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The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: A review

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Abstract

This work reviews the formation of ethylphenols (4-ethylphenol and 4-ethylguaiacol) from grape hydroxycinnamic acids in aging red wines by yeasts of the genera *Brettanomyces* and *Dekkera*. The physico-chemical factors that favour the growth of these undesirable yeasts, the techniques used to detect the presence of *Brettanomyces*/*Dekkera* species in wines, and the analytical techniques for monitoring the formation of volatile ethylphenols are all described. Finally, the advantages and disadvantages of the different options for controlling the growth of these yeasts are discussed.

Keywords: *Brettanomyces*; *Dekkera*; Red wines; 4-Ethylphenol; 4-Ethylguaiacol; Off-flavours

1. Introduction

In the food industry in general, and winemaking in particular, quality control ensures that finished products are more competitive, are of greater added value, and able to avoid some of the problems associated with export trade barriers.

Maturation and aging improves many red wines from a visual, olfactory and gustatory point of view. In addition, the aging period allows winemakers to control the appearance of their products on the market. However, when maturation and aging takes place in wooden barrels, organoleptic deviations from the optimum can occur due to undesirable yeasts etc. remaining in the pores of the wood after cleansing and sterilisation. This is particularly important when the aging process required is lengthy.

Winemakers are presently experiencing significant problems during the aging of their products, particularly those of high quality. One of the main reasons is that current trends have established such wines should undergo no form of treatment or stabilization process. These wines are not subject to clarification or physical treatments such as filtering, the idea being to maintain high concentrations of aromas, pigments and colloids, thus allowing the product to express its full potential. These new maturation methods have the advantages that they produce wines with a better colloidal density and structure, that contain more colouring material (which tends to stabilise), and which preserve for longer the characteristics of the grapes used. However, when wine is stored for long periods in wooden barrels it becomes more nutritionally attractive to slow-growing species such as *Brettanomyces bruxellensis*, *Brettanomyces anomalus*, *Saccharomyces bailli* and certain genera of lactic bacteria, all of which produce metabolic compounds (e.g., ethylphenols, acetic acid, etc.) that can cause deviations from the olfactory optimum. This is most noticeable in premium wines, and is a complex problem for today's wineries.

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The essential characteristics of the oak casks in which many wines are aged may actually encourage this problem. Although oak barrels can promote the organoleptic improvement of wine, they are porous containers and are difficult to clean – and even harder to sterilise. They therefore provide an environment in which undesirable microorganisms can survive and be transferred from wine to wine every time they are used. Although cleaning methods are becoming more sophisticated (water vapour, ozonization, etc.), the shape and microstructure of wooden barrels afford undesirable organisms a great degree of protection. Moreover, the high pH of red wine (a product of the long grape ripening periods allowed in the search for higher concentrations of varietal aromatic compounds), and the ever lower SO_2 levels demanded by the wine industry, add to these difficulties.

Together, these factors encourage the growth of wine-altering microorganisms. This, plus the fact that they are adapted to poor nutritional environments with high ethanol concentrations (indeed, *Brettanomyces* and *Dekkera* can even use ethanol as a carbon source), has allowed them to become established in many wineries.

2. The wine ecology of *Brettanomyces/Dekkera*

Brettanomyces and *Dekkera* are described in the literature as part of the microbiota of many fermented products, including, wine, cider, beer, kombucha and kefyr, etc. (Greenwalt, Steinkraus, & Ledford, 2000; Martens, Iserentant, & Verachtert, 1997; Morrissey, Davenport, Querol, & Dobson, 2004; Silva, Cardoso, & Geros, 2004; Teoh, Heard, & Cox, 2004; Wyder, Spillmann, & Puhan, 1997).

Both, *Brettanomyces* and *Dekkera* can grow during the red wine aging (Froudiere & Larue, 1988) and even after their bottling. These yeasts are rarely found during the alcoholic fermentation of the must (Wright & Parle, 1973) although they have been isolated alongside *Saccharomyces* (Froudiere & Larue, 1988). A few studies have reported the abnormal presence of *Brettanomyces* on grape clusters (Pretorius, 2000) and in wine storehouses (Peynaud & Domercq, 1956), and more commonly in vats, pumps, and on equipment that is difficult to sterilise (Fugelsang, 1998).

The appearance of anaerobiosis detains alcoholic fermentation by *Brettanomyces* – the so-called Custer Effect (negative Pasteur effect). Scheffers and Wikén (1969) introduced this concept and were the first to use it as a taxonomic criterion. Although the passage from aeorobiosis to anaerobiosis detains the growth of *Brettanomyces*, this can be reverted by oxygen or the addition of hydrogen acceptors such as carbonyl compounds (Wikén, 1967). The positive effect of adding oxygen on the growth of *Brettanomyces* in reducing conditions may explain why the micro-oxygenation technique favours the growth of these yeasts when undertaken during fermentation or aging (Lonvaud-Funel, 1999). Indeed, the naturally mild oxidising conditions encountered during aging in barrels can promote the growth of *Brettanomyces* and *Dekkera* species.

3. Metabolites of *Brettanomyces/Dekkera* with negative sensorial repercussions (*off-flavours*)

3.1. Volatile phenols

Volatile phenols greatly influence the aroma of wine. The most important molecules in this class are 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol (Chatonnet, Dubourdieu, Boidron, & Pons, 1992). Elevated concentrations of 4-ethylphenol in red wine are associated with disagreeable aromas often described as "phenolic", "leather", "horse sweat", "stable" or "varnish", etc. (Chatonnet, Dubourdieu, Boidron, & Lavigne, 1993; Chatonnet et al., 1992; Rodrigues, Gonçalves, Pereira-da-Silva, Malfeito-Ferreira, & Loureiro, 2001).

The origin of volatile phenols involves the sequential action of two enzymes (Fig. 1) on a hydroxycinnamic acid (ferulic, *p*-coumaric or caffeic acid) substrate. Hydroxycinnamate decarboxylase first turns these hydroxycinnamic acids into hydroxystyrenes (vinylphenols) (Edlin, Narbad, Gasson, Dickinson, & Lloyd, 1998), which are then reduced to ethyl derivatives by vinylphenol reductase (Dias et al., 2003). The enzyme that facilitates decarboxylation is present in a large number of bacteria, fungi, and yeasts, but the reduction step is only performed by the species *Dekkera bruxellensis*, *Dekkera anomala*, *Pichia guillermondii*, *Candida versatilis*, *Candida halophila* and *Candida mannitofaciens* (Chatonnet, Dubourdieu, & Boidron, 1995; Chatonnet, Viala, & Dubourdieu, 1997; Dias et al., 2003; Edlin, Narbad, Dickinson, & Lloyd, 1995).

Initially, the presence of ethylphenols in wine was attributed to lactic acid bacteria. Indeed, these are capable of producing significant quantities of vinylphenols, but under oenological conditions they only produce small amounts. Other yeasts present in wines, such as *Saccharomyces cerevisiae*, *Pichia* spp., *Torulaspora* spp. and *Zygosaccharomyces* spp. can produce 4-vinylphenol but do not reduce it to 4-ethylphenol (Dias et al., 2003).

The relationship between high concentrations of 4-ethylphenol in wines and the activity of *Brettanomyces* yeasts or the members of the sporulating genus *Dekkera*, was thoroughly studied during the 1990s (Chatonnet et al., 1995; Chatonnet et al., 1997; Cullere, Escudero, Cacho, & Ferreira, 2004; Fugelsang & Zoecklein, 2003; Kelly, 2003; Parish, Kelly, & Baldwin, 2003; Suárez-Lepe, 2001). Unlike *Brettanomyces* and *Dekkera*, neither *Sacch. cerevisiae* nor the lactic acid bacteria produce ethylphenol under oenological conditions. *D. bruxellensis* shows hydroxycinnamate decarboxylase and vinyl reductase activity under oenological conditions to the extent that the species is considered an undesirable yeast capable of producing high concentrations of 4-ethylphenol. The sensorial threshold of this compound is 230 $\mu g\, l^{-1}$, (Chatonnet, Boidron, & Pons, 1990), and therefore small quantities of 4-ethylphenol are appreciable in large quantities of wine. 4-ethylguaicol affects wine aromas to a lesser extent, but they are also related to the 'Brett character' of adulterated wines and have been associated

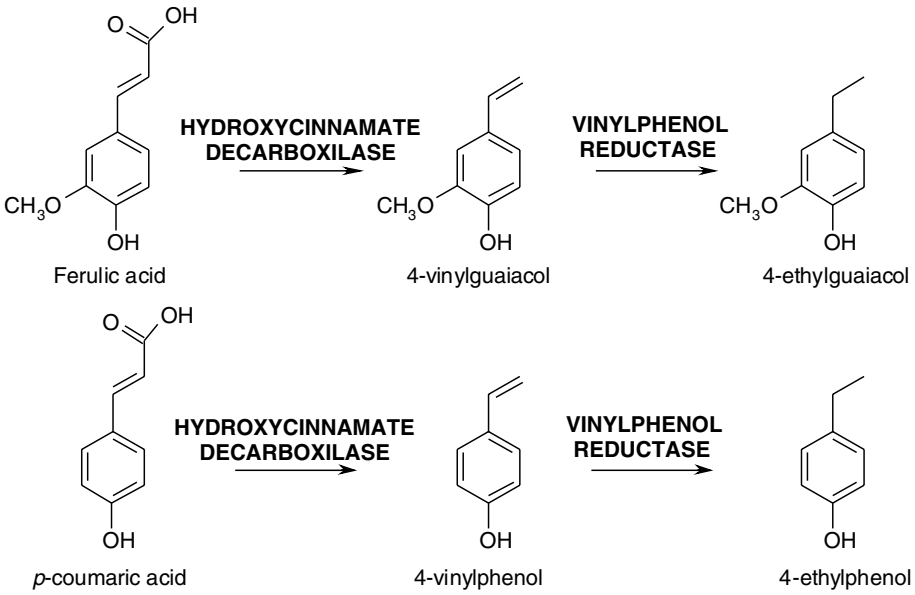

Fig. 1. Formation of ethylphenols from their hydroxycinnamic precursors.

with descriptive expressions such as "bacon" or "smoked". The sensorial threshold of these compounds is 47 $\mu g\,l^{-1}$ (Chatonnet et al., 1990). It has been reported (Licker, Acree, & Henick-Kling, 1999) that wines with high, medium and no Brett character have average 4-ethylphenol concentrations of 3.0, 1.74 and 0.68 $mg\,l^{-1}$, respectively.

Different strains of *Brettanomyces* can show great differences in their production of volatile phenols (Joseph & Bisson, 2004). The variety of grape used also affects the sensorial perception of ethylphenols. Physter and Mills (2004) indicate detection thresholds to be high in monovarietal Cabernet Sauvignon wines, and lower in Tempranillo wines.

3.2. Volatile acidity and tetrahydropyridines

Brettanomyces and *Dekkera* are acetic acid producers (Freer, Dien, & Matsuda, 2003; Freer, Dien, Matsuda, & Rothast, 2000; Suárez-Lepe & Iñigo, 2004). As well as stimulating the growth of *Brettanomyces*, oxygen also appears to stimulate its production of acetic acid. Under anaerobic conditions, however, acetic acid production in very low (Aguilar-Uscanga, 1998) or even nil (Blondin, Ratomahenina, Arnaud, & Galzy, 1982; Larue, Rozes, Froudiere, Couty, & Perreira, 1991).

Derivatives of amino acids such as 2-acetyl-1,4,5,6-tetrahydropyridine are though to be the cause of the "mousy" off-aroma (*gusto a ratón, goût de souris*) (Heresztyn, 1986) of some wines. Yeast strains differ significantly in the quantities of such derivatives they produce (Grbin & Henschke, 2000). The 'mouse' off-aroma is extraordinarily persistent and disagreeable, and produces an even worse taste in the mouth due to the neutralizing action of the saliva. The prevention of this aroma requires the control of undesirable yeast populations by avoiding unnecessary aerobiosis.

In addition to these compounds, which have been shown directly related to Brett character in adulterated wines, *Brettanomyces* and *Dekkera* produce a large variety of other volatile compounds with significant olfactory repercussions (Fig. 2).

3.3. Anthocyanin degradation

As well as producing volatile compounds with disagreeable aromas, *Brettanomyces* can, in favourable situations, hydrolyse anthocyanins, releasing glucose and destabilising the aglycone (Mansfield, Zoecklein, & Whiton, 2002). This may be the reason why wines contaminated by *Brettanomyces* have an undesirable colour.

4. Factors affecting the formation of volatile compounds by *Brettanomyces* and *Dekkera*

The formation of volatile phenols in wine depends on the presence of precursors and is proportional to the size of the *Brettanomyces*/*Dekkera* population (Gerbeaux, Jeudy, & Monamy, 2000). The latter authors have also shown that different strains of *B. bruxellensis* vary in their capacity to produce volatile phenols, although this is always greater when alcohol concentrations are lower (more are made at 12% v/v than at 14% v/v) and temperatures higher (e.g., more is produced at 18°C than at 13 °C). Little significance is attributed to the pH of the wine or the presence of residual sugars in this respect.

The intensity and temperature of maceration and the use of pectolytic enzymes have been studied as possible factors conditioning the formation of volatile phenols by *Brettanomyces* and *Dekkera* from hydroxycinnamic acids released from the grape skins (Gerbeaux, Vincent, & Bertrand, 2002).

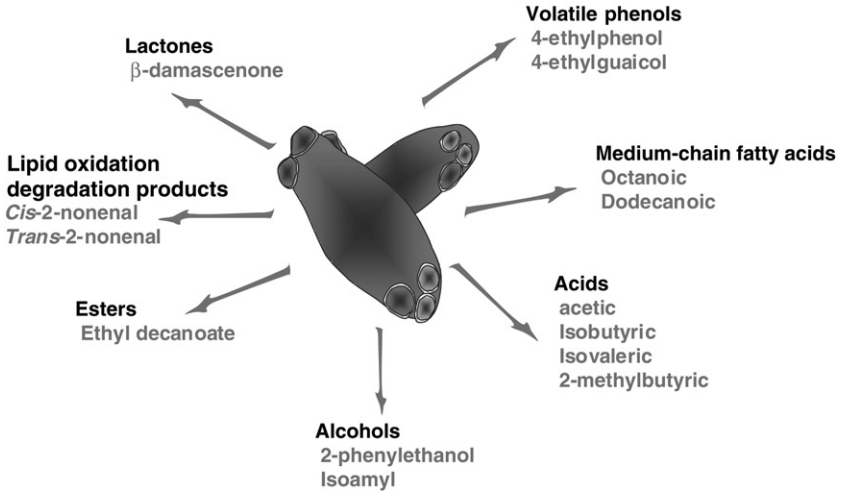

Fig. 2. Odour-active compounds generated by the metabolism of *Brettanomyces* spp.

The growth of *Brettanomyces*/*Dekkera* in synthetic media containing autolysed *Sacch. cerevisiae* has also been studied (Guilloux-Benatier, Chassagne, Alexandre, Charpentier, & Feuillat, 2001). Under these conditions it was observed that these contaminating yeasts grew easily, even in glucose concentrations of $<150\ mg\ l^{-1}$. However the quantity of ethylphenols formed was smaller than expected, probably because of the adsorption of the phenolic compounds by the cell wall fragments. More recent studies have reported the high capacity of yeast cell walls to adsorb phenolic compounds (Morata, Gómez-Cordovés, Colomo, & Suárez, 2005; Morata et al., 2003).

The isolation of *Dekkera* and/or *Brettanomyces* yeasts in wine is difficult because of their slow growth and reduced presence (Fugelsang, 1997; Kunkee & Bisson, 1993). However, selective media such as DBDM (*Dekkera* and/or *Brettanomyces* differential medium), can be used to isolate *Dekkera* spp. from wines in which they which make up <1% of the total microbiota (Rodrigues et al., 2001). This medium contains ethanol as the sole carbon source, cycloheximide and *p*-coumaric acid to reveal the presence of phenolic off-flavour producers (POFs), and bromocresol to disclose the production of acetic acid. Other media described in the literature, such as DG18, are much less selective (Deak et al., 2001).

Recently, Dias, Pereira-da-Silva, Tavares, Malfeito-Ferreira, and Loureiro (2003) studied the capacity of a number of yeasts present in wine microbiota to produce 4-ethylphenol from *p*-coumaric in model media. Molar conversions of 90% were reported for *D. bruxellensis*, *D. anomala* and *P. guillermondii*; other fermentative yeasts were incapable of producing 4-ethylphenol at these rates of conversion.

The use of old wooden casks can increase the presence of *Brettanomyces* and *Dekkera* species in wine because they are so difficult to clean and impossible to sterilize. To make matters worse, *Brettanomyces custersii* and *Dekkera intermedia* metabolise cellobiose, a disaccharide, forming the basic repeating unit of cellulose (a structural polysaccharide of wood) (Freer, 1991; Park, Hong, Kim, & Hong, 1999; Park, Hong, Kim, & Hong, 2000). The frequent re-use of these casks and the use of the micro-oxygenation technique to accelerate wine maturation, facilitates the polymerisation of wine pigments and the modification of the wine volatile profile (frequently associated with the use of oak chips or barrel aging), resulting in the proliferation of *Brettanomyces*/*Dekkera* (Aguilar-Uscanga, Delia, & Strehaiano, 2003; Ciani & Ferraro, 1997; Ciani, Maccarelli, & Fatichenti, 2003).

5. Detection of *Brettanomyces* and *Dekkera* in wines

Brettanomyces and *Dekkera* are characteristically small in size, have ogival cell walls and show branched structures in old cultures (Larue et al., 1991) (Fig. 3). In addition, many *Brettanomyces* strains form a surface film (Joseph & Bisson, 2004). However, neither their morphological features nor classical methods of identification are adequate for routine oenological microbiological work, nor do they identify viable populations (Millet & Lonvaud-Funel, 2000). Thus, in recent years, attempts have been made to develop techniques allowing the rapid and reliable identification of these species (Loureiro & Querol, 1999).

Fluorescence microscopy can quickly and accurately detect *Brettanomyces* and *Dekkera* previously probed with a fluorescent marker designed to seek out a fragment of their 26S ribosomal RNA (RNA-FISH hybridisation) (Stender, Fiandaca, Hyldig-Nielsen, & Coull, 2002; Stender et al., 2001). These authors assign this method a high sensitivity and specificity for *D. bruxellensis*.

Brettanomyces and *Dekkera* yeasts can also be identified by removing the fatty acids from their cell membranes, and, after their derivatisation as methyl esters, quantifying the type and amount of each by gas chromatography (Malfeito-Ferreira, Tareco, & Loureiro, 1997; Rozes, Garcia-Jares, Larue, & Lonvaud-Funel, 1992; Sancho, Jiménez-Jurado, Malfeito-Ferreira, & Loureiro, 2000).

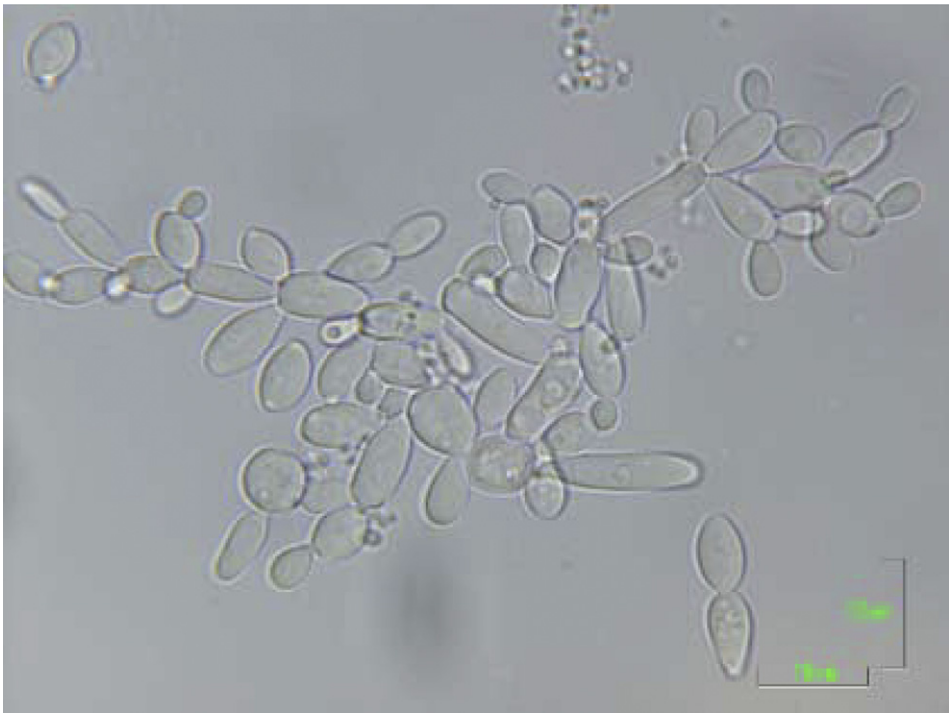

Fig. 3. Optical microscope image of a *Brettanomyces* strain (1000×).

However, molecular techniques such as PCR are the fastest, most sensitive and most specific. Generally, these methods focus on the amplification of specific fragments of ribosomal DNA and RNA.

Brettanomyces/*Dekkera* isolated from wine have been identified by the electrophoretic determination of their karyotypes and by the random amplification of polymorphic DNA (RAPD-PCR) (Mitrakul, Henick-Kling, & Egli, 1999). In addition, specific sequences of internal transcribed spacer (ITS) regions between ribosomal RNA genes (particularly ITS1 and ITS2) have been found that are different enough to identify four species of *Brettanomyces*/*Dekkera* (Egli & Henick-Kling, 2001; Esteve-Zarzoso, Belloch, Uruburu, & Querol, 1999).

Specific protocols that make use of the primers DB90F and DB394R (whose target is *loop* D1–D2 of gene 26S rRNA) are available for differentiating *B. bruxellensis* and *B. anomalus*. These primers only form amplification products with species of *Brettanomyces* (Cocolin, Rantsiou, Iacumin, Zironi, & Comi, 2004).

Nested amplification techniques (nested-PCR) that use two external and two internal primers can also been used. These detect *Brettanomyces* spp. directly in wine with high reproducibility and specificity, without the need for isolation and cultivation (Navascués & Rasines, 2003). This new technique is very versatile and allows rapid monitoring of *Brettanomyces* development from its initial phases.

Finally, the detection of ethylphenols in wine is a sign of *Brettanomyces*/*Dekkera* activity. However, this method has the drawback that by the time they are detected it may be too late to do anything about it. Nonetheless, a combination of gas chromatography and mass spectrometry (GC/MS) can identify and quantify both 4-ethylphenol and 4-ethylguaiacol with high sensitivity. Coupling this technique with sensorial analysis performed by a trained taster (GC/MS/*olfactometry*) is a powerful tool in wine quality control. Once chromatographic separation has taken place and the different volatile substances are isolated at the end of the capillary column (identified by mass spectrometry), the olfactory intensity and quality of the separated compounds are evaluated by the taster (Cullere et al., 2004).

6. Controlling *Brettanomyces*/*Dekkera* in wines

The remediation of problems caused by *Brettanomyces*/*Dekkera* has both a preventive and curative dimension (Table 1). A palliative solution is the fining of red wines before introducing them into their barrels. Murat and Dumeau (2003) report contaminating populations of *Brettanomyces* can be reduced by 40 to 2000-fold by treatment with fining proteins. The more fining agents used, the greater the reduction in the initial population; indeed, intense finings can almost entirely remove these yeasts. Some fining agents are more effective than others. It has also been noted that fining with casein or potassium caseinate can reduce ethylphenol levels if these are not too high (Ruiz-Hernández, 2003). Fining with liquid gelatine has been used to reduce *Brettanomyces* populations in Bordeaux red wine from an initial population of 1.2×10^4 cfu ml^{-1} to 270 and 170 cfu ml^{-1} with doses of 0.3 and 0.6 ml l^{-1}, respectively (Laffort info no. 9, 2003). However, fining is sometimes rejected by winemakers since it also reduces wine aroma and colour.

Filtration can also reduce the quantity of contaminating yeasts. However, this poses problems similar to those of fin-

Table 1
Optional methods for the control of *Brettanomyces* in wines

Treatment	Result	Drawback	Reference
Protein clarification			
• Gelatine • Egg white • Potassium caseinate • Caseins	Reduces *Brettanomyces* and *Dekkera* populations by flocculation	Loss of colour and aroma	Murat and Dumeau (2003), Ruiz-Hernández (2003)
Filtration			
• Membranes (0.45 μm) • Ultrafiltration	Reduces *Brettanomyces* and *Dekkera* populations by physical separation	Loss of colour and aroma	Calderón et al. (2004)
Physicochemical variables			
• Low aging temperature • low pH • Reduction of oxygen content • Avoidance of micro-oxygenation • High alcohol levels	Establishes physico-chemical conditions that reduce the viability of *Brettanomyces* and *Dekkera*	These variables can be difficult to modify in wines and may be incompatible with aging	Gerbeaux et al. (2000)
Reduction of precursor concentration			
• Low maceration temperature • Avoidance of pectolytic enzymes and enzymes with cinnamoyl sterase activity	Prevents the solubilization of hydroxycinnamic acids (the precursors of volatile phenols)	May cause a loss of colour and aroma	Gerbeaux et al. (2002)
Additives			
• SO_2 • DMDC • Chitosan • Sorbic acid • Benzoic acid • Fumaric acid • Ascorbic acid • Erythorbic acid	Inhibits the growth of *Brettanomyces* and *Dekkera* and prevents the conditions that favour the formation of ethylphenols	Some of these products are not authorised for use in the wine sector or are still experimental	Delfini et al. (2002), Gómez-Rivas et al. (2004)
High pressure processing			
• 400–500 MPa	Destroys microorganisms in wine without seriously affecting its sensorial properties	High equipment costs	Puig et al. (2003)
Biological techniques			
• Bacteriocins • Bacteriological enzymes • Zymocins	Inhibits the growth of *Brettanomyces* and *Dekkera*	The use of these techniques with wine is usually experimental	Toit and Pretorius (2000)
Genetic engineering			
• Transgenic yeasts	Genetically engineered yeasts that prevent the growth of *Brettanomyces* and *Dekkera*	Not currently allowed in winemaking	Toit and Pretorius (2000)

ing. In addition, to be effective, membranes with a pore size smaller than 0.45 μm must be used (Calderón, Morata, Uthurry, & Suárez, 2004), which causes a deterioration of the wine's colloidal structure and can reduce the intensity of its colour. Moreover, dormant, elongated forms of *Brettanomyces* cells may be able to pass through a 0.45 μm filter.

Controlling the variables that favour the growth of *Brettanomyces*, such as keeping the pH as low as possible, maintaining oxygen levels as low as possible, ensuring that the temperature in the wine storage facility is low (13 °C rather than 18 °C), and preventing macerations of too great an intensity, all help. Avoiding the extraction of ethylphenol precursors from the skins also helps prevent the production of undesirable aromas by these yeasts, should they grow. However, such precautions go against current red winemaking trends. For instance, intense macerations give body, structure, colour and aroma, and maturations in casks tend to be lengthy. In addition, a certain amount of micro-oxygenation is bound to take place during aging (through the barrel staves); sometimes this is even increased with the use of *cliqueurs*.

Certain additives can inhibit the growth of *Brettanomyces*. The most common is sulphur dioxide (SO_2), although it is hard to keep the concentration stable over prolonged aging periods in casks in which the environment is mildly oxidising. For instance, in a red wine at pH 3.65, initial

doses of free SO_2 of 15, 25, 30 and 35 mg l^{-1} are significantly reduced after four months of aging in barrels to 6, 11, 10 and 15 mg l^{-1}, respectively (Chatonnet, Boidron, & Dubourdieu, 1993). However, spoilage can be avoided by preventing the growth of undesirable *Brettanomyces/Dekkera* yeasts through the effective use of SO_2 and by reducing the oxygen available during the winemaking process, especially when transferring wine from one barrel to another during aging (du Toit, Pretorius, & Lonvaud-Funel, 2005). The use of between 0.5 and 0.8 mg l^{-1} of molecular SO_2 is recommended (Henick-Kling, Egli, Licker, Mitrakul, & Acree, 2000). It should be remembered that the molecular SO_2 content achieved is pH-dependent; 30 mg l^{-1} of free SO_2 releases 0.4 mg l^{-1} of molecular SO_2 at pH 3.7, and 0.8 at pH 3.4.

An alternative inhibitor is dimethyl dicarbonate (DMDC), commercially known as Velcorin® (Scott Laboratories, Petaluma, California). Although its effectiveness has been proven, a recent study showed that a dose of 400 mg/L cannot completely inhibit the growth of *B. anomalus*; it can only curtail it. In contrast, other fermentative yeasts are inhibited by dosages of 250–400 mg l^{-1} (Delfini et al., 2002).

It has also been shown that chitosan (a polysaccharide derived from chitin) has a selective effect on *Brettanomyces*, causing a delay in its latent phase in mixed cultures with *Sacch. cerevisiae* (Gómez-Rivas et al., 2004). *B. bruxellensis* and *Brettanomyces intermedius* cannot grow in the presence of 3–6 g l^{-1} of this chitosan, while this does not affect the development of *Sacch. cerevisiae*. Chitosan at 0.05–0.1% is known to delay spoilage by yeasts at 25 °C, in fact it even inactivates some species (Kiskó, Sharp, & Roller, 2005).

Certain weak acids, such as sorbic, benzoic and fumaric have antifungal activity and can be used against *Brettanomyces/Dekkera*, but their action is not selective and they are not authorized for use in winemaking. Characteristically, weak-acid preservatives do not kill micro-organisms but rather inhibit their growth, causing extended lag phases. Preservatives are more effective at low pH where solutions contain increased concentrations of undissociated acids. Using a thermodynamic and kinetic approach to model the action of a preservative on yeast, it was shown that inhibition depends more on the degree to which individual preservatives are concentrated within cells, than on the actual concentration of undissociated acid itself (Lambert & Stratford, 1999; Quintas, Leyva, Sotoca, Loureiro-Dias, & Peinado, 2005). Antioxidants such as ascorbic and erythorbic acids can be used to reduce the presence of oxygen during maturation, preventing ethylphenol formation.

Applying pressures of 400–500 MPa for 5–15 min at temperatures of 5–20 °C can reduce populations of certain yeasts (including *B. bruxellensis*) and lactic acid and acetic acid bacteria by more than 99.99% (Puig, Vilavella, Daoudi, Guamis, & Minguez, 2003) without causing major modifications to the wine's physicochemical properties, enzymatic activity, or sensorial properties. Not only is this effective, it reduces the dosage of SO_2 (an important bacteriostatic agent and antioxidant) required.

Biological control of contaminating yeasts and bacteria involves the use of various antimicrobial agents, bacteriolytic enzymes, zymocines and yeast strains with antimicrobial activity constructed by genetic engineering. However, in the wine environment they are not very effective and other techniques are usually required as well (Toit & Pretorius, 2000). Recently, two toxins active against *Dekkera/Brettanomyces* have been reported (Comitini, Ingeniis De, Pepe, Mannazzu, & Ciani, 2004). Produced by *Pichia anomala* (DBVPG 3003) and *Kluyveromyces wickerhamii* (DBVPG 6077), these toxins show a stable fungicidal effect against *D. bruxellensis* in wine for at least 10 days. They may have a future as antimicrobial agents against *Dekkera/Brettanomyces* during wine aging and storage.

7. Wood contamination and oak wood treatments

Brettanomyces/Dekkera has been found 8 mm down within the wood of barrel staves (Malfeito-Ferreira, 2005). These yeasts survive treatments where contact with SO_2 is limited, e.g., around bung holes, in the oak structure, and in yeast sediments (lees).

The age of the barrel greatly influences the growth of Brett populations during the long aging of red wines. Old barrels favour Brett contamination – oak wood is extremely porous and yeasts deep in the barrel staves are difficult to eliminate – and any ethylphenols in the mass of the wood are released (Chatonnet, Masneuf, Gubbiotti, & Dubourdieu, 1999). However, some authors (Lonvaud-Funel & Renauf, 2005) report that, due to their higher oxygen and sugar contributions, new barrels are even more likely to favour the maintenance of large Brett populations. It is probable that the wood pores become blocked as the barrels are used, so the oxygen arriving via them decreases.

Oak barrels that become contaminated with *B. bruxellensis* cannot be effectively sterilized. Neither careful washing followed by rinsing with sulphited water, nor shaving and firing, nor ozone treatment achieves sterilization (Pollnitz, Pardon & Sefton, 2000) – a result of the large internal volume and porous nature of oak barrels.

The sanitation of barrel wood requires at least 7 g of SO_2 gas per barrel. Filled wine barrels should receive 20–25 mg l^{-1} of free SO_2 (30–35 mg l^{-1} during hot summers) (Henick-Kling et al., 2000).

The following barrel sanitation procedures have been tested (Malfeito-Ferreira, 2005):

(a) Cold water rinse followed by three hot water rinses (70 °C).
(b) The same as the above plus filling with an aqueous solution of SO_2 (200 mg l^{-1}, pH 3) and storing for one month.
(c) Cold water rinse, followed by filling the barrel with hot water (90 °C for 10 min).
(d) Cold water rinse, followed by a hot water rinse (70 °C) and low pressure steam (10 min).

The most effective treatment was the last of these (d). The author recommended isolating Brett+ barrels to reduce the contamination of others during disinfection and wine pumping.

Ozone sanitation has been used with good results. The procedure commonly followed involves a high-pressure cold water wash followed by blasting with a stream of hot water (70 °C). The barrel is then rinsed for 2–3 min until all organic remains have been removed. It is then left to cool before treating with ozonated water (2–2.5 mg l^{-1}). One assay of ozonated water treatments reported that *Brettanomyces* population can be reduced by up to 99.99% (Coggan, 2003). When different doses of aqueous ozone (doses 1, 3 and 5 mg l^{-1} for 3 min) and ozone gas (600 or 1300 mg l^{-1} for 15, 30, 60 or 120 min) were assayed, the *Brettanomyces* population inside the oak wood staves was reduced by the latter but not by the former (Cantacuzene et al., 2003).

Ethylphenols appear in the wine after Brett contamination, but their concentration can be reduced by reverse osmosis and adsorption (Ugarte, Agosin, Bordeu, & Villalobos, 2005). The latter authors reduced the initial concentration (900 μl l^{-1} of 4-ethylphenol plus 4-ethylguaiacol) by 77% after a 3-h treatment involving reverse osmosis using an appropriate membrane with tangential-flow filtration equipment and a hydrophobic adsorbent resin. No significant reduction in wine colour, tannins, body (glycerol and diols) or ethanol was observed. However a reduction was seen in the concentration of aromatic compounds, especially methyl and ethyl vainillate and other esters.

PVPP and charcoal are adsorbents used in winemaking to reduce browning, off-odours and bitterness. Some wineries use these compounds to treat wines containing ethylphenols. The recommended doses are 0.015–0.24 g l^{-1} of charcoal for slight off-odours, and 0.12–0.96 g l^{-1} for more intense off-odours. PVPP (0.06–0.48 g l^{-1}) is used to remove Brett ethylphenols.

8. Formation of high-stability anthocyanin adducts in red wines

Another way to arrest the production of ethylphenols by *Brettanomyces*, and at the same time increase the formation of highly stable pigments during maturation, is to encourage the formation of derivatives, the precursors of which are hydroxystyrenes (vinylphenols) produced from the decarboxylation of hydroxycinnamic acids (e.g., ferulic, *p*-coumaric, caffeic, and sinapic acids). Fulcrand, Cameira-dos-Santos, Sarni-Manchado, Cheynier, and Favre-Bonvin (1996) proposed the formation of the malvidin derivative shown in Fig. 4 from 4-vinylphenol (a precursor of 4-ethylphenol).

Vinyl phenolic derivatives are highly stable due to their aromatic heterocyclic ring. This allows the sharing of the positive charge of anthocyanin with two oxygen molecules. Other pyranoanthocyanic derivatives, such as the vitisins, function in the same way (Bakker et al., 1997; Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998; Mateus, Silva, Vercauteren, & Freitas, 2001; Romero & Bakker, 1999; Vivar-Quintana, Santos-Buelga, Francia-Aricha, & Rivas-Gonzalo, 1999). In addition, since the formation of this ring involves carbon 4 of the anthocyanin, decolouration by SO_2 and reductions in colour intensity caused by high pH are less likely (Bakker & Timberlake, 1997).

The mechanism of formation of these compounds from the vinyl derivatives of different hydroxycinnamic acids (demonstrated using caffeic acid) has recently been reported (Fig. 5) (Schwarz, Wabnitz, & Winterhalter, 2003). This mechanism can be fully extrapolated to other vinyl derivatives of hydroxycinnamic acids. Derivates of vinylphenol (Cameira dos Santos, Brillouet, Cheynier, & Moutounet, 1996), vinylcatechol (Schwarz et al., 2003) and 4-vinylguaiacol (Hayasaka & Asenstorfer, 2002) have also been found in wine.

Strains of *Sacch. cerevisiae* that favour the formation of anthocyanin vinyl derivatives between malvidin-3-*O*-glucoside and 4-vinylguaiacol can be selected because of their hydroxycinnamate decarboxylase activity (Morata, Gómez-Cordovés, Calderón, & Suárez, 2006). This eliminates the hydroxycinnamic acid precursors of ethylphenol from wine, and forms highly stable, long-lasting pyranoanthocyanins during fermentation. During fermentation, vitisins A and B, pyranoanthocyanic molecules structurally similar to vinylphenol derivatives, are produced in significant amounts by selected yeast strains (Morata, Gómez-Cordovés, Colomo, & Suárez, 2003).

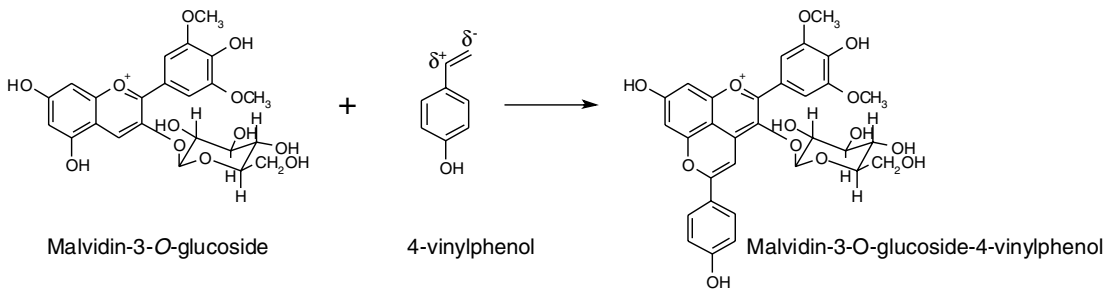

Fig. 4. Proposed decrease of unwanted 4-vinylphenol by reaction with anthocyanins of wine to highly stable products (Cameira dos Santos et al., 1996).

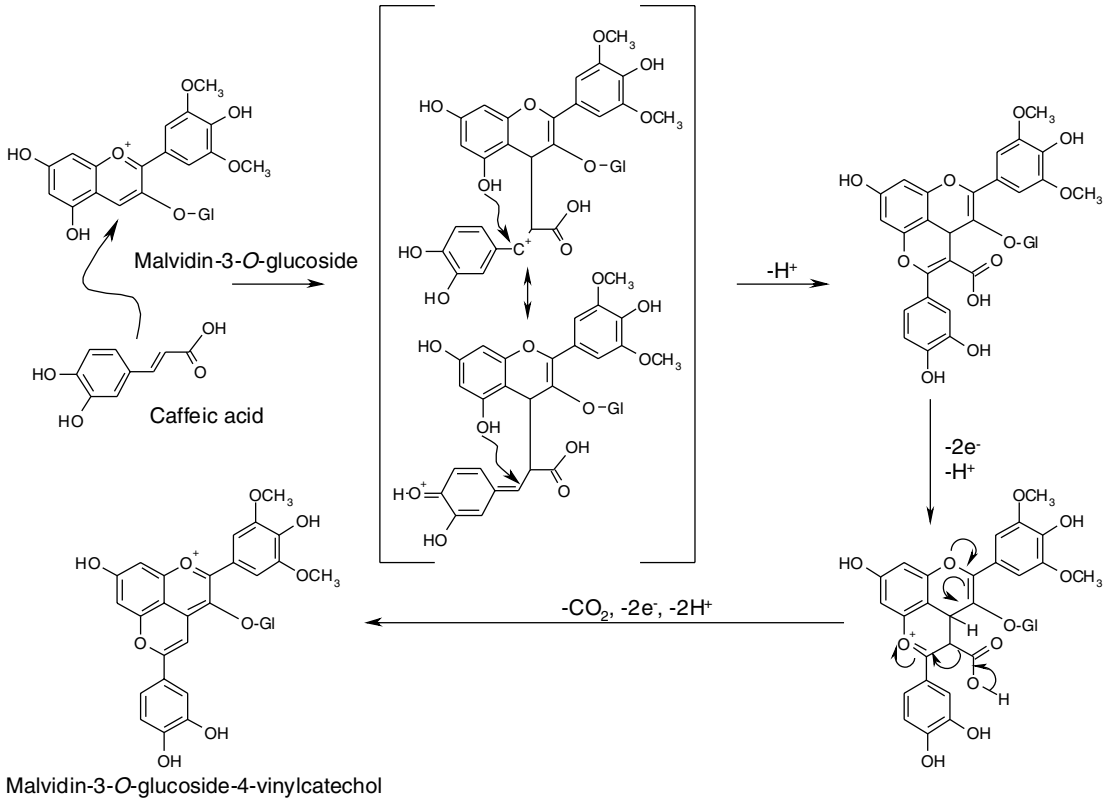

Fig. 5. Formation of high stability derivatives between malvidin-3-*O*-glucoside and 4-vinyl[2-hydroxy-phenol], extrapolated to other vinylphenols (adapted from Schwarz et al., 2003).

9. Conclusions

Currently, the organoleptic deterioration of wine caused by the formation of volatile ethylphenols (metabolic products of *Brettanomyces* and *Dekkera* species) is a serious economic problem, especially with respect to high quality wines that require long maturation periods in oak casks.

Techniques that help identify the presence of *Brettanomyces* and *Dekkera* species and prevent their growth are available. The combined use of these techniques may help to reduce the production of the undesirable aromas produced by these yeasts, although some might impede a wine from maturing as originally desired.

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