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Prevalence and Biodiversity of *Brettanomyces bruxellensis* in Wine from the North-West of Italy

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Abstract

Brettanomyces bruxellensis catalyses the transformation of hydroxycinnamic acid, a compound naturally occurring in grapes, producing volatile phenols thus reducing the organoleptic quality of wine. Volatile phenols can confer off-odours, in a concentration dependent way, described as phenolic, animal, mousy, wet wool, medicinal, smoky and spicy. In this paper we describe a multi disciplinary approach for the investigation of *B. bruxellensis* presence in some Italian wines. For the detection and quantification of *B. bruxellensis* in 87 different wines, we have applied culture independent and dependent method (quantitative PCR and traditional microbiological analysis). Moreover, Headspace Solid-Phase Microextraction method was used to quantify ethyl and vinyl phenols in wines. Statistical analysis performed on the results obtained showed that there was no correlation between culture-dependent and culture-independent methods. Strain biodiversity was investigated by SAU-PCR and a differentiation of the isolates based on the geographical origin was highlighted.

Keywords: Brettanomyces bruxellensis, quantitative PCR, ethyl phenols, biodiversity, SAU-PCR

Introduction

Wine is an alcoholic beverage typically made of fermented grape juice, in which there is a complex microbial ecology of opportunistic microorganisms, some of which could potentially grow and modify its chemical-physical and sensorial properties. Among all possible microbial contaminations of wines, the development of *Brettanomyces bruxellensis* (anamorph of *Dekkera bruxellensis)* is nowadays a major problem for the winemakers. It is a resistant species that survives even nutrients

deprivation and high ethanol content. It catalyses the transformation of hydroxycinnamic acid, naturally occurring in grapes producing volatile phenols (Benito et al. 2009). Its ability to convert hydroxycinnamic acids into volatile phenols can confer off-odours described as phenolic, animal, mousy, wet wool, medicinal, smoky and spicy and consequently it reduces the organoleptic quality of the final product (Chatonnet et al. 1992; Loureiro and Malfeito-Ferreira 2003; Romano et al. 2009). *B. bruxellensis* is a constant wine resident, but it develops mainly at the end of the alcoholic fermentation when other microbial species decline (du Toit et al. 2005). These yeasts are not among the dominant organisms in must during fermentation, but their presence might have a great influence on the final product. They are considered wine spoilage agents and their classical habitat in the winemaking environment is the winery and its equipments (Ciani and Ferraro 1997; Ciani et al. 2003) particularly barrel-ageing red wines. Recently they have been isolated in different part of the world: South Africa (Oleofse et al. 2009), USA (Stender et al. 2001), Italy (Cocolin et al. 2004a, Agnolucci et al. 2009), France (Tessoniere et al. 2009) and Spain (Ibeas et al. 1996). Yeasts of this genus have been isolated from wine (Ibeas et al. 1996; Agnolucci et al. 2009; Tessoniere et al. 2009), from sound and damaged grapes (Guerzoni and Marchetti 1987), from winery air samples (Connel et al. 2002) and in different stages of wine production such as alcoholic fermentation, malolactic fermentation and aging in barrels (Rodrigues et al. 2001).

Traditional methods to identify spoilage yeasts in wine are mainly based on microbiological culturing. Recently authors have described selective media developed by manipulating the type and concentration of selected antimicrobial agents and carbon sources to suppress the growth of other yeast species and bacteria (Rodrigues et al. 2001; Couto et al. 2005). These selective media are necessary to overcome difficulties for the recovery of *Dekkera/Brettanomyces* from materials heavily contaminated with other yeasts or moulds, which by growing faster prevent the detection of this slow growing microorganism. Culture dependent methods need from 7 to 15 days for the results. In recent times many efforts have been made in order to develop faster and innovative methods for the detection and enumeration of *Brettanomyces* yeast, able to give results before the

insurgence of the alteration. Methods based on different techniques have been described: in 2001, Stender et al. developed a method based on Fluorescent In Situ Hybridization with Peptide Nucleic Acid Probes; in 2003, Phister and Mills described a Real-Time PCR assay for the detection and enumeration of *Dekkera bruxellensis* directly in wine; in 2007, Hayashi et al. used a loop-mediated isothermal amplification method for the detection and identification of *Brettanomyces/Dekkera* spp. yeasts.

Since important economic losses may derive from wine spoilage by *Brettanomyces/Dekkera,* efforts towards the monitoring and control of these organisms in musts and wines are essential since they will allow winemakers to take prophylactic action in order to avoid problems before they arise.

In this paper we describe a multi disciplinary approach for the investigation of *B. bruxellensis* presence in some Italian wines. For the detection and quantification of *B. bruxellensis* in 87 different wines coming from Liguria and Piedmont regions in North-West Italy, we have applied quantitative PCR (qPCR) directly in the wine, as culture independent method, as well as traditional culture dependent method, using a selective medium (DBDM, Couto et al. 2005) to define the prevalence of *Brettanomyces* spp. Species-specific PCR and restriction analysis were performed on suspected colonies isolated from DBDM agar to identify *B. bruxellensis* and the molecular characterization of 196 isolated strains, belonging to this species, was performed for a better knowledge of strains biodiversity. Lastly, Headspace Solid-Phase Microextraction method (HS-SPME) was employed to quantify ethyl and vinyl phenols in wines.

Materials and methods

Wine samples

Eighty seven samples of red and white wines were collected, in a random way, directly from wineries or from the market in Piedmont and Liguria regions, North-West of Italy. They were

samples of Barbera, Nebbiolo, Barbaresco, Barolo, Dolcetto, Grenache, Rossese, Cabernet, Ciliegiolo, Ormeasco, Mosaico. More specifically, 46 wine were collected from Piedmont and they were represented by 26 from tanks before the bottling and 20 bottled. In the same way, from Liguria, one was from tank and 40 from bottles.

Yeast strains isolation and collection

Strain DSM 7001 of *D. bruxellensis* was used as references, purchased from DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Wine samples, at an appropriate sample dilution, were spreaded on plates containing DBDM agar (Dekkera/Brettanomyces Differential Medium: 6.7 g/L Yeast Nitrogen Base (Difco, Sparks, MD USA), 6% ethanol, 100 mg/L p-coumaric acid, 200 mg/L cloroamphenicol, 20 mg/L cycloheximide, 22 mg/L Bromocresol green, pH 5.4) (Rodrigues et al. 2001). Furthermore an enrichment procedure was carried out at 30 °C, in static conditions, adding 25 mL of YNB double strength broth (13.4 g/L Yeast Nitrogen Base (Difco), 10 g/L glucose, 100 mg/L p-coumaric acid, 200 mg/L cloroamphenicol, 20 g/L cycloheximide, pH 5.0) to 25 mL of each wine. After 14 days wines were spread on DBDM agar plates. A total of 196 isolates, exhibiting *Brettanomyces* typical morphology, after incubation at 30 °C for 14 days, were randomly selected from plates coming from the sampling and the enrichment procedure and purified by streak on Yeast Peptone Dextrose agar (2% glucose, 1% yeast extract, 2% peptone, 1.2% agar, all from Oxoid, Milan, Italy). Isolates were collected and stored at -80 °C in YPD broth plus glycerol 20% (v/v).

DNA extraction

Yeast isolates were grown for 48 hours at 30 °C in YPD medium. Nucleic acid extraction was performed as described by Cocolin et al. (2000).

Identification of *B. bruxellensis* by species-specific PCR and restriction analysis

One hundred nanograms of DNA were analyzed by species-specific (*B. bruxellensis* and *B. anomalus)* PCR amplification using primers DB90F (5-GAY ACT AGA GAG AGR RGG ARG GC-3, where Y= C or T and R= A or G) and DB394R (5-ACG AGG AAC GGG CCG CT GC-3) as described by Cocolin et al. (2004a). PCR amplification was performed in a 25 µL mixture containing 10 mM Tris-HCl (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, deoxynucleoside triphosphate (dNTPs) at a concentration of 0.2 mM, each primer at a concentration of 0.2 mM, 1.25 U of *Taq* polymerase (Applied Biosystems, Foster City, USA), and 1 µL of extracted DNA. The thermal cycler parameters were as follows: initial denaturation at 95°C for 1 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min, extension at 72 °C for 1.5 min; and final extension at 72 °C for 7 min. Amplification was carried out with a Bio-Rad thermalcycler (Milan, Italy). PCR products were electrophoresed in a 2% agarose gel in TAE buffer (0,04M Tris-Acetate, 1mM EDTA · Na_2 in H_2O, pH 8.3, Sigma Aldrich, Milan, Italy) and observed under UV light.

Eight microliters of *Brettanomyces*-specific PCR products was subjected to enzyme restriction analysis using the enzyme *Dde*I (Applied Biosystems, Milan, Italy), as described by Cocolin et al. (2004a). After digestion at 37 °C for 2 h, restriction fragments were separated in a 3% agarose gel in TBE buffer (130 mM Tris, 45 mM boric acid; 2.5 mM EDTA-Na_2 in H_2O; Sigma Aldrich) containing 0.5 µg/mL ethidium bromide (Sigma Aldrich). Gels were examined under UV illumination by using the UVI pro Platinum 1.1 Gel Software (Eppendorf, Milan, Italy).

SAU-PCR

Extracted DNA (200 ng), from strains identified as *B. bruxellensis*, was subjected to digestion using the *Sau3*A restriction endonuclease (Roche Diagnostics, Milan, Italy), as described by Corich et al. (2005). Restriction enzyme reactions were done in a final volume of 20 µL as suggested by the manufacturers. DNA was digested overnight at 37 °C and, for the PCR amplification, 1 µL of the restriction reaction was transferred into a 25 µL reaction volume containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 1 µM primer (see below) and 1.25 U *Taq* polymerase.

Prior to the amplification, a ‘‘filling’’ step was performed as follows: 25 °C for 5 min, ramp with a speed of 0.1 °C/s to 60 °C, and 30s at 60 °C. A preliminary low-stringency amplification cycle, characterized by a denaturation at 95°C for 1 min, annealing at 50 °C for 15s, ramp to 25 °C (0.1/°C s), followed by an increase to 50 °C (0.1 °C/s), and 30s at 50 °C, was carried out. The cycle continued with 35 repetitions of denaturation at 95 °C for 15s, annealing at 46 °C for 1 min and extension at 65 °C for 2 min. A final extension for 5 min at 65 °C was used at the end of the amplification. PCR products were separated on a 2% agarose gel in TBE (Sigma Aldrich), containing 0.5 μg/mL of ethidium bromide, for 120 min at 120V. After the run, gels were observed under UV illumination. Four primers were tested for their capability to differentiate *B. bruxellensis*: SAG1 (5’-CCGCCGCGATCAG-3’), SAG2 (5’-CCGCCGCGATCTC-3’), SCA1 (5’-CCGCCGCGATCCA-3’), SCA2 (5’-CCGCCGCGATCGT-3’) (Corich et al. 2005).

Extraction of nucleic acids from wine samples

One mL of each wine sample was used for nucleic acid extraction. After centrifugation at 13,400 rpm for 10 min at 4 °C, the pellets were subjected to the nucleic acids extraction by using the Master-Pure™ Complete DNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions. The nucleic acids were resuspended in 50 μl of sterile water and amplified.

Quantitative-PCR (qPCR) amplification conditions

Amplifications were performed in a final volume of 25 μL in the Chromo4 Real-Time PCR Detection System (Bio-Rad). One μl of DNA, extracted as described above, was amplified using the protocol described by Phister and Mills (2003). The specific primers used were: DBBRUXF (5’-GGATGGGTGCACCTGGTTTACAC-3) and DBRUXR (5’-GAAGGGCCACATTCACGAACCCCG-3). The FluoCycle SYBR Green master mix was used according to the manufactuer’s instructions (Euroclone, Milan, Italy). Reactions were performed in

0.2 mL MicroAmp optical tubes (Celbio, Milan, Italy) and each 25 µL reaction mixture contained the following: 1X SYBR Green mastermix, 900 nM DBRUXF, 300 nM DBRUXR and 1 µL DNA. Each reaction was performed in triplicate. The reactions were run using a modified protocol of Phister and Mills (2003): 40 cycles of denaturation at 95 °C for 15s, annealing and elongation at 75 °C for 15s. An initial 10 min denaturing step at 95 °C was used.

For quantification purposes, standard curves were constructed from serially diluted cells of *B. bruxellensis* strain DSM 7001 in wine. Before analysis the wine was determined to be negative for the presence of *B. bruxellensis* by both culture dependent and independent methods. The final concentration of the cells in the wine ranged from 10^7 to 10^2 colony forming units (cfu)/mL. The signals produced (threshold cycle, Ct) by the serial dilutions of *B. bruxellensis* in wine were plotted against the Log_{10} cfu and the standard curves were constructed.

SPME-GC/MS analysis

Volatile phenols (4-vinylphenol (4VP), 4-vinylguaiacol (4VG), 4-ethylphenol (4EP), and 4-ethylguaiacol (4EG) were quantified in all the wines collected in this study. For the extraction performed according to Boutou and Chatonnet (2007), 5 mL of the wine sample (pH 7), 5 mL of MilliQ water, 10 µL of a solution of internal standard (100 mg/L of 3,4 dimethyl-phenol) and 3 g of NaCl were added in a vial of 22 mL. For the Headspace Solid-Phase Microextraction (HS-SPME) a DVB/CARBOXEN/PDMS fiber of 1 cm of length was used for 1 hour at 45 °C, with stirring. For the analysis, a Shimadzu GC17-A gas chromatograph equipped with a Shimadzu QP5000 quadrupol mass spectrometer was used. Compounds were separated using a DB-WAX column (30 m x 0.25 mm i.d., 0.25 µm film thickness) using helium as carrier gas (1mL/min). Injections were performed for 5 minutes at 270 °C, in splitless mode. The GC program was: 35 °C for 2 minutes, gradient of 20 °C/min until 170 °C for 1 minute, gradient of 3 °C/min until 210 °C for 15 minutes. Detection and standards curves were achieved operating in electron impact mode (EI) with selection ion monitoring (SIM) mode and analytes were measured comparing peaks area of specific ions with

that of the internal standard (3,4 dimethylphenol). Quantification limits were: 13 µg/L for 4VG; 7 µg/L for 4VP; 9 µg/L for 4EG, 55 µg/L for 4EP. All the measures were carried out in duplicate.

Statistical analysis

Gels containing the SAU-PCR profiles of the *B. bruxellensis* strains were normalized, by using the 1 kb molecular ladder loaded in each gel, and subjected to Cluster Analysis with the BioNumerics software (Applied Maths, Kortrijk, Belgium). The Pearson product moment correlation coefficient was used to calculate the similarities in profile patterns, and dendrograms were obtained by means of the Unweighted Pair Group Method using the Arithmetic Average (UPGMA) clustering algorithm.

Correlations between traditional microbiological count, qPCR and ethyl-phenols quantification were determined by using the Microsoft Office Excel 2003 software. The analysis was performed for samples that gave positive responses to the methods considered in the specific correlation calculation. Samples that did not produce a colony on the agar plates, were negative in the qPCR assay and contained 4EP below the quantification limit, were not included in the statistical analyses. More specifically, 26 wine samples for traditional microbiological counts and qPCR, 27 wine samples for traditional microbiological counts and 4EP content and 24 wine samples for qPCR and 4EP content were correlated.

Results and discussion

B. bruxellensis has a well-characterized ability to grow in wines and to synthesize ethylphenols from their hydroxycinnamic precursors. Due to this metabolic capacity and the resultant phenolic off-odours, yeasts of this genus are globally recognized as one of the major and most complex spoilage issues for the wine industry. In the past years many efforts were made in order to develop

preventive methods aimed to avoid wine spoilage due to this yeast. Benito et al. (2009) tried to reduce the content of ethylphenols precursors favoring the formation of vinylphenolic pyranoanthocyanins. Puértolas et al. (2009) studied the inactivation of *B. bruxellensis* using the pulsed electric fields technology (PEF) but this technique could have negative effect even on the autochthonous microflora.

In the enological practice, the identification and enumeration of *Brettanomyces/Dekkera* are carried out using selective or differential microbiological media. However these methods have a limited efficacy, due to the low growth rate of the yeast and therefore to the time needed for the results.

Different types of rapid and reliable methods for the detection, quantification and characterization of *B. bruxellensis* in wine have been developed in the last years. Molecular methods as nested PCR (Ibeas et al. 1996), fluorescence *in situ* hybridization (Stender et al. 2001) have been used to determine the presence of this yeast in wine. Recently protocols based on qPCR have been used (Phister and Mills 2003; Tessonniere et al. 2009) permitting a significant time reduction for the response. These methods may allow detection and quantification of low amounts of *B. bruxellensis* cells, early and directly in wine before the insurgence of the alteration.

The goal of this study was to investigate the presence of *B. bruxellensis* in Italian wines coming from Piedmont and Liguria regions, in North West of Italy, and to study the correlation between ethyl phenols content and *Brettanomyces* incidence.

Modifying the protocol proposed by Phister and Mills (2003), the standard curve presented in Figure 1 was produced. As shown a good linearity range was obtained from 10^7 to 10^2 cfu/mL of wine, thereby giving the possibility to quantify *B. bruxellensis* in the wine samples used in this study, also when present in low numbers.

In the Table 1 the comparison of the results obtained by traditional microbiological methods and qPCR is reported. As shown, both approaches were able to detect *B. bruxellensis* in about half of the wines analyzed in this study. qPCR gave positive results for 10 more wines, when compared to the traditional plating. Maybe number of wine samples falling within each logarithm interval was

similar for both traditional colture dependent and independent methods, although by qPCR the number of wines was consistently higher. This was verified not only for wines with high counts (>5 Log cfu/mL) but also for the low contaminations (1-2 Log cfu/mL). As presented, culture-independent methods showed better results for the detection of low numbers of *B. bruxellensis*, thereby they should be considered as reliable methods for bottled wines where spoilage occurs with long storage periods. By traditional microbiological analysis it was determined that the 15% of the wines from Liguria resulted positive, in contrast to the 8% of the wines from Piedmont. This evidence underlines that, even if the 98% of the wines coming from Liguria were already bottled and ready for the consumers, *B. bruxellensis* populations were alive and metabolically active in wines coming from this region, not excluding the possibility of spoilage in the bottle (data not shown).

When the wines were subjected to HS-SPME-GC/MS for the quantification of all four phenols, results presented in Table 2 were obtained. 4VP and 4VG in all the wines were below the quantification limit of the method. About 70% of the wines showed very low contents of volatile phenols 4EP and 4EG, while about 20% of the wines had contents from 10 to 200 µg/L. About ten percent of the samples presented a high content of these molecules ranging between 200 and 400 µg/L. Only few wines showed a very high level of 4EP ranging from 400 to 800 µg/L. One sample had a 4EP content of about 1 mg/L. The results obtained in this study correlate well with the available literature on 4EP content in wines from other countries (Nikfardjam et al. 2009).

From a comparative analysis of the results obtained by culture dependent and independent methods and the ethyl phenols content, it was possible to classify the wine samples analyzed in four groups (Table 3). Twenty-eight wines, on a total of 87 analyzed, were positive for *B. bruxellensis* presence by both methods, and about 36% of them had an aggregate content above the olfactory threshold (Chatonnet at al. 1992). Wines of this group presented alive population of the spoilage yeasts: ten wines were already irremediably spoiled, however the other 18 wines presented low level of ethyl phenols, thereby corrective actions could be performed before the complete spoilage. Twenty-one

wines were positive by qPCR only and among these wines 9% of them had a high content of ethyl phenols. For these samples, it is possible to speculate that the negative results obtained by traditional microbiological counts were due to the presence of dead or stressed *B. bruxellensis* that did not allow its cultivation on the plates. Moreover it cannot be excluded the presence of viable but not culturable (VNBC) populations (Tessonniere et al. 2009). Cells in the VNBC state are metabolically active, but unable to undergo cellular division for growth in liquid or in agar, underestimating the risk of wine spoilage when only traditional microbiological methods are used in monitoring programs. Ten wines were positive by traditional microbiological analysis and most of them (90%) had an ethyl phenols level under the olfactory threshold (Chatonnet et al. 1992). In some cases the incongruency found could be attributed to the quantification limit of the qPCR. As a matter of fact counts below 10^2 cfu/mL could not be detected nor quantified. However it cannot be excluded an inhibition effect of wine components not totally eliminated during the DNA extraction process. Also other authors exploiting qPCR for *B. bruxellensis* detection and quantification faced similar results, being unable to clarify the differences found in samples detected positive by plate counts and negative by qPCR (Tessoniere et al. 2009). Finally, the last group consisted of 28 wines negative for *B. bruxellensis* presence and in all of them the undesirable compounds were below the quantification limit of the method. It is interesting to underline that the majority of the wines contained a level of ethyl phenols below the aggregate detection threshold, defined by Chatonnet et al. (1992) as the sum of ethyl and vinyl phenol and quantified in 426 µg/L. Only in the case of samples positive by both traditional microbiology plating and qPCR, half of the positive samples were characterized by a high amount of ethyl phenols as well.

In order to determine a possible correlation between the results obtained by traditional microbiological counts, qPCR and ethyl phenols content, statistical analysis were performed eliminating all the samples that gave a negative response for any of the three methods applied. From the results obtained, it can be concluded that there is no correlation between culture-dependent and culture-independent methods. More specifically, correlation coefficients were 0.38, -0.24 and 0.17

for traditional microbiological counts and qPCR, traditional microbiological counts and 4EP and 4EG content and qPCR and 4EP and 4EG content, respectively. This is most likely due to the number of variables that can affect the analysis. For instance the low number of cells can give a positive result by traditional method and negative by qPCR (counts below the detection limit of the method). Moreover, the presence of DNA from a dead population can give a positive result by qPCR, but negative results by traditional microbiological analysis. Based on the results obtained in this study, it is highlighted again how the spoilage by *Brettanomyces* spp. is not an easy process to monitor. Only the detection and quantification of ethyl phenols can give an appropriate view of the spoilage status of a wine, however it is a method that cannot be correlated in any way to the *Brettanomyces* presence in the wine. However, only by monitoring a wine for *Brettanomyces* presence it is possible to establish corrective actions in a timely manner to avoid the spoilage process. If only ethyl phenols are determined, there may be situations in which no definitive remediation could be carried out thereby resulting in important economic losses for the wineries.

From the plates of DBDM a collection of 196 isolates was created. Seventy-four and 122 were from wines coming from Piedmont and Liguria, respectively. One hundred-thirty two were collected from wines already bottled, while 64 isolates were from tanks. White wines did not present any suspected colony on the selective plates. After species-specific PCR and restriction enzyme analysis of the PCR products obtained, as described by Cocolin et al. (2004a), all of them were identified as *B. bruxellensis*. Based on this evidence it can be speculated that, although DBDM is a selective medium for *Brettanomyces* spp., in the wine samples analyzed the counts calculated in the wine could be referred to *B. bruxellensis* species only. As previously reported, *B. bruxellensis* is the principle responsible for the spoilage of wines (Chatonnet et al. 1992) and this aspect was again confirmed by the results of the isolation and identification of presumptive colonies found on the selective medium used in this study.

In order to investigate *B. bruxellensis* strain biodiversity, a fingerprinting method, namely SAU-PCR, was used. With this method, genomic DNA is first subjected to enzymatic cleavage using

Sau3A restriction enzyme, and then subjected to PCR using a primer constructed on the basis of the restriction site of the enzyme. The profiles obtained reflect the presence or the absence of *Sau3A* restriction site within the genome of the strain under investigation. This approach was previously used to characterize lactic acid bacteria populations (Corich et al. 2005), as well as to define the intraspecies diversity of *Saccharomyces cerevisiae* isolates obtained from former and modern wineries in Italy (Cocolin et al. 2004b). Four primers, as suggested by Corich et al. (2005), were used in the differentiation of the isolates, however only primer SAG1 could be efficiently used for the differentiation (data not shown). The dendrogram obtained by analyzing the SAU-PCR patterns is shown in Figure 2. At a similarity level of 70%, 12 clusters and 10 single-strains could be differentiated. Two groups were particularly numerous including 45 and 32 isolates (clusters 1 and 10, respectively) coming from both regions, while ten groups were smaller containing between 2 and 23 strains. Among 12 clusters, 9 consisted of strains from only one region (3 for Piedmont and 6 for Liguria), while only 3 had strains from both regions. The clusters from Liguria (identified in Fig. 2 by numbers 2, 3, 5, 6, 7 and 8) had mainly strains isolated from bottled wine, while the clusters 1, 4 and 10, and the ones containing isolates from Piedmont (9, 11 and 12) have a high percentage of strains isolated from tanks. It is interesting to notice that strains isolated from wines produced in the same winery, most of the times clustered differently, underlining a high strain biodiversity within the set of *B. bruxellensins* obtained in this study (data not shown).

In a previous study (Miot-Sertier and Lonvaud-Funel 2007), it was shown that endonuclease restriction analysis associated with pulsed field gel electrophoresis (PFGE) proved the best method for the study of *B. bruxellensis*. It is interesting to notice that the discrimination power of SAU-PCR used in this study allowed us to differentiate strains based on the geographical origin and on the type of samples from where strains were isolated.

Conclusions

The combination of the methods used in this study led us to the conclusion that an increase in the *B. bruxellensis* population is not directly correlated to a high content in ethyl phenols. A better knowledge of the environmental conditions that favor the production of ethyl phenols in *B. bruxellensis* will allow a greater understanding of the management of some technological variables to control the production of these unwanted phenol compounds during wine production.

Acknowledgements

This research was supported by funds from the Piedmont Region, Italy – research program CIPE 2006.

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Table 1. Comparative analysis of the results obtained by culture dependent and independent methods. Wines were grouped based on the level of the counts of *B. bruxellensis* detected by the two approaches used.

Log cfu/mL / Method of analysis	<1	1-2	2-3	3-4	4-5	>5
Culture dependent method	48	4	11	10	10	4
Culture independent method	38	6	14	14	9	6

Table 2. Results obtained by SPME-GC/MS on the quantification of 4-Ethyl-phenol and 4-Ethyl-guaiacol in the wines analyzed. The samples were classified based on the level of the two compounds (µg/L)

	<LOQ[a]	LOQ-200	200-400	400-600	600-800	800-1000	>1000
4-Ethyl-phenol	56	16	4	5	4	1	1
4-Ethyl-guaiacol	61	20	6	0	0	0	0

[a]Level of quantification

Table 3. Comparative analysis of the results obtained by culture dependent and independent methods and by chemical analysis. Wines were grouped based on the detection of *B. bruxellensis* by the two approaches used and in correlation to the levels of ethyl-phenols.

	Number of wine samples	Ethyl phenols range	
		Number of wines with <426 µg/L[a]	Number of wines with >426 µg/L
Positive by both culture dependent and independent methods	28	18	10
Positive by culture independent method only	21	19	2
Positive by culture dependent method only	10	9	1
Negative with both methods	28	28	0

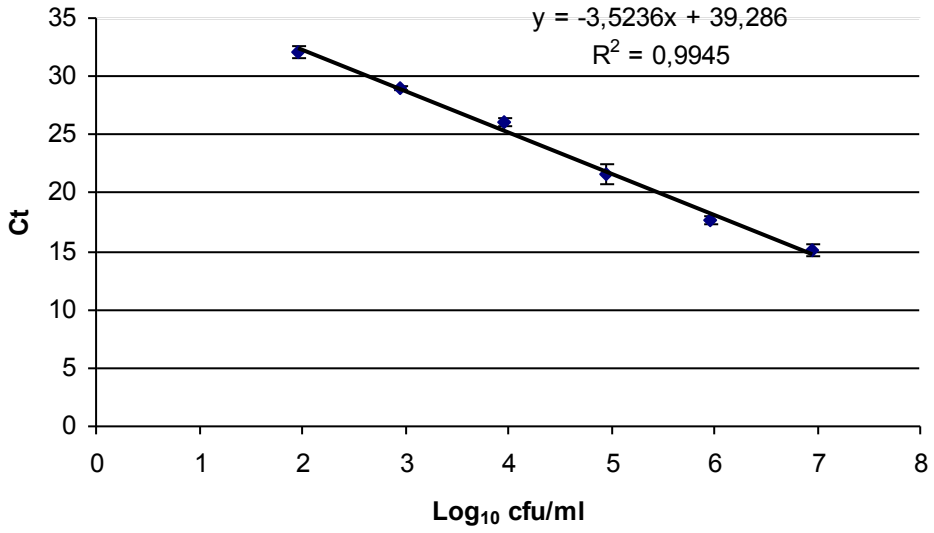
[a]Aggregate detection threshold for ethyl-phenols (Chatonnet et al. 1992)

Figure legends

Figure 1. Standard curve of *B. bruxellensis* constructed from cell dilutions in wine.

Figure 2. Biodiversity of *B. bruxellensis* isolated from the wines used in this study and achieved comparing the strain profile obtained by SAU-PCR using the primer SAG1 (Cocolin et al. 2004b). Analysis was performed using the BioNumerics Software (Applied Maths) using a similarity coefficient of 70%.

Figure 1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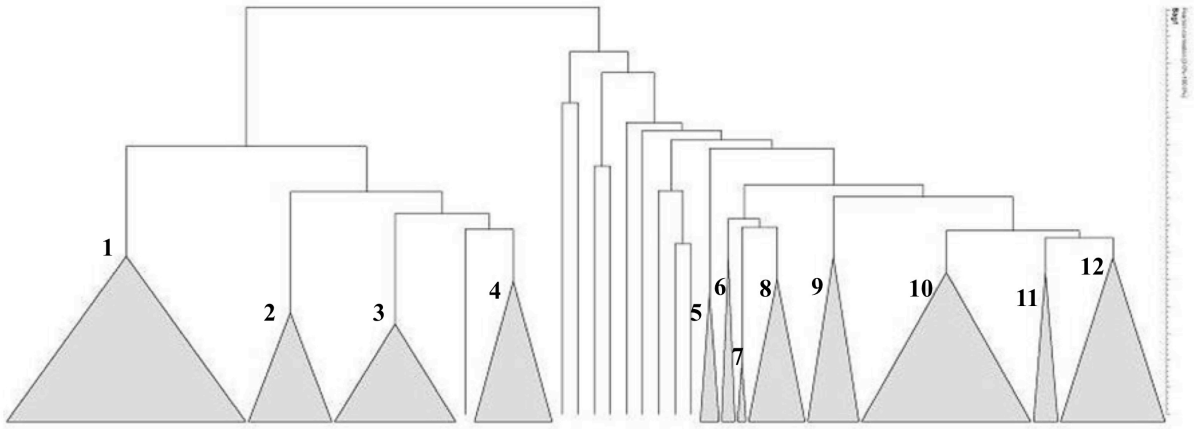
R2 = 0,9945
Ct
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
Log10 cfu/ml

Figure 2



CLUSTER NUMBER	1	2	3		4		5	6	7	8	9	10	11	12	TOTAL
NUMBER OF STRAINS	45	16	23	1	15	9	4	3	2	11	10	32	5	20	196
STRAINS FROM PIEDMONT	3	0	0	0	4	5	0	0	0	0	10	27	5	20	74
STRAINS FROM LIGURIA	42	16	23	1	11	4	4	3	2	11	0	5	0	0	122
STRAINS FROM TANK	4	0	0	0	4	3	0	0	0	0	9	22	4	18	64
STRAINS FROM WINE	41	16	23	1	11	6	4	3	2	11	1	10	1	2	132