THE ORIGIN OF *BRETTANOMYCES BRUXELLENSIS* IN WINES: A REVIEW

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Abstract

Aims: This work reviews the latest knowledge concerning the role of *Brettanomyces bruxellensis* in red wine alteration.

Results and conclusion: The origin of this yeast species and its place in the wine microbial consortium are discussed as well as microbial equilibriums with the other species, notably *Saccharomyces cerevisiae* and lactic acid bacteria. As a consequence, fermentations are described as key steps in *Brettanomyces* development management. Furthermore, the influence of ageing through the use of traditional winemaking practices is explained

Significance and impact of study: Finally, this paper emphases the need for a better understanding of chemical and microbial analysis together in order to better control this undesirable yeast and prevent the production of volatile phenols.

Key words: *Brettanomyces bruxellensis*, species, strains, wine, grape, volatile phenols, stabilization.

Résumé

Objectif : Ce travail propose un bilan des connaissances récemment acquises sur l'altération des vins rouges par la levure *Brettanomyces bruxellensis*.

Résultats et conclusion : L'origine des souches *Brettanomyces bruxellensis* présentes dans les vins et leur place au sein du consortium microbiologique œnologique est discutée. L'accent est mis sur les interactions de *B. bruxellensis* avec la matière première, le raisin, mais aussi avec les autres microorganismes du vin, et plus particulièrement, l'espèce *Saccharomyces cerevisiae* et les bactéries lactiques. Par conséquent, les fermentations se révèlent être des étapes décisives pour l'implantation des *B. bruxellensis* apportées par le raisin dans le vin. Ensuite, les influences de différentes opérations œnotechniques durant l'élevage sont détaillées et révèlent l'importance de la stabilisation microbiologique des vins durant cette période.

Signification et impact de l'étude : Finalement, ce travail soulève l'importance de la maîtrise des processus microbiologiques et d'une bonne connaissance des paramètres physico-chimiques des vins, en vue de contrôler le développement de *B. bruxellensis* et de prévenir la production des phénols volatils.

Mots clés : *Brettanomyces bruxellensis*, espèces, souches, vin, raisin, phénols volatils, stabilisation.

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A SHORT HISTORY OF *BRETTANOMYCES BRUXELLENSIS* IN WINES

Among all possible microbial alterations of wines, volatile phenols production by the yeast *Brettanomyces bruxellensis* is one of most feared by the winemaker and probably one of the most undesired by consumers. Different descriptors such as « medicinal », « smoked », « animal » or « spiced » are used to qualify the odours conferred by these compounds (HERESZTYN, 1986a, SUAREZ *et al.*, 2007). Even when those negative descriptors are not mentioned, these volatile phenols cause a loss in the fruity characters and varietals flavours of wine. Moreover, volatile phenols are not the only undesirable compounds produced by *B. bruxellensis*. Acetic acid (CIANI and FERRARO, 1997) conferring « sourness » and « piqué » notes (DUBOIS, 1993, 1994), decanoic acids bringing « soapy » odours (LICKER *et al.*, 1997) and the tetrahydropyridines characterized by the typical « mousy flavour » (HERESZTYN 1986b; SNOWDON *et al.*, 2006) can also be produced by this species.

Initially isolated from beer (CLAUSEN, 1905), *B. bruxellensis* was described in wines for the first time in the middle of the twentieth century by AGOSTINO (1950), BARRET *et al.* (1950) and PEYNAUD and DOMERCQ (1956). It is only relatively recently that wine microbiologists have been fully aware of its role in wine spoilage. In the 1990's, several studies focused on *B. bruxellensis* (FROUDIERE and LARUE 1988; LARUE *et al.*, 1991) and on volatile phenol production (CHATONNET *et al.*, 1992, 1995, 1997). *B. bruxellensis* was then described as the only species involved in the production of 4-ethylphenol and 4-ethylguaiacol, and its development in wines was systematically associated with cellar contamination and non-respect of hygiene recommendations. However, this simplistic view didn't eradicate spoilage by *B. bruxellensis*, thus showing the multifactorial causes of this wine alteration (figure 1).

B. bruxellensis naturally belongs to the oenological microbiological consortium (DELIA-DUPUY 1995; GILIS 1999). Previous studies showed that *B. bruxellensis* was particularly resistant to oenological constraints like alcohol concentrations (MEDAWAR *et al.*, 2003), SO_2 (DU TOIT *et al.*, 2005) and pH (RENOUF *et al.*, 2006a). Moreover it is not very demanding from a nutritional point of view (USCANGA *et al.*, 2000) and adapts well to oxygen absence or restriction (CIANI *et al.*, 2003). As a consequence, wines where *B. bruxellensis* is totally absent throughout the whole production process are rare and aiming for a complete absence of this microorganism in wine is unrealistic. Winemakers only have the option of limiting and controlling its multiplication. In this context, the question of the origin of *B. bruxellensis* is crucial to anticipate its multiplication.

TRACKING *BRETTANOMYCES BRUXELLENSIS*: IDENTIFICATION METHODS

Increasing yeast genome knowledge (WOOLFIT *et al.*, 2007) and molecular biology provide methods for the identification of oenological microorganisms (RENOUF *et al.*, 2007a). Spoilage microorganisms, including *B. bruxellensis*, are at the centre of detailed attentions

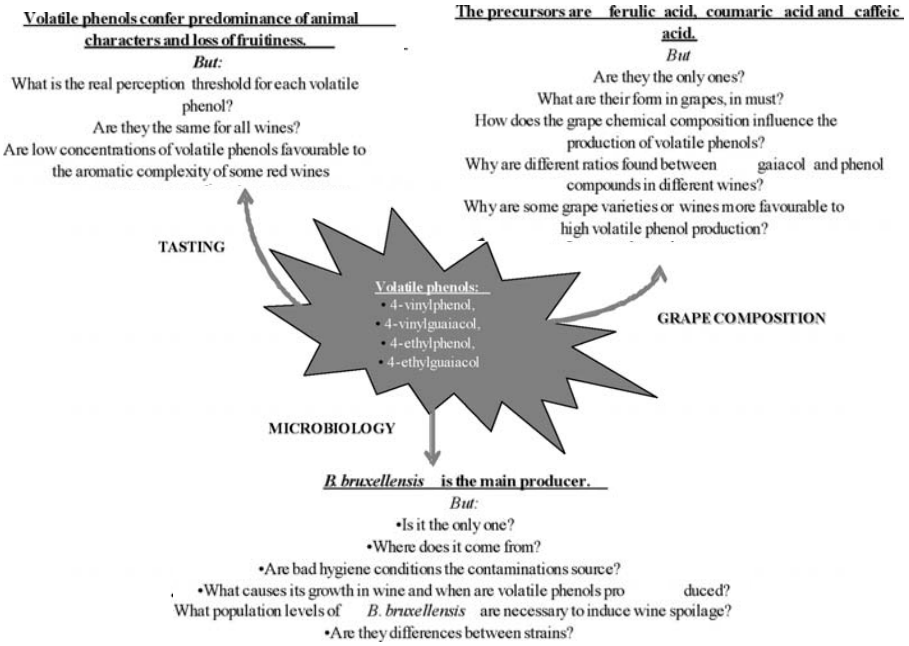

Figure 1 - Questionnable points still unresolved concerning *B. bruxellensis* and volatile phenol production in wine

since fast and effective detection methods are needed to limit their development. Phenotypic identification techniques (HEARD and FLEET, 1990; RODRIGUEZ *et al.*, 2001) are lengthy, hard and uncertain to achieve. Nowadays, molecular tools based on DNA analysis are used. It is indeed possible to detect and to identify specifically *B. bruxellensis* by a species-specific PCR targeting a DNA region of the *B. bruxellensis* genome (IBEAS *et al.*, 1996). The use of a nested-PCR in two stages improves the sensitivity and the specificity of the signal. This method is very effective and practical and can be used to quickly control the presence or the absence of *B. bruxellenxis* in a wine sample. Furthermore, quantitative real time PCR proposed by PHISTER and MILLS (2004) and by DELAHERCHE *et al.* (2004, 2007) makes it possible to detect specifically and instantaneously the presence and number of *B. bruxellensis* cells in wine. Threshold levels that have long been quite high have now been overcome (DELAHERCHE *et al.*, 2007). Other techniques used to identify *B. bruxellensis* rely on the amplification of ubiquitous yeast DNA regions followed by sequence polymorphism analysis. Species specific patterns are hence generated. The two most used techniques are PCR-RFLP (ESTEVE-ZARZOSO *et al.*, 1998) and PCR-DGGE (RENOUF *et al.*, 2007b) (table 1). The latter allows to analyze relatively complex microbial mixtures and is now widely used in microbial ecology studies (COCOLIN *et al.*, 2000; ERCOLINI, 2004; RENOUF *et al.*, 2007b).

All these methods are limited to the species level. More sensitive tools are needed to reach the strain level discrimination. Recent work (MIOT-SERTIER and LONVAUD-FUNEL, 2006) showed that RAPD-PCR, PCR fingerprinting with microsatellite oligonucleotide primers and SAU-PCR methods, which are generally used for other yeasts species (MITRAKUL *et al.*, 1999; DE BARROS LOES *et al.*, 1999; GARCIA-BERMEJO *et al.*, 2001), are not sufficient for *B. bruxellensis* strain discrimination. However, some of these methods are sufficient to study the genetic diversity of the species (MITRAKUL *et al.*, 1999; CURTIN *et al.*, 2007). Only an enzymatic restriction followed by a pulsed field gel electrophoresis enables a fine discrimination of *B. bruxellensis* isolates by providing a single profile for each one (MIOT-SERTIER and LONVAUD-FUNEL 2006; MIOT-SERTIER *et al.*, 2006).

BRETTANOMYCES BRUXELLENSIS IN THE VINEYARD

1- Brettanomyces and the grape microflora

Diverse and numerous bacteria (BAE *et al.*, 2006), yeast (RENOUF *et al.*, 2005a) and moulds (DOARÉ-LEBRUN *et al.*, 2006) colonize grape berries. This ecosystem varies according to the ripening stage (ROSINI *et al.*, 1982), the vine type (RENOUF *et al.*, 2005a), the geographic location of the winery (RENOUF *et al.*, 2006b) and also the agrichemical treatments (MONTEIL *et al.*, 1986). Population levels between species can be highly different (PRAKITCHAIWATTANA *et al.*, 2004). A grape berry carries between 10^4 and 10^6 microbial cells depending on its size, its maturity and its sanitary state (MORTIMER and POLSINELLI 1999; RENOUF *et al.*, 2005a). The grape microflora is mostly represented by a few major species, leaving minor ones barely perceptible. Minor species are indeed only represented by 1 to a few dozen cells per berry. They are therefore very easy to bypass, and for that reason, *B. bruxellensis* has for a long time escaped detection by wine microbiologists.

Progress in *B. bruxellensis* physiological knowledge (ROSE and HARRISSON 1971; GILIS 1999, USCANGA *et al.*, 2000; MEDAWAR, 2003) contributed to the development of media particularly favourable to its growth. These enrichment media make it possible to increase the concentration of a given species initially at a low level in a sample by unfavouring the growth of the major species. The use of such enrichment media intended

Table 1 - PCR methods used to identify *B. bruxellensis* in wine.

Molecular tools of identification by PCR	DNA region targeted by the PCR primers	References	identifying information
PCR species specific	*RAD4* gene	IBEAS et al. (1996)	Amplification or not
PCR-RFLP	5.8S rDNA gene and the two internal transcribed spacers (ITS1 and ITS2)	ESTEVE-ZARZOSO et al. (1999)	enzymatic restriction pattern (after amplicons digestion)
PCR-DGGE	D1/D2 domains of the rRNA 26S gene	COCOLIN et al. (2000)	Specific front of migration
Quantitative PCR	RAD4 gene	DELAHERCHE et al. (2004, 2007)	Real time melt curves analysis

for *B. bruxellensis* made it possible to highlight the vineyard origin of this yeast (BARBIN, 2006; BARBIN *et al.*, 2007; RENOUF and LONVAUD-FUNEL, 2007). Similar investigations had been previously carried out for *Saccharomyces cerevisiae* (MARTINI *et al.*, 1996). Taken together, studies on minor berry species show that all wine species are initially present on the grape surface, included within a microbial biofilm. Major grape species (*Cryptococcus* sp., *Aureobasidium pullulans*, *Sporobolomyces* sp. and others) do not usually play a big role in the winemaking process, but they are thought to have a central part in the preservation of the grape ecosystem (RENOUF *et al.*, 2005a).

2- Causes of Brettanomyces bruxellensis on grapes

B. bruxellensis detection changes during grape ripening. It is more frequent at harvest time than on green and immature berries (RENOUF and LONVAUD-FUNEL 2007). However, some plots are more sensitive than others. Moreover, different *B. bruxellensis* distributions seem to occur in relation to the plot's physical configuration (topography) or its environment (BARBIN *et al.*, 2007). Moisture and freshness constitute a particular microclimate, increasing the probability of *B. bruxellensis* detection (BARBIN, 2006). These observations are not surprising since temperature and water activity are two fundamental parameters in microbial development.

B. bruxellensis does not appear to be related to other microorganisms commonly found on grapes such as acetic bacteria, *Penicillium* sp. and *Aspergillus* sp. However, the simultaneous presence of *Brettanomyces* and *Botrytis* on grape has been noted. It is difficult to know if excessive moisture and heat favour *B. bruxellensis* and *Botrytis* at the same time or if there are direct interactions between these microbial species. Nevertheless, a correlation between the detection of the first and the presence of the second was established for grapes (BARBIN, 2006). Moreover the use of an anti-*Botrytis* treatment containing procymidone limits the presence of *B. bruxellensis* on berries. Procymidone, as well as dichofluanide, benomyl, iprodione and vinclozoline are the active substances found in vine antifungal treatments and have yeast inhibitory properties (NAVARRO *et al.*, 1999; STURM *et al.*, 2006). This could explain why in years where *Botrytis* development is favoured and treatments done, volatile phenol production due to *B. bruxellensis* occurs less during the early stages of winemaking. Hypotheses are still uncertain concerning the relation between *Botrytis* and *Brettanomyces*, but the sanitary state of the grape should be considered when exploring *B. bruxellensis* occurrence. On one hand, damaged grapes could enhance *B. bruxellensis* development on berries by liberating nutrients previously trapped in the berry (MORTIMER and POLSINELLI 1999). However, if damage is due to Botrytis, antifungal treatments could reduce yeast berry development. On the other hand, microbial biofilms containing *B. bruxellensis* will not be reduced on intact untreated berries. Hence, when considering the development of *B. bruxellensis* on grapes, one should not only consider the physical state of grapes but also the effect of antifungal treatments.

3- Brettanomyces bruxellensis in the vineyard: consequences in wines

In musts, the microbial consortium changes drastically. The most adapted microorganisms are favoured and their growth enhanced. Generally, this leads to alcoholic fermentation fully carried out by *Saccharomyces cerevisiae*. However, in some cases, *B. bruxellensis* can also be present at high levels, leading to phenolic off-flavours in the fermenting must (MIRAMBEAU *et al.*, 2007). Detailed analysis has shown that at the end of fermentation, *B. bruxellensis* populations could reach 10^6 CFU/mL and that several strains could be simultaneously present in one tank, with the neighbouring tanks being characterised by their own strains. This observation fits well with a vineyard-origin of the strains of *B. bruxellensis* responsible for the phenolic off-flavours observed. Although a detailed scenario of why these strains developed during fermentation is not yet available, several points can be considered. First, the presence of *B. bruxellensis* may be linked to a decline in the *Saccharomyces* population (RENOUF *et al.*, 2006c). However, drastic changes in the fermentation kinetics could not be observed, meaning that *B. bruxellensis* probably took over the fermentation activity. The decline of *Saccharomyces* could be attributed to the high sugar concentrations found in musts, leading to an ethanol stress higher than usual. This correlated well with observations showing the ethanol resistance of *B. bruxellensis* compared to *S. cerevisiae* (MEDAWAR *et al.*, 2003).

Strains isolated from the grape were compared with the strains isolated during fermentations, ageing and after bottling. Grape strains persist all along winemaking (MIOT-SERTIER *et al.*, 2006; RENOUF and LONVAUD-FUNEL, 2006). In laboratory experiments, these strains appear to produce important quantities of volatile phenols (RENOUF *et al.*, 2007c). Hence, a volatile phenol production can be linked to the presence of strains initially present on the grapes. Each year, the harvest brings its pool of *B. bruxellensis*, which can find favourable conditions for its growth during winemaking.

B. bruxellensis strains can be found in young wines or in those of more than thirty years old. Different strains can be found within several cellars. This suggests a great diversity range within *B. bruxellensis*, sometimes one strain representing one vineyard plot (RENOUF *et al.*, 2006b; BARBIN 2006).

Strains isolated from grapes could also be detected on tank surfaces and barrels, but only if the material has been previously used (RENOUF *et al*., 2006d). Similar observations were reported concerning *S. cerevisiae* strains during cider elaboration by SWAFFIELD and SCOTT (1995). So far, *B. bruxellensis* has never been found on the surface of or inside new material before its first contact with wine. Wine strains are therefore able to colonize oenological material progressively through wine contact. Nevertheless, one should not think used material is the cause of contamination, but rather its bad maintenance and cleaning. These results underscore the importance of a regular and effective maintenance of the barrels before their re-use. One effective method consists in applying hot water under pressure in all directions in the barrel, draining it and sulphurizing it (RENOUF *et al.*, 2006d).

Hence, *B. bruxellensis* strains can have multiple origins (grapes, cellar, and even atmosphere and insects). Within all these possible sources, finding the initial one raises an important issue. To gain insight on this important topic, it is necessary to pursue with the study of strain identification within the *B. bruxellensis* species. Indeed, considering yeast strain diversity can make a major contribution to know the species distribution in a given environment.

BRETTANOMYCES DURING WINEMAKING

1. From vine to wine: the importance of fermentations

Unlike most grape berry yeast species which are sensitive to osmotic pressure and SO_2, *B. bruxellensis* finds, after pressing, an environment more favorable to its growth (NISHING *et al.*, 1985).

In the fresh grape must, the fermentative species *B. bruxellensis* is able to degrade glucose and fructose but also oligosaccharides with a degree of polymerization from 2 to 9 (FREER 1991; CHATONNET *et al*., 1999). Nevertheless, *B. bruxellensis* is less adapted than *S. cerevisiae* to rich media like grape must (ABBOTT *et al.*, 2004). The massive addition of exogenous strains of *S. cerevisiae* selected for their oenological qualities intensifies this domination (RENOUF *et al.*, 2006c).

In laboratory tests, sugars consumption kinetics vary according to the *B. bruxellensis* strain considered (BARBIN, 2006). In the cellar, *B. bruxellensis* is generally able to grow with very low sugar concentrations (GEROS *et al.*, 2000). Hence, glucose and fructose full consumption, leaving residual concentration of less then 300 mg/L during alcoholic fermentation, is an essential factor when limiting *B. bruxellensis* development in wine. Small quantities of dissolved oxygen and the poor quantities of available nitrogen or vitamins (AGUILAR-USCANDA, 1998) are not limiting growth factors for *B. bruxellensis*.

B. bruxellensis is finally not very demanding from a nutritional point of view. This species can grow on synthetic media containing only ethanol as an organic carbon source (RODRIGUEZ *et al.*, 2001; SILVA *et al.*, 2004; CONTERNO *et al.*, 2006). Ethanol assimilation is not direct; it is first transformed into acetate, which is then used as a substrate (GILIS, 1999). Thus acetic acid, and ethanol, can be products of, but also substrates for *B. bruxellensis* (GILIS *et al.*, 1999). Glucose and fructose act as catabolic repressors able to direct, or not, according to their concentration, the use of ethanol and acetic acid (SANFACON *et al.*, 1976). As for ethanol, *B. bruxellensis* is less sensitive than *S. cerevisiae* to acetic acid (ABBOTT *et al.*, 2004).

These metabolic properties, that can vary greatly according to the strain considered (CONTERNO *et al.*, 2006), explain why *B. bruxellensis* is able to develop in must, then in wine. To prevent its growth, it is crucial to point out the oenological practices that will have an impact on its multiplication. During the initial winemaking stages, two parameters are essential for *B. bruxellensis* growth: SO_2 added to the harvest and initial cold maceration. SO_2 and low temperatures are a priori judicious to alter microbial development. But recent investigations (RENOUF *et al.*, 2006c) show that these early winemaking practices act on sensitive species (*Candida* sp., *Hanseniaspora* sp., *Metschnikowia* sp.,) while other like *Pichia* sp., *Torulaspora* sp. (ALVES-ARAUJA *et al.*, 2004) and *B. bruxellensis* can resist. Hence, when the first disappear, the latter, which should be more cryotolerant, are able to develop and take advantage of the ecological vacancy that is offered to them. Environmental constraints can therefore select *B. bruxellensis* by default because this latter is more resistant.

Similar observations are made during alcoholic fermentation. Firstly, *B. bruxellensis* seems not to be sensitive to the killer properties of certain commercial strains of *S. cerevisiae* contrary to other yeast found in fermenting must (ZAGORC *et al.*, 2001; PEREZ *et al.*, 2004). COMITINI *et al.*, (2004) reported the production of anti-*Brettanomyces* toxins by *Pichia anomala* and *Kluyevromyces wickerhamii*. But these last species are rarely dominant in the AF microflora. So far, nothing has been reported concerning the possible antagonistic behaviour of some *S. cerevisiae* strain towards *B. bruxellensis*.

B. bruxellensis is more resistant to ethanol than *S. cerevisiae*. Thus, *B. bruxellensis* is one of the rare species able to develop when alcoholic fermentation is completed and the medium impoverished in fermentable

sugars and concentrated in ethanol (RENOUF *et al.*, 2006). Sometimes, as a consequence of a high initial sugar concentration, high ethanol concentration and other unfavourable conditions for *Saccharomyces cerevisiae*, the latter declines, leaving the alcoholic fermentation unfinished. The more ethanol resistant species *B. bruxellensis* can then grow favoured by the regression of *Saccharomyces* cerevisiae and by the high sugar concentration still left in the must. This explains why sluggish fermentations are often followed by *B. bruxellensis* development. However, this switch in the dominant species is not systematically correlated with a drastic change in the fermentation kinetics. In this case, the winemaker only notices an early phenolic off-flavour occurrence, signalling the presence of high *B. bruxellensis* populations (MIRAMBEAU *et al*., 2007). When this occurs, an early devatting is recommended to contain the growth of. *B. bruxellensis* which is preferentially concentrated in the press wines (RENOUF, 2006).

After alcoholic fermentation, the second fermentation, called malolactic fermentation, performed by lactic acid bacteria and mainly the *Oenococcus oeni* species (LONVAUD-FUNEL, 1999) often occurs. This is the second key stage for *B. bruxellensis* development in wine. Multiplication of *B. bruxellensis* is frequently observed during the growth of *O. oeni* and L-malic acid fermentation (GERBAUX *et al.*, 2000). A correlation was established between the length of malolactic fermentation and the *B. bruxellensis* biomass developed in the wine. The longer the malolactic fermentation, the higher the produced biomass (RENOUF *et al.*, 2005B). This could result from direct interactions between the bacteria and yeast cells or from indirect interactions. Hence malolactic fermentation kinetics is a determining point

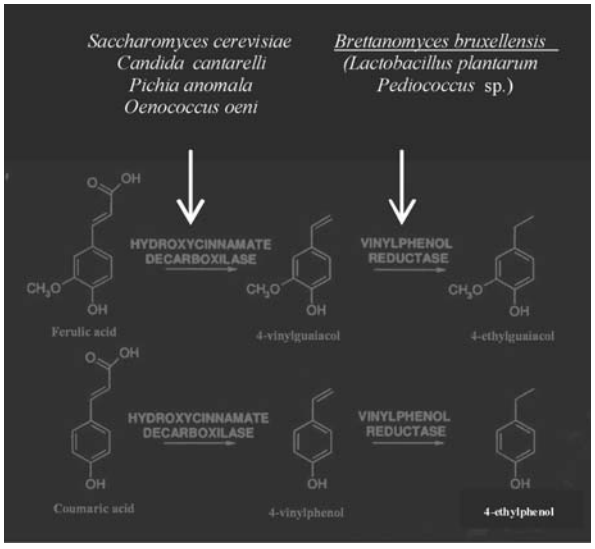

Figure 2 - Volatile phenol production from coumaric acid and ferulic acid and the principal species implicated in their synthesis.

at which one should monitor *B. bruxellensis* development. Moreover, fast malolactic fermentation is encouraged in order to protect the wine as soon as possible by SO_2 addition. Co-inoculation techniques (AVEDOVECH *et al.*, 1992; SIECZKOWSKI, 2004; MURAT *et al.*, 2007) consisting in the addition of a malolactic starter two or three days after the initial *S. cerevisiae* strain inoculation are carried out in this perspective (MURAT *et al.*, 2007). This provides an alternative to the Brettanomyces problems arising from sluggish malolactic fermentations (GINDREAU and AUGUSTIN, 2007).

After fermentations, sulphiting accentuates the decline of the fermentative species. SO_2 is a crucial element inhibiting *B. bruxellensis* (GERBAUX *et al.*, 2000; DUTOIT *et al.*, 2005). Even if some intraspecific difference of sensitivity can be noted (DU TOIT and PRETORIUS, 2005; CONTERNO *et al.*, 2006), in general, for *B. bruxellensis*, a minimum of 0.625 mg/L molecular SO_2 is required (HENICK-KLING *et al.*, 2000). Based on correspondence between molecular SO_2, free SO_2, and pH, that implies a concentration of 60 mg/L free SO_2 for a wine with a pH of 3.8 at 15 °C (SUDRAUD and CHAUVET, 1985). The SO_2 effectiveness depends on the pH, but also on the level of phenolic compounds (BARBE *et al.*, 2000). Free SO_2 must be regularly adapted to the pH and the combination phenomena.

2- From barrel to bottle : ageing, a key step to prevent Brettanomyces development

The majority of the oenological species in must or during fermentations can produce 4-vinylphenol and 4-vinylguaiacol, notably the main fermentative species: *S. cerevisiae* and *O. oeni* (RENOUF *et al.*, 2006f) (figure 2). But *B. bruxellensis* is the only species able to produce important quantities of 4-ethylphenol and 4-ethylguaiacol (figure 3). Within each species, the volatile phenol synthetic capacities are strain-dependant (RENOUF *et al.*, 2007c; CONTERNO *et al*., 2006).

Production is more effective during aging, when *B. bruxellensis* is the principal residual species. Monitoring *B. bruxellensis* population and volatile phenol production shows that the quantities synthesised are directly linked with *B. bruxellensis* accumulation (figure 4). As a consequence, relatively low and latent populations (10^2-10^3 CFU/mL) over long periods are as prejudicial as transitory high levels. That underlines the importance of permanent microbiological surveys during aging to reduce the *B. bruxellensis* population to the lowest possible level. Some practices are more favourable than others for the reduction of microbial populations. The following examples aim to illustrate the impact of these practices with regard to the risk of *B. bruxellensis* development.

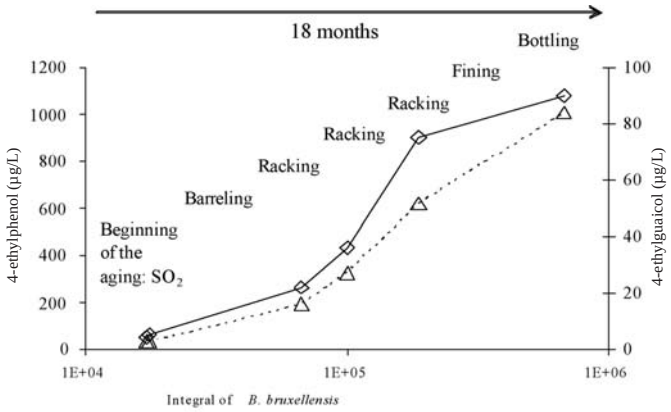

Figure 3 - Evolution of 4-ethylphenol (left axis) and 4-ethylguaiacol (right axis) during aging and correlation with accumulation of *B. bruxellensis* in wine.

3. Microbial stabilization procedures

When barreling, the choice of the barrels should not be made on microbiological considerations. Suitably maintained used barrels are not more favourable to microbial development than new barrels. On the contrary, the latter are more permeable to oxygen and can bring new substrates. They can lead to the maintenance of high levels of acetic acid bacteria and yeast, notably *B. bruxellensis*, during the first period of their use (RENOUF and LONVAUD-FUNEL, 2005). Regular rackings, which eliminate the sedimented cells, contribute to lower yeast populations and in particular *B. bruxellensis* (RENOUF and LONVAUD-FUNEL, 2004). Thus, the reincorporation of lees during ageing can sometimes bring *B. bruxellensis* in wines. As a consequence, this practice should be considered only after a microbial analysis showing the absence or a low level of *B. bruxellensis* in lees. This factor is all the more important if the alcoholic fermentation was chaotic. Fining is also beneficial because it helps flocculation of *B. bruxellensis* and cell elimination with sediments (MILLET, 2001; MURAT and DUMEAU, 2003).

These traditional methods thus ensure a progressive reduction of the microflora. More radical treatments like heat treatments (COUTO *et al.*, 2005) or filtration (UBEDA *et al.*, 1999; RENOUF *et al.*, 2007d) are effective against *B. bruxellensis*. Wines in which the intrinsic microbial ecosystem is imbalanced are more favourable to contamination. These microbial stabilisation methods should be considered only before bottling, the probability of re-contamination being then lower.

Chemical alternatives to SO_2 can also be considered. DMDC (dimethyldicarbonate or Velcorin® or E242) is a chemical conservative which shows remarkable antimicrobial activities (PORTER *et al.*, 1982; OUGH *et al.*, 1988). It inhibits the enzymes involved in glycolysis (TEMPLE and OUGH, 1978). Its effectiveness in the stabilization of sweet wines has been shown (THRELFALL and MORRIS, 2002; DIVOL *et al.*, 2005). Its action is transitory since it is very quickly hydrolyzed in wine into methanol and ethyl acetate (OUGH and LANGBEHN 1976; PETERSON and OUGH 1979; DELFINI *et al.*, 2002). Thus, it cannot completely replace SO_2 which has more remanence. It could be used as an alternative to sterilizing filtration or heating. Today, the use of DMDC in the European Union is only allowed in wines having a residual sugar content higher than 5 g/L and for a maximum amount of 200 mg/L, given its degradation products (law 643/2006, April 27, 2006). At the laboratory scale, the minimum inhibitory concentration of DMDC for *B. bruxellensis* is 150 mg/L in grape juice. For once *B. bruxellensis* seems to be more sensitive than other microbial species to stabilization practice (RENOUF *et al.*, 2008).

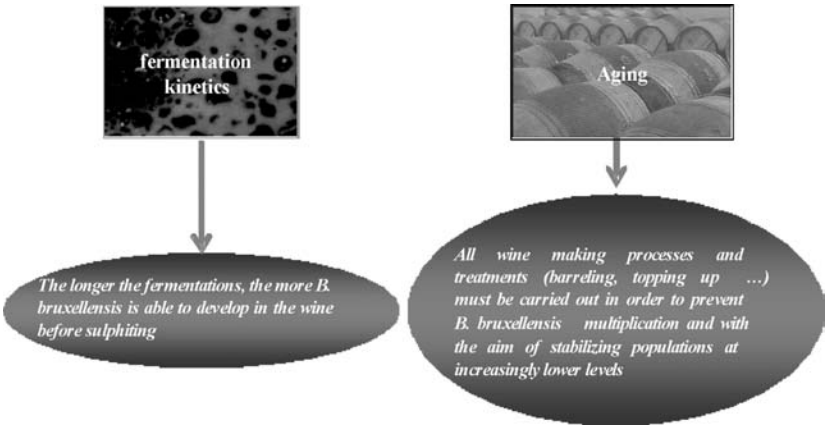

Figure 4 - Two key steps in the prevention of *B. bruxellensis* development.

Table 2 - Factors to monitor and principal methods used to prevent the development of *B. bruxellensis* in the wines.

	Beneficial conditions for the growth of *B. bruxellensis*	Conditions limiting the growth of *B. bruxellensis*	Reference
SO_2	Free SO_2 limited by high pH values or combination with phenolic compounds	0.625 mg/L of molecular SO_2	LICKER *et al.* 1997
Racking	-	Every three months	RENOUF and LONVAUD-FUNEL 2004
Fining	-	With white eggs or gelatin at the end of aging	MURAT and DUMEAU 2003,
Filtration	-	Retention threshold lower than 1.0 µm	RENOUF *et al.* 2007d
DMDC addition	-	The minimum inhibitory concentration of DMDC is estimated at 150 mg/L (grape juice, laboratory conditions).	RENOUF *et al.* 2008
Thermal treatment	-	Several minutes at 40°C but thermal parameters can be strongly variable (population levels, pH, alcohol, phenolic index…)	COUTO *et al.* 2005

CONCLUSION

The yeast *B. bruxellensis* is at the centre of current microbiological considerations in wine, because the volatile phenols that it produces confer off-odors which are disliked by consumers and which mask the required fruity character of wine. Previous investigations have pointed out the bad hygienic conditions of the cellar as the main factor of *B. bruxellensis* development in wine. But despite many progresses in this domain, spoilage by volatile phenols still remains an important issue, showing the multifactorial causes of *B. bruxellensis* occurrence in wine. It is now known that *B. bruxellensis* initially comes from the vineyard and notably from the grape.

During winemaking, *B. bruxellensis* remains, despite constraining oenological conditions (alcohol content, pH, SO_2). If the fermentative species usually predominant are unfavoured, the microbial balance will bend in favour of *B. bruxellensis* development. Fast fermentations will allow early SO_2 addition, protecting the wine. The second crucial step occurs during ageing, where *B. bruxellensis* should be carefully monitored and traditional oenological practices used to reduce its occurrence (figure 4, tableau 2). But, in addition to these practical considerations, efforts should be carried out to better understand volatile phenol production.

In fact, most oenological yeast (CHATONNET *et al.*, 1993; RODRIGUES *et al.*, 2001) and bacteria species (BAUMES *et al.*, 1986; CAVIN *et al.*, 1993; CHATONNET *et al.*, 1995, 1997; RENOUF *et al.*, 2007f) are able to produce 4-vinylguaiacol and 4-vinylphenol. However, the metabolic path stops there, without further transformation into 4-ethylguaiacol and 4-ethylphenol. Moreover, *S. cerevisiae* is unable to form 4-vinylguaiacol and 4-vinylphenol the in presence of phenolic compounds (CHATONNET *et al.*, 1989). Hence *B. bruxellensis* is one of the only species able, in oenological conditions, to form 4-ethylguaiacol and 4-ethylphenol.

From a physiological point of view, these transformations are still the object of research (DIAS *et al.*, 2003). Two principal hypotheses can be given to explain why *B. bruxellensis* degrades hydroxycinnamic acids. The first postulates that the yeast recovers energy from this decarboxylation/reduction in the form of an electron gradient allowing ATP production. The second theory involves a detoxification procedure. Phenolic acids deteriorate the plasmic membrane by destructuring the phospholipid bi-layer. *B. bruxellensis* might therefore degrade them to decrease their inhibiting action on cell maintenance.

In the cellar, the highest production of 4-ethylphenol and 4-ethylguaiacol is most frequently observed during aging and only more rarely during fermentations. The production of volatile phenols can probably be related to three parameters (figure 5): quality of the substrates, oenological practices and microbial developments.

Obviously relations exist between these parameters. For example, sugar or L-malic acid concentrations in musts influence the fermentations and the microbial developments, which are also affected by the oenological operations. The microbial surveys should also be considered. Once the date of the grape harvest is fixed, the grapes should be analyzed (chemical data and indigenous microbial populations). On the basis of this information, fast and total fermentations should be

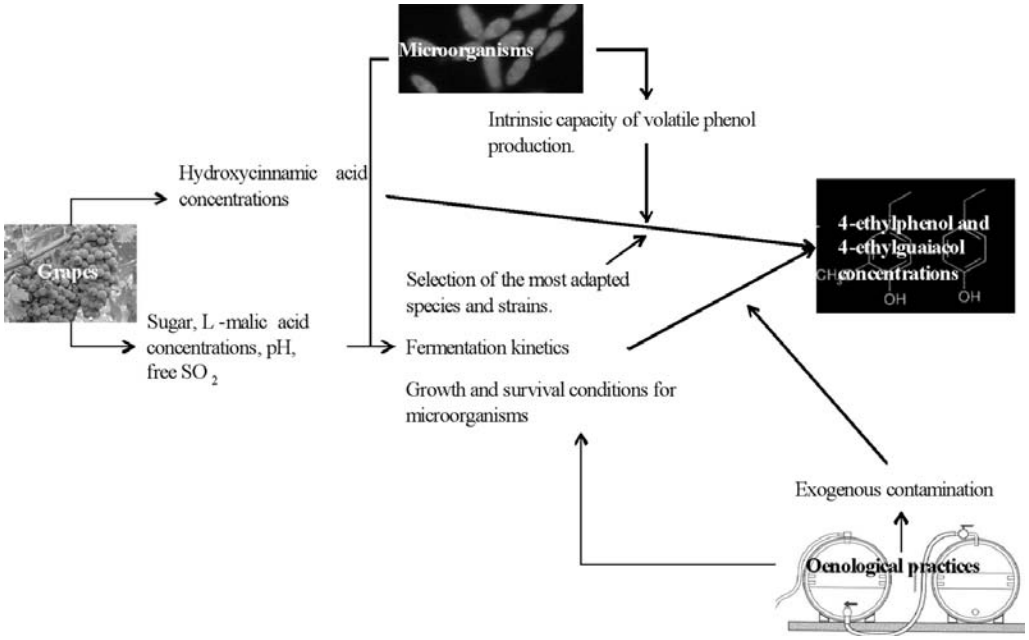

Figure 5 - Compilation of the various factors intervening in the volatile phenols production in wines

favoured in order to quickly sulphite the new wine once the fermentative species decline.

Theoretically, the strain factor should also be taken into account (CONTERNO *et al.*, 2006). None of the *B. bruxellensis* strains has the same intrinsic production capacities, neither the same development faculties in wine. Fundamental studies on the physiological behaviour of *B. bruxellensis* strains are needed to progress in the comprehension of this diversity. They should be associated with a better substrate characterization. Indeed, the concentration of phenolic acids in general, and hydroxycinnamic acids in particular could play an important role in volatile phenol production. However, this aspect has hardly been investigated and little information exists on whether or not grape and wine hydroxycinnamic acid concentration is a limiting factor in volatile phenol synthesis. From a sensorial point of view, PHISTER and MILLS (2004) have suggested that olfactory thresholds are higher in monovarietal Cabernet-Sauvignon wines than in Tempranillo wines. Hence, a better characterization of the relationship between the microorganisms, grape substrates, and the sensorial analysis is needed in order to have a full picture of the volatile phenol presence in wines.

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