Review

Spoilage yeasts in the wine industry

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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.

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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
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-

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<th>Table 1</th>
<th>Contamination and spoilage yeast recovered from foods and beverages</th>
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<tr>
<td><strong>Most frequent contaminants</strong>&lt;sup&gt;a&lt;/sup&gt; (Deak and Beuchat, 1996)</td>
<td><strong>Spoilage species</strong> (Pitt and Hocking, 1985)</td>
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<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Brettanomyces intermedium&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><em>Debaryomyces Hansenii</em></td>
<td><em>Candida holmi</em></td>
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<td><em>Pichia anomala</em></td>
<td><em>Candida krusei</em></td>
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<td><em>Pichia membranifaciens</em></td>
<td><em>Debaryomyces hansenii</em></td>
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<td><em>Rhodotorula glutinis</em></td>
<td><em>Kloeckera apiculata</em></td>
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<td><em>Rhodotorula mucilaginosa</em></td>
<td><em>Pichia membranifaciens</em></td>
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<tr>
<td><em>Torulaspora delbrueckii</em></td>
<td><em>Saccharomyces cerevisiae</em></td>
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<td><em>Klyveromyces marxianus</em></td>
<td><em>Schizosaccharomyces pombe</em></td>
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<td><em>Issatchenkia orientalis</em></td>
<td><em>Zygosaccharomyces bailii</em></td>
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<td><em>Zygosaccharomyces bailii</em></td>
<td><em>Zygosaccharomyces bisporus</em></td>
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<td><em>Candida parapsilosis</em></td>
<td><em>Zygosaccharomyces rouxii</em></td>
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<td><em>Candida guilliermondii</em></td>
<td><em>Hansenula anomala</em></td>
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<td><em>Candida albida</em></td>
<td><em>Klyveromyces marxianus</em></td>
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<td><em>Candida tropicalis</em></td>
<td><em>Pichia fermentans</em></td>
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<td><em>Saccharomyces exigus</em></td>
<td><em>Sporobolomyces roseus</em></td>
</tr>
<tr>
<td><em>Pichia fermentans</em></td>
<td><em>Torulaspora delbrueckii</em></td>
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<tr>
<td><em>Trichosporon pullulans</em></td>
<td><em>Hansenula pullulans</em></td>
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<tr>
<td><em>Hansenula uvarum</em></td>
<td><em>Candida zeylanoides</em></td>
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<sup>a</sup> Species presented by decreasing level of occurrence.

<sup>b</sup> Anamorph of *D. bruxellensis*. 
garded as spoilers (e.g., \textit{Rhodotorula} spp.) and dangerous spoilage species are not necessarily frequent contaminants (e.g., \textit{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 \textit{Pichia anomala} (Plata et al., 2003)) or the early stage of fermentation (e.g., acetate production by \textit{Kloeckera apiculata}/\textit{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 (\textit{Saccharomyces cerevisiae}), able to referment sweet bottled wines; (ii) \textit{Zygosaccharomyces bailii}; (iii) film-forming yeasts (\textit{Hansenula}, \textit{Kloeckera}, \textit{Pichia}, \textit{Metchnikowia}, \textit{Debaryomyces}); (iv) \textit{Brettanomyces} spp. Similarly, Sponholz (1992) highlighted the problems due to ester production and film formation by species of \textit{Hansenula}, \textit{Candida} and \textit{Pichia}, while referring to \textit{Z. bailii} and \textit{Brettanomyces} spp. as the most dangerous wine spoilage yeasts. The relevance of \textit{Z. bailii} and \textit{Brettanomyces} spp. was also stressed by Boulton et al. (1996) and Fugelsang (1997), who described their spoilage abilities in detail. \textit{Schizosaccharomyces pombe} and \textit{Saccharomycodes ludwigi}, 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 \textit{D. bruxellensis}, \textit{Z. bailii}, and \textit{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 \textit{Z. bailii} due to its sorbic acid and sulphite tolerance at high ethanol levels (Malfeito-Ferreira et al., 1989). Furthermore, strains of \textit{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. Heresztyzn (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 guilliermondi 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 pathogenic 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

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<td>Culture media for the enumeration of particular foodborne yeasts</td>
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<td>DBDM</td>
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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 techni-
ques 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, biolumines-
cence, 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-Oli-
veira 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 conse-
quence, various miniaturised and simplified identifi-
cation methods have been developed. However,
they use the same approach as the classical identifi-
cation methods, being time consuming, even when
procedures are automated and computerised, and
often result in false identifications (Deak and Beu-
chat, 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). Pres-
ently, physiological and biochemical tests are still
being developed and used. The simplified identifi-
cation 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 devel-
oped for bacterial identification. Techniques like re-
striction fragment length polymor-phism (RFLP) of
mitochondrial DNA, chromosomal DNA electropho-
resis, 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 molec-
ular 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 micro-
biologists.

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

<table>
<thead>
<tr>
<th>Technique</th>
<th>Target species</th>
<th>Biomass source</th>
<th>Reference</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFLP-mtDNA restriction analysis</td>
<td><em>S. cerevisiae</em> and <em>Saccharomyces</em> spp.</td>
<td>broth cultures or plate colonies</td>
<td>Guillamon et al. (1994), Epifanio et al. (1999), Gutiérrez et al. (1999), Esteve-Zarzoso et al. (2000), Comi et al. (2000), Torija et al. (2002)</td>
<td>based on Querol et al. (1992a,b)</td>
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<tr>
<td>with several restriction enzymes</td>
<td></td>
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<tr>
<td>RFLP-mtDNA restriction analysis</td>
<td><em>S. cerevisiae</em> and several wine related species</td>
<td>broth cultures in microcentrifuge tubes</td>
<td>López et al. (2001)</td>
<td>improvement of Querol et al. (1992a,b) technique; time: 25, 5 h based on White et al. (1990)</td>
</tr>
<tr>
<td>with <em>Hinf</em> I and <em>Dde</em> I</td>
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<tr>
<td>PCR-RFLP of the 5.8S, 18S or 28S ITS regions of the rRNA gene, followed by restriction with several enzymes</td>
<td>Several yeast species</td>
<td>plate colonies or broth cultures</td>
<td>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)</td>
<td>based on Esteve-Zarzoso et al. (1999) and Querol et al. (1992a,b)</td>
</tr>
<tr>
<td>RFLP-mtDNA restriction analysis</td>
<td><em>S. cerevisiae</em> and several wine species</td>
<td>plate colonies</td>
<td>Gutiérrez et al. (2001)</td>
<td>based on Querol et al. (1992a,b)</td>
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<tr>
<td>PCR-RFLP of 18S rRNA and ITS 1 region</td>
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<tr>
<td>RFLP-mtDNA restriction analysis; electrophoretic karyotyping by CHEF; PCR amplification of δ sequences</td>
<td><em>Saccharomyces</em> spp. starters</td>
<td>plate colonies</td>
<td>Fernández-Espinar et al. (2001)</td>
<td>based on Querol et al. (1992a,b)</td>
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<tr>
<td>RFLP-mtDNA restriction analysis</td>
<td><em>S. cerevisiae</em></td>
<td>broth cultures</td>
<td>Constantí et al. (1998)</td>
<td>based on Querol et al. (1992a,b)</td>
</tr>
<tr>
<td>PCR-RFLP of the 5.8S-ITS region of the rRNA gene</td>
<td>several yeast species</td>
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<tr>
<td>PCR fingerprinting with (GAC)$_3$ and (GTG)$_3$ primers and NTS region amplification and restriction with <em>Hae</em>III and <em>Msp</em>I</td>
<td><em>S. cerevisiae</em> and <em>K. apiculata</em></td>
<td>broth cultures</td>
<td>Caruso et al. (2002)</td>
<td>based on Baleiras-Couto et al. (1995, 1996)</td>
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<tr>
<td>Electrophoretic karyotyping by CHEF</td>
<td>several species</td>
<td>plate colonies</td>
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<tr>
<td>RFLP-PCR of rDNA</td>
<td><em>Dekkera</em>/ <em>Brettanomyces</em> spp.</td>
<td>broth cultures</td>
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<tr>
<td>Nested PCR of DNA</td>
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<td>RAPD-PCR of total DNA</td>
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<tr>
<td>AFLP selective PCR amplification of restriction fragments of total DNA</td>
<td>several species</td>
<td>plate colonies or broth cultures</td>
<td>Alguacil et al. (1998)</td>
<td>based on Ibeas et al. (1996)</td>
</tr>
<tr>
<td>Staircase electrophoresis of low-molecular-weight RNA profiles</td>
<td>several species</td>
<td>not described</td>
<td></td>
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<tr>
<td>PCR amplification of DNA SSRs</td>
<td><em>S. cerevisiae</em> starters</td>
<td>broth cultures</td>
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<tr>
<td>PNA FISH targeting rRNA</td>
<td><em>D. bruxellensis</em></td>
<td>plate colonies</td>
<td>Stender et al. (2001)</td>
<td>probes based on the sequence of D1–D2 region of 26S rDNA</td>
</tr>
</tbody>
</table>

(continued on next page)
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 Dekkeria 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, Klyuyveromyces, 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, Metschnikowia 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, A. pullulans, 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 other 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 sanitisation 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 mumified 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*<sub>ww</sub>, 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 sublethral 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; *Malfieito-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* (*Malfieito-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 pathogens 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 (Lourenço and Querol, 1999; Loureiro, 2000). Briefly, it is possible to separate the yeasts 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).
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 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)

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

* 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,
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 to 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. bailii 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. bailii, S. cerevisiae, and S. ludwigii per bottle. Spoilage was assessed by visual observation of growth after 8 weeks. This author concluded that 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. bailii, 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,
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

<table>
<thead>
<tr>
<th>Table 6</th>
<th>Bottled wine microbial specifications used by wineries and wholesalers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winery ¹</td>
<td>Acceptable levels</td>
</tr>
<tr>
<td>1</td>
<td>&lt;1:100 ml total counts</td>
</tr>
<tr>
<td>2</td>
<td>&lt;100/ml yeast</td>
</tr>
<tr>
<td>3</td>
<td>&lt;1/ml yeast</td>
</tr>
<tr>
<td>4</td>
<td>&lt;30:750 ml total counts</td>
</tr>
<tr>
<td>5</td>
<td>&lt;25:200 ml total counts</td>
</tr>
<tr>
<td>6</td>
<td>&lt;2:250 ml yeast</td>
</tr>
<tr>
<td>7</td>
<td>&lt;100:100 ml total counts</td>
</tr>
<tr>
<td>8</td>
<td>&lt;75:100 ml total counts</td>
</tr>
<tr>
<td>9</td>
<td>&lt;10:100 ml yeast</td>
</tr>
<tr>
<td>10</td>
<td>0–5:100 ml yeast</td>
</tr>
<tr>
<td>11</td>
<td>0:500 ml yeast</td>
</tr>
<tr>
<td>12</td>
<td>1:500 ml yeast</td>
</tr>
<tr>
<td>500 ml yeast</td>
<td>sugar &lt;2 g/l, or sugar &gt;2 g/l and free SO₂ &gt;40 mg/l</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Wholesaler</th>
<th>Unacceptable levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;100:100 ml total counts</td>
</tr>
<tr>
<td>2</td>
<td>&gt;200:100 ml total counts</td>
</tr>
<tr>
<td>3</td>
<td>&gt;100:100 ml yeast</td>
</tr>
<tr>
<td>4</td>
<td>&gt;1000:100 ml yeast</td>
</tr>
<tr>
<td>5</td>
<td>&gt;0:100 ml yeast</td>
</tr>
<tr>
<td>6</td>
<td>&gt;200:100 ml yeast</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 7</th>
<th>Procedures used by wineries when microbial specifications are not met</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winery ³, ⁸, ¹⁰</td>
<td>hold for a period of time and retest</td>
</tr>
<tr>
<td>⁴, ¹¹</td>
<td>hold for enough time to meet specifications or to reprocess the affected product</td>
</tr>
<tr>
<td>⁶</td>
<td>a second set of bottles is analysed the following day to confirm yeast presence</td>
</tr>
<tr>
<td>⁷</td>
<td>hold and repeat; if still over limits, hold until values are lower than 10% of the specification</td>
</tr>
</tbody>
</table>

Note to Table 6:

¹ 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 hygienic 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

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![Diagram](https://via.placeholder.com/150)

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
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 obsoleted 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.

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