling and to hold all final product produced after the occurrence of such problem.

To define microbiological criteria, it is advisable to have uniform analytical procedures—sampling, sample volume, diluents, culture media, and incubation conditions. Currently, methods are somewhat variable which makes it difficult to compare results. OIV has published standard methods for use in the wine industry (Anon., 1998), but they are surprisingly rarely used in the industrial routine. Commercial contracts established with wholesalers also include microbiological criteria (Table 6) accepted by wineries. Alternatively, wineries propose an attainable specification, which is accepted and checked by wholesalers. It would not be reasonable to establish commercial specifications more severe than those used in the wineries, although wholesaler 4 demands "nil" contamination in wines (Table 6). As mentioned before, it is important to standardise analytical procedures in order to compare microbiological results, but only one wholesaler establishes the conditions of microbiological analysis (Table 6).

5.2.3. The concept of wine susceptibility and its usefulness for predicting wine stability

As far as yeast is concerned, oenologists have the following fundamental priorities: (i) to assure that bulk wine is not deteriorated by the activity of contaminating yeasts; (ii) to assure that bottled wine is microbiologically stable or, in case, it is not; (iii) to predict its shelf life. To predict the microbiological stability of wine, whether bulk, bag-in-box or bottled, is not an easy task, even though deterioration caused by yeasts is a rare event, as it depends on many factors (Fig. 1). In fact, wine susceptibility to yeast colonization is strongly dependent on the hygieneic quality of the grapes and on their degree of ripeness. Furthermore, wine susceptibility is also dependent on the contamination of grapes with polluting chemicals. Given the

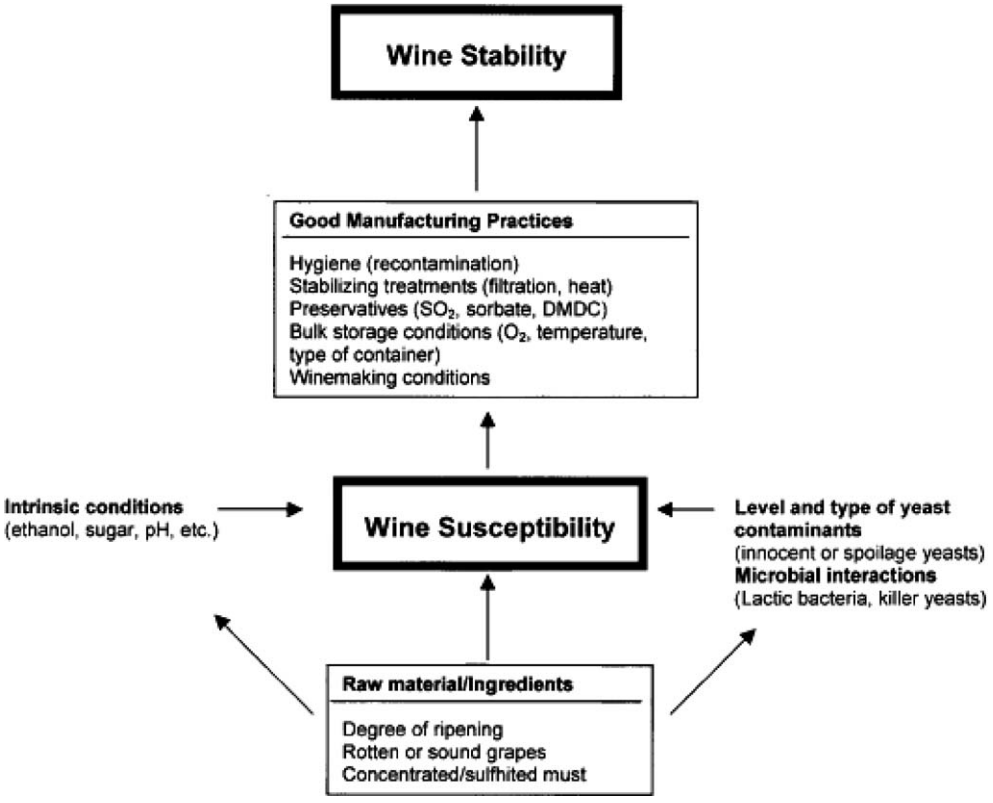

Fig. 1. Factors affecting wine stability.

impossibility to know and much less to measure the effect of pollutants and microbial metabolites on yeast activity, it is especially useful to define the concept of "wine susceptibility." This can be done for pre-established experimental conditions, as the resistance of a wine against the colonization by one or more yeast strains with known wine-spoiling capacities. The appropriate choice of standard yeast strains, with different wine-spoiling capacities, from the most virulent (certain strains of *Z. bailii*, *S. cerevisiae*, and *S. ludwigii*) to the less virulent (e.g., *C. stellata* or *C. parapsilosis*), would allow a range of wine susceptibility to be defined. The concept of "wine susceptibility," which has been referred to by several authors, was studied, although with different purposes, by Thomas (1983). A total of 80 yeast strains, isolated from wine bottling stores and samples of spoiled wines were each inoculated in duplicate into 80 table wines. The inoculated wines were incubated statically for up to 28 days at 25 °C and monitored daily for visible growth. According to the results obtained, the author grouped the tested yeasts according to their wine-spoiling potential, but did no examine the correlation between susceptibility to yeast spoilage and the chemical composition of the wines. In Fig. 2, a range of wine susceptibility against spoilage by yeasts is shown based on ethanol content and presence or absence of residual sugar. Although the proposed range has little practical use, given that most wines have less than 14 % (v/v) ethanol, it illustrates the great potential that this approach may have in the definition of microbiological criteria for the wine industry and in the assessment of yeast spoilage. The subdivision of the three groups with higher susceptibility against colonization by spoilage yeasts referred in Fig. 2 would be enough to significantly improve the present situation. For that, it will be necessary to use predictive microbiology and to develop mathematical models that take into account, besides the quantification of wine susceptibility, the ethanol content, residual sugar concentration, pH, oxygen, preservative levels (sulphite and sorbic acid), and the initial microbial load. Perhaps this is the way to overcome the difficulties presented by Whiteley (1979), who developed several equations to predict wine stability, that could not be generalised because wine susceptibility was quite variable even for equivalent levels of ethanol, sulphite, and sorbic acid.

6. Conclusions and future trends

From the several aspects covered in this review, the main conclusions may be summarised as follows:

– knowledge of the microbial ecology of grapes, particularly damaged grapes, wineries and vectors has

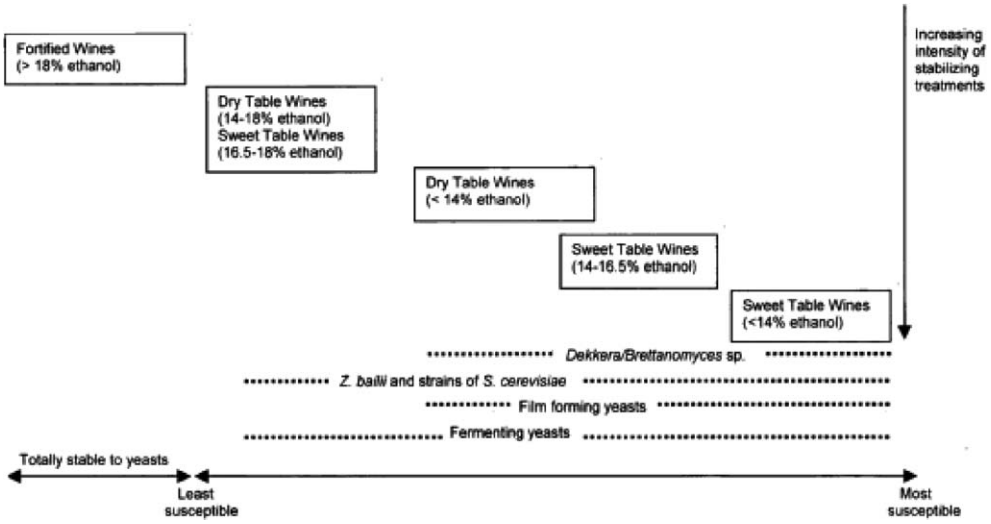

Fig. 2. The susceptibility of wines to the yeast colonisation. (Note: dry table wines have less than 2 g/l reducing sugars.)

many gaps that do not enable a full understanding of the origin and dissemination of spoilage yeasts in wines;
- some yeast species are of great concern in wine industry, namely, *Dekkera*/*Brettanomyces* spp. in red wines matured in oak barrels and in wines originating from poor sanitary quality grapes;
- microbiological criteria used in industry are, as a rule, old-fashioned, arbitrary, and established without scientific background;
- scientific research has not yet been able to produce the knowledge needed to solve the problems caused by spoilage yeasts;
- considering the currently available methods, the wine industry cannot implement efficient and appropriate HACCP systems for spoilage yeasts.

Therefore, developments of future research should be aimed to:

- provide a better knowledge of the yeast microflora of damaged and dried grapes;
- elucidate the role of insects as spoilage yeast colonisers of grapes and wineries;
- transfer the rapid molecular typing techniques from research laboratories to the industry laboratories;
- quantify wine susceptibility to yeast colonisation;
- improve predictive models of wine spoilage.

This review also identifies the needs of the wine industry that continues to use inappropriate or obsolete methodologies to monitor spoilage yeasts. Future measures should be implemented aiming to improve the wine spoilage risk management:

- to avoid the dissemination of spoilage yeasts in the winery;
- to apply adequate zymological control to each type of wine;
- to standardise microbiological criteria, namely, sampling by attributes, using standard analytical methods and appropriate specifications.

All proposed measures could be much more easily implemented if researchers and industry worked more closely in the future.

Acknowledgements

We are indebted to the colleagues that forwarded our inquiry to wine and wholesalers companies as well as to those answering to our request on procedures of microbiological control.

References

Alguacil, M., Fidalgo, M., Jiménez, J., Lozano, J.I., Neva, M.A., Perdigones, F., 1998. Detección de *Brettanomyces*/*Dekkera* en instalaciones de vendimia mediante PCR. Aliment., Equipos Tecnol. 10, 81–85.

Amerine, M.A., Kunkee, R.E., 1965. Yeast stability tests on dessert wines. Vitis 5, 187–194.

Andrews, S., 1992. Specifications for yeasts in Australian beer, wine and fruit juice products. In: Samson, R.A., Hocking, A.D., Pitt, J.I., King, A.D. (Eds.), Modern Methods in Food Mycology. Elsevier, Amsterdam, pp. 111–118.

Anonymous, 1996. Food Micromodel Software (version 2). Food Micromodel, Leatherhead, UK.

Anonymous, 1998. Recueil des Méthodes Internationales D'analyse des Vins et des Moûts. Office International de la Vigne et du Vin, Paris, France. O.I.V., ed.

Anonymous, 1999. The Australian Wine Research Institute (Eds.), 1999. The Code of Good Manufacturing Practice for the Australian Grape and Wine Industry. Glen Osmond, Australia.

Arias, C.R., Burns, J.K., Friedrich, L.M., Goodrich, R.M., Parish, M.E., 2002. Yeast species associated with orange juice: evaluation of different identification methods. Appl. Environ. Microbiol. 68, 1955–1961.

Augustyn, O., Kock, J., Ferreira, D., 1992. Differentiation between yeast species, and strains within a species, by cellular fatty acid analysis 5. A feasible technique? Syst. Appl. Microbiol. 15, 105–115.

Baleiras-Couto, M.M., Vogels, J.T.W.E., Hofstra, H., Huis in't Veld, J.H.J., van der Vossen, J.M.B.M., 1995. Random amplified polymorphic DNA and restriction enzyme analysis of PCR Amplified rDNA in taxonomy: two identification techniques for foodborne yeasts. J. Appl. Bacteriol. 79, 525–535.

Baleiras-Couto, M.M., Hartog, B.J., Huis in't Veld, J.H.J., Hofstra, H., van der Vossen, J.M.B.M., 1996. Identification of spoilage yeasts in a food-production chain by microsatellite polymerase chain reaction fingrprinting. Food Microbiol. 13, 59–67.

Barros-Lopes, M., Rainieri, S., Henschke, P.A., Landridge, P., 1999. AFLP fingerprinting for analysis of yeast genetic variation. Int. J. Syst. Bacteriol. 49, 915–924.

Beech, F.W., Davenport, R.R., 1970. The role of yeasts in cider making. In: Rose, A.H., Harrison, J.S. (Eds.), The Yeasts, vol. 3. Academic Press, New York, pp. 73–146.

Belin, J.-M., 1972. Recherches sur la répartition des levures à la surface de la grappe de raisin. Vitis 11, 135–145.

Beuchat, L.R., 1993. Selective media for detecting and enumerating foodborne yeasts. Int. J. Food Microbiol. 19, 1–14.

Beuchat, L.R., Copeland, F., Curiale, M.S., Danisavich, T., Gangar, V., King, B.W., Lawlis, T.L., Likin, R.O., Okwusoa, J., Smith, C.F., Townsend, D.E., 1998. Comparison of the simplate total plate count method with petrifilm, redigel, and conventional pour-plate methods for enumerating aerobic microorganisms in foods. J. Food Prot. 61, 14–18.

Beuchat, L.R., Scouten, A.J., Jablonska, J., 2002. Influence of composition of diluent on populations of yeasts and moulds recovered from raw fruits. Lett. Appl. Microbiol. 35, 399–402.

Bisiach, M., Minervini, G., Zerbetto, F., 1986. Possible integrated control of grapevine sour rot. Vitis 25, 118–128.

Blancard, D., Gravote, E., Jailloux, F., Fermaud, M., 1999. Etiologie de la pourriture acide de la vigne dans le Sud-ouest de la France. Proceedings of the OILB Meeting Working Group "Integrated Control in Viticulture", Sub-Group Fungal and Bacterial Diseases, 1–4 March, Florence, Italy, 51–54.

Boekhout, T., Kurtzman, C.P., O'Donnell, K., Smith, M.T., 1994. Phylogeny of the yeast genera hanseniaspora (Anamorph *Kloeckera*), *Dekkera* (Anamorph *Brettanomyces*), and eeniella as inferred from partial 26S ribosomal DNA nucleotide sequences. Int. J. Syst. Bacteriol. 10, 781–786.

Boekhout, T., Robert, V., Smith, M., Stalpers, J., Yarrow, D., Boer, P., Gijswijt, G., Kurtzman, C., Fell, J., Guého, E., Guillot, J., Roberts, I., 2002. Yeasts of the World—Morphology, Physiology, Sequences and Identification. CD-ROM from ETI Information Services, Wokingham, UK.

Bouix, M., Grabowski, A., Charpentier, M., Leveau, J.Y., Duteurtre, B., 1999. Évaluation rapide de la contamination des moûts par cytrométrie en flux. J. Int. Sci. Vigne Vin 33, 25–32.

Boulton, B.R., Singleton, V.L., Bisson, L.F., Kunkee, R.E., 1996. Principles and Practices of Winemaking. Chapman and Hall, New York.

Cai, J., Roberts, I.N., Collins, M.D., 1996. Phylogenetic relationship among members of the ascomycetous yeast genera *Brettanomyces*, *Debaryomyces*, *Dekkera*, and *Kluyveromyces* deduced by small-subunit rRNA gene sequences. Int. J. Syst. Bacteriol. 4, 542–549.

Carreira, A., Loureiro, V., 1998. A differential medium to detect *Yarrowia lipolytica* in 24 hours. J. Food Mycol. 1, 3–12.

Caruso, M., Capece, A., Salzano, G., Romano, P., 2002. Typing of *Saccharomyces cerevisiae* and *Kloeckera apiculata* strains from Aglianico wine. Lett. Appl. Microbiol. 34, 323–328.

Cavin, J., Andioc, P., Etievant, P., Divies, C., 1993. Ability of wine lactic bacteria to metabolize phenol carboxylic acids. Am. J. Enol. Vitic. 1, 76–80.

Chaskes, S., Tyndall, R.L., 1975. Pigment Production by *Cryptococcus neoformans* from *para*- and *ortho*-diphenols: effect of the nitrogen source. J. Clin. Microbiol. 1, 509–514.

Chatonnet, P., Dubourdieu, D., Boidron, J.N., Pons, M., 1992. The origin of ethylphenols in wines. J. Sci. Food Agric. 60, 165–178.

Chatonnet, P., Boidron, J., Dubourdieu, D., 1993. Influence des conditions d'élevage et de sulfitage des vins rouges en barriques sur le teneur en ácide acétique et en ethyl-phenols. J. Int. Sci. Vigne Vin. 27, 277–298.

Chatonnet, P., Dubourdieu, D., Boidron, J.N., 1995. The influence of *Brettanomyces*/*Dekkera* spp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. Am. J. Enol. Vitic. 46, 463–468.

Chatonnet, P., Viala, C., Dubourdieu, D., 1997. Influence of polyphenolic components of red wines on the microbial synthesis of volatile phenols. Am. J. Enol. Vitic. 48, 443–448.

Cocolin, L., Bisson, L.F., Mills, D.A., 2000. Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiol. Lett. 189, 81–87.

Cocolin, L., Heisei, A., Mills, D.A., 2001. Direct identification of the indigenous yeasts in commercial wine fermentations. Am. J. Enol. Vitic. 52, 49–53.

Cocolin, L., Manzano, M., Rebecca, S., Comi, G., 2002. Monitoring of yeast population changes during a continuous wine fermentation by molecular methods. Am. J. Enol. Vitic. 53, 24–27.

Comi, G., Maifreni, M., Manzano, M., Lagazio, C., Cocolin, L., 2000. Mitochondrial DNA restriction enzyme analysis and evaluation of the enological characteristics of *Saccharomyces cerevisiae* strains isolated from grapes of the wine-producing area of Collio (Italy). Intern. J. Food Microbiol. 58, 117–121.

Connel, L., Stender, H., Edwards, C.G., 2002. Rapid detection and identification of *Brettanomyces* from winery air samples based on peptide nucleic acid analysis. Am. J. Enol. Vitic. 53, 322–324.

Constantí, M., Reguant, C., Poblet, M., Zamora, F., Mas, A., 1998. Molecular population dynamics: effect of sulphur dioxide and inoculum on must fermentation. Int. J. Food Microbiol. 41, 169–175.

Davenport, R.R., 1974. Microecology of yeasts and yeast like organisms associated with an English vineyard. Vitis 13, 123–130.

Davenport, R.R., 1976. Microflora of fruit juice products. Long Ashton Report, University of Bristol, UK.

Davenport, R.R., 1980. An outline guide to media and methods for studying yeasts and yeast-like organisms. In: Skinner, F., Passmore, S., Davenport, R. (Eds.), Biology and Activities of Yeasts. Academic Press, London, pp. 261–278.

Davenport, R.R., 1986. Unnaceptable levels for yeasts. In: King, A., Pitt, J., Beuchat, L., Corry, J. (Eds.), Methods for the Mycological Examination of Food. Plenum Press, New York, pp. 214–215.

De Boer, E., Beumer, R., 1999. Methodology for detection and typing of foodborne microorganisms. Int. J. Food Microbiol. 50, 119–130.

Deak, T., Beuchat, L.R., 1996. Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, USA.

Deak, T., Reichart, O., 1986. Unnaceptable levels of yeasts in bottled wine. In: King, A., Pitt, J., Beuchat, L., Corry, J. (Eds.), Methods for the Mycological Examination of Food. Plenum Press, New York, pp. 215–218.

Delfini, C., 1988. Refermentation potential in bottled sweet wines of yeasts adapted to sulfur dioxide. Chem. Mikrobiol. Technol. 11, 137–142.

Dennis, C., Buhagiar, R., 1980. Yeast spoilage of fresh and processed fruits and vegetables. In: Skinner, F., Passmore, S., Davenport, R. (Eds.), Biology and Activities of Yeasts. Academic Press, London, pp. 123–134.

Dias, L., Dias, S., Sancho, T., Stender, H., Querol, A., Malfeito-Ferreira, M., Loureiro, V., 2003. Identification of yeasts origi-

nated from wine related environments and capable of producing 4-ethylphenol. Food Microbiol. (in press).
Di Stefano, R., 1985. Gli etil fenoli nei vini. Vignevini 5, 35–38.
Dlauchy, D., Tornai-Lehoczli, J., Péter, G., 1999. Restriction enzyme analysis of PCR amplified rDNA as a taxonomic tool in yeast identification. Syst. Appl. Microbiol. 22, 445–453.
Donèche, B.J., 1992. Botrytized wines. In: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology. Harwood Academic Pubs, pp. 327–351.
Donnelly, D.M., 1977a. Airborne microbial contamination in a winery bottling room. Am. J. Enol. Vitic. 28, 176–181.
Donnelly, D.M., 1977b. Elimination from table wines of yeast contamination by filling machines. Am. J. Enol. Vitic. 28, 182–184.
Egli, C.M., Henick-Kling, T., 2001. Identification of *Brettanomyces*/*Dekkera* species based on polymorphism in the rRNA internal transcribed spacer region. Am. J. Enol. Vitic. 52, 241–247.
Egli, C.M., Edinger, W., Mitrakul, C., Henick-Kling, T., 1998. Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and chardonnay wines. J. Appl. Microbiol. 85, 779–789.
Epifanio, S., Gutierrez, A.R., Santamaría, M.P., López, R., 1999. The influence of enological practices on the selection of wild yeast strains in spontaneous fermentation. Am. J. Enol. Vitic. 50, 219–224.
Erickson, J., 1993. Hydrophobic membrane filtration method for the selective recovery and differentiation of *Z. bailii* in acidified ingredients. J. Food Prot. 563, 234–238.
Esch, F., 1992. Yeast in soft drinks and concentrated fruit juices. Brygmesteren 4, 9–20.
Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. 49, 329–337.
Esteve-Zarzoso, B., Gostíncar, A., Bobet, R., Uruburu, F., Querol, A., 2000. Selection and molecular characterixation of wine yeasts isolated from the 'El Penedès' area (Spain). Food Microbiol. 17, 553–562.
Fernández, M., Úbeda, J.F., Briones, A.I., 2000. Typing of non-*Saccharomyces* yeasts with enzymatic activities of interest in winemaking. Int. J. Food Microbiol. 59, 29–36.
Fernández-Espinar, M.T., López, V., Ramón, D., Bartra, E., Querol, A., 2001. Study of the authenticity of commercial wine yeast strains by molecular techniques. Intern. J. Food Microbiol. 70, 1–10.
Fleet, G.H., 1992. Spoilage yeasts. Crit. Rev. Biotechnol. 12, 1–44.
Fleet, G.H., 1999. Microorganisms in food ecosystems. Int. J. Food Microbiol. 50, 101–117.
Florenzano, G., Balloni, W., Materassi, R., 1977. Contributo alla ecologia dei lieviti *Schizosaccharomyces* sulle uve. Vitis 16, 38–44.
Freydiere, A.-M., Guinet, R., Boiron, P., 2001. Yeast identification in the clinical microbiology laboratory: phenotypical methods. Medic. Mycol. 39, 9–33.
Fugelsang, K., 1997. Wine Microbiology. The Chapman and Hall Enology Library, New York.
Gadoury, D.M., Seem, R.C., Wilcox, W., Henick-Kling, T., 2002. Effect of powdery mildew on bunch rots, berry microflora, and juice and wine quality. Proceedings of the 4th International Workshop on Powdery and Downy Mildew in Grapevine. Department of Plant Pathology, University of California (Pubs.), Davis, pp. 64–65.
Granchi, L., Bosco, M., Messini, A., Vincenzini, M., 1999. Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. J. Appl. Microbiol. 87, 949–956.
Gravot, E., Blancard, D., Fermaud, M., Lonvaud, A., Joyeux, A., 2001. La pourriture acide. Phytoma 543, 36–39.
Guerzoni, E., Marchetti, R., 1987. Analysis of yeast flora associated with grape sour rot and of the chemical disease markers. Appl. Environ. Microbiol. 53, 571–576.
Guerzoni, M.E., Piva, M., Gardini, F., 1985. Proposal of a rapid HS-GLC method for microbiological control of food. Lebensm.-Wiss. Technol. 18, 128–132.
Guillamón, J.M., Barrio, E., Huerta, T., Querol, A., 1994. Rapid characterization of four species of the *Saccharomyces* Sensu-Stricto complex according to mitochnodrial DNA patterns. Int. J. Syst. Bacteriol. 44, 708–714.
Guillamón, J.M., Sabaté, J., Barrio, E., Cano, J., Querol, A., 1998. Rapid indentification of wine yeasts species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Arch. Microbiol. 169, 387–392.
Gutiérrez, A.R., Santamaria, P., Epifanio, S., Garijo, P., López, R., 1999. Ecology of spontaneous fermentation in one winery during 5 consecutive years. Lett. Appl. Microbiol. 29, 411–415.
Gutiérrez, A., Epifanio, S., Garijo, P., López, R., Santamaría, P., 2001. Killer yeasts: incidence in the ecology of spontaneous fermentation. Am. J. Enol. Vitic 52, 352–356.
Harrigan, W., 1998. Laboratory Methods in Food Microbiology. Academic Press, San Diego.
Heard, G.M., Fleet, G.H., 1986. Evaluation of selective media for enumeration of yeasts during wine fermentation. J. Appl. Bacteriol. 60, 477–481.
Henschke, P.A., Thomas, D.S., 1988. Detection of wine-spoiling yeasts by electronic methods. J. Appl. Bacteriol. 64, 123–133.
Heras-Vazquez, F., Mingorance-Cazorla, L., Clemente-Jimenez, J.M., Rodriguez-Vico, F., 2003. Identification of yeast species from orange fruit and juice by RFLP and sequence analysis of the 5.8S rRNA gene and the two internal transcribed spacers. FEMS Yeast Res. 3, 3–9.
Heresztyn, T., 1986. Metabolism of volatile phenolic compounds from hidroxycinnamic acids by *Brettanomyces* yeast. Arch. Microbiol. 146, 96–98.
Hock, S., 1990. Coping with *Brettanomyces*. Pratical Winery and Vineyard 1/2, 26–31.
Hocking, A.D., 1996. Media for preservative resistant yeasts: a collaborative study. Int. J. Food Microbiol. 29, 167–175.
Ibeas, J.I., Lozano, I., Perdigones, F., Jiménez, J., 1996. Detection of *Dekkera*–*Brettanomyces* strains in sherry by a Nested PCR method. Appl. Environ. Microbiol. 62, 998–1003.
Ingram, M., 1958. Yeasts in food spoilage. In: Cook, A.H. (Ed.), The Chemistry and Biology of Yeasts. Academic Press, London, pp. 603–633.
Kalathenos, P., 1995. Predictive Modelling of Wine Spoilage Mi-

croorganisms. PhD thesis. Institute of Food Research, Dept. of Microbiology. Reading, UK.

King, A.D., Pitt, J.I., Beuchat, L.R., Corry, J.E. (Eds.), 1986. Methods for the Mycological Examination of Food. Plenum Press, New York.

Kish, S., Sharf, R., Margalith, P., 1983. A note on a selective medium for wine yeasts. J. Appl. Bacteriol. 55, 177–179.

Kopke, C., Cristovão, A., Prata, A.M., Silva Pereira, C., Figueiredo-Marques, J.J., San-Romão, M.V., 2000. Microbiological control of wine. The application of epifluorescence microscopy method as a rapid technique. Food Microbiol. 17, 257–260.

Kühle, A., Jespersen, L., 1998. Detection and identification of wild yeasts in lager breweries. Int. J. Food Microbiol. 43, 205–213.

Kuniyuki, A.H., Rous, C., Sanserson, J.L., 1984. Enzyme-linked immunosorbent assay (ELISA) detection of *Brettanomyces* contaminants in wine production. Am. J. Enol. Vitic. 35, 143–145.

Kunkee, R., Bisson, L., 1993. Winemaking yeasts, 2nd ed. In: Rose, A.H., Harrison, J.S. (Eds.), The Yeasts, vol. 5. Academic Press, London, pp. 69–127.

Kurtzman, C.P., Fell, J.W. (Eds.), 1998. The Yeasts, A Taxonomic Study, 4th ed. Elsevier, Amsterdam.

Laureano, P., D'Antuono, I., Malfeito-Ferreira, M., Loureiro, V., 2003. Effect of different sanitation treatments on the numbers of total microorganisms and of *Dekkera bruxellensis* recovered from the wood of wine ageing barriques. Abstracts of the 23rd International Specialised Symposium on Yeasts, Budapest.

López, V., Querol, A., Ramón, D., Fernández-Espinar, M.T., 2001. A simplified procedure to analyse mitochnodrial DNA from industrial yeasts. Int. J. Food Microbiol. 68, 75–81.

López, V., Fernández-Espinar, M.T., Barrio, E., Ramón, D., Querol, A., 2002. A new PCR-based method for monitoring inoculated wine fermentations. Int. J. Food Microbiol. 81, 63–71.

Loureiro, V., 2000. Spoilage yeasts in food and beverages: characterisation and ecology for improved diagnosis and control. Food Res. Int. 33, 247–256.

Loureiro, V., Querol, A., 1999. The prevalence and control of spoilage yeasts in foods and beverages. Trends Food Sci. Technol. 10–11, 356–365.

Makdesi, A.K., Beuchat, L.R., 1996a. Evaluation of media enumerating heat-stressed, benzoate-resistant *Zygosaccharomyces bailii*. Int. J. Food Microbiol. 33, 169–181.

Makdesi, A.K., Beuchat, L.R., 1996b. Performance of selective media for enumerating *Zygosaccahromyces bailii* in acidic foods and beverages. J. Food Prot. 59, 652–656.

Malfeito-Ferreira, M., Lopes, J., Loureiro, V., 1989. Infecting yeasts in Portuguese bottled white wines. Proceedings of the XIIIth International Symposium on Yeasts. Leuven, Belgium, 34–35.

Malfeito-Ferreira, M., Tareco, M., Loureiro, V., 1997. Fatty-acid profiling: a feasible typing system to trace yeast contamination in bottling plants. Int. J. Food Microbiol. 38, 143–155.

Malfeito-Ferreira, M., Rodrigues, N., Loureiro, V., 2001. The influence of oxygen on the "horse sweat taint" in red wines. Ital. Food Bev. Technol. 24, 34–38.

Marchetti, R., Guerzoni, M., Gentile, M., 1984. Recherche sur l'étiologie d'une nouvelle maladie de la grappe: la pourriture acide. Vitis 23, 55–65.

Martini, A., 1993. Origin and domestication of the wine yeast *Saccharomyces cerevisiae*. J. Wine Res. 4, 165–176.

Martini, A., Federici, F., Rosini, G., 1980. A new approach to the study of yeast ecology of natural substrates. Can. J. Microbiol. 26, 856–859.

Martini, A., Ciani, M., Scorzetti, G., 1996. Direct enumeration and isolation of wine yeasts from grape surfaces. Am. J. Enol. Vitic. 47, 435–440.

Mian, M.A., Fleet, G.H., Hocking, D., 1997. Effect of diluent type on viability of yeasts enumerated from foods or pure culture. Int. J. Food Microbiol. 35, 103–107.

Millet, V., Lonvaud-Funel, A., 2000. The viable but non-culturable state of wine micro-organisms during storage. Lett. Appl. Microbiol. 30, 136–141.

Mills, D.A., Eric, A.J., Cocolin, L., 2002. Yeast diversity and persistence in botrytis-affected wine fermentations. Appl. Environ. Microbiol. 68, 4884–4893.

Minarik, E., 1983. Levures de contamination des vins embouteillé. Bull. O.I.V. 56 (628), 414–419.

Mitrakul, C.M., Henick-Kling, T., Egli, C.M., 1999. Discrimination of *Brettanomyces*/*Dekkera* yeast isolates from wine by using various DNA finger-printing methods. Food Microbiol. 16, 3–14.

Molina, F.I., Shen, P., Jong, S., 1992. Validation of the species concept in the genus *Dekkera* by restriction analysis of genes coding for rRNA. Int. J. Syst. Bacteriol. 43, 32–35.

Mortimer, R., Polsinelli, M., 1999. On the origins of wine yeast. Res. Microbiol. 150, 199–204.

Mossel, D.A., Struijk, C.B., 1992. The contribution of microbial ecology to management and monitoring of the safety, quality and acceptability (SQA) of foods. J. Appl. Bacteriol. (Symp. Suppl.) 73, 1S–22S.

Mossel, D.A., Corry, J.E., Struijk, C.B., Baird, R.M., 1995. Essentials of the Microbiology of Foods. Wiley, Chichester.

Neradt, F., 1982. Sources of reinfections during cold-sterile bottling of wine. Am. J. Enol. Vitic. 33, 140–144.

Pallman, C.L., Brown, J.A., Olineka, T.L., Cocolin, L., Mills, D.A., Bisson, L., 2001. Use of WL medium to profile native flora fermentations. Am. J. Enol. Vitic. 52, 198–203.

Perry-O'Keefe, H., Stender, H., Broomer, A., Oliveira, K., Coull, J., Hyldig-Nielsen, J.J., 2000. Filter-based PNA in situ hybridization for rapid detection, identification and enumeration of specific micro-organisms. J. Appl. Microbiol. 90, 180–189.

Peynaud, E., Domercq, S., 1955. Sur les especes de levures fermentant selectivement le fructose. Ann. Inst. Pasteur 89, 346–351.

Peynaud, E., Domercq, S., 1956. Sur les Brettanomyces isolés de raisins et de Vins. Arch. Mikrobiol. 24, 266–280.

Phaff, H.J., Stammer, W.T., 1980. Specifity of natural habitats for yeasts and yeast-like organisms. In: Skinner, F., Passmore, S., Davenport, R. (Eds.), Biology and Activities of Yeasts. Academic Press, London, pp. 79–102.

Pitt, J.I., Hocking, A.D., 1985. Fungi and Food Spoilage. Academic Press, Sydney, Australia.

Plata, C., Millán, C., Mauricio, J.C., Ortega, J.M., 2003. Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. Food Microbiol. 20, 217–224.

Pollnitz, A., Pardon, K., Sefton, M., 2000. Quantitative analysis of

4-ethylphenol and 4-ethylguaiacol in red wine. J. Chromatogr. A 874, 101–109.

Querol, A., Barrio, E., Huerta, T., Ramón, D., 1992a. Molecular monitoring of wine fermentations conduced by active dry yeast strains. Appl. Environ. Microbiol. 58, 2948–2953.

Querol, A., Barrio, E., Ramón, E., 1992b. A comparative study of different methods of yeast strains of *Saccharomyces cerevisae* characterization. Syst. Appl. Microbiol. 58, 2948–2953.

Querol, A., Fernández-Espinar, M.T., Barrio, E., 2003. Genetic variability of yeast in wine fermentation. In: Aroa, D.K., Bridge, P.D., Bhatnagar, D. (Eds.), Handbook of Fungal Biotechnology, 2nd ed., chap. 38. in press.

Quesada, M.P., Cenis, J.L., 1995. Use of random amplified polymorphic DNA (RAPD-PCR) in the characterization of wine yeasts. Am. J. Enol. Vitic. 46, 204–208.

Rale, V.B., Vakil, J.R., 1984. A note on an improved molybdate agar for the selective isolation of yeasts from tropical fruits. J. Appl. Bacteriol. 56, 409–413.

Rankine, B.C., Pilone, D.A., 1973. *Saccharomyces bailii*, a resistant yeast causing serious spoilage of bottled table wine. Am. J. Enol. Vitic. 24, 55–58.

Redzepovic, S., Orlic, S., Sikora, S., Majdak, A., Pretorius, I.S., 2002. Identification and characterization of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* strains isolated from Croation vineyards. Lett. Appl. Microbiol. 35, 1–6.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A., 2000. Handbook of Enology, vol. 1. The Microbiology of Wine and Vinification. Wiley, Chichester.

Rodriguez, S.B., 1987. A system for identifying spoilage yeast in packaged wine. Am. J. Enol. Vitic. 38, 273–276.

Rodrigues, N., Gonçalves, G., Malfeito-Ferreira, M., Loureiro, V., 2001. Development and use of a differential medium to detect yeasts of the genera *Dekkera*/*Brettanomyces*. Int. J. Food Microbiol. 90, 588–599.

Romano, P., Suzzi, G., Comi, G., Zironi, R., 1992. Higher alcohol and acetic acid production by apiculate wine yeasts. J. Appl. Bacteriol. 73, 126–130.

Rosini, G., Federici, F., Martini, A., 1982. Yeast flora of grape berries during ripening. Microbiol. Ecol. 8, 83–89.

Sabate, J., Cano, J., Esteve-Zarzoso, B., Guillamón, J.M., 2002. Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes. Microbiol. Res. 157, 1–8.

Samson, R.A., Hocking, A.D., Pitt, J.I., King, A.D. (Eds.), 1992. Modern Methods in Food Mycology. Academic Press, London.

Sancho, T., Giménez-Jurado, G., Malfeito-Ferreira, M., Loureiro, V., 2000. Zymological indicators: a new concept applied to the detection of potential spoilage yeast species associated with fruit pulps and concentrates. Food Microbiol. 17, 613–624.

Schuller, D., Côrte-Real, M., Leão, C., 2000. A differential medium for the enumeration of the spoilage yeast *Zygosaccharomyces bailii* in wine. J. Food Prot. 63, 1570–1575.

Schütz, M., Gafner, J., 1993. Analysis of yeast diversity during spontaneous and induced alcoholic fermentations. J. Appl. Bacteriol. 75, 551–558.

Sponholz, W.-F., 1992. Wine spoilage by microorganisms. In: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology. Harwood Academic Publishers, pp. 395–419.

Stender, H., Kurtzman, C., Hyldig-Nielsen, J., Sorensen, D., Broomer, A., Oliveira, K., Perry-O'Keefe, H., Sage, A., Young, B., Coull, J., 2001. Identification of *Dekkera bruxellensis* (*Brettanomyces*) from wine by fluorescence in situ hybridization using peptide nucleic acid probes. Appl. Environ. Microbiol. 67, 938–941.

Stender, H., Fiandaca, M., Hyldig-Nielsen, J.J., Coull, J., 2002. PNA for rapid microbiology. J. Microbiol. Methods 48, 1–17.

Stummer, B., Francis, I., Markides, A., Scott, E.S., 2002. Proceedings of the 4th International Workshop on Powdery and Downy Mildew in Grapevine. Dept. of Plant Pathology, University of California, Davis, pp. 62–63.

Techera, A.G., Jubany, S., Carrau, F.M., Gaggero, C., 2001. Differentiation of industrial wine yeast strains using microsatellite markers. Lett. Appl. Microbiol. 33, 71–75.

Thomas, D.S., 1983. Susceptibility of wines to yeast spoilage. Long Ashton Report, University of Bristol, UK.

Thomas, D.S., 1993. Yeasts as spoilage organisms in beverages, 2nd ed. In: Rose, A.H., Harrison, J.S. (Eds.), The Yeasts, vol. 5. Academic Press, London, pp. 517–561.

Thomas, D.S., Ackerman, J.C., 1988. A selective medium for detecting yeasts capable of spoiling wine. J. Appl. Bacteriol. 65, 299–308.

Thomas, D.S., Davenport, R.R., 1985. *Z. bailii*—a profile of characteristics and spoilage activities. Food Microbiol. 2, 157–169.

Torija, M.J., Rozès, N., Poblet, M., Guillamón, J.M., Mas, A., 2002. Effects of fermentation on the strain population of *Saccharomyces cerevisiae*. Int. J. Food Microbiol. 80, 47–53.

Török, T., Mortimer, R.K., Romano, P., Suzzi, G., Polsinelli, M., 1996. Quest for wine yeasts—an old story revisited. J. Ind. Microbiol. 17, 303–313.

Tucknot, O.G., Davis, P.A., Rosser, P.R., 1981. Taints in fermented beverages. Annual Report of the Long Ashton Research Station, University of Bristol.

Tudor, E.A., Board, R.G., 1993. Food spoilage yeasts, 2nd ed. In: Rose, A.H., Harrison, J.S. (Eds.), The Yeasts, vol. 5. Academic Press, London, pp. 435–516.

Valderrama, M.J., Silóniz, M.I., Gonzalo, P., Peinado, J.M., 1999. A differential medium for the isolation of *Kluyveromyces marxianus* and *Kluyveromyces lactis* from dairy products. J. Food Prot. 62, 189–193.

Van der Vossen, J.M., Hofstra, H., 1996. DNA based typing, identification and detection systems for food spoilage microorganisms: development and implementation. Int. J. Food Microbiol. 33, 35–49.

Van der Walt, J.P., van der Kerken, A.E., 1958. The wine yeast of the cape: part I. A taxonomical survey of the yeasts causing turbidity in South African table wines. Antoine Van Leeuwenhoek 24, 239–252.

Van der Walt, J.P., van der Kerken, A.E., 1961. The wine yeasts of the cape: part V. Studies on the occurrence of *Brettanomyces intermedius* and *Brettanomyces schanderlii*. Antonie van Leeuwenhoek 27, 81–90.

Van der Westhuizen, T., Augustyn, O., Khan, W., Pretorius, I.S., 2000a. Geographical distribution of Indigenous *Saccharomyces*

cerevisiae strains isolated from vineyard in the coastal regional of the Western Cape in south Africa. South Afr. J. Enol. Vitic. 21, 3–9.

Van der Westhuizen, T., Augustyn, O., Khan, W., Pretorius, I.S., 2000b. Seasonal variation of indigenous *Saccharomyces cerevisiae* strains isolated from vineyard of the Western Cape in south Africa. South Afr. J. Enol. Vitic. 21, 10–15.

Vaughan-Martini, A., Martini, A., 1995. Facts, myths and legends on the prime industrial microorganism. J. Ind. Microbiol. 14, 514–522.

Vaz-Oliveira, M., Barros, P., Loureiro, V., 1995. Analyse microbiologique du vin. Tecnhiques des tubes multiples pour l'enumeration de micro-microorganisms dans les vins. Feuilles Verts O.I.V. 987, Paris.

Veal, D.A., Deere, D., Ferrari, B., Piper, J., Attfield, P.V., 2000. Fluorescence staining and flow cytometry for monitoring microbial cells. J. Immunol. Methods 243, 191–210.

Velásquez, E., Cruz-Sánchez, J.M., Rivas-Palá, T., Zurdo-Pineiro, J.L., Mateos, P.F., Monte, E., Martínez-Molina, E., Chordi, A., 2001. YeastIdent-Food/ProleFood, a new system for the identification of food yeasts based on physiological and biochemical tests. Food Microbiol. 18, 637–646.

Warth, A.D., 1985. Resistance of yeast species to benzoic and sorbic acid and sulphur dioxide. J. Food Prot. 48, 564–569.

White, T., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Gelfand, D., Sininsky, J., White, T. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp. 315–322.

Whiteley, L., 1979. The Microbiological Spoilage of White Wines. Second report to the Consortium of wine companies. Long Ashton, Bristol, UK.

Wium, H., Malfeito-Ferreira, M., St. Aubyn, A., Loureiro, V., 1990. Rapid characterization of yeasts contaminants associated with sparkling wine production. Industrie delle Bevande 19, 504–506.

Zohre, D.E., Erten, H., 2002. The influence of *Kloeckera apiculata* and *Candida pulcherrima* yeasts on wine fermentation. Proc. Biochem. 38, 319–324.