Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Brettanomyces bruxellensis evolution and volatile phenols production in red wines during storage in bottles

J. Coulon[1], M.C. Perello[2], A. Lonvaud-Funel[2], G. de Revel[2] and V. Renouf[2]*

1 MICROFLORA, ISVV, Université de Bordeaux, Villenave d'Ornon, France
2 UMR Œnologie, ISVV, Université de Bordeaux, Villenave d'Ornon, France

Keywords
alteration,*Brettanomyces bruxellensis*,spoilage prediction,volatile phenols,wine.

Correspondence
Joana Coulon, MICROFLORA, ISVV, Université de Bordeaux, 210 Chemin de Leysotte, CS50008, 33882 Villenave d'Ornon, France.
E-mail: microflora@oenologie.u-bordeaux2.fr

2009/0603: received 2 April 2009, revised and accepted 10 September 2009

doi:10.1111/j.1365-2672.2009.04561.x

Abstract

Aims: The presence of *Brettanomyces bruxellensis* is an important issue during winemaking because of its volatile phenols production capacities. The aim of this study is to provide information on the ability of residual *B. bruxellensis* populations to multiply and spoil finished wines during storage in bottles.
Methods and Results: Several finished wines were studied. *Brettanomyces bruxellensis* populations were monitored during two and a half months, and volatile phenols as well as chemical parameters regularly determined. Variable growth and volatile phenols synthesis capacities were evidenced, in particularly when cells are in a noncultivable state. In addition, the volatile phenol production was clearly shown to be a two-step procedure that could strongly be correlated to the physiological state of the yeast population.
Conclusions: This study underlines the importance of minimizing *B. bruxellensis* populations at the end of wine ageing to reduce volatile phenols production risk once the wine in bottle.
Moreover, the physiological state of the yeast seems to have an important impact on ethyl-phenols production, hence demonstrating the importance of taking into account this parameter when analysing wine spoilage risks.
Significance and Impact of the Study: Little data exist about the survival of *B. bruxellensis* once the wine in bottle. This study provides information on the alteration risks encountered during wine storage in bottle and reveals the importance of carrying on further studies to increase the knowledge on *B. bruxellensis* physiology.

Introduction

Grape juice transformation in wine is the result of the activity of diverse micro-organisms. In the vineyard, grape berries are already covered by filamentous fungi, yeast and bacteria (Renouf *et al.* 2005). In the cellar, yeast and particularly the *Saccharomyces cerevisiae* species, degrade glucose and fructose, producing ethanol and CO_2 during the alcoholic fermentation (AF) as well as numerous other substrates, thus transforming grape must into wine. Then, in red wine, lactic acid bacteria (LAB), mainly represented by the *Oenococcus oeni* species, carries on the malolactic fermentation (MLF) by metabolizing L-malic acid into L-lactic acid and CO_2 (Lonvaud-Funel 1999). However, micro-organisms do not always yield positive effects and some can be prejudicial to the wine quality, for example by producing off-odours like the yeast *Brettanomyces bruxellensis* (Loureiro and Malfeito-Ferreira 2003). Among the different undesirable compounds produced by *B. bruxellensis* (Hereszyn 1986; Romano *et al.* 2008), volatile phenols are, without any doubt, the most prejudicial (Chatonnet *et al.* 1992). Their production leads to a loss of freshness and fruitiness, and concentrations above their limit perception threshold (426 $\mu g\ l^{-1}$ for a 1 : 10 mix of ethyl-phenol and ethyl-guaiacol, Chatonnet *et al.* 1992) can confer a horsy defect to the wine, which are highly detrimental to its quality and unwanted by consumers. In order to provide enough

information and efficient solutions to winemakers, microbiologists investigate to better understand the origin *B. bruxellensis* in wine (Suárez *et al.* 2007), its growth conditions (Alguilar Uscanga *et al.* 2000; Fugelsang and Zoecklein 2003; Renouf *et al.* 2006a; Garcia Alvarado *et al.* 2007), volatile phenols production pathways and regulations (Dias *et al.* 2003; Benito *et al.* 2009a; Tchobanov *et al.* 2008; Harris *et al.* 2009; Godoy *et al.* 2009) and genetic (Conterno *et al.* 2006; Curtin *et al.* 2007; Woolfit *et al.* 2007) or physiological characteristics (Medawar *et al.* 2003; Vigentini *et al.* 2008). *Brettanomyces bruxellensis* is probably the most well-adapted yeast species to dry red wine. It is somewhat resistant to SO_2, high ethanol content, oxygen and sugar depletion (Barata *et al.* 2008; Benito *et al.* 2009b). It can be particularly active during sluggish alcoholic or MLFs, benefiting from the decline of the fermentative species (Renouf *et al.* 2006b). It can also grow during wine ageing despite SO_2 additions and the use of techniques such as fining (Murat and Dumeau 2003; Du Toit *et al.* 2005) and racking (Renouf and Lonvaud-Funel 2004), which contribute to the microbial stabilization of wine. Anti-microbial actions like heat treatments (Couto *et al.* 2005a) and filtration (Ubeda and Briones 1999; Millet and Lonvaud-Funel 2000) do have an effect on the survival and growth of *B. bruxellensis*, but their action is only transitory and does not protect the wine from further *B. bruxellensis* contaminations and development. It appears clearly that the most efficient way to prevent wine spoilage by *B. bruxellensis* is to control its development through winemaking management and a preventive attitude, such as carefully monitoring prefermentation and fermenting phases and stabilization practices during ageing. But, at the end of barrel ageing, before bottling, residual population of *B. bruxellensis* can often be detected (Nisiotou and Gibson 2005; Renouf *et al.* 2006b; Curtin *et al.* 2007). Although populations are usually too low at this point to synthesize ethyl-phenols, they could further develop and spoil the wine during bottle storage, when the winemaker can no longer intervene. The objective is to study the impact of residual *B. bruxellensis* populations present initially, even at low levels, on volatile phenols concentrations in finished wine stored in bottles.

Materials and methods

Wine collection and preparation

Brettanomyces bruxellensis growth and volatile phenols production was studied from 13 different bottled wines originating from different appellations located in the Bordeaux area. Their origins are listed in Table 1. For each wine, five marketed bottles (750 ml) were homogenized and aliquoted into 150 ml bottles under sterile conditions. All 150 ml bottles were air-tight sealed and stored at 20°C. *Brettanomyces bruxellensis* populations and ethyl-phenols levels were measured at regular intervals. For each analytical point, three samples of each wine were used.

Table 1 List of red wines studied

Wines	Appellation	Vintage
A	Pauillac	1998
B	Margaux	2000
C	Pomerol	2001
D	Saint-Emilion	2001
E	Pessac-Léognan	2003
F	Pauillac	2003
G	Saint-Emilion	2005
H	Pauillac	2006
I	Pauillac	2006
J	Pauillac	2006
K	Saint-Emilion	2006
L	Margaux	2006
M	Saint-Emilion	2006
N	Pauillac	2006

Microbial analyses

Enumeration of the B. bruxellensis yeast populations
Selective media plate counts were carried out to determine the non-*Saccharomyces* yeast population. Media was prepared as initially described by Millet and Lonvaud-Funel (2000) with changes as mentioned by Renouf *et al.* (2006a). For culture determinations, three platings were performed for each sample. Samples were either filtered onto a nitrocellulose 0·45- μm filter (Millipore) and then the filter deposited onto the solid media or direct plating was performed using sterile glass beads with 0·1 ml undiluted or diluted wine. For the first two determinations, the three platings per sample were performed as follows: 100 ml of wine filtered, 1 ml of wine filtered and direct plating of 0·1 ml of a 1/10 diluted wine. Further determinations were performed the same way for stable or small populations. For growing populations, determinations were performed by filtering 1 ml of wine and plating 0·1 ml of diluted wine (1/10 and 1/100) or plating 0·1 ml of 1/10, 1/100 and 1/1000 serially diluted wine. For all determinations, the homogeneity of the colony counts taking into account the filtering or the dilutions was verified, and the plate containing in between 15 and 150 UFC was selected for the population enumeration.

The use of a high cycloheximide concentration (Sigma–Aldrich) prevented the growth of the *Saccharomyces* species during the seven days necessary for the development of *B. bruxellensis* colonies. The concentration

of cycloheximide (500 mg l^{-1}) was chosen for this medium in accordance with results of a study by Zott (2008) which demonstrated that *B. bruxellensis* is the predominant wine yeast species able to grow at this concentration on solid nutritive medium. Moreover, as shown by several authors (Nisiotou and Gibson 2005; Curtin *et al.* 2007; Renouf *et al.*2007) at the end of the ageing and in the bottle, *B. bruxellensis* is, by far, the predominant residual yeast species in wine. Therefore, in the wines studied, the non-*Saccharomyces* population was assimilated in this work with the *B. bruxellensis* population.

Determination of the B. bruxellensis population by q-PCR
Brettanomyces bruxellensis populations were also analysed and quantified by Q-PCR. First, the wine DNA microflora was isolated according to the method described by Delaherche *et al.* (2004), except that microbial cells were harvested by filtering 50 ml of wine onto a 0·45 μm nitrocellulose membrane. Real time amplifications were carried out as described by Delaherche *et al.* (2004), solely using 2 μl DNA template in a 25 μl reaction mixture. For each run, the amplification baseline was adjusted by including a control using 50 ng of a calibrated pure *B. bruxellensis* strain DNA preparation (*B. bruxellensis* strain L0417, IOEB Collection). *Brettanomyces bruxellensis* populations were then estimated by plotting the resulting Ct onto a linear regression giving the equivalence in between the PCR signal and the *B. bruxellensis* cell number in the reaction mixture (Delaherche A., personal communication). This linear regression was performed using 86 naturally contaminated wines instead of one serial diluted contaminated wine as described by Delaherche *et al.* (2004). All these changes allowed decreasing the quantification threshold from 10^4 UFC ml^{-1} as initially described to 10 UFC ml^{-1}. Finally, results were expressed as CFU equivalents ml^{-1} of wine by taking into account the wine sample volume used for the extraction and the matrix volume used in the PCR. All results represent three independent PCR performed using a single wine extraction. Means and errors bars were calculated based on these triplicates. Although it can be discussed that PCR method tend to detect living as well as dead cells, with this method, dead yeast cells account for less than 1% of total yeast cells. This important feature has been evidenced by Zott (2008) for different yeast species, as well as Coulon (personal communication) for *B. bruxellensis* specifically. In these investigations, experiments to evidence this phenomena were conducted and showed that (i) free DNA could not be detected with this method, more than likely because the wine is filtered on a 0·45- μm membrane as a first step of the extraction method and (ii) pure yeast cultures added to wine and killed either rapidly or slowly in different ways (heat treatment, DMDC application and SO_2 application) could not be detected by q-PCR. These results were confirmed by plate counts and épifluorescence estimations.

Chemical analyses

D-glucose and D-fructose were measured at least twice by enzymatic methods (R-Biopharm, St Didier, France). Ethanol, pH, free SO_2 and total SO_2 were determined according to the international methods for wines and musts analysis published by the Organisation Internationale de la Vigne et du Vin (*OIV*, 2 July 2007). Free SO_2 and total SO_2 were measured three times and the mean calculated. Molecular SO_2 was calculated according to the formula provided by Blouin (1992), taking into account the ethanol content, pH, wine temperature and free SO_2 concentration. Volatile phenols concentrations (vinyl-phenol, vinyl-gaiacol, ethyl-phenol and ethyl-guaiacol) were measured according to the protocol described by Romano *et al.* (2008) using a solid-phase micro-extraction (SPME) associated to a GC-MS chromatography. The sum of vinyl-phenols and ethyl-phenols was then calculated by adding the concentrations of the vinyl and ethyl derivatives, respectively. Results are expressed as means of triplicates measurements on one sample.

Results

Evolution of chemical parameters

pHs ranged from 3·60 to 3·94 (Table 2). Molecular SO_2 ranged from 0·52 to 0·16 mg l^{-1}, the highest concentrations being found as expected in the youngest wines. All wines contained residual glucose and fructose concentrations (Table 2), but none of them had concentrations above 350 mg l^{-1}. During storage, active SO_2 decreased in all wines. After 80 days, the highest value (0·29 mg l^{-1}) was found in one of the wines with the lowest pH. Glucose and fructose were partially consumed in most wines, indicating that a microbial activity had occurred (A, B, D, E, F, G, I, H, K, L, M and N). However, ethyl-phenols production was detected even in wines with stable glucose and fructose concentrations (Table 3).

No clear relation between ethyl-phenol synthesis and chemical parameters usually considered like initial residual sugars and SO_2 could be shown among all wines (Fig. 1). Moreover, sugar consumption was not related to ethyl-phenol synthesis (Table 3).

B. bruxellensis development and volatile phenols evolution

All wines initially contained residual *B. bruxellensis* populations, whether detected by q-PCR, on selective media,

Table 2 Wines composition before and after storage

	Beginning of experiment					After conservation (80 days at 20°C)		
	pH	Ethanol %/vol	Glucose mg l^{-1}	Fructose mg l^{-1}	Active SO_2 mg l^{-1}	Glucose mg l^{-1}	Fructose mg l^{-1}	Active SO_2 mg l^{-1}
A	3·80	11·9	230 ± 10	90 ± 47	0·16 ± 0·01	120 ± 35	30 ± 9	0·13 ± 0·02
B	3·62	12·3	50 ± 4	30 ± 1	0·29 ± 0·06	20 ± 6	30 ± 10	0·19 ± 0·02
C	3·74	13·06	50 ± 10	20 ± 2	0·52 ± 0·03	40 ± 20	10 ± 10	0·19 ± 0·02
D	3·82	13·0	160 ± 6	20 ± 4	0·20 ± 0·03	110 ± 42	20 ± 9	0·13 ± 0·02
E	3·60	12·0	150 ± 9	120 ± 3	0·15 ± 0	80 ± 23	50 ± 4	0·11 ± 0·01
F	3·78	12·8	60 ± 6	50 ± 4	0·19 ± 0·02	40 ± 7	20 ± 3	0·14 ± 0·02
G	3·79	13·2	130 ± 4	50 ± 2	0·40 ± 0·07	70 ± 9	10 ± 2	0·13 ± 0·02
H	3·77	12·7	150 ± 8	50 ± 8	0·22 ± 0·06	50 ± 5	30 ± 4	0·17 ± 0·02
I	3·93	12·4	120 ± 4	80 ± 9	0·22 ± 0·02	60 ± 20	40 ± 20	0·19 ± 0·02
J	3·94	12·9	70 ± 9	20 ± 9	0·24 ± 0·07	70 ± 7	10 ± 10	0·17 ± 0·02
K	3·70	13·4	90 ± 7	240 ± 6	0·40 ± 0·01	50 ± 6	40 ± 4	0·29 ± 0·03
L	3·81	12·7	140 ± 2	90 ± 4	0·24 ± 0·04	60 ± 5	30 ± 2	0·20 ± 0·02
M	3·83	12·4	140 ± 3	70 ± 8	0·31 ± 0·01	120 ± 35	50 ± 9	0·19 ± 0·02
N	3·76	12·4	110 ± 9	50 ± 4	0·36 ± 0·04	50 ± 3	30 ± 7	0·26 ± 0·03

or both. During wine storage, populations increased in almost all wines and volatile phenols were synthesized. Ethyl-phenols are initially present in all wines. Because no microbial enumeration data was available prior our study, one cannot specify the microbial species implicated in volatile phenol production before the experiment. During the experiment, ethyl-phenols synthesis continued to occur. This production could reasonably be attributed to *B. bruxellensis* in all wines. Indeed, epifluorescence analysis carried on systematically with the first two microbial determinations showed that when micro-organisms could be detected, they corresponded to warhead shaped yeast or acetic acid bacteria (data not shown). This production could clearly be fractioned in a two-step process, a first period concerning vinyl-phenols synthesis, taken over in the second step by ethyl-phenols production (Fig. 2). According to *B. bruxellensis* development, wines could be categorized into three groups. Examples of each group are shown Fig. 2. The first group (Group 1) comprised wines A, D, E and G. No cultivable population was found in these wines. However, *B. bruxellensis* was detected at low levels by q-PCR. It survived at the same level and declined except in wine D where it transitorily reached 3×10^3 cells ml^{-1}. The fact that a population decline could be observed in this group confirms the observation stated in the Materials and Methods that mainly living yeast cells and not dead cells are detected. In these four wines, ethyl-phenols concentrations were initially among the highest of all wines. However, these are also the wines where volatile phenol production during the experiment was the lowest (Table 3). These results indicate that a microbial activity had already occurred prior to the experiment, perhaps explaining *B. bruxellensis* tempered development and low phenol synthesis latter on.

The second group (B, C, H, I, J, K, L, M and N) showed progressively increasing *B. bruxellensis* populations, as enumerated by q-PCR. Figure 2 shows the example of wines C and I, but all wines of this group harboured a similar yeast growth behaviour (data not shown). These populations could also be determined by colony count. After 40 days, cultivable populations seemed to diminish. This change can de attributed to a change in the cultivability of the populations, but as the colony count difference is of 1 log or less, the uncertainty of the culture method cannot be totally excluded. Because total viable populations enumerated by PCR still continued to grow, but to a lesser extent than previously, the cultivability decrease is more than likely the phenomenon sustaining this observation. After 80 days, cultivable populations started to increase again. Volatile phenols increased progressively and importantly throughout the wines conservation. In addition, it is noteworthy that precisely after 40 days of conservation, the accumulation of vinyl-phenols stops while ethyl-phenols still continue to evolve. For most wines of this group, ethyl-phenols were initially at low concentrations but increased in large amounts together with *B. bruxellensis* development. The third group (Fig. 2, Group3) is only represented by wine F which contained the highest ethyl-phenol initial concentration. In this case, the initial cultivable *B. bruxellensis* population first started to decrease then multiplied after 40 days (no colony on a 1 ml filtered wine solid media). Q-PCR enumeration showed a continuously growing population, but with once again, a slower development when populations could not be cultivated on solid media. This growing population was able to synthesize high amounts of ethyl-phenols (Table 3), reaching at the end of the experiment more than 5000 $\mu g\ l^{-1}$. Moreover, the

Table 3 Ethyl-phenols and vinyl-phenols evolution during wines storage. Ethyl-phenols production was calculated by subtracting ethyl- and vinyl-phenols concentrations after and before the experiment. Sugar consumption was calculated by subtracting glucose and fructose concentrations after and before the experiment

		Days after beginning of experiment			
	Ethyl-phenols* (μg l^{-1})	0	130	Ethyl-phenols production (μg l^{-1})	Sugar consumption (mg l^{-1})
Group 1					
A	4-EP + 4-EG	778 ± 63	1360 ± 120	695	170
	4-VP + 4-VG	10 ± 2	123 ± 4		
D	4-EP + 4-EG	605 ± 42	659 ± 44	131	60
	4-VP + 4-VG	0 ± 0	77 ± 6		
E	4-EP + 4-EG	640 ± 98	785 ± 60	162	240
	4-VP + 4-VG	53 ± 14	70 ± 6		
G	4-EP + 4-EG	508 ± 14	657 ± 74	234	100
	4-VP + 4-VG	0 ± 0	85 ± 14		
Group 2					
B	4-EP + 4-EG	916 ± 70	2876 ± 59	2161	30
	4-VP + 4-VG	49 ± 14	250 ± 30		
C	4-EP + 4-EG	745 ± 33	2258 ± 110	1740	20
	4-VP + 4-VG	56 ± 4	283 ± 74		
H	4-EP + 4-EG	325 ± 122	1670 ± 32	1574	120
	4-VP + 4-VG	41 ± 25	270 ± 63		
I	4-EP + 4-EG	38 ± 4	2013 ± 160	2211	100
	4-VP + 4-VG	5 ± 1	241 ± 37		
J	4-EP + 4-EG	49 ± 10	1670 ± 102	1776	0
	4-VP + 4-VG	5 ± 1	160 ± 19		
K	4-EP + 4-EG	21 ± 32	1391 ± 143	1505	240
	4-VP + 4-VG	5 ± 7	140 ± 38		
L	4-EP + 4-EG	30 ± 8	1699 ± 88	1814	160
	4-VP + 4-VG	5 ± 2	160 ± 58		
M	4-EP + 4-EG	17 ± 3	1731 ± 0	2182	40
	4-VP + 4-VG	5 ± 2	473 ± 0		
N	4-EP + 4-EG	293 ± 27	1823 ± 93	1701	80
	4-VP + 4-VG	19 ± 7	190 ± 47		
Group 3					
F	4-EP + 4-EG	1220 ± 462	5530 ± 201	4771	50
	4-VP + 4-VG	0 ± 0	461 ± 43		

*4-EP, 4-ethyl-phenol; 4-EG, 4-ethyl-gaiacol; 4-VP, 4-vinyl-phenol; 4-VG, 4-vinyl-gaiacol.

vinyl-phenols concentration, which decreases after 40 days in Group 1 and Group 2, continues to increase until 80 days of storage in this wine. This example confirms the link between growing cells and high ethyl-phenols production.

Discussion

Wines studied in this work could be categorized into three different groups. The first one (Group 1) showed mostly no growing cells, not cultivable on rich nutritive agar media containing cycloheximide, although this media is generally used to enumerate this species (Rodriguez *et al.* 2001; Couto *et al.* 2005b; Zott 2008). In these wines, ethyl-phenol concentrations were initially among the highest all wines, and increased during storage less than twofold. In the second and third groups (Group 2 and Group 3), *B. bruxellensis* multiplied faster, yielding high populations and producing higher amounts of ethyl-phenols. In these wines, ethyl-phenols concentrations increased from 3- to 104-fold during storage. *Brettanomyces bruxellensis* populations were partially cultivable, representing in average 10% of the total viable population. This experiment highlights a relationship between the physiological state of *B. bruxellensis*, and its capacity to produce volatile phenols. Cultivable populations seem indeed able to synthesize higher amounts of ethyl-phenols than viable but noncultivable cells. This questions the fact on whether it is the cell's state that influences its ethyl-phenol production capacities or whether it is the ethyl-phenols synthesis that confers better growing capacities to the yeast cells. Answers to that question might be found by having a closer look at the volatile

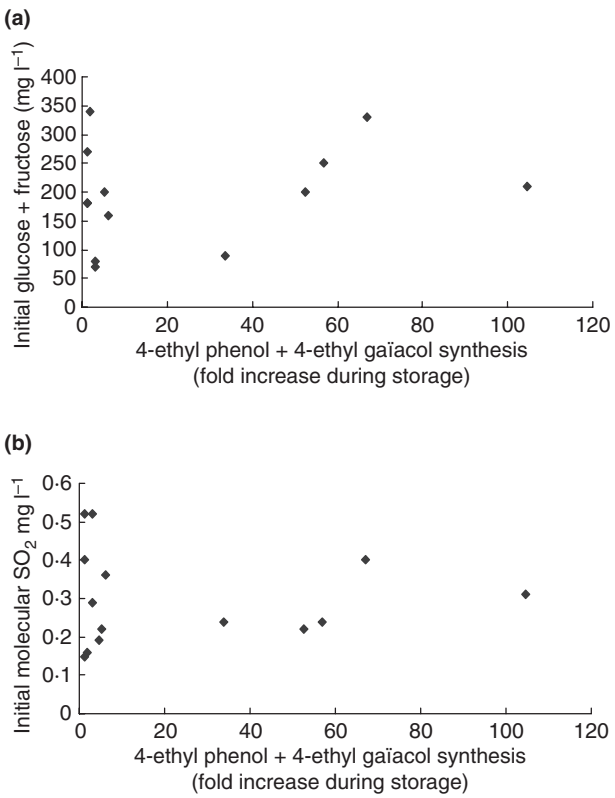

Figure 1 Correlation between ethyl-phenols synthesis and initial glucose + fructose concentration (a) and molecular SO_2 (b).

phenols sequence production. This experiment clearly points out a two-step synthesis, were vinyl-phenols first accumulate, yielding substrate for the second part of the reaction leading to ethyl-phenols synthesis. This sequential ethyl-phenol production could systematically be correlated to the *B. bruxellensis* physiological state. Indeed, maximum vinyl-phenol concentrations were found with fast-multiplying cells. They then decreased, indicating that the yeast metabolism is centred on ethyl-phenol production. This was generally accompanied by a population regression or/and a noncultivable state shift. Other authors had already noted that ethyl-phenols synthesis occurred during the late exponential phase or stationary growth phase in synthetic media (Dias *et al.* 2003; Harris *et al.* 2008) or wine (Romano *et al.* 2008). Vinyl-phenols production is the result of the decarboxylation of cinnamic acids. This type of reaction is known, mostly in LAB, to enhance the cell proton-motive force highly enough to drive ATP synthesis through the FO-F1-ATPase (Poolman 1993). One of the most known examples in oenology is L-malate into L-lactate decarboxylation during the MLF of LAB. Hence, it could be conceived that vinyl-phenols synthesis would enhance the ATP cell pool, providing enough energy for cell multiplication. Moreover, as shown in this study, other energy sources than glucose and fructose must be used by cells. However, no study has yet clearly shown a direct relationship in between ATP synthesis and cinnamic acid metabolism. Alternatively, a more indirect consequence of this decarboxylation can be considered. It has been shown in *S. cerevisiae* that yeast enhance their H^+ efflux through the plasma membrane H^+-ATPase activity to counteract the proton-motive dissipating force of weak acids like cinnamic acids, resulting in an inhibition of yeast growth (Chambel *et al.* 1999). Hence, cinnamic acid decarboxylation could be an alternative route for minimizing the uncoupling effect of this toxic compound and maintaining the growing abilities of *B. bruxellensis* cells. The second step of the reaction which concerns vinyl-phenols transformation into ethyl-phenols, is a reduction, probably participating in maintaining the cell's redox balance. This reaction doesn't act directly on the cell's energy provision, thus explaining why cell multiplication is less active when ethyl-phenols synthesis takes over vinyl-phenols production. Moreover, the *B. bruxellensis* growth kinetics, cell state and ethyl-phenols production could be linked to the initial ethyl-phenols concentration, as shown by comparing wines of Group 1 and Group 2. Indeed, in wines containing high initial amounts of ethyl-phenols, where cinnamic acids have already been metabolized, cells were usually noncultivable. In wine where these molecules have already been synthesized, *B. bruxellensis* seems less prone to produce ethyl-phenols, maybe because of precursors depletion. However, this is not always true, as shown in wines B, C and F of Group 2 and Group 3; although ethyl-phenol concentrations were initially very high, they still increased importantly and cells remained partially cultivable. These observations highlight two points that are crucial yet poorly understood in ethyl-phenols synthesis by *B. bruxellensis* in wines. First, the strain differences should be further studied. It has indeed been shown that different *B. bruxellensis* strains harbour various growth and ethyl-phenols synthesis abilities (Conterno *et al.* 2006; Vigentini *et al.* 2008; Oelofse *et al.* 2009). Because wines were collected from different wine production facilities, this factor more than likely participates in ethyl-phenol evolution. Strain enumeration and determination by genetic analysis (Miot-Sertier and Lonvaud-Funel 2007; Curtin *et al.* 2007) should hence be further investigated to gain insight about the diversity of the strain microbial consortia. Second, focus also needs to be made on ethyl-phenols precursors and their kinetic conversion into ethyl-phenols. Unfortunately, this point still lacks uncertainties, mainly because cinnamic acid availability is often not considered (Salameh *et al.* 2007). Indeed, bioconversion of ethyl-phenols precursors, like *p*-coumaric, by *B. bruxellensis* is highly dependent on the growth media (Dias *et al.* 2003) and cinnamic acid ratios (Romano *et al.* 2008). Moreover,

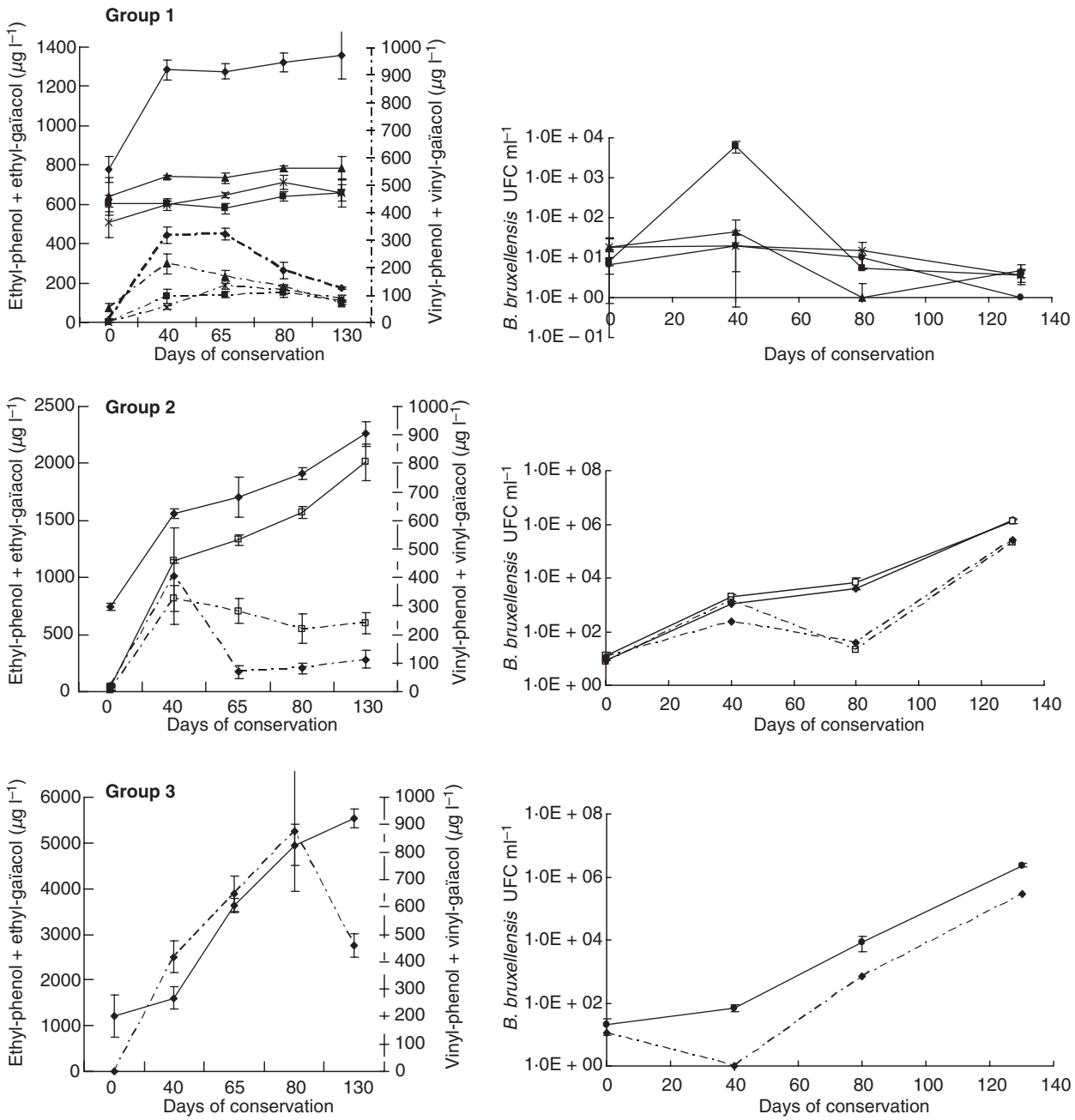

Figure 2 Evolution of ethyl-phenols and *Brettanomyces bruxellensis* population during wine conservation. Wines were separated into three different groups, according to their growth and ethyl-phenols production. For group 2, only two examples of wines are shown (C and I), however, ethyl-phenol and *B. bruxellensis* had similar evolutions for all wines of this group (B, C, H, I, J, K, L, M and N). Ethyl-phenols (—) and vinyl-phenols (–·–). *B. bruxellensis* population was estimated either by q-PCR (—) or by selective media plating (–·–). Group 1: —◆—, A; —▲—, E; —■—, D; —×—, G. Group 2: —◆—, C; —□—, I. Group 3: —◆—, F.

vinyl-phenol reductase and cinnamate decarboxylase, the two enzymes involved in ethyl-phenol production in *B. bruxellensis* are precursor inducible, but these precursors also have an inhibitory effect on *B. bruxellensis* growth (Harris *et al.* 2008). This underlines the importance of concentrating further studies on free cinnamic acid estimations, although variations in wines can be noted because of the instability, esterification and cell-adsorption of *p*-coumaric acid (Salameh *et al.* 2007). This study shows that the prediction of volatile phenol production risks is

more linked to the cells state (cultivable or not), initial ethyl-phenols concentration and yet undetermined chemical parameters than traditional measurements like molecular SO_2 or glucose and fructose concentrations whose concentration were not linked to ethyl-phenol synthesis capacities. For a given wine, variable SO_2 and sugars concentration have been shown to have an influence on ethyl-phenols synthesis (Chatonnet *et al.* 1993). But when considering different wines, these parameters are of less importance and no fixed values can be given than will guarantee the absence of the ethyl-phenols synthesis. This study confirms as it has previously been observed that *B. bruxellensis* is not very demanding from a nutritional point of view and that it can grow with other energy sources than glucose and fructose (Alguilar Uscanga *et al.* 2000; Conterno *et al.* 2006). Finally, the fact that *B. bruxellensis* development was observed indicates that it was initially present in all wines after bottling, even at low levels. Wine manipulation needed for the experiment may have enhanced its development; although wines were air-tight conserved, the different lots came from bottled wines that were initially homogenized then fractioned, which inevitably introduced air. This points out the importance of protecting the wines from air in the cellar, especially just before bottling.

References

Alguilar Uscanga, M.G.A., Delia, M.L. and Stehaiano, P. (2000) Nutritional requirements of *Brettanomyces bruxellensis*: growth and physiology in batch and chemostat cultures. *Can J Microbiol* **46**, 1046–1050.

Barata, A., Caldeira, J., Botelheiro, R., Pagliara, D., Malfeito-Ferreira, M. and Loureiro, V. (2008) Survival pattern of *Dekkera bruxellensis* in wines and inhibitory effect of sulfur dioxide. *Int J Food Microbiol* **121**, 201–207.

Benito, S., Palomero, F., Morata, A., Calderón, F. and Suárez-Lepe, J.A. (2009a) A method for estimating *Dekkera/Brettanomyces* populations in wines. *J Appl Microbiol* **106**, 1743–1751.

Benito, S., Palomero, F., Morata, A., Calderón, F. and Suárez-Lepe, J.A. (2009b) Factors affecting the hydroxycinnamate decarboxylase/vinylphenol reductase activity of *Dekkera/Brettanomyces*: application for *Dekkera/Brettanomyces* control in red wine making. *J Food Sci* **74**, 15–22.

Blouin, J. (1992) Techniques d'analyses des moûts et des vins. Dujardin Salleron Ed.

Chambel, A., Viegas, C.A. and Sa-Correia, I. (1999) Effect of cinnamic acid on the growth and on plasma membrane H^+-ATPase activity of *Saccharomyces cerevisiae*. *Int J Food Microbiol* **50**, 173–179.

Chatonnet, P., Dubourdieu, D., Boidron, J.-N. and Pons, M. (1992) The origin of ethylphenols in wine. *J Sci Food Agric* **60**, 165–178.

Chatonnet, P., Boidron, J.N. and Dubourdieu, D. (1993) Influence des conditions d'élevage et de sulfitage des vins rouges en barriques sur leurs teneurs en acide acétique et en éthyl-phénols. *J Int Sci Vigne Vin* **27**, 277–298.

Conterno, L., Joseph, C.M.L., Arvik, T.J., Henick-Kling, T. and Bisson, L. (2006) Genetic and physiological characterization of *Brettanomyces bruxellensis* strains isolated from wine. *Am J Enol Vitic* **57**, 139–157.

Couto, J.A., Neves, F., Campos, F. and Hogg, T. (2005a) Thermal inactivation of wine spoilage yeast *Dekkera/Brettanomyces*. *Int J Food Microbiol* **104**, 337–344.

Couto, J.A., Barbosa, A. and Hogg, T. (2005b) A simple cultural method for the presumptive detection of the yeasts *Brettanomyces/Dekkera* in wines. *Lett Appl Microbiol* **41**, 505–510.

Curtin, C.D., Bellon, J.R., Henschke, P.A., Godden, P.W. and Barros-Lopez, M.A. (2007) Genetic diversity of *Dekkera/Brettanomyces bruxellensis* isolates from Australian wineries. *FEMS Yeast Res* **7**, 471–481.

Delaherche, A., Claisse, O. and Lonvaud-Funel, A. (2004) Detection and quantification of *Brettanomyces bruxellensis* and « ropy » *Pediococcus damnosus* strains in wine by real time polymerase chain reaction. *J Appl Microbiol* **97**, 910–915.

Dias, L., Pereira-Da-Silva, S., Tavares, M., Malfeito-Ferreira, M. and Loureiro, V. (2003) Factors affecting the production of 4-ethylphenol by the yeast *Dekkera bruxellensis* in oenological conditions. *Food Microbiol* **20**, 377–384.

Du Toit, W., Pretorius, I. and Lonvaud-Funel, A. (2005) The effect of sulphur dioxide and oxygen on the viability and cultivability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. *J Appl Microbiol* **98**, 862–871.

Fugelsang, K.C. and Zoecklein, B.W. (2003) Population dynamics and effects of *Brettanomyces bruxellensis* strains on pinot noir (*Vitis vinifera* L.) wines. *Am J Enol Vitic* **54**, 294–300.

Garcia Alvarado, Y., Mendez Acona, J., Mata Jimenez, M.T., Gómez Rodriguez, J. and Alguilar Uscanga, M.G. (2007) Modeling of yeast *Brettanomyces bruxellensis* growth at different acetic acid concentrations under aerobic and anaerobic conditions. *Bioprocess Biosyst Eng* **30**, 389–395.

Godoy, L., Garrido, D., Martínez, C., Saavedra, J., Combina, M. and Ganga, M.A. (2009) Study of the coumarate decarboxylase and vinylphenol reductase activities of *Dekkera bruxellensis* (anamorph *Brettanomyces bruxellensis*) isolates. *Lett Appl Microbiol* **48**, 452–457.

Harris, V., Ford, C.M., Jiranek, V. and Grbin, P.R. (2008) *Dekkera* and *Brettanomyces* growth and utilisation of hydroxycinnamic acids in synthetic media. *Appl Microbiol Biotechnol* **78**, 997–1006.

Harris, V., Ford, C.M., Jiranek, V. and Grbin, P.R. (2009) Survey of enzyme activity responsible for phenolic off-flavor production by *Dekkera* and *Brettanomyces* yeast. *Appl Microbiol Biotechnol* **81**, 1117–1181.

Hereszyn, T. (1986) Formation of substituted tétrahydropyridines by species of *Brettanomyces* and *Lactobacillus* isolated from mousy wines. *Am J Enol Vitic* **37**, 127–132.

Lonvaud-Funel, A. (1999) Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie Van Leeuwenhoek* **76**, 317–323.

Loureiro, V. and Malfeito-Ferreira, M. (2003) Spoilage by yeasts in the wine industry. *Int J Food Microbiol* **86**, 23–50.

Medawar, W., Strehaiano, P. and Delia, M.L. (2003) Yeast growth: lag phase modelling in alcoholic media. *Food Microbiol* **20**, 527–532.

Millet, V. and Lonvaud-Funel, V. (2000) The viable but non-culturable state of wine microorganisms during storage. *Lett Appl Microbiol* **30**, 136–141.

Miot-Sertier, C. and Lonvaud-Funel, A. (2007) Development of a molecular method for the typing of *Brettanomyces bruxellensis* (*Dekkera bruxellensis*) at the strain level. *J Appl Microbiol* **102**, 555–562.

Murat, M.L. and Dumeau, F. (2003) Impact of fining on populations levels of certain spoilage microorganisms in red wines. *Aust NZ Grapegr Winemaker* **478**, 92–94.

Nisiotou, A.A. and Gibson, G.R. (2005) Isolation of culturable yeasts from market wines and evaluation of the 5,8s-ITS rDNA sequences analysis for identification purposes. *Lett Appl Microbiol* **41**, 454–463.

Oelofse, A., Lonvaud-Funel, A. and du Toit, M. (2009) Molecular identification of *Brettanomyces bruxellensis* strains isolated from red wine and volatile phenol production. *Food Microbiol* **26**, 377–385.

OIV, http://news.reseau-concept.net/images/oiv/client/RECEUIL_2007_Vol1.pdf.

Poolman, B. (1993) Energy transduction in lactic acid bacteria. *FEMS Microbiol Rev* **12**, 125–148.

Renouf, V. and Lonvaud-Funel, A. (2004) Racking are key stages for the microbial stabilization of wines. *J Int Sci Vigne Vin* **38**, 219–224.

Renouf, V., Claisse, O. and Lonvaud-Funel, A. (2005) Numeration, identification and understanding of yeast and bacteria ecosystem on grape berry surface. *Aust J Grape Wine Res* **11**, 316–327.

Renouf, V., Falcou, M., Miot-Sertier, C., Perello, M.C., de Revel, G. and Lonvaud-Funel, A. (2006a) Interactions between *Brettanomyces bruxellensis* and other yeast species in the initial stages of winemaking. *J Appl Microbiol* **100**, 1208–1219.

Renouf, V., Claisse, O. and Lonvaud-Funel, A. (2006b) Inventory and monitoring of wine microbial consortia. *Appl Microbiol Biotechnol* **75**, 149–164.

Renouf, V., Perello, M.C., de Revel, G. and Lonvaud-Funel, A. (2007) Survival of wine microorganisms in the bottle during storage. *Am J Enol Vitic* **58**, 379–386.

Rodriguez, N., Gonçalves, G., Pereira-da-Silva, S., Malfeito-Ferreira, M. and Loureiro, V. (2001) Development and use of a new medium to detect yeasts of the genera *Dekkera/Brettanomyces*. *J Appl Microbiol* **90**, 588–599.

Romano, A., Perello, M.C., de Revel, G. and Lonvaud-Funel, A. (2008) Growth and volatile compound production by *Brettanomyces/Dekkera bruxellensis* in red wine. *J Appl Microbiol* **104**, 1577–1585.

Salameh, D., Brandam, C., Medawar, W., Lteif, R. and Sthrehaiano, P. (2007) Highlight on the problems generated by *p*-coumaric acid analysis in wine fermentations. *Food Chem* **107**, 1661–1667.

Suárez, R., Suárez-Lepe, J.A., Morata, A. and Calderón, F. (2007) The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: a review. *Food Chem* **102**, 10–21.

Tchobanov, I., Gal, L., Guilloux-Benatier, M., Remize, F., Nardi, T., Guzzo, J., Serpaggi, V. and Alexandre, H. (2008) Partial vinylphenol reductase purification and characterization from *Brettanomyces bruxellensis*. *FEMS Microbiol Lett* **284**, 213–217.

Ubeda, J.F. and Briones, A.I. (1999) Microbiological quality control of filtered and non-filtered wines. *Food Control* **10**, 41–45.

Vigentini, I., Romano, A., Compagno, C., Merico, A., Molinari, F., Tirelli, A., Foschino, R. and Volonterio, G. (2008) Physiological and oenological traits of different *Dekkera/Brettanomyces bruxellensis* strains under wine-model conditions. *FEMS Yeast Res* **8**, 1087–1096.

Woolfit, M., Rozpędowska, E., Piškur, J. and Wolfe, K.H. (2007) Genome survey sequencing of the wine spoilage yeast *Dekkera (Brettanomyces) bruxellensis*. *Euk Cell* **6**, 721–733.

Zott, K. (2008) Les levures non-*Saccharomyces*: dynamique, caractérisation et interaction avec *Saccharomyces* durant les étapes pré-fermentaires et la fermentation alcolique. Thèse de doctorat. Faculté d'œnologie. Université V. Segalen Bordeaux2.