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TESI DI DOTTORATO

Biodiversity of *Dekkera/Brettanomyces* in Apulian wines: isolation, characterization of representative biotypes and assessment of control strategies based on biotechnological resources management

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SUMMARY

ABSTRACT

Keywords: *Brettanomyces bruxellensis*, Wine, Spoilage, Sau-PCR, Biodiversity, Volatile phenols, VBNC state.

BACKGROUND

Among all the possible microbial contaminations of wine, development of *Brettanomyces bruxellensis* yeast is the most dreaded by winemakers. Indeed, growth of yeasts belonging to *Dekkera*/*Brettanomyces* during wine manufacturing, can seriously affect the quality of the final product, especially during aging.

Spoilage yeasts of the genus *Brettanomyces* or its teleomorph *Dekkera* are well adapted to survive during the winemaking process, due to their ethanol tolerance and relative resistance to the normal concentrations of sulphur dioxide found in wine.

To prevent the development of this yeast in wine, it is important to know the biodiversity of *Brettanomyces bruxellensis*. A more detailed study of these aspects could help winemakers to learn more about this yeast and to implement preventive and fighting measures to try to reduce the economic losses caused by *B. bruxellensis.*

AIM OF THE STUDY

The specific objectives of this work were to isolate *Brettanomyces* strains from several wineries located in Puglia and to characterize representative biotypes, in order to evaluate control strategies based on biotechnological resources management.

MATERIAL and METHODS

Yeasts of the genus *Brettanomyces* were selected in tank, barrel and fermenting must of several Apulian wines. The yeast strains were identified and genetically characterized using restriction pattern analysis of the internal transcribed spacer region (5.8S-ITS), species-specific primers and restriction analysis. Biodiversity of selected *B. bruxellensis* strains was studied with Sau-PCR. Potential spoilage and VBNC behavior of different biotype were studied with gas chromatograph and flow cytometry.

The cells of *Brettanomyces* were quantified in wine with Real Time PCR. To reduce the volatile phenols in wine and the growth of *Brettanomyces bruxellensis* different preparations of yeast cell wall and chitosan, respectively, were used.

RESULTS

Forty-eight isolates of *B. bruxellensis*, obtained from several wines collected in Apulia (Southern Italy), were genetically characterized using an integrated approach, including a strain biodiversity analysis by Sau-PCR. Furthermore, the production of volatile phenols was assessed in wine and in synthetic medium, confirming oenological spoilage potential of the analyzed strains. The present work represents the first survey on molecular and technological characterization of strains belonging to the genus *Brettanomyces* in Apulian wines. The results obtained indicate a remarkable genetic variability of the *B. bruxellensis* identified strains, and corroborate the evidence of a high level of genotypic and phenotypic polymorphism within *B. bruxellensis* species. Moreover, observations reported suggest that, at least within our region, strains from wines produced in the same geographical areas often clustered differently, indicating a complex intra-specific biodiversity in the regional wine environments. Diversity in volatile phenols production reflects intraspecific biodiversity highlighted by Sau-PCR analysis.

Entry and exit of these strains from the viable but not cultivable state (VBNC) and resuscitation character were analyzed. Increasing concentrations of free SO_2 induced the entrance in the VBNC state. Strains were able to "resuscitate" once that SO_2 was removed. A strain-dependent character was observed for the VBNC behavior. Indeed, VBNC entrance and 'resuscitation' varied as a function of strain, rather than in reason of free SO_2 concentration. These findings suggest VBNC behavior as a new key criterion for the characterization of spoilage potential of *B. bruxellensis* strains. From an industrial point of view, these outcomes are important to design physicochemical and biotechnological strategies for *B. bruxellensis* control of in wine.

This study also reported methods for reducing the presence of *B. bruxellensis* in wine and the volatile phenols produced using different preparations of chitosan and yeast cell walls. Yeast cells wall exert an adsorbing action against lipophilic species such as ethylphenol, thus favoring a partial or total reduction of molecules responsible for off-flavors. In contrast, chitosan is able to directly reduce the growth of *B. bruxellensis*.

RIASSUNTO

Parole chiave: *Brettanomyces bruxellensis*, Vino, *Spoilage*, Sau-PCR, Biodiversità, Fenoli volatili, *VBNC state*.

INTRODUZIONE

Tra tutte le possibili contaminazioni del vino, lo sviluppo del lievito *Brettanomyces bruxellensis* è il più temuto dai vinificatori. Infatti, la crescita di lieviti appartenenti al genere *Dekkera/Brettanomyces* durante la produzione del vino, in particolare durante l'invecchiamento, può compromettere seriamente la qualità del prodotto finale.

Il lievito *spoilage* del genere *Brettanomyces* o il suo telomorfo *Dekkera* si adatta bene a sopravvivere durante il processo di vinificazione, caratteristica imputabile alla tolleranza all'etanolo e alla relativa resistenza alle normali concentrazioni di solfiti presente nel vino.

Per prevenire lo sviluppo di questo lievito nel vino, è importante conoscere la biodiversità intraspecifica di *Brettanomyces bruxellensis* nei *terroir* di riferimento. Uno studio più approfondito di questi aspetti potrebbe essere di aiuto per gli enologi per una maggiore comprensione del potenziale *spoilage* e per attuare misure di prevenzione e di contrasto allo scopo di minimizzare le ingenti perdite economiche causate da *B. bruxellensis*.

SCOPO DEL LAVORO

L'obiettivo principale di questo lavoro è stato quello di isolare ceppi di *Brettanomyces* da diverse aziende vinicole situate in Puglia e caratterizzare alcuni biotipi rappresentativi della biodiversità intraspecifica analizzata, allo scopo di valutare strategie di controllo basate sulla gestione delle risorse biotecnologiche in enologia.

MATERIALI E METODI

Lieviti del genere *Brettanomyces* sono stati isolati in vasche inox e botti contenenti mosti in fermentazione e vini di diversi areali pugliesi. I ceppi di lievito sono stati identificati e geneticamente caratterizzati attraverso l'analisi del profilo di restrizione dell'*internal transcribed spacer region* (5.8S-ITS). La biodiversità dei ceppi di *B. bruxellensis* isolati è stata studiata mediante Sau-PCR. E' stato valutato, successivamente, il potenziale *spoilage* e il comportamento nello stato vitale ma non coltivabile (viable but not cultivable state, VBNC) dei differenti biotipi attraverso analisi cromatografiche e citofluorimetriche.

E’ stata stimata la popolazione di *Brettanomyces* in vino sono state applicate anche metodiche di Real Time PCR. Per ridurre i fenoli volatili nel vino e la crescita di *Brettanomyces bruxellensis* sono state utilizzate rispettivamente diverse preparazioni di scorze di lievito e chitosano.

RISULTATI

Quarantotto isolati di *B. bruxellensis*, ottenuti da diversi vini pugliesi (Sud Italia), sono stati geneticamente caratterizzati utilizzando un approccio integrato, che include, accanto ad indagini molecolari specie-specifiche, un'analisi della biodiversità intraspecifica. Inoltre, per un panel di ceppi rappresentativo di tale biodiversità, è stata valuta la produzione di fenoli volatili in vino e in mezzo sintetico, misurando il potenziale *spoilage* dei ceppi analizzati. Sotto questo profilo, il presente lavoro rappresenta non solo la prima indagine sulla caratterizzazione molecolare e tecnologica di ceppi appartenenti al genere *Brettanomyces* nei vini pugliesi, ma anche uno dei primi studi che valuta il potenziale *spoilage* di ceppi di *B. bruxellensis* rappresentanti la biodiversità intraspecifica di *B. bruxellensis* all'interno di una regione. I risultati ottenuti indicano una notevole variabilità genetica dei ceppi di *B. bruxellensis* isolati, corroborando l'evidenza di un elevato livello di polimorfismo genotipico e fenotipico all'interno della specie *B. bruxellensis*. Inoltre, le osservazioni riportate suggeriscono che, almeno nella nostra regione, ceppi isolati da vini prodotti nelle stesse aree geografiche spesso sono raggruppati in modo diverso, indicando una complessa biodiversità intra-specifica negli ambienti in relazione alle produzioni di vini regionali. La diversità della produzione di fenoli volatili riflette la biodiversità intraspecifica evidenziata dalla Sau-PCR. La diversità dei ceppi legata ai diversi 'potenziali *spoilage*' aumenta la rilevanza industriale di questo studio, consentendo la progettazione di nuove strategie fisiche, chimiche e biotecnologiche per limitare l’incidenza di questo rilevante *spoilage* microbico.

Nel caso dei ceppi rappresentativi selezionati si è analizzato l’ingresso e l'uscita (attitudine a “resuscitare”) dallo stato vitale ma non coltivabile (VBNC). E’ stato confermato che crescenti concentrazioni di SO_2 libera inducono l'ingresso nello stato VBNC. I ceppi sono stati in grado di "resuscitare" dopo rimozione della SO_2. È stato osservato, per il comportamento VBNC, un carattere ceppo-dipendente. Infatti, ingresso e uscita dallo stato VBNC variano in maniera rilevante in funzione del ceppo. Questi risultati suggeriscono un comportamento VBNC come nuovo criterio chiave per la caratterizzazione del potenziale spoilage dei ceppi di *B. bruxellensis*. Da un punto di vista industriale, questi risultati sono importanti per progettare strategie fisico-chimiche e biotecnologiche per il controllo di *B. bruxellensis* in vino.

Sono stati anche riportati metodi per ridurre la presenza di *B. bruxellensis* e fenoli volatili prodotti nel vino utilizzando diverse preparazioni di chitosano scorze di lievito. Le scorze di lievito esercitano un'azione adsorbente contro le specie lipofile quali etilfenolo, favorendo così una riduzione parziale o totale delle molecole responsabili degli odori sgradevoli. Al contrario il chitosano è in grado di ridurre direttamente la crescita di *B. bruxellensis*.

1. INTRODUCTION

The alcoholic or primary fermentation in wine is a natural process performed by native yeasts, mainly belonging to *Saccharomyces* spp., which considerably contribute to the chemical and organoleptic properties of the final product. Additionally, most red and some white grape wines undergo a secondary fermentation denoted as malolactic fermentation (MLF) (Davis *et al*., 1985). The MLF is carried out by wine lactic acid bacteria (LAB) such as *Oenococcus oeni*, a dominant bacterial species which is well adapted to the harsh wine conditions and confers the finest oenological malolactic characteristics (Alexandre *et al*., 2004; Fugelsang and Edwards, 2007; Massera *et al*., 2009). However, other microbial species may occur in the complex consortia established in the must/wine environment, they are able to compromise the quality of wine. Yeasts belonging to the genus *Brettanomyces/Dekkera* are generally considered the main oenological spoilage microbes (Wedral *et al*., 2010). Currently, five species are recognized as members of this genus: *Brettanomyces bruxellensis*, *B. anomalus*, *B. custersianus*, *B. naardenensis* and *B. nanus* (Zuehlke *et al*., 2013). Currently, only strains of *B. bruxellensis* were found able to produce several compounds that are detrimental for the organoleptic quality of the wine, including some classes of volatile phenols which derive from the sequential conversion of specific hydroxycinnamic acids such as ferulate and p-coumarate (ferulic and p-coumaric acids). Hydroxycinnamate decarboxylase first transforms these hydroxycinnamic acids into vinylphenols, which are then respectively reduced to 4-ethylphenol and 4-ethylguaiacol by vinylphenol reductase (Edlin *et al*., 1998; Dias *et al*., 2003; Suárez *et al*., 2006). Volatile phenols greatly influence the aroma of wine. In fact, elevated concentrations of 4-ethylphenol are associated with unpleasant aromas described as “phenolic”, “horse sweat”, “stable” or “leather” (Suárez *et al*., 2006). The sensorial threshold of these compounds is 230 µg/L for 4-ethylphenol and 47 µg/L for 4-ethylguaiacol (Chatonnet *et al*., 1990; Suárez *et al*., 2006). Analytical methods have been developed either to monitor metabolites responsible for wine depreciation (Pollnitz *et al*., 2000) or to detect and estimate the population of these spoilage yeasts (Benito *et al*., 2009). The isolation and identification of *Brettanomyces* species

in wine may be performed by selective media (Suárez *et al*., 2006), PCR specific amplifications with subsequent enzymatic digestion (Cocolin *et al*., 2004) or real-time PCR approach, which allows a fast determination and enumeration of *Brettanomyces* directly in wine (Phister and Mills 2003; Tofalo *et al*., 2012). Moreover, several techniques, such as nested PCR (Ibeas *et al*., 1996), restriction fragment length polymorphism analysis (PCR-RFLP) (Mitrakul *et al*., 1999), PCR-denaturing gradient gel electrophoresis (DGGE; Renouf *et al*., 2006), infrared spectroscopy (Oelofse *et al*., 2010), and macrorestriction analysis of the chromosomal DNA by pulsed-field gel electrophoresis (REA-PFGE; Miot-Serties and Lonvaud-Funel, 2007) have been also used to study intraspecific differences among *B. bruxellensis* strains. Recently, a Sau-PCR method was also successfully employed to study *B. bruxellensis* biodiversity in a given geographic area (Campolongo *et al*., 2010).

B. bruxellensis could be detected in vineyards, soil, grape must, fermentation tanks and barrels (Rodrigues *et al*., 2001). According to Rodriguez *et al*. (2001), it is mainly found in oak barrels, where wine is placed for aging with the aim to improve its sensory proprieties. This presence in barrels imposes a problem for all producers of fine wineries. The critical characteristic of oak barrels is their porosity and *Brettanomyces* is able to settle in the pores of the wood and survive even after washing. Following this way, *Brettanomyces* can also move from one wine to another during the aging process (Garde-Cerdán *et al*., 2008). Although a large number of culture-dependent techniques are available to assess the presence of this undesired yeast during the vinification processes, in several cases *Brettanomyces* is undetectable, while the corresponding final products are affected by phenolic odors typical of the 'Brett' character (Serpaggi *et al*., 2012). This phenomenon has been explained studying the aptitude of *Brettanomyces* cells to enter in a Viable But Not Culturable (VBNC) state), a physiological condition characterized by a cells inability to divide in a bacteriological medium, even if they are still alive and maintain the metabolic activities and cell functions (Agnolucci *et al*., 2010; Divol *et al*., 2005; du Toit *et al*., 2005; Kell *et al*., 1998). *B. bruxellensis* cells are able to enter in the VBNC state as a response to several form of chemical

and environmental stress conditions, such as elevated osmotic concentrations (e.g. seawater), starvation, oxygen concentration, incubation outside the temperature range of growth, or exposure to white light (Oliver 2000), probably as an adaptive strategy for long-term survival of microbes under adverse environmental conditions. However, *Brettanomyces* cells can return to cultivable state through a process called 'resuscitation' that reverse the physiological changes that connote VBNC cells (Li *et al*., 2014). VBNC entrance poses criticalities in managing microbial stability and microbial safety in food (Li *et al*., 2014; Millet and A. Lonvaud-Funel 2000). The presence of foodborne bacteria capable to enter in a VBNC state is well known (Ordax *et al*., 2009). In contrast, few studies investigate the VBNC behavior in yeasts (Salma *et al*., 2013). Referring to the winery environment, recent studies have shown that sulphur dioxide, an antimicrobial agent used in food preservation, induces the VBNC state in *B. bruxellensis* (Agnolucci *et al*., 2010; du Toit *et al*., 2005; Serpaggi *et al*., 2012). Indeed, a concentration of molecular SO_2 of 0.8 mg/L may induce a VBNC state in *Brettanomyces* after two days. The strains may 'resuscitate', returning to a cultivable state, by increasing the pH in order to remove the SO_2 from the medium (Serpaggi *et al*., 2012). In fact, resuscitation process can often start by simply removing the stress that initially induced the VBNC response (du Toit *et al*., 2005, Oliver 2005). Some studies report that resuscitation can be induced by conditions such as nutrient addition (Roszak *et al*., 1984), temperature upshift (Nilsson *et al*., 1991) and heat shock (Ravel *et al*., 1995). However, most of the resuscitation processes were successful if the VBNC state is only harbored for a short period (Bogosian *et al*., 2001; McDougald *et al*., 1998; Serpaggi *et al*., 2012). Whereas it has been suggested that several yeast species in wine may enter in VBNC when exposed to sulfite stress (Salma *et al*., 2013), the VBNC state of *B. bruxellensis* is a major threat for wine industry. Indeed, if the presence of this yeast during production process is underestimated even after laboratory analysis, final product could be affected, thus leading to a major economic loss due to its organoleptic features, unwanted and unaccepted by consumers.

1.1 *Brettanomyces* yeast

1.1.1 Taxonomy and morphology

Yeasts of the genus *Brettanomyces* are known since 1904 as involved in second fermentation of beers (Peynaud and Domercq, 1956) and have been put, since then, in most fermented beverages such as beer, wine or cider (Chatonnet, *et al*., 1995; Hereztyn, 1986; Larue, *et al*., 1991).

The genus *Brettanomyces* is considered the imperfect form of the genre *Dekkera* belonging to the ascomycetes. Five species of the genus *Brettanomyces* are currently recognized: *B. bruxellensis*, *B. anomalus*, *B. custersianus*, *B. naardenensis*, *B. nanus*. But in wines, *bruxellensis* and *anomalus* species are the most encountered (Mitrakul, *et al*., 1999; Rodrigues *et al*., 2001; Martorell *et al*., 2006). From the five species currently known, the species primarily associated with winemaking is *B. bruxellensis* (*D. bruxellensis*) (Egli and Henick-Kling, 2001; Stender *et al*., 2001; Cocolin *et al*., 2004), although *B. anomalus* (*D. anomala*) and *B. custersianus* isolations from must fermentations have been reported in two instances (Querol *et al*., 1990; Esteve-Zarzoso *et al*., 2001). Generic *Brettanomyces* term will be used for the rest of the writing.

The morphology of this yeast may change depending on the culture of environmental conditions (Aguilar *et al*., 2000). They can be spheroids, ogival, cylindrical, or elongated. Although smaller than *Saccharomyces*, they are easily visible under a microscope optics. These cells multiply by multipolar budding (Van Der Walt 1970; Shung Chang *et al*., 1985) which leaves leading to scarring typical cellular forms. Sometimes we encounter even a reminiscent form of a small spoon (Aguilar *et al*., 2000; Medawar *et al*., 2003) (Figure 1, Suárez *et al*., 2007). It can be found in the form of small isolated cells (2.0 to 7.0) x (3.5 to 22.0 microns), in pairs or forming small chains sometimes even adopting a form of pseudo-non septate mycelium (Smith and Grinsven, 1984; Shung-Chang *et al*., 1985).

This polymorphism make them difficult to screen and identify by observation under a microscope.

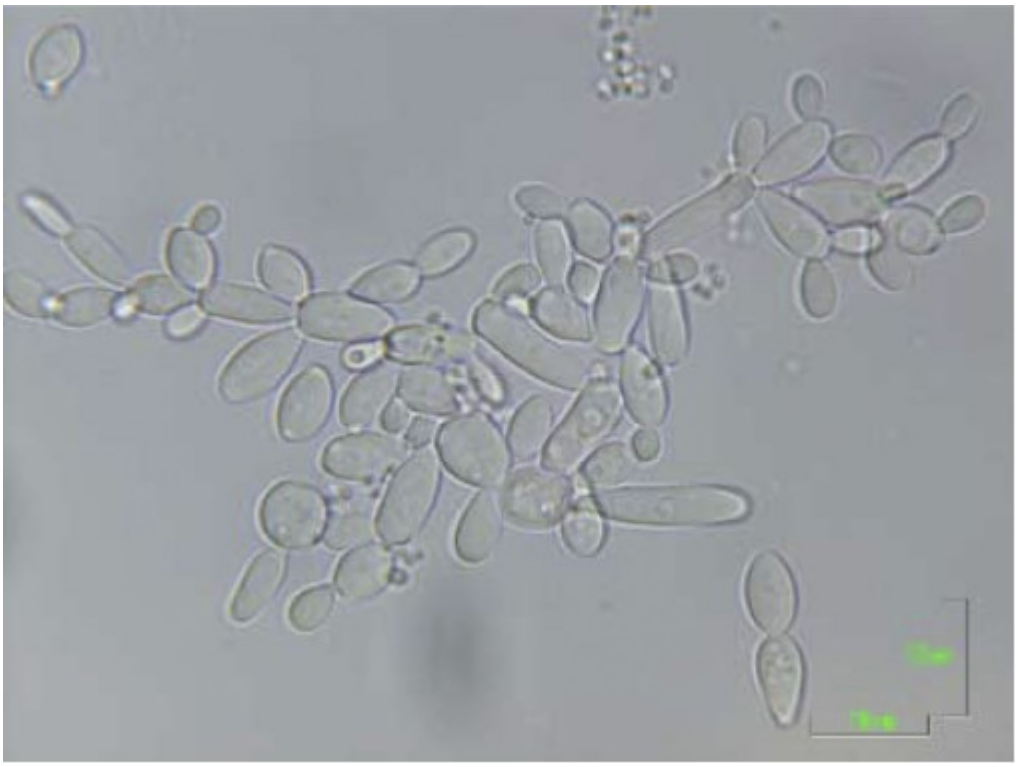


Fig. 1 Optical microscope image of a *Brettanomyces* strain (1000x). (Suárez *et al*., 2007)

1.1.2 Physiology and metabolism

Conterno *et al*., showed the ability of different *Brettanomyces* strains to use glucose, galactose and fructose as carbon sources (Conterno *et al*., 2006). This study also showed that some strains could grow with ethanol as sole carbon source. This ability was also demonstrated by Gilis *et al*., (1999) and Silva *et al*. (2004).

In addition to the ability to use most of hexoses and ethanol as sources carbon, *Brettanomyces* can develop with only 1 g/L of sugar in the medium (Silva *et al*., 2004).

Although *Brettanomyces* yeasts ferment sugars relatively slowly compared to other yeasts (Peynaud and Domercq, 1956; Sponholz, 1997), they are able to fermented grape juice and form various amounts of ethanol, 3.5 to 11% (v / v) ethanol.

1.1.3 Nitrogen sources

Like other organisms, nitrogen is essential for multiplication of *Brettanomyces*, but it can be provided to the cell in inorganic or organic form. The nitrogen in the form of inorganic ions and ammonium, in a very small amount, may be sufficient to the growth of the yeast.

Yeast extract appears as the major factor for growth, as it brings many amino acids and other forms of organic nitrogen. Although his absence does not prevent the growth of yeast, the maximum biomass concentration synthesized is proportional to the intake of yeast extract in the culture medium (Aguilar *et al*., 2000). This ingredient also provides magnesium ions and phosphate which however do not appear to be essential for the development of *Brettanomyces*. A small amount of nitrogen supplied by the yeast extract is sufficient to yeast growth.

Brettanomyces needs very little nitrogen to develop (Guilloux-Benatier *et al*., 2001), especially because these compounds are abundantly present in grapes, so they do not limit cell growth.

Vitamins are important to the development of *Brettanomyces*, such as biotin and thiamine (Conterno *et al*., 2006).

Different studies on the nutritional needs of *Brettanomyces* therefore show that this yeast can grow in conditions of low nutrients.

1.1.4 Effect of oxygen

As for *S. cerevisiae*, oxygen stimulates growth of *Brettanomyces* during alcoholic fermentation. The yeast can grow four times faster than anaerobic (Gilis, 1999). Indeed, in the absence of oxygen, glucose is consumed after a long lag phase, with a slow speed (Aguilar, 1998; Ciani *et al*., 2003). Under these conditions, the production of acetic acid is very low (Blondin *et al*., 1982). More recent works on the effect of oxygen show that production of acetic acid by *Brettanomyces* is favored by the increased intake oxygen (Aguilar *et al*., 2003).

The decrease of the rate of fermentation in the absence of oxygen is now understood as directly related to the limited capacity of these yeasts to re-oxidize their cofactors (Rodríguez Vaquero *et al*., 2007).

In presence of oxygen, on a glucose-rich medium, three different phases of growths were observed: a first phase during which *Brettanomyces* consumes glucose and multiplies, then it produces ethanol and acetic acid when glucose is completely consumed; the second phase consists of a conversion from acetic acid to ethanol without growth of the yeast. Then the final phase consists of acetic acid consumption accompanied by a resumption of growth, which is a fermentation phase (Wijsman *et al*., 1984).

1.1.5 Effect of ethanol

Tolerance to ethanol has been studied extensively in several *Brettanomyces* strains. Over 30 different strains and different origins were tested by Conterno *et al*. (2006) for a 10% content (v / v) ethanol in synthetic medium and all strains were tolerant to ethanol (Conterno *et al*., 2006). Another study demonstrated the strength of thirty strains contents above 13.5% (v / v) in synthetic medium and wine (Barata *et al*., 2008). Although it has been shown that the growth rate and biomass yield may be greatly reduced by the presence of ethanol (Medawar, *et al*., 2003), yeast *Brettanomyces* has a high tolerance to ethanol.

1.1.6 Effect of pH

In 1999, Gillis showed that the growth of *Brettanomyces* is possible with pH between 2.6 and 4.5 (Gilis, 1999). In 2004, Silva *et al*. determined an unchanged rate of specific growth between pH 3.5 and pH 4.5 (Silva *et al*., 2004). Finally, Conterno *et al*. (2006) showed that thirty-three strains of various origins can grow at pH 2.0 (Conterno *et al*., 2006). These converging studies demonstrate that the pH commonly found in wine has no effect on the proliferation of *Brettanomyces.*

1.2 The problem *Brettanomyces* in wine

The rigid-chemical environmental conditions at the end of alcoholic fermentation allow the survival of a limited number of yeast and bacteria. These conditions are determined by high concentrations of ethanol, content of fermentable sugar and acid pH. If not controlled, the metabolic activity of these microorganisms can irreparably modify the chemical composition of wine by changing the sensory properties of the final product. The risk of wine spoilage by bacteria decreased in a significant way because of the advances in wine technology and implementation of good manufacturing practices throughout winemaking process; despite this, yeast contaminations still represent a serious threat of spoilage (Agnolucci *et al*., 2009). The yeasts of the species *Brettanomyces/Dekkera bruxellensis* are the most responsible for the production of volatile phenols, causing significant economic losses in the wine industry. The development in wines of *Brettanomyces/Dekkera*, in fact, determines organoleptic changes, due to the appearance of phenolic and animals odor that in some specific conditions can remind the scents of farm, horse sweat, medicine, animal skin and leather (Suárez *et al*., 2007). During the last period *Brettanomyces/Dekkera* has been studied by several research groups with the aim to increase knowledge and establish appropriate control measures. Investigation on genome variability among individuals represents an important tool to assess their biodiversity and to study their environmental diffusion. The safeguard of microbial strains with technological interest is becoming a strategic activity in food and wine industries. Studies on the environmental diffusion and the incidence of *Dekkera/B. bruxellensis* species have been reported by different authors, focusing on the identification, isolation and genotyping of indigenous and reference strains (Martorell *et al*., 2006; Conterno *et al*., 2006; Curtin *et al*., 2007; Agnolucci *et al*., 2009). *B. bruxellensis* yeasts have developed numerous options in order to adapt and survive to the changing status of the environment. On the other hand, molecular profiling, carried out with several techniques (Martorell *et al*., 2006; Conterno *et al*., 2006; Curtin *et al*., 2007; Agnolucci *et al*., 2009; Vigentini *et al*.,

2011), has shown a high genetic variability among strains. Therefore, different genetic groups of *B. bruxellensis* isolates could be related to distinct physiological characteristics. The severity of *B. bruxellensis* related spoilage in wine arises from its ability to survive in spite of the unfavorable environment and the sanitation practices applied to the wood barrels. Such characteristics make the cellar contaminations invasive and difficult to treat. Its capability to grow in wine during barrel aging can be partially ascribed to its high resistance to SO_2, to the lack of microbial competitors in the post-fermentation winemaking stage and to its capability to produce both α and β-glucosidase, two enzymes which allow cellobiose, a disaccharide found in toasted wood, to be metabolized (Mansfield, 2002). The control of *Dekkera/Brettanomyces bruxellensis* in must, wine and wine contact surfaces is relevant for wine producers in order to reduce their economic losses; wine industries are constantly seeking to optimize current methods or to find new approaches without modifying the sensorial properties of the final product (Couto *et al*., 2005).

1.3 Organoleptic deviations caused by *Brettanomyces:* volatile phenols

The main molecules that alter the quality of wine are volatile phenols. These compounds give the wine often unpleasant aromas described as ‘plastic’, ‘glue’, ‘sweaty leather’, ‘barnyard’, ‘spicy’ and ‘clove-like’. The transformation of these precursors by *Brettanomyces bruxellensis* occurs through two enzymes (Figure 2, Oelofse *et al*., 2008)

- The first enzyme (cinnamate decarboxylase) allows the transformation of cinnamic acid, para-cumaric and ferulic acid in 4-vinyl phenol (4VP) and 4-vinyl guaiacol (4VG) that are not aromatic.

- The second enzyme (vinyl phenol reductase) ensures the transformation of vinyl phenol in 4-ethyl phenol and 4- ethyl- guaiacol (4EG), the volatile phenols (Suarez *et al*., 2007; Tchobanov *et al*., 2008).

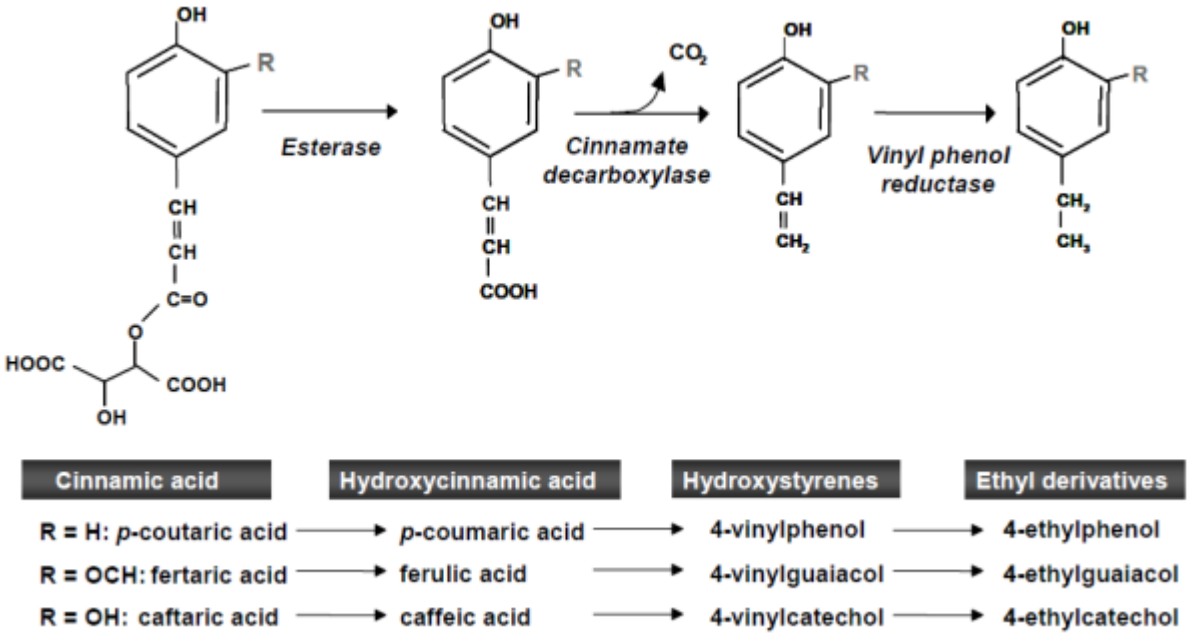


Fig 2. Formation of volatile phenols via the decarboxylation of hydroxycinnamic acids (Oelofse *et al*., 2008)

Although other yeasts are capable to transform acid para-cumaric in 4VP, *Brettanomyces* is the only one to be able to reduce VP in 4EP and 4EG (Chatonnet *et al*., 1995). Other microorganisms like *Lactobacillus brevis* and *Pediococcus pentosaceus* are able to decarboxylate acid para-cumaric in 4VP (Chatonnet *et al*., 1995). *Brettanomyces* can produce several milligrams of ethyl-phenols per liter of wine and this concentration is directly proportional to the size of the population of *Brettanomyces* (Chatonnet *et al*., 1995).

The threshold of perception of ethyl phenols is 425 µg/l for a report 4EP/4EG of 10/1 in a red wine. Beyond this threshold, these substances can be clearly seen by the consumer and aroma and flavor of wine quality is affected. The phenol character is very marked from 600 µg/l (Chatonnet *et al*., 1992); (Romano *et al*., 2009). It remains difficult to determine a critical concentration of contamination for these compounds. Indeed, depending on the individual, the wine matrix or strain of *Brettanomyces*, the concentration thresholds detected of volatile phenols are different (Suárez *et al*., 2007; Rayne and Eggers, 2008; Conterno *et al*., 2006; Harris *et al*., 2009). It has been found that

the threshold concentrations of these compounds (Table 1, Oelofse *et al*., 2008; Curtin *et al*., 2005), especially the ethyl derivatives, vary substantially and the perception of the individual aromas is greatly influenced by the wine style, cultivar and the consumer's perceptive abilities.

This is why it is important to limit uncontrollable production of the different compounds presented above, and should be important to detect *Brettanomyces* in wine rapidly and specifically.

Compound	Concentration in red wine (µg/L)	Aroma threshold (µg/L)	Aroma descriptor
4-Vinylphenol	8.8–4.3	440*/600**	Phenol Medicinal
4-Vinylguaiacol	0.2–15	33*/110**	Clove-like
4-Ethylphenol	118-3696	30-60**	Horsy
4-Ethylguaiacol	1–432	20***	Spicy, clove
4-Ethylcatechol	27–427	10*	Phenol Band-Aid® Medicinal Banyard

*model wine, **red wine, ***water

Tab. 1 Aroma threshold values of volatile phenols in wine (Oelofse *et al*., 2008; Curtin *et al*., 2005)

1.4 Control of *Brettanomyces bruxellensis* in wine

The presence of *B. bruxellensis* may cause many troubles in the production of foods and drinks, and in particular in winemaking processes (Guerzoni and Marchetti, 1987; Renouf and Lonvaud-Funel, 2007). This is because it fails to transform hydroxycinnamic acids in ethyl- and vinyl- derivatives, thus representing a major problem for the cellar (Fugelsang and Zoecklein, 2003). These compounds give unpleasant odors to wine, thus reducing its aroma and quality (Fugelsang, 1997). Because of that, *B. bruxellensis* is considered the major cause of spoilage in wine (Fugelsang, 1997; Loureiro and Malfeito-Ferreira, 2003). Nowadays, there are investigations in progress, in order to find ways to prevent and treat the development of *B. bruxellensis* in wine. A remedy that could apparently solve the problem is the fining of red wine before putting it in barrels (Murat and Dumeau, 2003). However, this remedy is often rejected by winemakers because it reduces the

flavor and color of wine (Suàrez *et al*., 2007). Another method could be filtration using membranes with pore sizes smaller than 0.45 μm (Calderon *et al*., 2004). This method, however, could reduce the intensity of the wine, going to deteriorate its colloidal structures, and also cells of *B. bruxellensis* in Viable but not Cultivable (VBNC) state appear to have a more elongated and smaller form than normal cells and may cross the membrane in a undisturbed way (Millet and Lonvaud-Funel 2000). Another remedy used to control *B. bruxellensis* is the additive SO_2 which, thanks to its potential antimicrobial is ideal for storing wine (Romano and Suzzi, 1992), was found to be able to inhibit the growth of *B. bruxellensis* (Du Toit *et al*., 2005). Recent studies (Agnolucci *et al*., 2010; Serpaggi *et al*., 2012) have shown that the SO_2 induces *B. bruxellensis* in the state VBNC, but remain in a dormant state, while maintaining all the metabolic and physiological activities. Another way could be the inhibitory dimethyl dicarbonate (DMDC). Its use has been audited during the wine making process (Renoulf *et al*., 2007). In finished wines, 120 mg/L of DMDC cause inhibition of *B. bruxellensis* while 250 mg/L can ensure the elimination of the yeast. Other studies have shown that the effectiveness of DMDC depends on the level of ethanol present in wine (Malfeito-Ferreira *et al*., 2004) and is not dependent on pH values present in wine (Threlfall and Morris, 2002). Moreover, there are weak acids that have antifungal action and that can be used against *B. bruxellensis*, but they are not selective and they are not allowed to be used in vinification processes (Suàrez *et al*., 2007). From the microbiological point of view, studies about toxins against *Brettanomyces* are in progress. Comitini *et al*., (2004) have reported two toxins produced by *Pichia anomala* (DBVPG 3003) and *Kluyveromyces wickerhamii* (DBVPG 6007) that have an action against *B. bruxellensis*. Recently, Oro *et al*., (2014) studied the antimicrobial activity of different strains of *Metschnikowia pulcherrima* on wine yeasts, including *B. bruxellensis*, advising, as well, a biotechnological approach to improve the flavor profile of wine.

A summary of all the scientific investigations on the control of *Brettanomyces* yeasts can be seen in **Table 2**. (Oelofse *et al*., 2008)

Treatment	Reference
Protein clarification	Murat & Dumeau (2003)
Gelatine	Ruiz-Hernández (2003)
Egg white	
Potassium caseinate	
Caseins	
Filtration	Millet & Lonvaud-Funel (2000)
Membranes (0.45 µm)	Calderón *et al* . (2004)
Ultrafiltration	
Physicochemical variables	Gerbeaux *et al* . (2000)
Low aging temperature	
Low pH	
Reduction of oxygen content	
Avoidance of micro-oxygenation	
High alcohol levels	
Reduction of precursor concentration	Gerbeaux *et al* . (2002)
Low maceration temperature	
Avoidance of pectolytic enzymes and enzymes with cinnamoyl esterase activity	
Additives	Ison & Gutteridge (1987)
SO_2	Delfini *et al* . (2002)
DMDC	Renouf *et al* . (2007)
Chitosan	Gómez-Rivas *et al* . (2004)
Sorbic acid	
Benzoic acid	
Fumaric acid	
Ascorbic acid	
Erythorbic acid	
High pressure processing	Puig *et al* . (2003)
400-500 MPa	
Biological techniques	Du Toit & Pretorius (2000)
Zymocins/killertoxins	Comitini *et al* . (2004)
Bacteriological enzymes	
Genetic egineering	Du Toit & Pretorius (2000)
Transgenic yeasts	
Other alternatives	Guilloux-Benatier (2001)
Ozone	Coggan (2003)
Reverse osmosis	Cantacuzene *et al* . (2003)
Absorbents	Chassagne *et al* . (2005)
PVPP	Yap *et al* . (2007)
Charcoal	
Ultrasonics	

Tab. 2 Summary of investigation on *Brettanomyces* control (Oelofse *et al*., 2008).

1.5 Intraspecific biodiversity of *Brettanomyces bruxellensis*

Brettanomyces bruxellensis is considered the main spoilage yeast in wine (Chatonnet *et al*., 1992). This yeast, which has a genetic variation, is able to grow and survive the stressful conditions of wine (Vigentini *et al*., 2013). In fact, *B. bruxellensis* is characterized by a high degree of intraspecific polymorphism, probably due to an increase of the mutation during asexual state or from a fusion of different genomes (Curtin *et al*., 2007; Agnolucci *et al*., 2009; Hellborg and Piskur 2009).

Conterno and colleagues (2006) have observed differences in strains of *B. bruxellensis* isolates (47 strains). Some of them seem grouped according to the geographical origin but there are also similarities between isolates from different regions. For example, in a group there is only one isolate from New York, another one from California and the other ones are all isolated from Europe (Conterno *et al*., 2007); In another group, 81% of the isolates were from America but in this group there are also two strains isolated from Portugal, one from France and one from Thailand. Even Campolongo *et al*. (2010) and Di Toro *et al*. (2015) have shown that isolated coming respectively from two different regions or from the same region formed different groupings. In the study of isolates from the same region, only a group was formed by strains isolated from wines from the same city, all other groups were formed by strains isolated from wines from cellars of several cities (Di Toro *et al*., 2015). Campolongo *et al*., (2010) by isolating strains from cellars of Piedmont and Liguria and studying the biodiversity through the SAU-PCR, obtained 12 clusters, nine of which were formed by strains of a single region and three strains of both regions. Using different methods of study (**Table 3**), it can be observed that strains from different locations grouped together. Probably because a different group of *B. bruxellensis* spread throughout the world (Arvik *et al*., 2002). This could be explained because the same winemaking practices or the same type of wines may select similar strains of *B. bruxellensis* or more strains of *B. bruxellensis* could be disseminated thanks to blends of wine or winemaking practices cooperatives (Conterno *et al*., 2006). According

to Curtin *et al*., 2007, there are several vectors that may carry strains of *B. bruxellensis* like insects which could transport between wine cellars (Van der Walt and Van Kerken 1961), or this could be related to the adaptation to the wine environment (Curtin *et al*., 2007). In fact, Curtin and colleagues (2007) observed different genotypes, studying a large number of *B. bruxellensis* isolated from Australian wines (**Table 3**). The genotype I was found in the majority of Australian wine regions and probably they were isolated well adapted to the conditions of Australian wine, the genotypes IV and V were found in isolates from wine cellars of colder areas. In contrast to these studies (Conterno *et al*., 2006; Curtin *et al*., 2007; Agnolucci *et al*., 2009; Campolongo *et al*., 2010; Di Toro *et al*., 2015), Vigentini *et al*., (2012) did not find predominant genotypes, probably because of the technique used has not always provided a complete intraspecific differentiation. The authors even observed that, in a country like Italy, distinct populations of *B. bruxellensis* may be found, and more strains similar to each other were often isolated from the same winery. However, as reported by some authors,is sometimes impossible to identify a grouping based on geographical origin (Albertin *et al*., 2014). The air produced during fermentation may be a diffusion carrier (Bokulich *et al*., 2013). The same genotype from several wineries, located in different geographical areas, could be explained by the exchange of equipment for winemaking contaminated with *B. bruxellensis* (Albertin *et al*., 2014).

The biodiversity of this yeast could lead to a diversity regarding the appearance of *Brettanomyces* spoilage. Conterno *et al*. (2006), clumped 47 isolates of *B. bruxellensis* in 6 clusters. No correlation was found between physiological traits and clusters (**Table 3**). In contrast, Di Toro *et al*., (2015) reported as the diversity between the strains was not only genetic but also related to their potential spoilage and their different production of volatile phenols. A more detailed study of these aspects could help winemakers to learn more about this yeast and implement preventive and fighting measures to try to reduce the huge economic losses caused by *B. bruxellensis*.

Geographic Area	Molecular technique	Number of strains	Numbers of cluster	Reference
Argentina, Belgium, New Zealand, Portugal, Missouri	Alignment of the sequenced portion of 26S rDNA	47	6	Conterno *et al*., 2006
31 region of Australia	MseI-C/PstI-AC primer pair for AFLP genotype groups	244	8	Curtin *et al*., 2007
Piedmont and Liguria (Italy)	SAU-PCR	196	12 cluster and 10 single strains	Campolongo *et al*., 2010
Italy, France, Belgium, Germany, South Africa.	mtDNA RFLP analysis	23	5 cluster and 1 single strain	Vigentini *et al*., 2012
Puglia (Italy)	SAU-PCR	48	7	Di Toro *et al*., 2014
France, Australia, South Africa	Eight microsatellite markers.	18	3	Albertin *et al*., 2014

Tab 3. Summary of genetic studies on *Brettanomyces bruxellensis* strains

1.6 Intraspecific biodiversity and spoilage potential

Lately winemakers are putting more attention to the presence of the yeast *Brettanomyces bruxellensis* in wine. This yeast makes the wine unacceptable to consumers because it is capable to produce volatile phenols that strongly influence the flavor of the finished product (Suarez *et al*., 2007). Among these compounds, the most important are 4-ethylphenol, 4-ethylguaiacol, 4-vinylphenol and 4-vinylguaiacol (Chatonnet *et al*., 1992), which give an unpleasant aroma often described as stable, leather, medicine, horse sweat when they are in high concentrations (Rodrigues *et al*., 2001; Chatonnet *et al*., 1993; Chatonnet *et al*., 1992). The threshold of perception of these compounds is for the 4-ethylphenol of 230 µg/L, while for the 4-ethyl guaiacol of 47 µg/L (Chatonnet *et al*., 1990). According to Joseph and Bisson (2004), large differences in the production of these compounds can be shown by different strains of *B. bruxellensis*. Indeed, Gerbeaux *et al*.,

(2000) have shown that strains of *B. bruxellensis* are different in their ability to produce volatile phenols, although this capacity increases when alcohol concentrations are lower (we note more production at 12% v/v than at 14% v/v) and temperatures increased. Recently Di Toro *et al*. (2015) have studied the ability of seven strains of *B. bruxellensis*, genetically different, to produce 4-ethylphenol and 4-ethylguiacol in a synthetic medium with the addition of precursors (100mg/L of p -cumaric acid; or 100mg/L of ferulic acid; or 100mg/L of both), glucose (2 g/L or 20g/L) and ethanol. In synthetic medium supplemented with glucose and p-coumaric acid, all strains tested were able to produce 4-ethylphenols and transform the entire p-coumaric acid added. Moreover, in synthetic medium supplemented with glucose, ethanol and p-coumaric acid, all strains produce 4 ethylphenols but in lower concentrations compared to synthetic medium without ethanol. These results are in agreement with those previously reported by Dias *et al*. (2003) and Oelofse *et al*. (2009); both authors showed that the production of 4-ethylphenol was lower in the medium containing ethanol. The amount of ethylphenols produced was different from strain to strain, and all strains have a considerable "potential spoilage" in reason of their 4-ethylphenol/4-ethylguaiacol production (Di Toro *et al*., 2015).

1.7 Critical steps of contamination in winemaking

Due to its ability to grow in harsh environments and withstand the environmental conditions of wine, *Brettanomyces* yeast is an enemy that must be controlled throughout winemaking process, from grape harvest to bottling. It is therefore important to know the steps and critical contamination points for better protection throughout winemaking.

1.7.1 The harvest

For a long time, contamination of grapes by *Brettanomyces* was not highlighted, despite strong evidence of attendance, as this yeast represents a minority on berries which combines a wide variety of microorganisms (Prakitchaiwattana *et al.*, 2004; Renouf *et al.*, 2007). The presence of *Brettanomyces* on grape berry is connected either to the vineyard or the variety or the vintage (Renouf *et al*., 2006). Its presence seems more dependent on environmental stresses (temperature, sunlight, rain, pesticide treatments) like all other microorganisms. The chemical composition of the grape also plays an important role (sugar, malic acid, tartaric acid, pH and nitrogen), although no correlation between these parameters and the presence of *Brettanomyces* could be demonstrated in a study conducted on several vineyards and at different time of ripeness (Renouf *et al*., 2006).

Moreover, in 1999, Chatonnet *et al*. showed a link between contamination with *Brettanomyces* and parcels of vines near wineries releases (Chatonnet *et al*., 1999). But what is certain is that the grapes with rot, and in particular the ones associated with *Botrytis*, are often contaminated with *Brettanomyces* (Barbin 2006).

1.7.2 Alcoholic fermentation (AF) and malolactic (MFL) fermentation

Brettanomyces yeasts are rarely detected during alcoholic fermentation. This is a step in winemaking that has generally low risks. In contrast, it is at the end of this stage that risks increase. Indeed, if the end of the FA drags on, *Brettanomyces* can implant and develop (Renouf *et al*., 2006). The presence of residual sugar at the end of FA may further promote the growth of the yeast spoilage (Chatonnet *et al*., 1995).

After the AF, the yeast *S. cerevisiae* interrupts its activity and *O. oeni* takes its place to start MLF. But sometimes the transition between these two microorganisms is too long, thus leaving the

possibility to *Brettanomyces* to settle in the middle with its resilience at difficult conditions, such as the high content of ethanol or nutrient poverty (Renouf *et al*., 2008; Aguilar *et al*., 2000).

The study of Murat and Gindreau shows a strong relationship between the duration of the period of latency before MLF and contamination of *Brettanomyces* in wine. It is therefore advisable to accelerate the onset of MLF to avoid contamination by *Brettanomyces* (Murat and Gindreau, 2006).

1.7.3 Wine aging

It is important to note that even a wine that has undergone AF and normal MLF may undergo development of *Brettanomyces*. Indeed, it has been shown that a perfectly sterile wine (filtered), with no sugar residues, still allows *Brettanomyces* to develop (Chatonnet *et al*., 1992).

Aging in oak

This aging method presents a number of risks for the development of *Brettanomyces*.

The first point is the exchange of oxygen between the outside and the wine (Vivas and Glories, 1993) including the use of new oak barrels. This oxygen supply leads to decrease the effectiveness of sulphites and stimulates growth of *Brettanomyces*. In addition, new wood releases more of monosaccharides, easily assimilated by yeast (del Alamo *et al*., 2000).

The use of used barrels reduces the risk of oxygen exchange since the pores are often obstructed by aging preceding wines (Lonvaud-Funel and Renouf, 2005).

However, these barrels are already contaminated with *Brettanomyces* as it is difficult to clean between two farms (Chatonnet and Dubourdieu, 1999), especially the yeast can nest to a depth of 8 mm in wood (Suarez *et al*., 2007).

Oxygen supply

In addition to location directly on the wood of the barrel, the exchange of oxygen can also occur at the bung. Different positions of the plug have been tested, but none allows a sufficient limitation of the exchange (Chatonnet *et al*., 1992). Racking is also a critical step because it brings a lot of oxygen in the wine, which stimulates the growth of yeast. Transfer contamination risk is also increased during filling (Gilis, 1999; Renouf *et al*., 2007).

The micro-oxygenation and the use of a clicker (device for one-time contribution oxygen in the tank) may have critical points about the growth of *Brettanomyces*. Indeed, a study conducted in tanks (Gilis, 1999) showed that the point mass oxygenation could accelerate the growth of *Brettanomyces* unlike a micro-oxygenation continues.

Temperature

The diffusion of oxygen through the wood of the barrel increases when temperatures rise, especially during the hottest time of the year. This increase in distribution may reduce the effect of sulfitage into the barrel, thus promoting the development of *Brettanomyces*. In addition, temperatures above 20 °C feel favorite yeast growth *Brettanomyces* (Chatonnet *et al*., 1993).

1.7.4 Preparation for bottling

Sensitive points of the bottling line are mainly the spout of the printer, the bell, the tank and the pipes (Ruyskensvelde *et al*., 2006).

Total microbial population before bottling is typically 10^3 to 10^4 CFU/ml. The main species found are: *Acetobacter aceti*, *S. cerevisiae*, *O. oeni*, *Pediococcus parvulus* and *B. bruxellensis* (Renouf *et al*., 2007). While conditions within the bottle are hostile to the development of microorganisms (microaerobiosis, low pH, high ethanol, very low nutrient concentrations), they can harbor bacteria

and live yeast even after decades (Renouf *et al*., 2007). Among these microorganisms commonly found in bottle, we should include *Brettanomyces*, thanks to its ability to withstand harsh environments like wine.

1.8 Methods for detection and identification of *Brettanomyces*

To control the risk of damage to the wine due to *Brettanomyces*, a first objective is to develop tools allowing contamination assessment to act as quickly possible and to avoid deterioration of the wine.

1.8.1 Identification methods

As regards the methods of identification, the goal is to differentiate *Brettanomyces* other yeasts to perform diversity studies. These methods have drawbacks, because *Brettanomyces* must be first isolated in pure strains and lately cultivated; this procedure often requires more than 10 days. In addition, these methods are not quantitative.

Fatty acid profiles

Among these methods, there is a first account identification through the fatty acid profile. *Brettanomyces* can be identified with a fatty acid profile database of different yeasts determined by Gas Chromatography (Rozes *et al*., 1992). However, the equipment required for this method is not suitable for an extension of the method, and the results are dependent on culture conditions since the composition of fatty acid membrane varies depending on environmental conditions.

Molecular methods

For twenty years, molecular methods are at the service of the detecting microorganisms in complex media. PCR (Polymerase Chain Reaction) allows highly accurate detection and is fast enough for a

Microorganism, even a particular strain in a complex environment with low and diverse population. This method is based on the detection of a specific DNA Many molecular techniques using PCR were developed for the *Brettanomyces* detection in order to reduce the time needed to identify it.

Stender (Stender *et al*., 2001) has developed nucleic acid peptides which are complementary sequence on a single target rRNA 26S *Brettanomyces*, coupled to a FISH (fluorescent in situ hybridization) method.

Mitrakul (Mitrakul *et al*., 1999) showed that the PCR-RAPD (Random Amplification of Polymorphic DNA) can allow the identification of *Brettanomyces* at the species and of the strain.

Then there is also the LAMP (Loop-mediated isothermal amplification) technique established by Notomi (Notomi *et al*., 2000) and applied to *Brettanomyces* in 2007 (Hayashi *et al*., 2007), using the ITS regions of rDNA *Brettanomyces*.

In 2006, Miot-Sertier and Lonvaud-Funel implemented REA-PFGE (restriction enzyme analysis and gel electrophoresis field pulse) method to discriminate 9 strains of *B. bruxellensis* (Miot-Sertier and Lonvaud-Funel, 2006). In 2007, a study was performed on 244 strains of *Brettanomyces bruxellensis* isolated from red wines made in 31 Australian different cellars (Curtin *et al*., 2007). The method used was the AFLP (amplified fragment length polymorphism) and showed that three genotypes were majority in the Australian wines. Another study of biodiversity has showed *Brettanomyces* at a high level of polymorphism of the strains isolated from a small Italian region (Agnolucci *et al*., 2009), through the use of methods and RAPD (Restriction analysis of mitochondrial DNA).

All these developments have improved the conventional PCR in efficiency and accuracy, while previous methods offer a high level of discrimination. However, they are only qualitative, and are not specifically developed to be used directly on wine. Finally, still little (or no) analysis

laboratories are trained in these techniques and can perform these analysis as a routine, because it is often mostly too heavy to be put in place for quick identification of *Brettanomyces*.

1.9 Methods of detection and enumeration

Methods have been developed specifically to detect and quantifying *Brettanomyces* in wine. The speed, sensitivity and low detection represent limits for the performance of the method.

Monitoring of volatile phenols

Monitoring the formation of volatile phenols may denounce the presence of *Brettanomyces* (Dominguez *et al*., 2002). However, this method cannot determine whether this appearance is proportional to the amount of yeast (Medawar *et al*., 2003) and the concept of threshold is difficult to implement since the presence of volatile phenols is not always synonymous with aromatic wine spoilage.

Selective media

It is possible to use different culture media (solid or liquid) which determine a physiological characteristic of the species sought. For *Brettanomyces*, the criteria most often applied are:

- acidification in aerobiosys
- synthesis of ethyl-phenols
- assimilation of various carbon sources such as ethanol
- resistance to actidione (cycloheximide).

These cultures have the advantage of being quantitative. But this type of selective detection lacks of specificity. In addition, time required to obtain results is often too long for effective response in case of contamination since takes a period exceeding 7 days.

Molecular methods

The criteria of molecular methods make these methods rapid, sensitive and reliable.

Molecular methods as the specific labeling of DNA and the quantitative PCR provide greater specificity but have disadvantages. The PNA FISH (Hybridization fluorescence in situ using peptide probes of nucleic acid probes) technique is very specific since it allows the marking of the 26S rDNA of *Brettanomyces* to fluorescence. It consists of using nucleotide polymers linked by peptide bonds as probe as marked in their N-terminus by a carboxyfluorescein.

These fluorescent peptides enter cells easily because they are not loaded in contrast to nucleic acids. Being complementary to the D1-D2 variable region 26S ribosomal DNA of *Brettanomyces*, PNA-FISH fix it and make the cells fluorescent and therefore easily detectable by epifluorescence microscopy. Although it is very effective and sensitive, this method is expensive and time consuming to set up (Stender *et al*., 2001).

Quantitative PCR is an easier way to apply, specific and rapid but requires training of technical staff (Phister and Mills, 2003; Delaherche *et al*. 2004; Tessonnière *et al*., 2009). Based solely on DNA, this method detects *Brettanomyces* whether living or not. This method relies on the use of a fluorescent agent which binds to the DNA. In the first cycles, the increase in Fluorescence causes an exponential kinetics. The cycle is equal to an overrun of fluorescence; detection threshold is used, using a proportional relationship, to calculate the initial amount of matrix.

The difficulty in developing a robust and reliable method of quantitative PCR or PNA-FISH probes hybridization is due to the need for a constant efficiency in DNA extraction from wine. In addition, the DNA must be of sufficient purity so that the performance of the PCR reaction is not affected.

Flow Cytometry

The method of marking and enumeration by flow cytometry is a simple and fast, but it is not specific for *Brettanomyces*. Flow cytometry is a method to differentiate and count cells by measuring simultaneously several characteristics of the cell when it passes the laser:

- The distribution of the incident light

- The emission of fluorescence

In general, two probes are used for cell counting:

- FSC (forward scatter), which characterizes the particle size

- FL1 which characterizes the intensity of the fluorescence emitted at a wavelength data.

Cytometer consists of three different systems:

- A fluid system allowing laminar flow cell suspension to go one by one before the laser;

- An optical system: laser beam and different filters for selecting appropriate wavelengths;

- An electronic system: a photomultiplier tube detects the emitted light, digitizer converts it into an electrical signal and digital signal and finally the computer manages and saves data.

The fundamental representation cytometry data is diagrammatically dispersion which is used to represent the appearance of the cell population. This technique allows a multiparameter analysis of cells, and can be used with various markers (DNA, viability, membrane potential, specific probes ...).

The method of counting by flow cytometry requires background noise related to the presence of interfering particles in the sample is minimized. For products liquid, filtration removes these particles.

Flow cytometry is used in quality control of fermented products (Gunasekera *et al*., 2000), beer (Kobayashi *et al*., 2007), wine (Bouix *et al*., 1999) and cider (Herrero *et al*., 2006). Attempts to control the fermentation of wines also conducted (Malacrino *et al*., 2001; Chaney *et al*., 2006; Farthing *et al*. 2007; Rodriguez and Thornton, 2008).

1.10 Viable But Non-Culturable (VBNC)

1.10.1 Definition

The state defined as "Viable But Non Culturable" (VBNC) has been proposed for the first time in 1982, by the laboratory of Rita Colwell of the University of Maryland. This team showed that cells of *Escherichia coli* and *Vibrio cholerae* were still alive, although they were not able to grow on an agar medium (Xu *et al*., 1982).

It has been described that, to be considered as viable, yeast cells had to maintain membrane integrity, enzymatic activity, and the ability to have a metabolic activity (Guillou *et al*., 2001). Generally, the viable cells are able to form colonies on agar media and when they lose this ability, then they are usually considered dead. This particular metabolic state called VBNC describes cells that are still metabolically active but unable to multiply, especially on agar media that normally allow quantification. This metabolic state has been proposed as a cells' strategy to withstand harsh environmental conditions (Oliver, 1993), in particular for bacteria not forming spores, which have no survival systems provided by endospores. It is important to note that to state that a cell is in VBNC state, it is necessary that the latter is able to become cultivable if environmental conditions improve: it is the "resuscitation".

Since the first study (Xu *et al*., 1982), the number of species of bacteria determined to be able to enter in this particular state have been increasing (Colwell, 2000; Oliver, 2000; Oliver, 2005) and belong to both, Gram-positive and Gram-negative bacteria (**Table 4**).

Technically, a population income VBNC state because of environmental stress conceptualized as follows:

1- cultivable population falls, becoming undetectable by traditional culture methods (agar, even non-selective),

2- a viable population stabilizes, thus revealing a low mortality

3- total population remains stable over time, indicating that the cells do not die since cell lysis result in a decrease in the number of undamaged cells (**Figure 3**: Population evolution Profile by counting methods). An observation Microscopic cells VBNC shows that cells maintain their integrity.

Aeromonas salmonicida	*Lactobacillus plantarum*	*Serratia marcescens*
Agrobacterium tumefaciens	*Lactococcus lactis*	*Shigella dysenteria*
Alcaligenes eutrophus	*Legionella pneumophila*	*S. flexneri*
Aquaspirillum sp.	*Listeria monocytogenes*	*S. sonnei*
Burkholderica cepacia	*Micrococcus flavus*	*Sinorhizobium meliloti*
B. pseudomallei	*M. luteus*	*Streptococcus faecalis*
Campylobacter coli	*M. varians*	*Tenacibaculum sp.*
C. jejuni	*Mycobacterium tuberculosis*	*Vibrio angutilarum*
C. lari	*M. smegmatis*	*V. campbelli*
Cytophaga allerginae	*Pasteurella piscida*	*V. cholerae*
Enterobacter aerogenes	*Pseudomonas aeruginosa*	*V. fisheri*
E. cloacae	*P. fluorescens*	*V. harveyi*
Enterococcus faecalis	*P. putida*	*V. mimicus*
E. hirae	*P. syringae*	*V. natriegens*
E. faecium	*Ralstonia solanacearum*	*V. parahaemolyticus*
Escherichia coli (including EHEC)	*Rhisobium leguminosarum*	*V. proteolytica*
Francisella tularensis	*R. meliloti*	*V. shiloi*
Helicobacter pylori	*Rhodococcus rhodochrous*	*V. vulnificus (types 1 et 2)*
Klebsiella aerogenes	*Salmonella enteritidis*	*Xanthomonas campestris*
K. pneumoniae	*S. typhi*	
K. planticola	*S. typhimurium*	

Tab. 4 Bacteria described as being in VBNC state (Oliver, 2005)

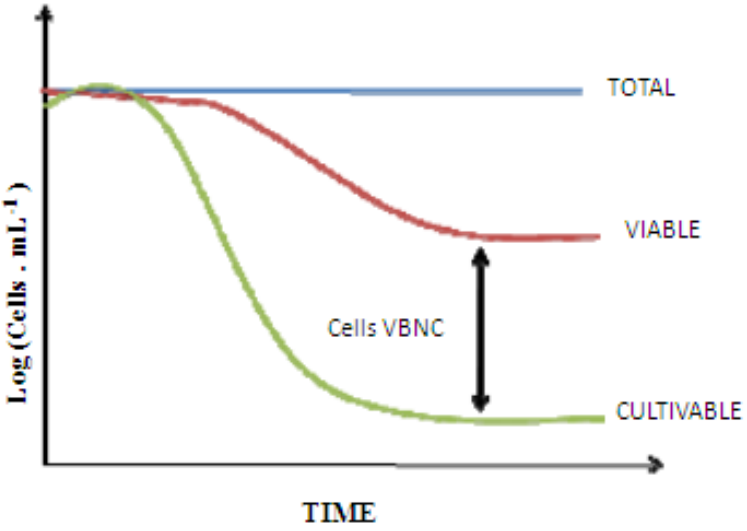


Fig 3 Populations evolution profile

1.10.2 Induction of VBNC state

Different environmental stresses can cause the entry of bacteria into VBNC state. Nutritional deficiency (Cook and Bolster, 2007), osmosis (Asakura *et al*., 2008; Chiu *et al*., 2008), oxygen (Kana *et al*., 2008), most of the food preservatives (Quiros *et al*., 2009; Cunningham *et al*., 2009) are all in the VBNC state. In unfavorable conditions, cells can enter VBNC condition and survive with limited metabolic activity without multiplying. So many changes have likely occurred within the cells to enable them to survive in this state.

In *Brettanomyces bruxellensis*, it has been shown that sulphur dioxide induces the VBNC state (Agnolucci *et al*., 2010; du Toit *et al*., 2005; Serpaggi *et al*., 2012). Serpaggi *et al*., showed that a concentration of molecular SO_2 of 0.8 mg/L, may induce a VBNC state in *Brettanomyces* after two days.

1.10.3 Resuscitation

To be considered VBNC, a population must be able to exit this condition and regain its ability to multiply: this is called "Resuscitation" (Oliver, 1993).

The hypothesis of the VBNC state is based on the fact that cells are able to recover their ability to multiply, thus regaining normal metabolism when environmental conditions improve. This resuscitation was the subject of a strong debate (Barer, 1997; Bogosian *et al*., 1998; Bogosian and Bourneuf, 2001; Nyström, 2001), since some authors suggest that the recovery of cultivability is due to the presence and the resumption of growth by few cells in the normal residual metabolism of a mainly VBNC population. However, the recovery of cells division in a VBNC population has been described unambiguously for many bacteria (Dhiaf *et al*., 2008; Zhong *et al*., 2009). A study by Whitesides and Oliver 1997 showed that even in the presence of less than 0.0001 cells in a sample, VBNC cells recovered their growth capacity (Whitesides and Oliver, 1997).

Resuscitation was demonstrated *in vitro*, *in vivo* and *in situ* (Oliver, 2005). To certain species, removal of the environmental stress can be sufficient to cause VBNC output, whereas in other bacteria it is necessary to add nutrients to the medium to generate it. For example, bacterium *V. vulnificus* which enters into VBNC at low temperatures, can emerge simply by increasing the culture temperature (Oliver and Bockian 1995). For other pathogenic bacteria, injecting VBNC cells into a host cell causes a resuscitation. This is the case for *Legionella pneumophila* (García *et al*., 2007).

In *Brettanomyces bruxellensis*, it has been shown that sulphur dioxide induces the VBNC state in this yeast and that the strains may ‘resuscitate’, returning to a culturable state, by increasing pH in order to remove SO_2 from the medium (Serpaggi *et al*., 2012).

2. AIM OF THE STUDY

The main aim of this study is to enhance quality of the finished wine product via an improvement management and to reduce the presence of *Brettanomyces bruxellensis* yeast in wine. The following phases have been scheduled:

- *Dekkera/Brettanomyces* strains colture isolation from Apulian wines
- Genotipical analysis for strain differentiation and correlation of biodiversity with geographical origin
- Capacity of different strains to produce volatile phenols in wine
- Rapid identification of *B. bruxellensis* in wine using Real Time PCR
- Study of *B. bruxellensis'* growth in presence of ethanol and SO_2
- Use of chitosan and yeast cell wall to reduce the growth of *Brettanomyces bruxellensis* in wine and volatile phenols production respectively.
- Screening of yeasts (*M. pulcherrima*) isolated in wine to evaluate their inhibitory power on *B. bruxellensis*.
- Microvinification test to control *B. Bruxellensis* development.

3. MATERIALS AND METHODS

3.1 Yeast isolate sampling and growth conditions

Samples of this study were chosen to have representative data covering the main Apulian wine production areas (**Figure 4**).

Fig 4 Localization of sampling grape localities

Samples were collected from fermenting musts, wines, ageing wines (tanks and barrels) (**Table 5**) produced by wineries located in the following seven different areas of Apulia region producing wine with Denomination of Controlled Origin: San Severo, Tavoliere, Cacc'e Mmitte, Rosso Barletta, Castel del Monte, Salice Salentino, Primitivo di Manduria. The *Brettanomyces* strains

were isolated from wines using a modified WLN agar medium (Wallerstein Laboratory Nutrient media 60 g/L, sorbic acid 0.25 g/L, trehalose 5 g/L, p-coumaric acid 100 mg/L, agar 15 g/L, cycloheximide 30 mg/L and pH 5.5). WLN plates were incubated over a period of 5-11 days at 30 °C (Oelofse *et al*., 2009). One colony was randomly selected from each contaminated sample.

Strains	Source	*Origin*	**Strains**	Source	*Origin*	**Strains**	Source	*Origin*
UniFg 1	Barrel	*Foggia*	**UniFg 17**	Tank	*Foggia*	**UniFg 33**	Barrel	*Taranto*
UniFg 2	Barrel	*Foggia*	**UniFg 18**	Tank	*Foggia*	**UniFg 34**	Barrel	*Taranto*
UniFg 3	Tank	*Bari*	**UniFg 19**	Tank	*Foggia*	**UniFg 35**	Tank	*Lecce*
UniFg 4	Tank	*Taranto*	**UniFg 20**	Tank	*Taranto*	**UniFg 36**	Tank	*Barletta*
UniFg 5	Tank	*Taranto*	**UniFg 21**	Barrel	*Lecce*	**UniFg 37**	Tank	*Foggia*
UniFg 6	Barrel	*Bari*	**UniFg 22**	Barrel	*Bari*	**UniFg 38**	Tank	*Lecce*
UniFg 7	Barrel	*Bari*	**UniFg 23**	Barrel	*Barletta*	**UniFg 39**	Tank	*Foggia*
UniFg 8	Barrel	*Lecce*	**UniFg 24**	Barrel	*Barletta*	**UniFg 40**	Barrel	*Lecce*
UniFg 9	Barrel	*Bari*	**UniFg 25**	Barrel	*Taranto*	**UniFg 41**	Barrel	*Bari*
UniFg 10	Tank	*Foggia*	**UniFg 26**	Tank	*Bari*	**UniFg 42**	Barrel	*Bari*
UniFg 11	Tank	*Foggia*	**UniFg 27**	Tank	*Foggia*	**UniFg 43**	Barrel	*Taranto*
UniFg 12	Tank	*Barletta*	**UniFg 28**	Tank	*Foggia*	**UniFg 44**	Barrel	*Lecce*
UniFg 13	Tank	*Barletta*	**UniFg 29**	Tank	*Barletta*	**UniFg 45**	Tank	*Taranto*
UniFg 14	Tank	*Taranto*	**UniFg 30**	Tank	*Foggia*	**UniFg 46**	Tank	*Barletta*
UniFg 15	Fermenting must	*Taranto*	**UniFg 31**	Barrel	*Lecce*	**UniFg 47**	Tank	*Foggia*
UniFg 16	Fermenting must	*Foggia*	**UniFg 32**	Barrel	*Taranto*	**UniFg 48**	Tank	*Barletta*

Tab. 5 Sources and Origins of isolated strains

3.2 Strains identification

Brettanomyces spp. strains were identified following two different PCR methods, which respectively employed the ITS1/ITS4 and DB90/DB394 primer pairs. The first primer set, ITS1/ITS4, was used for RFLP analysis of 5.8S rRNA gene and the two ribosomal internal transcribed spacers, according to Pham *et al*. (2011). The cells were resuspended in 100 µL of reaction mix containing 0.5 µM primers ITS 1 (5 'TCCGTAGGTGAACCTGCGG 3'), 0.5 µM

primer ITS4 (5 'TCCTCCGCTTATTGATATGC 3'), 10 µM dNTP, 1.5 mM MgCl and 1X buffer. 1 unit of Taq polymerase (Qiagen) was then added to each tube and amplification was carried out according to the following thermal cycle: initial denaturation 95 ° C for 5 minutes, 35 cycles of denaturation at 94 ° C for 1 minute, annealing at 55 ° C for 2 minutes and extension at 72 ° C for 2 minutes; final extension at 72 ° C for 10 minutes. The amplification products were digested with the restriction enzymes *Hae III*, *Hinf I*, *Cfo I* (M-Medical, Italy) and then analysed by agarose gel electrophoresis.

The yeasts RFLP profiles were identified using the yeast-ID database (CECT-IATA, Spanish Type Culture Collection, Universitat de València, València, Spain). The PCR method with the DB90/DB394 primer pair was performed as described by Cocolin *et al*. (2004).

The amplification was carried out in 50µl (final volume) of a mixture containing 10 mM Tris HCl (pH 8), 50 mM KCl, 1.5 mm $MgCl_2$, dNTP at a concentration of 0.2 mM, each primer at a concentration of 0.2 µM, 1,25 Units of Taq polymerase (Qiagen) and 2µl of DNA extract (about 100ng). The amplification parameters were as follows: initial denaturation at 94 ° C for 10 minutes; 35 cycle of denaturation at 94 ° C for 1 minute, annealing at 55 ° C for 1 minute, extension at 72 ° C for 1 minute; and a final extension at 72 ° C for 10 minutes.

In order to distinguish between *B. bruxellensis* and *B. anomalus* strains, a restriction analysis with the endonuclease *DdeI* was also performed. The *Brettanomyces*-specific PCR product was subjected to restriction analysis as described by Cocolin *et al*. (2004). The amplicons corresponding to three different strains (Unifg 14, Unifg 47, Unifg 39) were also sequenced (PRIMM, Italy) to confirm the assignment of the yeast species.

3.3 Sau-PCR analysis

Genomic DNA (200 ng) extracted from strains identified as *B. bruxellensis* was digested using *Sau3AI* restriction endonuclease, as previously described (Corich *et al*., 2005). DNA was digested overnight at 37°C and one microliter of the digestion mixture was used as template for the PCR

amplification with primer SAG1 (Campolongo *et al*., 2010). PCR products were separated on a 2% agarose gel in TBE (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, for 120 min at 120 V. Electrophoresis gels were scanned with Versadoc system (Bio-Rad, Richmond, CA, USA), converted to TIFF images, compared and analyzed with FPQuestTM software (BioRad Laboratories, Richmond, USA); then, the Sau-PCR electrophoretic patterns were grouped, and analysed for the similarity and through the Dice coefficient. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA).

3.4 Volatile phenol production

Seven representative strains of *B. bruxellensis* were cultivated in a media described by Oelofse *et al*. (2009). The base media was composed of YNB (Yeast Nitrogen Base) without amino acids 6.7 g/L (Sigma), casamino acids 3.0 g/L, biotin 0.6 g/L, thiamine 1.6 g/L and hydroxycinnamic acid precursors 100 mg/L [p-coumaric acid (p-CA) or ferulic acid (FA)]. The base medium was evaluated with different carbon source compositions. The capital letters symbolize the carbon source type: A, glucose 20 g/L and B, glucose 2 g/L and ethanol 8% (v/v). The roman numbering indicates the specific precursor added: I, p-CA; II, FA; III, p-CA and FA. The pH value was adjusted to 3.6 using o-phosphoric acid. One hundred milliliter of medium were inoculated with 100 µL of each *B. bruxellensis* strains at OD_{600}nm = 1.0. Cultures were incubated at 25°C for 7 days until late exponential/beginning stationary phase was reached. Volatile phenols production was evaluated in red wine with alcohol 13%, pH 3.8 and sterilized by filtration (0.45 µm; Millipore, France). Before direct inoculation in wine, strains were pre-conditioned in YNB media A (without p-CA and FA) added with progressive incremental amounts of sterile red wine. Briefly, 9 mL of each *B. bruxellensis* strain at OD_{600}nm = 1.0 were suspended in a medium base: red wine mixture in proportion 9:1. The ratio among inoculated base medium and red wine ratio was progressively adjusted to 7:3, 1:1 and 3:7 over a 2 days period, by incremental addition of sterile red wine.

Prior to direct inoculation in wine, cells were centrifuged at 4000 rpm and suspended in the base media to give a final OD_{600} = 0.6. Then, one milliliter of this suspension was used to inoculate 80 mL of sterile red wine added with p-CA and FA at 100 mg/L each. Incubation was carried out at 25 °C without agitation and samples were taken for volatile phenol analysis after 7 and 30 days.

3.5 Chromatographic analysis of hydroxycinnamic acids

Synthetic wine samples were extracted by liquid-liquid extraction with diethyl ether. Briefly, five milliliters of wine were adjusted to pH 2 and then extracted twice with 5 mL of diethyl ether by mixing the mixture for ten minutes. The organic phase was dried over Na_2SO_4 and then the solvent was evaporated under nitrogen. The dry residue obtained was dissolved in 0.5 mL of 1:1 methanol–water and 20 µL of each extract were analyzed by HPLC (Cabrita, Palma, Patao, and Freitas, 2011). The HPLC apparatus consisted of an Agilent 1100 equipped with a photodiode array detector. The wavelength used for quantification of hydroxycinnamic acids were 280 and 320 nm. Separation was achieved according to Li *et al*., (2008) with the following modifications: a Phenomenex-Luna column (5µ C18; 100 Å; 250 x 4.6 mm) was used and the temperature of the column was set at 30 °C. A gradient elution program was utilized with a mobile phase consisting of acetonitrile (solution A) and 1% (v/v) H_3PO_4 in water (solution B) as follows: isocratic elution, 100% B, 0-30 min; linear gradient from 100% B to 85% B, 30-55 min; linear gradient from 85% B to 50% B, 55-80 min; linear gradient from 50% B to 30% B, 80-82 min; post time, 10 min before the next injection. The flow rate of the mobile phase was 1.0 mL/min, and the injection volume was 20 µL. All hydroxycinnamic acids were quantified using a calibration curves produced using commercial standards (Sigma-Aldrich, USA).

3.6 Chromatographic analysis of volatile phenols

Extraction of free volatile phenols in wine was carried out by means of liquid-liquid extraction according to Cabrita *et al*. (2011). An Agilent gas chromatograph model 6890N coupled to a mass

selective detector model 5973 inert was used. An amount of 1µL of extract was injected in splitless mode on a DB-Wax capillary column (60m×0.25mm i.d.; 0.25µm film thickness). Oven temperature program was: 90 °C (5min) – 3 °C/min – 230 °C (25min). Injector temperature was 250 °C. Mass detector conditions were: electronic impact (EI) mode at 70 eV; scanning rate: 1 scan/s; mass acquisition range: 40–450 amu. Helium was used as carrier gas under a flow rate of 1 mL/min. Peak identifications were based on comparison of their mass fragmentation with those of pure standards and/or with those reported in the commercial library Nist98 (Agilent, USA). The quantitative analysis of positively identified compounds was performed by total ion current using the calibration curves proposed for these compounds.

3.7 Bioinformatic tools and GenBank accession numbers

Analyses of DNA were carried out using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and MEGA 4 software (Tamura, Dudley, Nei, and Kumar, 2007). The accession numbers of the D1/D2 26S rDNA sequence of the *Brettanomyces bruxellensis* Unifg 14, Unifg 47, Unifg 39 strains are KF841628, KF841629 and KF841630, respectively.

3.8 Analysis of the VBNC state

B. bruxellensis strains were grown on YPD agar at 28 °C for 5 days. VBNC studies were performed in a synthetic wine medium (10% v/v ethanol, 3 g/1 D-L malic acid, 0.01% acetic acid, 0.1 g/L potassium sulphate, 0.025 g/L magnesium sulphate, 1 g/L yeast extract, 1.5 g/L glucose, 1.5 g/L fructose) as described by Serpaggi *et al*. (Serpaggi *et al*., 2012). The pH was adjusted at pH 3.5 and the medium was sterilized by filtration through a 0.2 µm filter (Millipore, France). A YPD-grown colony was used to inoculate 10 mL of this synthetic wine, which was then incubated at 28 °C for 3 days. The biomass produced was used to inoculate one liter of synthetic wine that was incubated until a cell density of about 10^7 CFU/mL was reached. Five aliquots consisting of hundred

milliliters of this last culture were separately added with incremental amounts of 67.4% (w/v) sodium metabisulphite to respectively give a final concentration of 15.2, 23, 30, 40 and 46 mg/L of free SO_2. The above addition respectively corresponded to a final concentration of 0.4, 0.6, 0.8, 1 and 1.2 mg/L of molecular SO_2. The culture containing the middle concentration of molecular SO_2 (0.8 mg/L) was chosen as model to study the 'resuscitation' phenomenon. Cells were returned to a culturable state after the removal of sulphur dioxide from the medium. This was achieved by adding NaOH until the pH of the medium reached the value 4.0, it being the value sufficient to completely remove the molecular sulphur dioxide. All the trials were performed in duplicate.

3.9 Culturability and viability assays

In order to determinate the total viable and culturable populations, aliquots of *B. bruxellensis* cultures were removed at various times after incubation at 28 °C in synthetic wine medium. Cell culturability was assessed by a plate assay using YPD agar. The percentage of cells that were culturable was expressed in respect to total cell count. Cell viability was determined by staining with fluorescein diacetate (FDA). Cells were stained for 15 minutes with FDA, at a final concentration of 15 mM, in 0.5 M sodium phosphate buffer at pH 7. The suspension of FDA-stained cells was analyzed by flow cytometry with a Guava EasyCyte Plus SSC4C cytometer (Guava Technologies Inc., USA). Green fluorescence was read using the PMT3 detector with a 525 nm ± 30 nm bandpass filter. Measurements were processed with GuavaCytosoft software and the results expressed as the number of viable yeast per milliliter of the original sample.

3.10 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 nucleic acid extraction by using the DNA Power Soil Isolation Kit (Mobio Laboratories, Inc) following manufacturer instructions.

For the quantification of *B. bruxellensis* cells, a qPCR was performed in accordance with Phister and Mills (2003).

Initially, the cells of *B. bruxellensis* were determined in wines artificially contaminated with known concentrations of *B. bruxellensis*. From the serial dilutions of contaminated wines, DNA were extracted and subjected to amplification.

3.11 Quantitative-PCR amplification conditions

Quantitative-PCR (qPCR) amplifications were performed in a final volume of 20 µL in "Applied Biosystems 7300 Real-Time PCR System". The specific primers used were DBBRUXF (5'-GGATGGGTGCACCTGGTTTACAC-3)/DBRUXR (5'-GAAGGGCCACATTCACGAACCCCG-3) (Phister and Mills 2003), Rad 1 (GTT CAC ACA ATC CCC TCG ATC AAC) /Rad 2 (TGC CAA CTG CCG AAT GTT CTC) (Tessonnière *et al*., 2009) and Brett 1 (CGA AGA AGT TGA ACG GCC GCA TTT G)/Brett 2 (TCT TCG ATA TGC CGT CCA AAA GCT C) (Delaherche *et al*. 2004). The reaction mixture contained the following: 1X SYBR Green mastermix (Invitrogen, Milano, Italy), 300 nM of each forward and reverse primer, and 3 µL DNA. Each reaction was performed in triplicate. The reactions were run using a modified protocol (Phister and Mills 2003): 40 cycles of denaturation at 95°C for 15 sec, annealing and elongation at 75°C for 15 sec. 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 in wine. The final concentration of 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 log10 CFU and the standard curves were constructed.

3.12 Screening for tolerance to SO_2 and ethanol

In order to analyse the tolerance to SO_2 and ethanol, strains of *Brettanomyces bruxellensis* were screened in *Brettanomyces* medium (BM) (Vigentini *et al*., 2013), a defined minimal medium (Conterno *et al*., 2006) with 5% (v/v) ethanol and acidified to pH 3.6 with orthophosphoric acid, 85% (v/v). Growth tests were conducted in 96-well microplates with 0.2 mL of medium at 18 and 23°C in static condition. To avoid an excessive evaporation of the culture medium (defined as a greater than 10% v/v loss), microplate were closed with sterile lids and externally sealed with laboratory film.

Cells at approximately 0.4 OD_{650} in BM were directly inoculated at 1% (v/v) in each well. Six different concentrations of molecular SO_2 (0.1; 0.2; 0.4; 0.6; 0.8; 1mg/L) at different pH (3; 3.2; 3.4; 3.6; 3.8; 1) and six different concentrations of ethanol (9; 10; 11; 12; 13 and 14%) at different pH (3; 3.2; 3.4; 3.6; 3.8; 1).

3.13 Yeast cell wall to reduce the volatile phenols in wine

The products under investigation are several preparations of yeast cell walls (A40, A80, A40 + 40, B40, A40-50; wine before adding yeast hulls), whose effectiveness was assessed after 12 and 24h. Uninoculated wine was used as control. Volatile phenols were quantified by gas chromatography.

3.14 Use of chitosan to reduce the growth of *Brettanomyces bruxellensis*

Chitosan is a linear polysaccharide composed of two repeating units (D-glucosamine units (GlcN) and N-acetyl-D-glucosamine (GLcNAc) units) randomly distributed a long the polymer chain and linked by b(1-4)-bonds. The chitosan preparation used is a powder with particles whose diameter is lower that 50 μm, product of the deacetylation of chitin extracted from *Aspergillus niger* and produced by Lallemand (Italian branch): No Brett Inside (commercial available products).

The wine samples (50mL) were contaminated with different concentrations of *Brettanomyces bruxellensis* (10^2 to 10^6). For each contaminated wine, two concentrations of chitosan (4 g/hL and 10 g/hL) were tested and were added prior to cell inoculation. For each experiment, a control was carried out with the same inoculum in a wine without any added chitosan. The experiments were carried out at 20°C. At different times (T0, T2, T5, T9, T12), samples were streaked on YPD plates to control the growth of *Brettanomyces bruxellensis.* The whole was carried out in triplicate and results were reported as an average value of two replicates..

3.15 Inhibition of *Brettanomyces bruxellensis*

Twenty *Metschnikowia pulcherrima* strains, belonging to the Collection of Department of Agricultural Sciences, Food and Environment of University of Foggia, were evaluated for their potential antimicrobial activities. Fifty *Brettanomyces bruxellensis* strains, belonging to the same collection, were used as sensitive strains.

The *M. pulcherrima* strains were pre-grown in YPD broth at 30°C, until the logarithmic phases (Oro *et al*., 2014).

The potentially sensitive strains were pre-grown in 5mL YPD broth at 30 °C for 24 h.

Approximately 10^5 CFU/mL of all of the sensitive strains were uniformly suspended in 20 mL YPD agar and immediately poured into sterile Petri plates.

After solidification, *M. pulcherrima* strains (7 µL) were spotted on plates. The plates were incubated at 30°C for 3 days. After this time, the color of the *M. pulcherrima* colonies in the spots and the presence of inhibition halos against each sensitive strain were evaluated.

Positive antimicrobial results were visualized as the inhibition of growth of the sensitive strain, which was seen as a clear zone surrounding the seeded yeast.

3.16 Microvinification assays

It was also developed a protocol winemaking experimental means to control the development of *Brettanomyces bruxellensis*. Microvinifications were carried out in a must of Nero di Troia. The experimental protocol involved the use of various associations of indigenous strains, previously isolated, approaches in single or multi strain and various associations *Saccharomyces*- Non-*Saccharomyces* (*Candida zemplinina*, *Hanseniaspora uvarum*, *Hanseniaspora guilliermondi*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*) and Lactic Acid Bacteria (*L. plantarum*). All strains belonging to the Collection of Department of Agricultural Sciences, Food and Environment of University of Foggia, except two commercial starter of *S. cerevisiae* (EP2 Maurivin and Sauvignon Maurivin). For each test was simulated environmental contamination media from *B. bruxellensis*, obtained by inoculating the must (100mL) used for tests with a mix of seven strains of *B. bruxellensis* (later called Bretta Mix) with a final concentration of $2 \bullet 10^3$ CFU/mL. The presence of *Brettanomyces bruxellensis* with plate count were monitored to 30 days on WLN agar medium.

Yeast (*S. cerevisiae* and non-*Saccharomyces*) and bacteria (*L. plantarum*) has been inoculated in grape juice with a final concentration of $2 \bullet 10^6$ CFU/mL.

4. RESULTS AND DISCUSSION

4.1 Isolation of *Brettanomyces* yeasts in Apulian wines

A screening procedure was carried out for *Brettanomyces/Dekkera* spp. isolation from post-alcoholic fermentation of red wine. Wine samples were directly applied onto agar plates containing modified WLN medium (Oelofse *et al*., 2009). Forty-eight colonies were then isolated and putative *Brettanomyces* clones were identified by PCR- RFLP analysis of the ITS1/ITS4 rDNA fragment. The results obtained indicated that all the isolates belonged to the genus *Brettanomyces* (Esteve-Zarzoso *et al*., 1999; Pham *et al*., 2011). Restriction with either *HaeIII* or *HinfI* generated a couple of fragments with a length of 350 and 100 bp, or 250 and 200 bp, respectively (**Figure 5**). Interestingly, the res

triction analysis with *CfoI* gave two different restriction profiles, the former was composed of 3 fragments (230, 130 and 80 bp) while the latter consisted of 4 fragments (240, 120, 80 and 50 bp). These findings are in accordance with those previously reported by Pham *et al*. (2011).

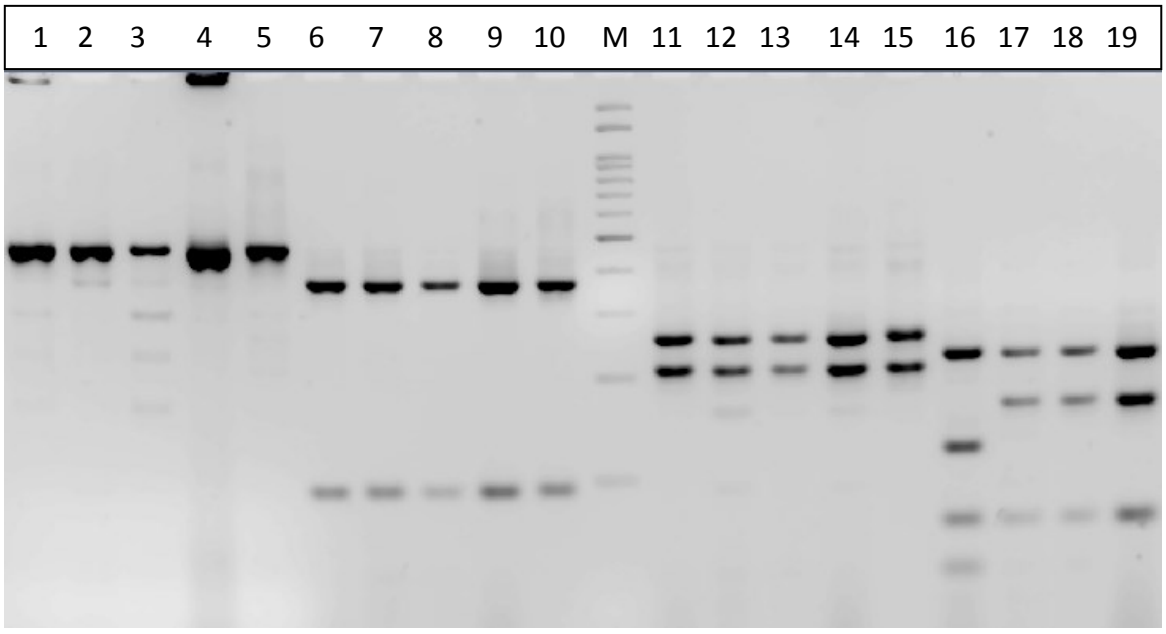


Fig 5. Analysis RFLP of the putative strains of *Brettanomyces* spp. Lane 1-5 ITS amplification product, lane 6-10 restriction analysis of the amplification product with the enzyme *HaeIII*, lane M 100 bp marker, lane 11-15 restriction analysis of the amplification product with the enzyme *HinfI*, lane 16-19 restriction analysis of the amplification product with the enzyme *CfoI*.

The restriction analysis of the amplicons obtained by the PCR assay with the specific primer pair DB90F/DB394R was performed to confirm whether the identified strains belonged to either *B. bruxellensis* or *B. anomalus* species (**Figure 6**)

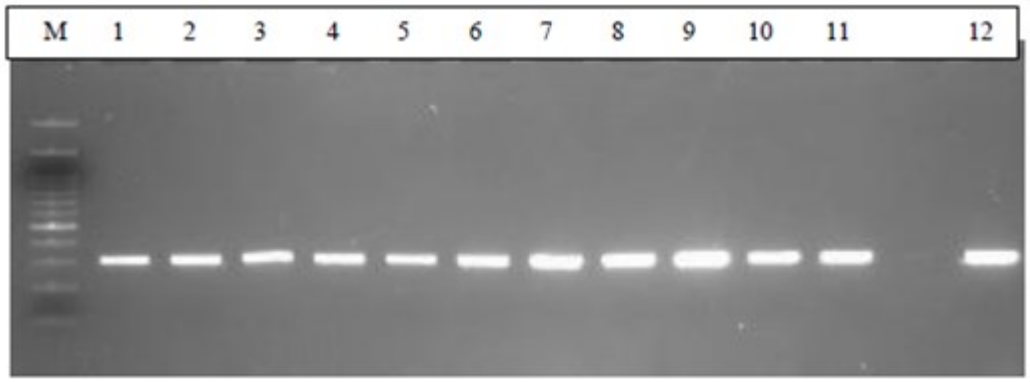


Fig 6. Amplification with primers DB90F and DB394R on genomic DNA of putative strains of *Brettanomyces bruxellensis/Brettanomyces anomalus*. M - marker 100bp.

As reported in **Figure 7**, the restriction pattern expected for *B. bruxellensis* using *DdeI* as restriction enzyme digestion, consisting in two different restriction fragments of about 154 bp and 129 bp (Figure 7), thus indicating that all the isolated strains belonged to *B. bruxellensis*. To further verify such identification, the sequences of DB90F/DB394R fragments were determined and resulted to be identical to those from *B. bruxellensis* type strain (Sturm *et al*., 2014), thus confirming that the isolates identified in Apulian wines belonged to *B. bruxellensis* species. Our findings confirmed that *B. bruxellensis* is the only species belonging to the *Brettanomyces* genus associated with the wine environment.

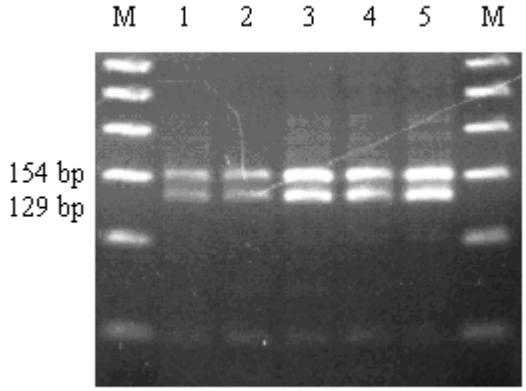


Fig. 7. Representative restriction patterns of the PCR products obtained with specific primers DB90F and DB394R produced by digestion with *DdeI*. Line 1, Unifg8; Line 2, Unifg14; Line 3, Unifg23; Line 4, Unifg29; Line 5, Unifg37. M, marker 50 bp Promega.

4.2 Biodiversity within the isolated *B. bruxellensis* strains

In the present study, a Sau-PCR approach was employed to assess the biodiversity of *B. bruxellensis* strains isolated from Apulian wines. The number of bands generated by Sau-PCR was in the range 9-16, with an average of 11 band per strain. The majority of the 48 strains showed unique Sau-PCR pattern, with only six exceptions in which identical pattern were generated (Figure 8). As shown by the dendrogram obtained by analyzing the Sau-PCR patterns (**Figure 8**), seven clusters could be differentiated at a similarity level of 70%. Two groups included 9 and 13 isolates (clusters 1 and 4, respectively). Additionally, smaller groups, which include from three to seven isolates, were observed. Among the seven clusters, only one (cluster 2, with 6 isolates) was composed of strains isolated only from cellars of the same geographical area within the Apulian region (San Severo, Foggia, Italy). Indeed, apart from such cluster, the isolates from wines produced in the same cellar clustered differently, thus highlighting a complex intra-specific biodiversity in the wine environments. Sau-PCR fingerprinting method (Corich *et al*., 2005), has been already used to define the intraspecific diversity of *B. bruxellensis* strains in wines from the

North-western of Italy (Campolongo *et al*., 2010) and our results corroborate the trends reported in that study. Indeed, Campolongo and coworkers (2010) differentiated 12 clusters and 10 individual strains (with a 70% coefficient of similarity). Nine out of the 12 clusters identified, consisted of strains isolated from the same region. *B. bruxellensis* biogeography in winemaking areas has been receiving increasing attention, often with controversial results. In cross-national studies, Conterno *et al*., (2006) found a certain linkage between clusters and geography of isolation, while Vigentini *et al*. (2012) detected a poor correlation of clusters and the geographic location of isolation. Curtin *et al*., (2007), studying biodiversity in 31 winemaking regions of Australia, highlighted the presence of three genotypes found across multiple winemaking regions. At the regional level, Campolongo *et al*. (2010), highlighted a good correlation of genetic clusters with region of isolation (Piedmont/Liguria, Italy). Studying intraspecific biodiversity at level of winemaking areas, we found, according to Campolongo *et al*. (2010), that strains isolated from the same area could be grouped into different clusters (with one exception), suggesting a high biodiversity between strains of *B. bruxellensis*. As observed by Curtin *et al*. (2007), these controversial findings might be explained by potential vectors (e.g. wine transported between wineries located in different geographic regions, insects), but also by adaptation phenomena to the wine environment in multiple location.

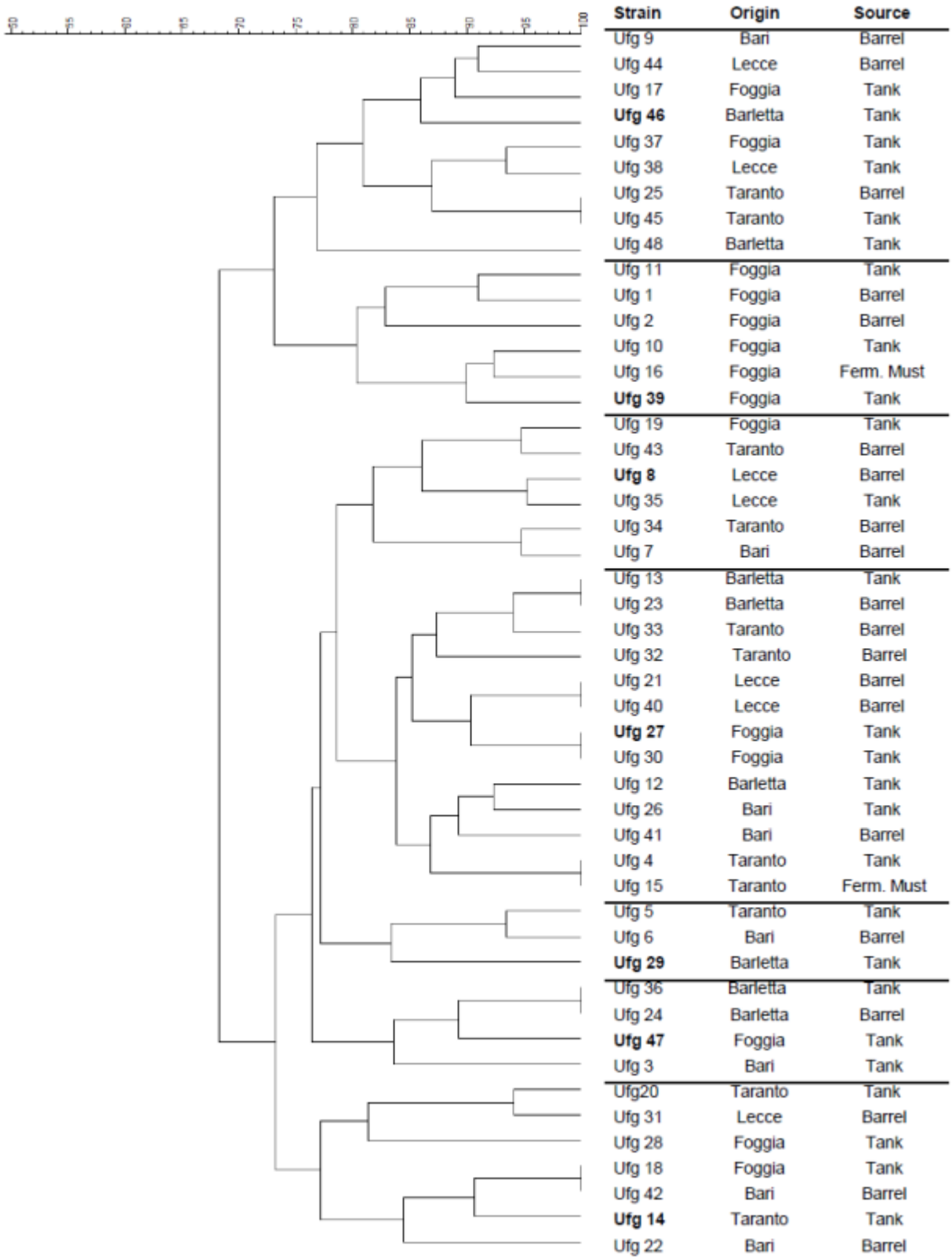


Fig 8. Dendrogram from Sau-PCR profiles The relationship between the isolated strains of *B. bruxellensis* is reported. The strains used for further analysis are in bold and underlined

4.3 Volatile phenol production

With the aim to analyze the ability of the identified isolates to produce volatile phenols, seven strains, representative of each of the clusters obtained by Sau-PCR analysis were chosen: Unifg 8 = strain A, cluster 3; Unifg 14 = strain B, cluster 7; Unifg 39 = strain C, cluster 2; Unifg 47 = strain D, cluster 6; Unifg 29 = strain E, cluster 5; Unifg 27 = strain F, cluster 4; Unifg 46 = strain G, cluster 1. Strains A, B, C, D, E, F and G of *B. bruxellensis* were inoculated both in wine and in synthetic media and samples were analyzed for volatile phenol content after 7 (synthetic media and wine) and 30 days (wine) (Figure 9). After seven days, in the (negative) controls, we found a 4-etylphenol (4-EP)/4-ethylguaicol (4-EG) content of 120/100 µg/L (synthetic medium AI), 100/83 µg/L (synthetic medium AII), 100/86 µg/L (synthetic medium AIII), 11/100 µg/L (synthetic medium BI), 100/110 µg/L (synthetic medium BII), 100/88 µg/L (synthetic medium BIII), and 78/11 µg/L (wine). In the basic medium supplied with glucose (20 g/L) and p-coumaric acid (p-CA, 100 mg/L), all the analysed strains produced 4-etylphenol in a range of 1410 µg/L and 1900 µg/L, after seven days of incubation. Strain E produced the greatest amounts of ethyl phenols (1900 µg/L). All strains were able to produce 4-ethylphenol and to transform the added p-coumaric acid (**Figure 9**). Strain B was able to transform all the added p-coumaric acid into 4-ethylphenol.

In synthetic medium BI (containing 2 g/L glucose, 8% (v/v) ethanol, and 100 mg/L p-coumaric acid) the production of 4-ethylphenol was lower than in media A, although, even in these conditions, all strains were able to produce 4-ethylphenols but not all the p-coumaric acid was transformed. This finding is in agreement with Dias *et al*. (2003) and Oelofse *et al*. (2009), who both showed that the maximum conversion rates of p-coumaric acid to 4-ethylphenol is lower in the ethanol-containing media. In contrast, when the precursor of volatile phenol was ferulic acid, the production of 4-ethylguaiacol in media A was not different from that observed in media B. Indeed, in some cases, such as for strain C, the production of 4-ethylguaiacol increased in presence of ethanol, i.e. 2750 µg/L and 3760 µg/L in media A and B, respectively (**Figure 9**). It was interesting to observe that the analysed strains produced different levels of volatile phenols and transformed

different amounts of their precursor. Such variability was much more evident when both precursor of volatile phenol, i. e. p-coumaric acid and ferulic acid, were simultaneously added to the media). In this case, the production of volatile phenols ranged from 1140 µg/L to 3030 µg/L.

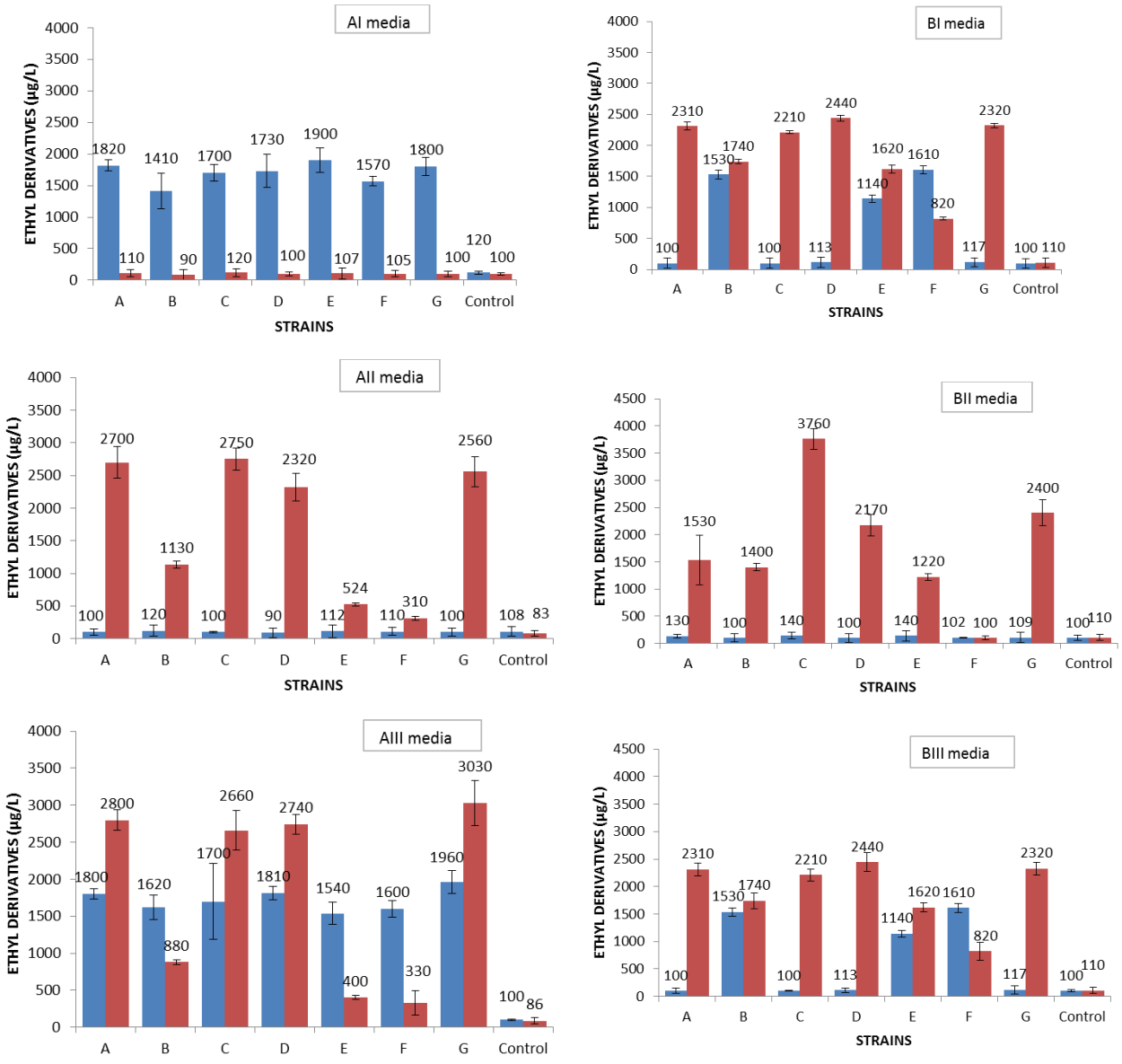


Fig 9 Volatile phenol analysis of *B. bruxellensis* strains in AI medium (containing glucose and 100 mg/L p-coumaric acid), AII medium (containing glucose and 100 mg/L ferulic acid), AIII medium (containing glucose and 200 mg/L of p-cumaric acid and ferulic acid together); BI medium (containing glucose-ethanol and 100 mg/L p-coumaric acid), BII medium (containing glucose-ethanol and 100 mg/L ferulic acid) and BIII medium (containing glucose-ethanol and 200mg/L of p-cumaric acid and ferulic acid). The values are average of three independent replicates. Blue, 4-ethyl phenol and red, 4-ethylguaiacol.

The wines inoculated with strains D and F showed a similar volatile phenol profile seven days after inoculation, with a production of 4-ethylphenol of 100 µg/L and 114 µg/L, respectively (**Figure 10**). In contrast, 4-ethylguaiacol was undetectable seven days after inoculation. When incubation was extended to 30 days, a significantly increased production of 4-ethylphenol was observed. (**Figure 10**). For instance, when testing strain D, a production of 100 µg/L and 1200 µg/L 4-ethylphenol was observed after seven and thirty days from inoculation, respectively. The same trend was recognized for strain A, with a production of 540 µg/L 4-ethylphenol seven days after inoculation, while an increased level of 4-ethylphenol (1539 µg/L) was measured thirty days after inoculation. Strain A produced greater amounts of 4-ethylphenols compared to the other strains, thus overcoming the detection threshold of 425 µg/L, as stated by Chatonnet *et al*. (1995). Indeed, strain A was likely to reproduce the situation that can be usually found in a wine that is naturally contaminated by *Brettanomyces*. Concerning minor differences in spoilage potential, as reported in **Figure 10**, strains D and F produced similar amounts of 4-ethylphenol after 7 days whereas after 30 days, strain D produced significant higher amount of 4-ethylphenol compare with strain F. These differences in spoilage potential were observed also with strains A and B. In the case of the strain B and E, after 30 days, we registered an increase in the production of 4-ethylphenol as reported in **Figure 10**. In contrast to 4-ethylphenol, low concentrations of 4-ethylguaiacol were produced by all the analysed strains. Very low concentrations of 4-ethylguaiacol were produced by all the analysed strains, even after 30 days of incubation. Overall, even if with tangible differences, all the analysed strains displayed a considerable 'spoilage potential' in reason of their 4-etylphenol/4-ethylguaiacol production.

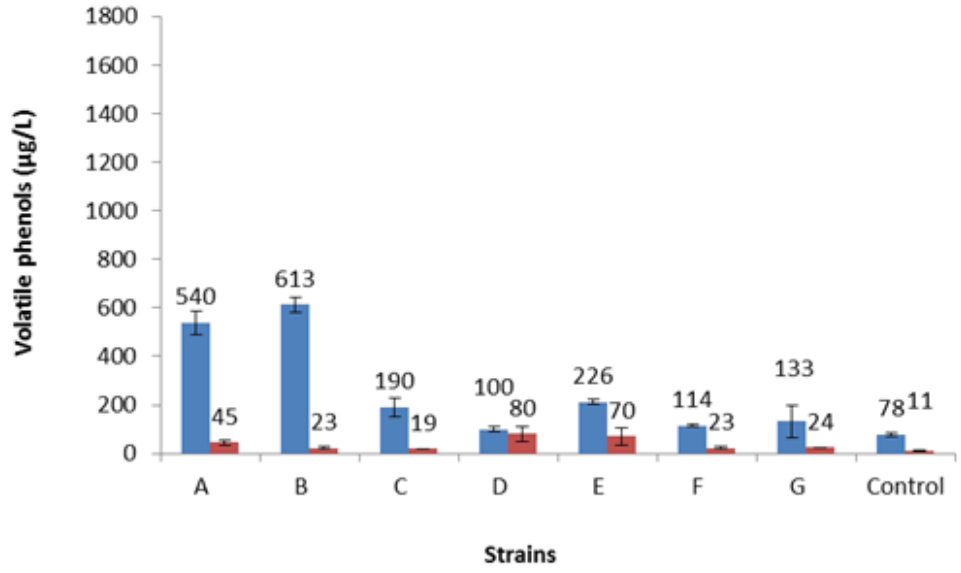


Fig. 10a Volatile phenol analysis of *B. bruxellensis* strains in wine after 7 days. The values are average of three independent replicates. Blue, 4-ethyl phenol and red, 4-ethylguaiacol.

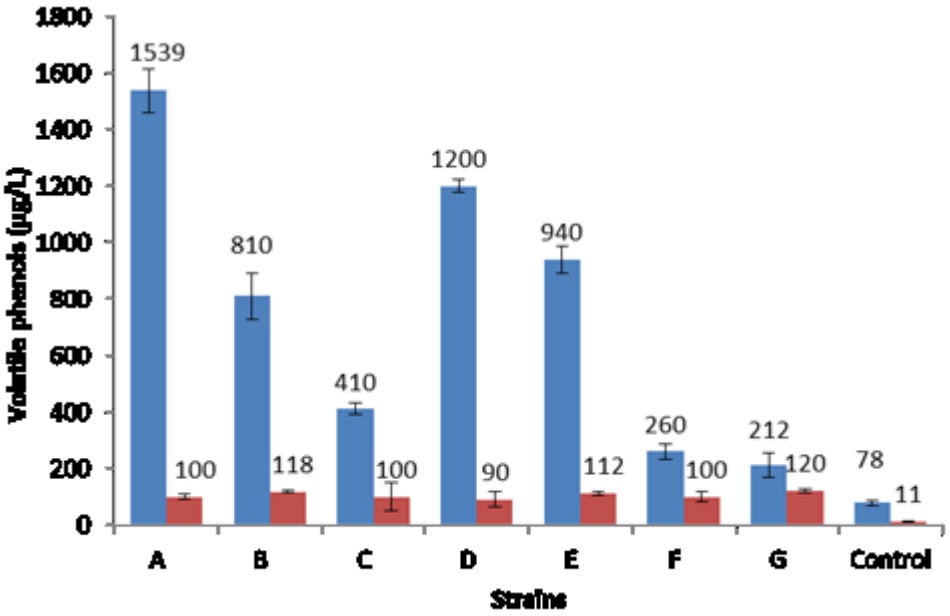


Fig. 10b Volatile phenol analysis of *B. bruxellensis* strains in wine after 30 days. The values are average of three independent replicates. Blue, 4-ethyl phenol and red, 4-ethylguaiacol.

4.4 VBNC analysis

In order to investigate the correlation between *B. bruxellensis* strain diversity and its VBNC state in wine, we analysed the VBNC behaviors of seven strains representative of intraspecific biodiversity of *B. bruxellensis* isolated from Apulian (Southern Italy) wines (Di Toro *et al*., 2015). To trigger the VBNC physiological state in *B. bruxellensis*, SO_2 was used as 'inducing' factor under wine-like stress conditions (Agnolucci *et al*., 2010; Divol *et al*., 2005; du Toit *et al*., 2005; Serpaggi *et al*., 2012). Total and viable cells were observed by flow cytometry and viable and culturable cells were monitored by colonies counting on YPD-agar medium.

All seven strains analysed by flow cytometry remained viable for the entire experiment with no statistical no statistically significant difference among the strains: from this point of view, all the losses in culturability (**Figure 11**) provided a measure of VBNC phenomena.

When cultured in a wine-like medium in absence of SO_2, a slight decrease in culturability is observed: all the strains showed a slight entrance in VBNC state, probably due to ethanol content and starvation condition (**Figure 11**; blue lines). It appeared clear the role of SO_2 as 'VBNC inducer': increasing concentrations of this molecule in the medium increase the number of non-culturable cells (**Figure 11**). Moreover, it is important underline that this sensitivity varied in a strain-dependent manner. About the 'resuscitation' character of these strains, they were differently able to exit from the VBNC state. The ability of cells to exit from this state was investigated choosing as model the 0.8 mg/L concentration of molecular SO_2, which was reduced to 0 by increasing the pH from 3.5 to 4.0 (Salma *et al*., 2013; Serpaggi *et al*., 2012). One day after removal of the stress, all the strains showed a concentration of culturable cells ranging from 10^3 to 10^4 CFU/mL, which was lower than that observed before the entry in the VBNC state. We found that the strain A was culturable at a concentration of molecular SO_2 equal to 0.4 mg/L until 10 days (**Figure 11**). At 0.6 mg/L of molecular concentration of SO_2, culturability was lowered to $2.60 \bullet 10^2$ CFU/mL after only 2 days and decreased at eighth day, until reached a concentration of 10 CFU/mL. Complete loss of culturability was observed after three days at either 0.8 and 1.2 mg/L

SO_2 concentrations. However, the removal of SO_2 allowed an immediate resuscitation, with a recover of culturability that reached up to $3•10^2$ CFU/mL (**Figure 11**). Strains B and C remained cultivable at a concentration of molecular SO_2 equal to 0.4 mg/L and 0.6 mg/L until 10 days. After stress was removed, there is a recovery phase in which the strain B reached an high level of culturability ($5.97•10^5$ CFU/mL) (**Figure 11-B**). Strain C immediately lost the culturability at a concentration of 1.2 mg/L of molecular SO_2, already after two days, after three days at 1 mg/L of SO_2 and after 10 days at a concentration of 0.8 mg/L, then it returned culturable after the SO_2 (0.8 mg/L concentration of molecular SO_2) was removed (**Figure 11-C**). In contrast, strain D was cultivable only in presence of 0.4 mg/L of SO_2 ($2.•10^4$ CFU/mL). Concentrations of molecular SO_2 equal to 0.6, 0.8, 1 and 1.2 mg/L, made the strain non-culturable after only two days. However, even in this case, removing the stress allowed the strain to return in a culturable state ($7.51•10^4$ CFU/mL) (**Figure 11-D**). The strains E and F were culturable, until 10 days, in presence of molecular SO_2 equal to 0.4 or 0.6 mg/L. Culturability was already lost after three days at a concentration of 0,8 mg/L, 1 mg/L and 1,2 mg/L of SO_2. A resumption of culturability up to a value of $1.02•10^4$ CFU/mL and $1.10•10^4$ for strains E and F, respectively, was observed after the SO_2 stress (0.8 mg/L concentration of molecular SO_2) was removed **(Figure 11-E** and **F**). Finally, strain G remained culturable in presence of molecular SO_2 equal to 0.4 mg/L and 0.6 mg/L up to $2.65•10^2$ CFU/mL and $2.87•10^1$ CFU/mL respectively after 10 days and it lost culturability after 9 days at the concentration of 0.8 mg/L and after 8 days at the concentration of molecular SO_2 equal to 1 mg/L and 1.2 mg/L. The strain returned in a culturable form (up to $1.30•10^4$ CFU/mL) after the stressing condition (0.8 mg/L concentration of molecular SO_2) was eliminated (**Figure 11-G**).

The aptitude of *B. bruxellensis* to survive when exposed to wine-like stress conditions entering a viable but not culturable state (VBNC) has already been described by several authors (Agnolucci *et al*., 2010; du Toit *et al*., 2005; Fleet 1999; Palkova *et al*., 2006; Serpaggi et al., 2012).

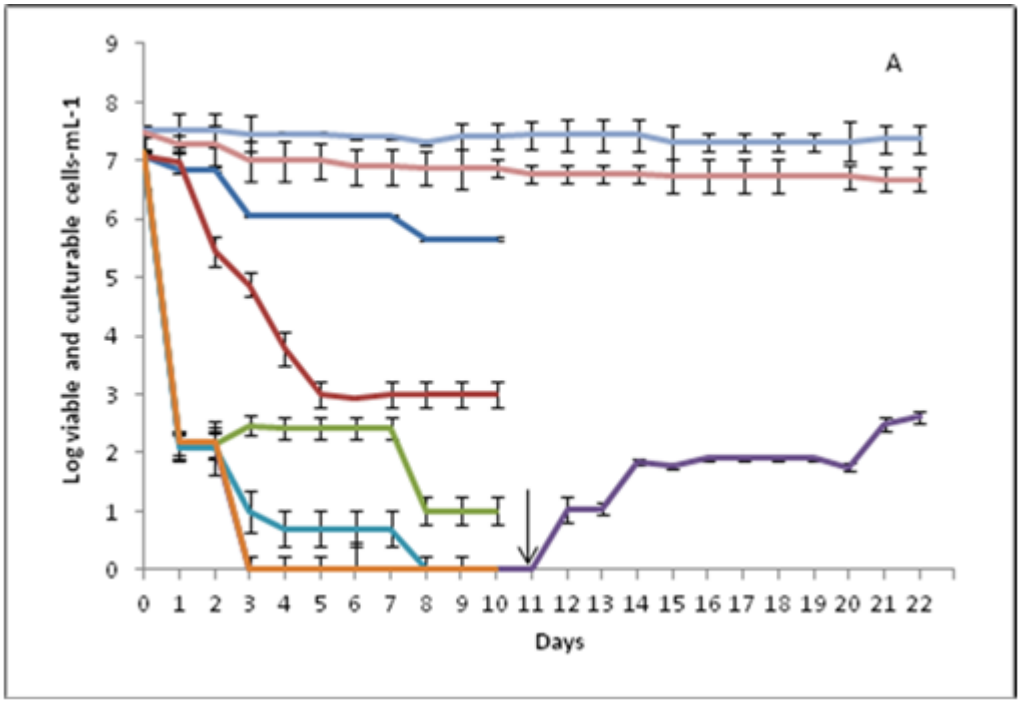
When cultured in a wine-like medium in absence of SO_2, all the strains showed a slight entrance in VBNC state, probably due to ethanol content and starvation condition. Serpaggi and co-workers

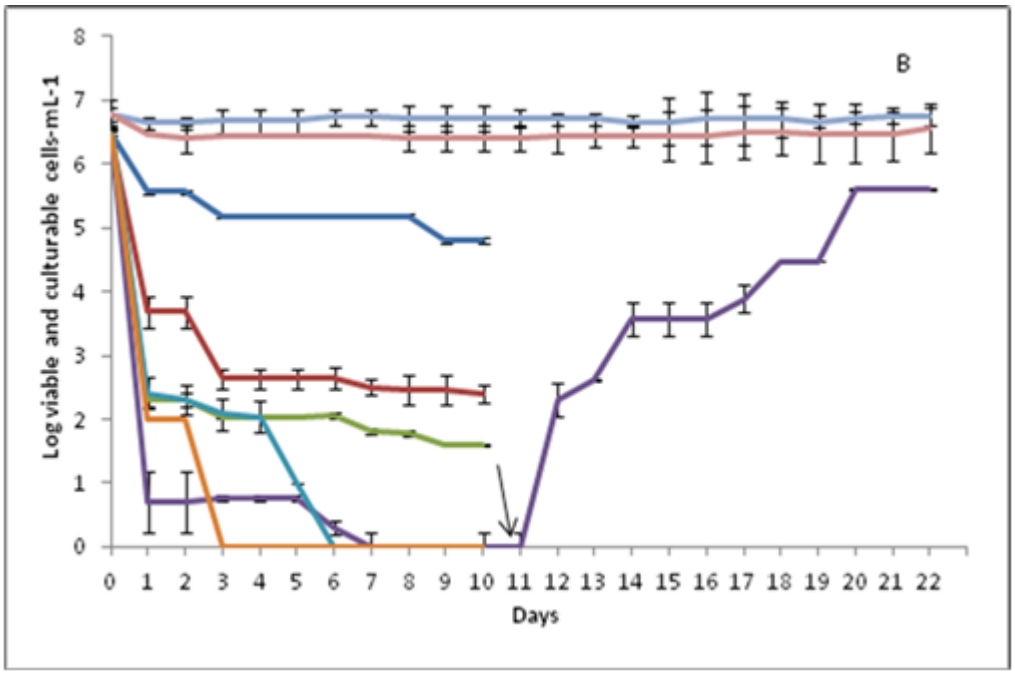
(Serpaggi *et al*., 2012), analysing the VBNC behaviour of *B. bruxellensis* strain LO2E2, did not describe this phenomenon. This finding could be explained by a strain dependent sensitivity to wine-like stress factors caused by the synthetic medium. In presence of SO_2, the pattern changed in a strain-dependent manner and this was strictly related to the molecular SO_2 concentration. Vigentini and collaborators (Vigentini *et al*., 2013), analyzing 108 strains of *B. bruxellensis*, highlighted strain-dependent sensitivity to the SO_2 concentration. In accordance, we observed that the strains analysed have different VBNC behaviour as a function of molecular SO_2 concentration, corroborating the evidence of a strain-dependent sulphite sensitivity character within the *B. bruxellensis* species. The 'resuscitation' character has been widely debated (Bogosian *et al*., 2001; Bogosian *et al*., 1998; Nyström 2003). According to these authors, the recovery of culturability is due to the presence and sudden growth of a few residual cells with a normal metabolism in a population predominantly non- culturable. Recently, Salma et al. (Salma *et al*., 2013) demonstrated that the removal of environmental stresses was sufficient to induce the exit from the VBNC state. Indeed, the recovery of the culturable cultivable state was shown as a true 'resuscitation' character and not a simple growth of a few residual cells with a normal metabolism.

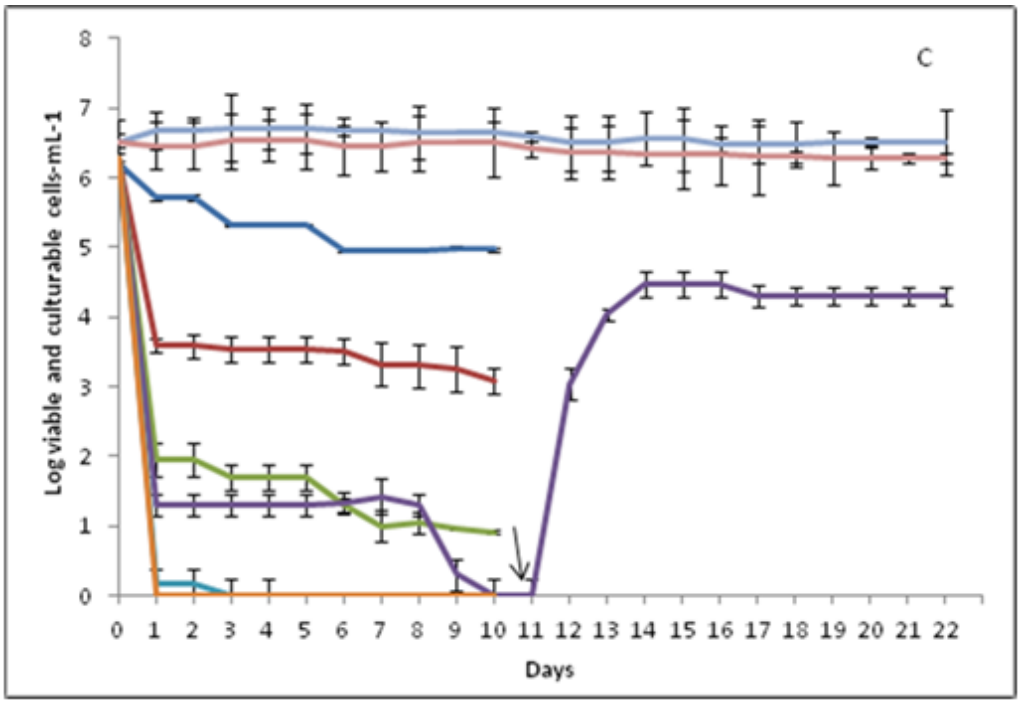
We found that the ability of the analysed *B. bruxellensis* strains to enter in a VBNC state and even to 'resuscitate' is greatly flexible. Our findings are in accordance with other studies (Agnolucci *et al*., 2010; du Toit *et al*., 2005; Serpaggi *et al*., 2012) on the variability of strains sensitivity to SO_2. In addition, our results confirm the partial evidences reported by Zuehlke and Edwards (2013) and by Agnolucci *et al*. (2014) on the strain-dependent variability of VBNC state. We reported, for the first time, conclusive evidence that VBNC behaviour in *B. bruxellensis* varies in a strain-dependent manner, as a function of molecular SO_2 concentration. To the best of our knowledge, it is the first demonstration of VBNC state as a strain-dependent character in yeast. This variability concern several phases of VBNC state: entrance in VBNC state, rapidity of the entrance, influence of molecular SO_2 concentration, percentage of 'resuscitation'. Moreover, our findings may explain an apparent dichotomy: Divol and Lonvaud-Funel (2005) found that SO_2 removing was not sufficient

to exit from the VBNC, while Serpaggi *et al*. (2012) reported a complete ability of *B. bruxellensis* strains to recover culturability after stressor was removed. In fact, the strain-dependent character of 'resuscitation' percentage shed new light on this phenomena. Our results confirm the high level of phenotypic polymorphism existing in *B. bruxellensis* species, already evidenced at the level of sulphite sensitivity/tolerance (Curtin *et al*., 2012; Vigentini *et al*., 2013).

Our outcomes clearly indicate VBNC behaviour as a new key standard in the characterization of spoilage potential of *B. bruxellensis* strains. From an industrial point of view, the results are important to design physicochemical and biotechnological strategies for *B. bruxellensis* control in regional wines.




B
Log viable and culturable cells-mL-1
Days


C
Log viable and culturable cells-mL-1
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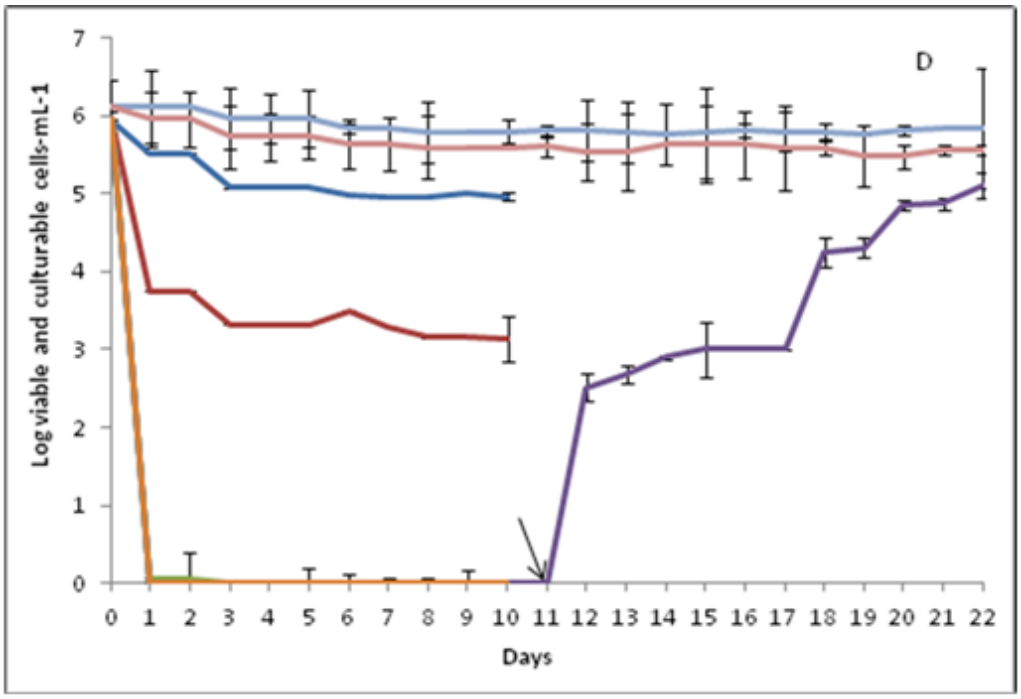
D
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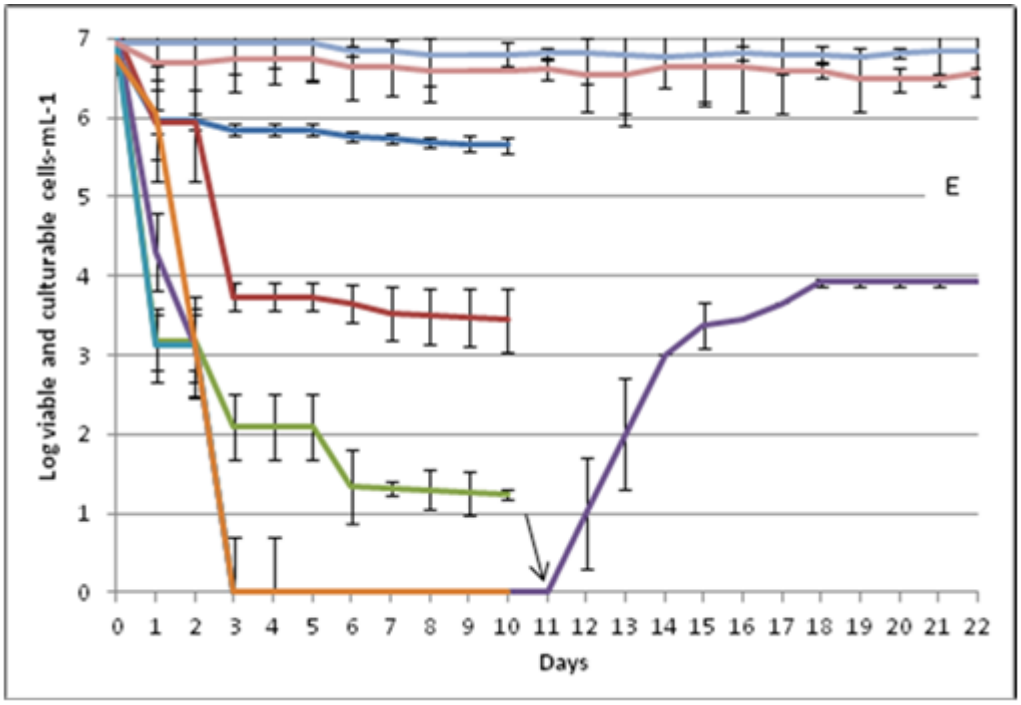
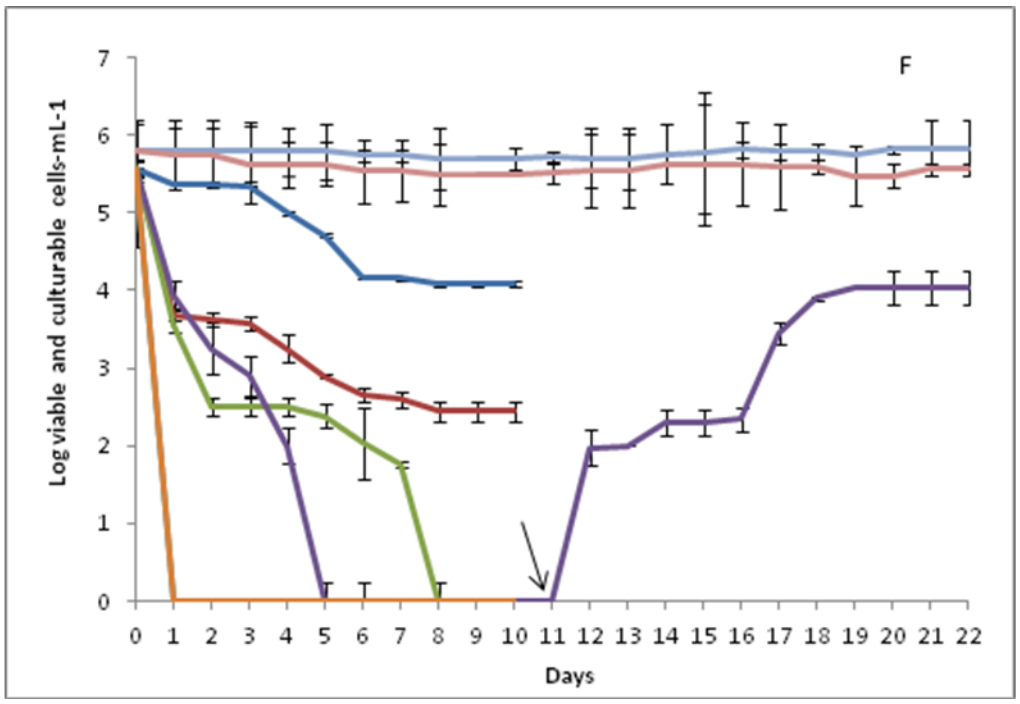

E
Log viable and culturable cells-mL-1
Days



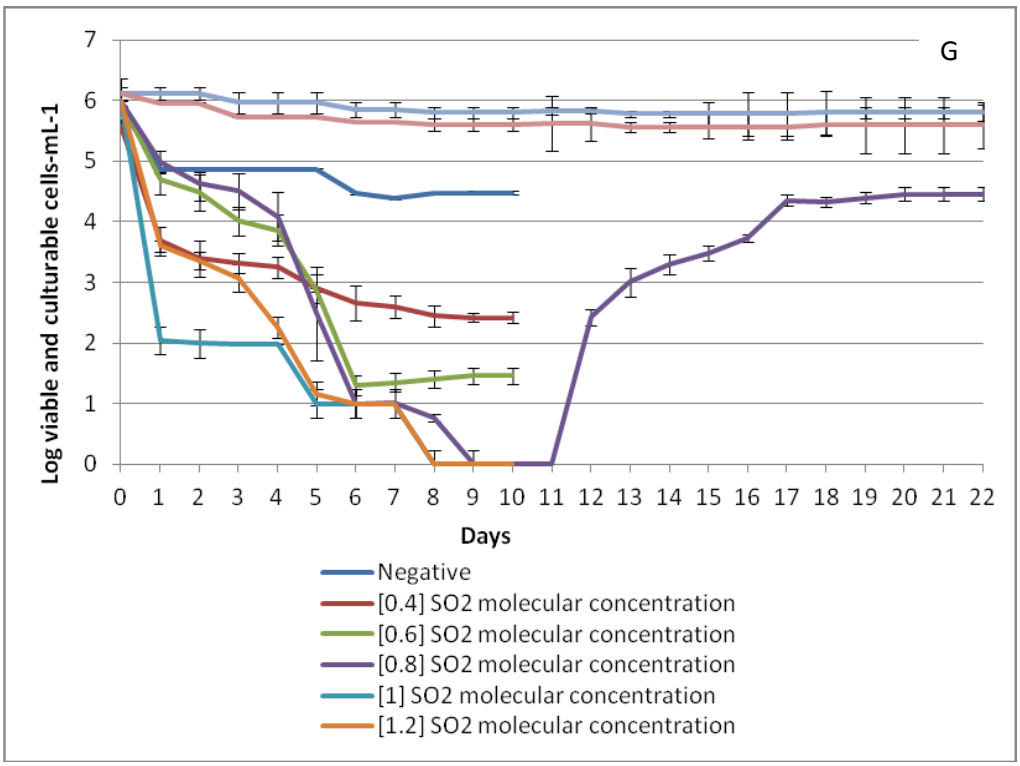


Fig. 11 Total, viable and culturable cells of samples A, B, C, D, E, F and G monitored by flow cytometry and colonies count on YPD-agar medium, at different concentrations of molecular SO_2 added to 10^7 cells/mL in stationary phase. The arrow indicates the point in which the SO_2 was removed by pH increase to 4.0. The values reported are the means of two independent experiments.

The differents lines indicated the different growth curves of *B. bruxellensis* in absence and in presence of different concentrations of molecular SO_2, as listed in the legenda at the end of "G" graphic, which can be applied also to the other graphics.

4.5 Rapid identification of *Brettanomyces bruxellensis* in wine

Fast and sensitive methods are needed to detect microorganisms in wine, thus allowing operators to control the dynamics of the process and to avoid phenomena of spoilage. It is known that traditional microbiology techniques do not allow determining the cells of *Brettanomyces bruxellensis*, which are often non-cultivable on standard laboratory media, although they are present and metabolically active.

In the first phase we extracted and quantified DNA from pure culture of *B. bruxellensis*. The concentration of the extracted DNA was measured by reading absorbance at 260 nm in a spectrophotometer.

DNA concentration of 50.5 ± 1.2 mg / mL (mean of three replicates) was measured. Subsequently we staged, as mentioned, a series of seven decimal dilutions from extracted DNA (in duplicate). This series was used for a preliminary method evaluation, in terms of sensitivity and range of cell concentration that the system primer probe is able to detect.

We succeeded in constructing the standard curve (**Figure 12**) using the data that relate the threshold cycle with the concentrations of DNA standard. This curve shows on the abscissa the logarithm of the concentration of DNA (known concentration) and in ordinate the Ct value. The calibration curve thus obtained related the threshold cycle with the DNA concentration of *B. bruxellensis* present in the reaction.

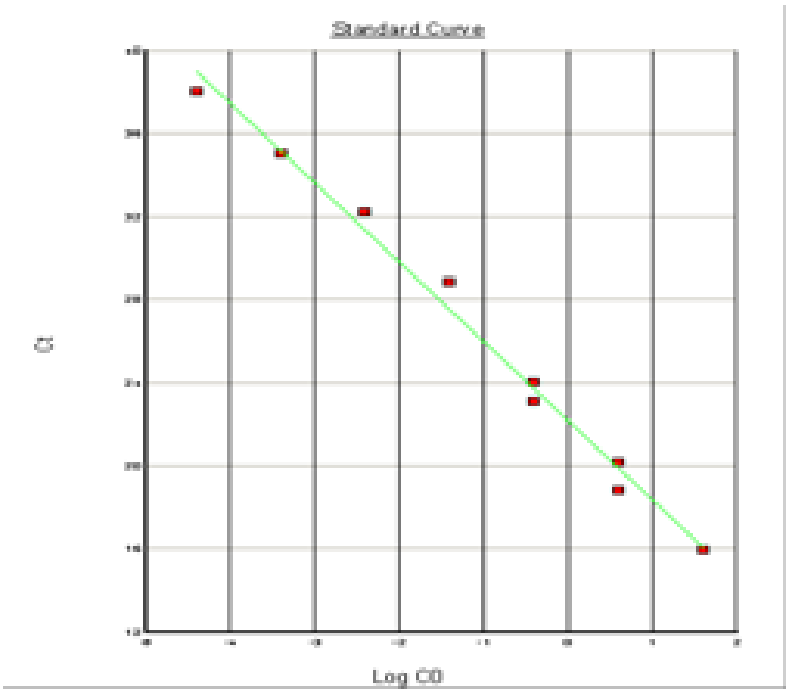


Fig 12 Standard curve for calibration of the QPCR method. In abscissa the logarithm of the DNA concentration of the standard (µg/mL) and in ordinate the cycle threshold (Ct).

Initially, the determination of *B. bruxellensis* was performed on samples of wine artificially contaminated with specific concentrations of a *B. bruxellensis* strain. From the serial dilutions of artificially contaminated wines, we extracted the respective genomic DNA, which were subjected to amplification. In **Table 5**. data obtained from samples of DNA extracted from wine are shown.

Sample	Ct	DNA Concentration (ng/µL)	UFC/mL
A	18,07	10,51	376
B	17,64	10,40	372
C	18,79	10,70	382
D	18,05	10,51	375
E	18,27	10,57	378
F	18,69	10,68	381
G	35,06	0,0004	/
H	38,02	0,00004	/

Tab 5. Parameters related to the wine samples (Ct, concentration of DNA and the corresponding concentration of cells of *B. bruxellensis*)

Primers Rad1 and Rad2 were found to be the most accurate for the identification of *B. bruxellensis* cells. Primer pairs Brett1/Brett2 and DBrux R/DBrux F were eliminated because their aspecifity.

4.6 Tolerance of *Brettanomyces bruxellensis* to SO_2 and ethanol

The most effective way to prevent *B. bruxellensis* spoilage in wine is to control its development and ethylphenols' production, through a precautionary approach to winemaking management. Therefore, understanding the factors that control the growth of this microorganism during wine production is crucial in order to improve the design of microbial resources and reduce the development of *B. bruxellensis* in wine. The main 'stressors' in wine are usually low pH value, high ethanol concentration, low temperature and presence of sulfites (SO_2). Thus, we evaluated the growth of *B. bruxellensis* at 18 ° C and 23 ° C, using different ethanol [range 9-14%] concentrations and pH value [range 3.0-4.0]. Furthermore, the effect of SO_2, [range 0.1-1.0 mg/L] and pH [range 3.0-4.0] was analysed. In general, tolerance to wine stressors was strains-specific (see **Figure 13, 14**, **15** and **16**). pH strongly influenced the growth of the strains analysed with no growth observed at pH 3 (data not shown). Some strains were already sensitive to low concentrations of SO_2 (**Figure 13**), while others were able to grow even at higher SO_2 concentrations (**Figure 14**). As reported in **Figure 15** and **16**, ethanol influenced growth of *B. bruxellensis* strains mainly affecting the duration of the lag phase and the respective entry in the exponential phase.

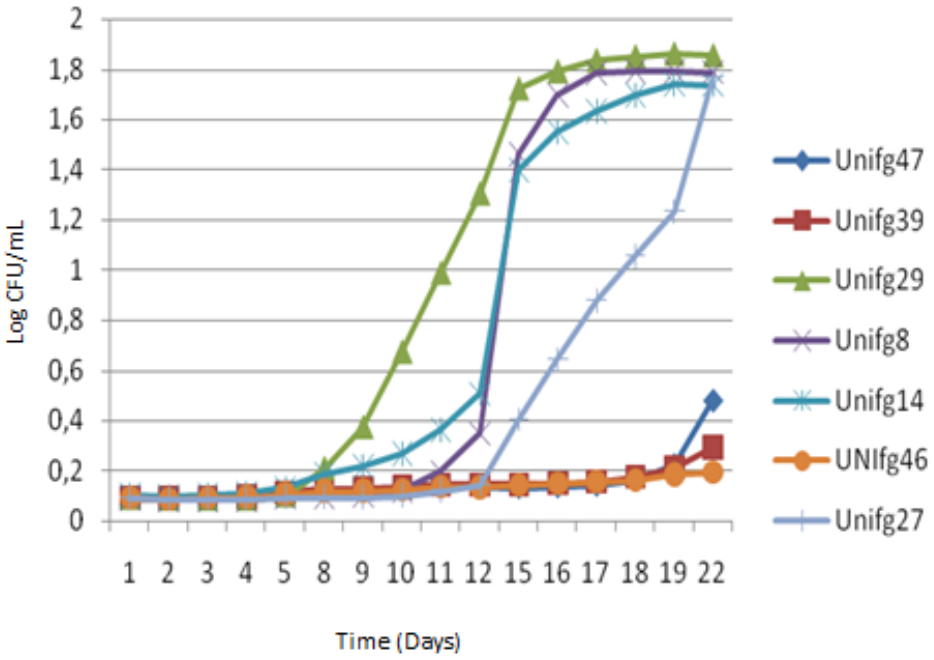


Fig 13. Growth curves of *B. bruxellensis* strains in medium at pH 3.4 and a concentration of SO_2 molecular equal to 0.2 mg / L

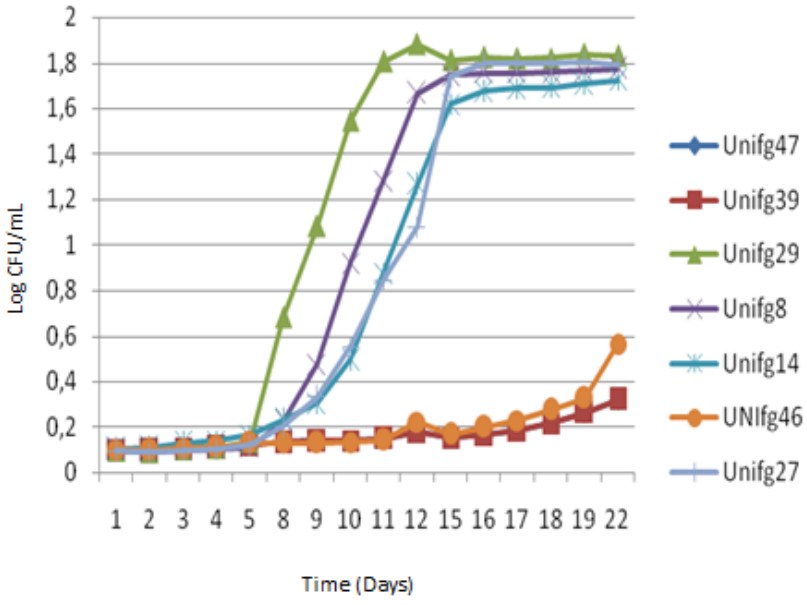


Fig 14. Growth curve of *B. bruxellensis* strains in medium at pH 3.4 and a concentration of SO_2 molecular equal to 0.2 mg / L

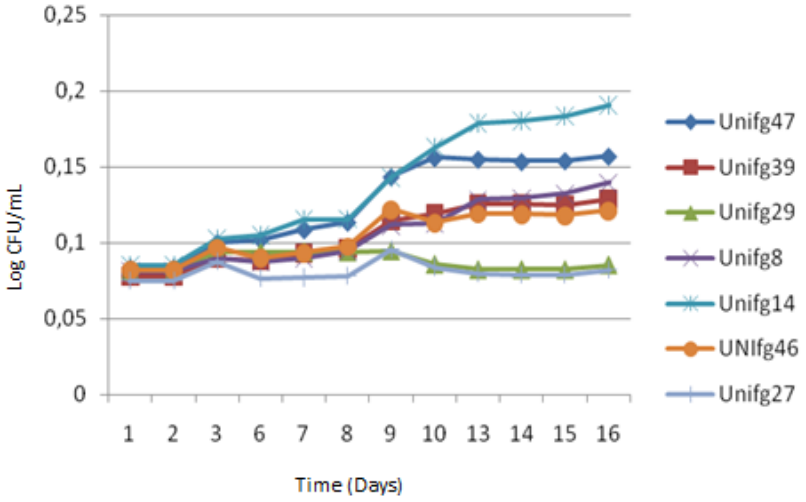


Fig 15. Growth curves of *B. bruxellensis* strains in medium at pH 3.2 and a concentration of ethanol equal to 9%

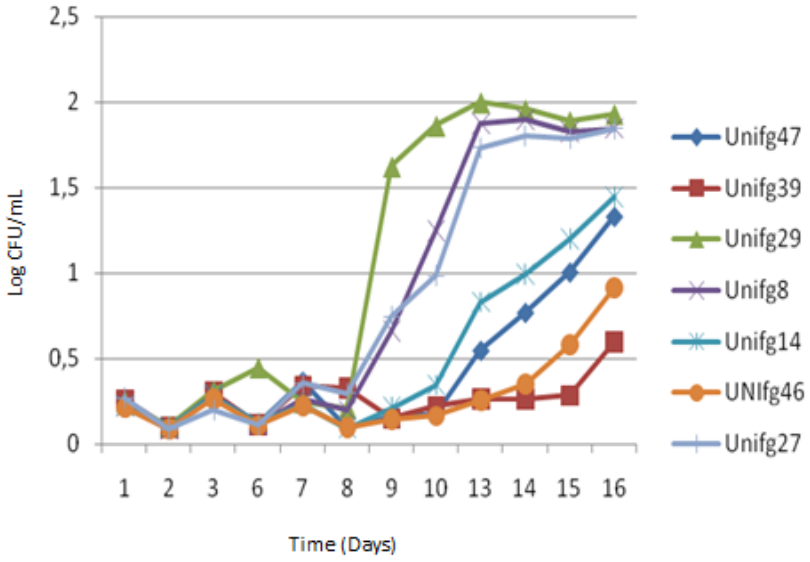


Fig 16. Growth curves of *B. bruxellensis* strains in medium at pH 3.2 and a concentration of ethanol of 12%

4.7 Reduction of volatile phenols in wine by yeast cell wall.

Ethylphenols, 4-ethylphenol and 4-ethylguaiacol, are molecules produced by yeasts of the genus *Brettanomyces* spp. If they are present in wines in concentrations above the threshold of perception, they determine the appearance of characteristic and unpleasant odours (defined as animal and phenolic odours). These substances are produced starting from hydroxy-cinnamic acids (p-coumaric and ferulic), odourless precursors in the grapes.

In order to develop some corrective actions, the use of technological approaches commercially available (e.g. yeast hulls) and their ability to reduce the ethylphenol formed were analysed. The products investigated were several preparations of yeast cell walls (A40, A80, A40 + 40, B40, A40-50; whose effectiveness was assessed after 12 and 24h.

All wines samples were subjected to a determination of ethylphenols, using the gas chromatograph GC-MS, preceded by a step of samples extraction using SPE method (solid phase extraction). As reported in Table 6 and 7 and in **Figure 17**, a decrease of ethylphenols analysed was observed in samples where the cell-wall preparation were used, decreased compared to the controls either after 12 h or after 24 h of treatment. The cell-wall preparation 50-A40 (circled in **Figure 17**), was found to be the best solution; in fact a 12 h of treatment, resulted in a complete removal of 4-ethylguaiacol (4-EG), and a reduction of approximately 26% of 4-ethylphenol (4-EP), while a 24 h treatment reduced 4-EG and 4-EF respectively of 28% and 47% compared to 24 h control. Similar considerations should be made for the A80 cell-wall preparation; A40 + 40; B40, and during 12 h treatment, reduced 4-EP and 4-EG respectively of 79% -97% -94% compared to 12 h control. Finally, we can observe that the yeast cell walls exert an adsorbing action against lipophilic species, such as ethylphenols, favouring a partial or total reduction of molecules responsible for off-flavours. According to Nieto-Rojo *et al*., (2014) 4-Ethylguaiacol and 4-ethylphenol show a fast sorption process, indicating that they become fixed to the functional groups of the molecules which make up the surface of the yeast walls. Vasserot *et al*., (1997) also reported a rapid process of anthocyanin sorption by yeast lees, and this would confirm that on the yeast walls exist surface

groups capable of fixing different substances. Similar kinetics were obtained by Aksu and Donmez (2003) for several yeasts used as sorbents, and various organic compounds suggest that the uptake of hydrophobic solutes (such as 4-EG and 4-EP) occurs predominantly on the surface.

Samples		4 ethylguaiacol	4 ethylphenol
Controll 12h	**µg/mg**	0,046	0,249
	±Ds	0,006	0,144
A40 12h	**µg/mg**	0	0,129
	±Ds	0	0,017
A80 12h	**µg/mg**	0,037	0
	±Ds	0,012	0
A40+40 12h	**µg/mg**	0,045	0
	±Ds	0,021	0
B40 12 h	**µg/mg**	0,043	0
	±Ds	0,022	0
50-A40 12h	**µg/mg**	0	0,064
	±Ds	0	0,009
Wine before yeast walls addition	**µg/mg**	0	0,639
	±Ds	0	0,086

Tab 6 Yeast wall preparations used during 12 h treatment

Samples		4 ethylguaiacol	4 ethylphenol
Control 24h	**µg/mg**	0,094	0,220
	±Ds	0,017	0,046
A40 24h	**µg/mg**	0,041	0,154
	±Ds	0,010	0,009
A80 24h	**µg/mg**	0	0,129
	±Ds	0	0,021
A40+40 24h	**µg/mg**	0,066	0,286
	±Ds	0,010	0,081
B40 24h	**µg/mg**	0,084	0,281
	±Ds	0,011	0,030
50-A40 24h	**µg/mg**	0,026	0,104
	±Ds	0,006	0,008

Tab 7 Yeast wall preparations used during 24 h treatment

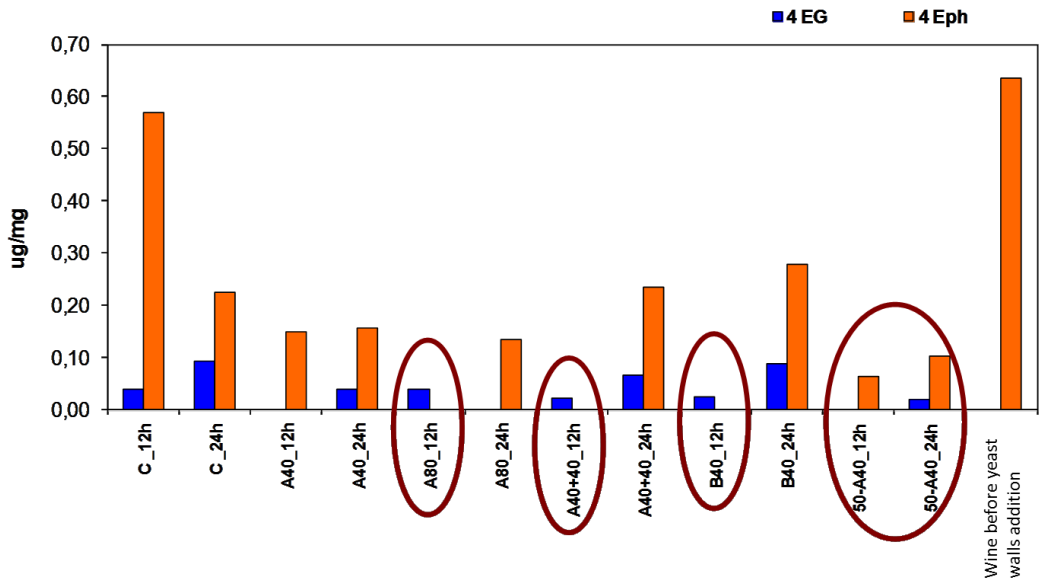


Fig 17 Volatile phenols concentration (µg/mg/) after different treatments. EG (blue), Ethilguaiacol; Eph (orange), Ethilphenol.

4.8 Reduction of *B. bruxellensis* growth in wine by chitosan

Furthermore, a reduction in growth of *B. bruxellensis* in wine was observed when chitosan was used. Two different doses of chitosan (4 g/hl and 10 g/hl) were tested in combination with different concentrations of *B. bruxellensis* (from 10^6 to 10^2 CFU). Wine without *B. bruxellensis* and chitosan and wine with different concentrations of *B. bruxellensis* but without chitosan were used as controls. Growth of *B. bruxellensis* was negatively affected by chitosan (**Figure 18**). Indeed, of *B. bruxellensis* already decreases after few days in presence of chitosan. After 9 and 12 days no further *B. bruxellensis* growth was noted.

Our results showed that for *B. bruxellensis* yeast in stationary phase, the studied chitosan had a negative effect on their viability as it drastically decreased the living cell concentrations after 24 h, according to Taillandier *et al*. (2014). The effect was linked to added chitosan concentration. Chitosan activity should be related to adsorption phenomena of cells on the polymer. This has

already being suggested by some authors, for others, yeasts from *Saccharomyces* genus. Zakrzewska *et al*. (2007) have suggested that adsorption occurred between chitosan and phospholipids of the wall and membrane.

Other studies reported the leakage of intracellular components as proteins and potassium as an effect of chitosan on bacteria cells (Chung and Chen 2008; Kong *et al*., 2010).

For *B. bruxellensis* in growth phase, after a viability loss measured during the first 24 h of contact time, the living cells started to increase slightly at 168 h but remained to a very low concentration compared to the control ($0,8*10^6$ vs $6*10^6$ cells ml^{-1}) even for the lowest concentration of chitosan. Ferreira *et al*. (2013) reported that a chitosan concentration of 0,75 g l^{-1} was enough to inactivate a strain of *B. bruxellensis* in wine after 2-h contact time, but a concentration of 1,5 g l^{-1} only led to a 3-log reduction for another strain. Roller and Covill (1999) reported results for 7 yeasts from 4 genera tested in apple juice in the presence of chitosan glutamate: after a cellular death during the first 2 days of contact time, the yeast of some species started to grow if the concentration of chitosan was inferior to 0,5 g l^{-1}. They made the hypothesis of the recovery of injured cell after 6 days. Actually in real winemaking conditions, the situation could be controlled by racking off the wine after a few days of contact time before the possible recovery of yeast cells.

The impact of fungal origin chitosan on *B. bruxellensis* has also been tested under winemaking conditions at winery scale and was also efficient in reducing the concentration of viable cells (Pic *et al*., 2011).

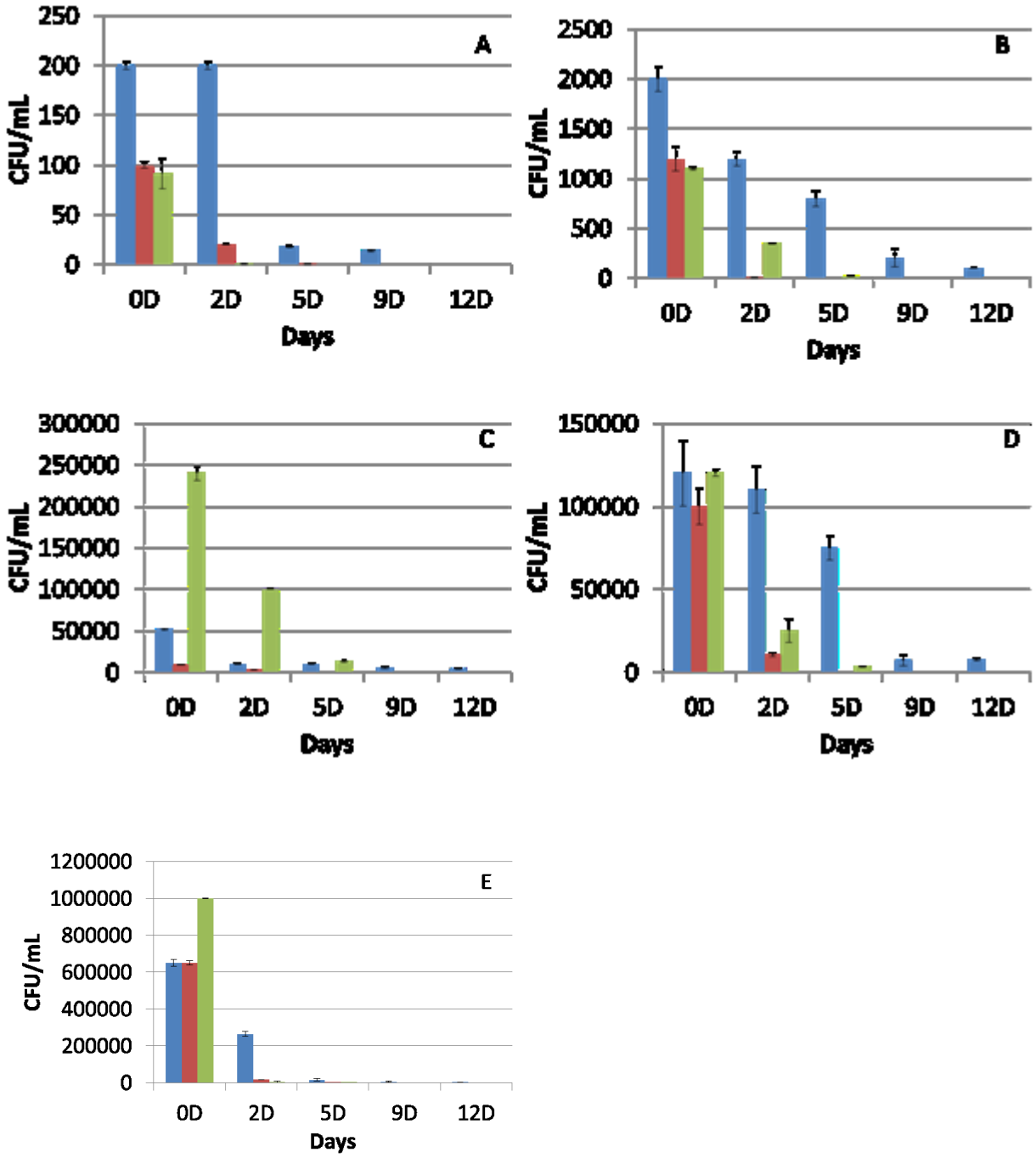


Fig 18. Concentrations of *Brettanomyces bruxellensis* after chitosan treatment. Blue bar represents the control (wine inoculated with *B. bruxellensis* without chitosan); red bar: wine inoculated with *B. bruxellensis* and 4g/hL of chitosan; green bar: is wine inoculated with *B. bruxellensis* and 10g/hL of chitosan. Different concentrations of *B. bruxellensis* are reported: A (10^2 CFU/ml), B (10^3 CFU/ml), C (10^4 CFU/ml), D (10^5 CFU/ml) and E (10^6 CFU/ml).

4.9 Inhibition tests

In winemaking, an interesting application of biological activities is seen by the use of killer yeast to control the proliferation of spoilage micro-organisms during the prefermentation phase (Comitini *et al*., 2004; Santos *et al*., 2009; Comitini and Ciani 2010). Yeasts are interesting micro-organisms for use in biological control, and, in particular, they are relatively easy to handle and show economy of manipulation and cultivation (Pimenta *et al*., 2009).

Furthermore, the potentially of wine microorganisms to control growth of *B. bruxellensis* was analysed. YEPD plates were inoculated with *B. bruxellensis* strains and several strains of *M. pulcherrima* yeast were spotted. The halo formed around the spot after 24-48 hours of incubation indicated that the yeast or bacterium was able to inhibit the growth of *B. bruxellensis*. As reported in **Figure 19,** some strains of the yeast *M. pulcherrima* showed an inhibitory effect.

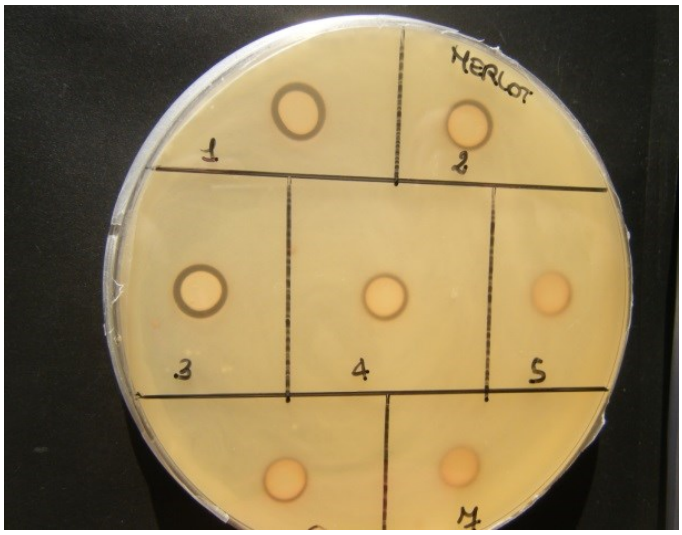


Fig 19 Example of *Brettanomyces bruxellensis* strain inhibited by *M. pulcherrima* strains. The arrows indicate the inhibition zone

The red or maroon pigment (pulcherrimin) of *M. pulcherrima* has been the subject of numerous previous studies. This pigment forms reddish halos around colonies of *M. pulcherrima.* The reduction in halo size observed when the medium was supplemented with $FeCl_3$ suggests that the

cells do not secrete the pigment but instead secrete a soluble, diffusible precursor that forms the pigment in the medium when it encounters iron. At low iron concentrations the precursor diffuses further from the yeast colony before it is immobilized by iron, resulting in a wider but paler halo. At higher iron concentrations the halos are smaller because the precursor molecules do not diffuse as far before they bind sufficient iron for pigment production. Since the pigment in these cases is concentrated in a smaller area, the color of the resulting halos is more intense. At even higher concentrations of iron, no halo is produced, suggesting that the entire precursor pool is converted to pigment within the cells. At these concentrations the yeast colonies are dark red (maroon) (Sipiczki *et al*., 2006).

The inhibition activity of *M. pulcherrima* appears to be related to the diffusion of pulcherriminic acid as precursor of pulcherrimin, which immobilizes the iron in the growth medium. The antimicrobial activity of *M. pulcherrima* could be associated with this secretion of pulcherriminic acid, which forms a red pigment when it is linked to the iron ions available in the medium as previously reported by Sipiczki (2006), Türkel and Ener (2009) and Oro (2014).

4.10 Microvinification test

An experimental vinification protocol has been performed to control *B. Bruxellensis* development.

We used different associations of autochthonous and commercial strains. Autochthonous strains, previously isolated and tested in synthetic medium and grape juicewith single strain or multi strain approach, wer selected for their fermentative performances. For each test we simulated a mean environmental *B. Bruxellensis* contamination, obtained by inoculating must with seven *B. bruxellensis* strains mix (lately called Bretta mix) with a final concentration equal to $2 \bullet 10^3$ UFC/mL.

Wine making process was monitored for a period of about 30 days by counting on WL nutrient agar plate, modified as to make it selective for *B. bruxellensis* development. It could not develop at the beginning of alcoholic fermentation, in tests performed with *B. bruxellensis*, probably because of competition with *Saccharomyces cerevisiae*. The only exception was the control; as no further yeast was inoculated, *B. bruxellensis* could fastly develop in the first fifteen days.

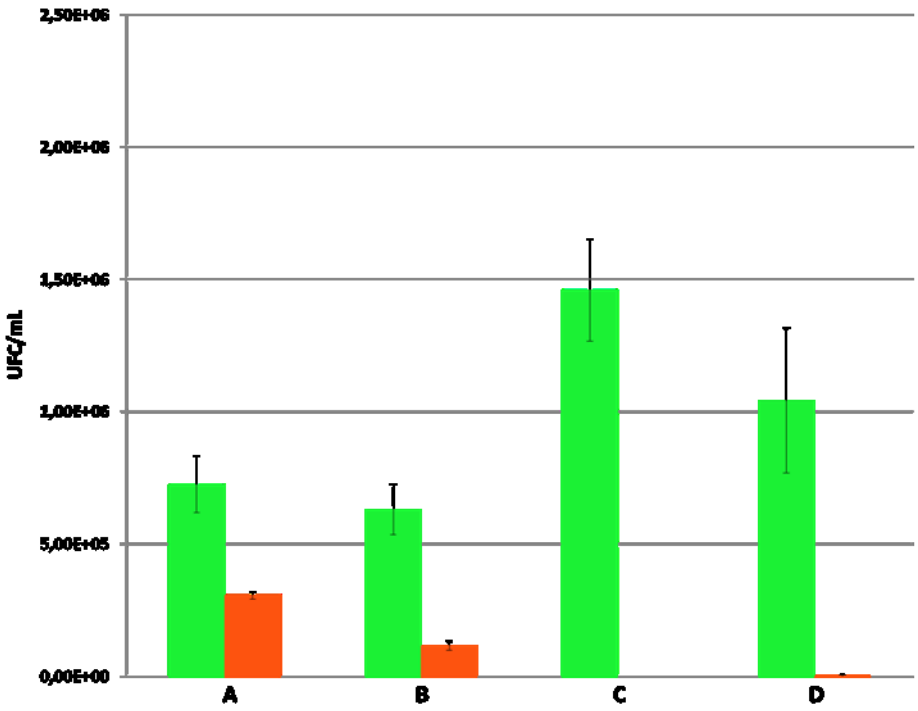


Fig 20 Count of *B. bruxellensis*, at two different stages of sampling, starting from alcoholic fermentation. T14 (green), T28 (red). A) S. Cerevisiae 16 + mix Bretta, B) *S. cerevisiae* 16 – *S. Cerevisiae* E4 + mix Bretta, C) *S. Cerevisiae* 16 – *S. Cerevisiae* E4+ *S. cerevisiae* EP2 (Maurivin) + mix Bretta, D) *S. cerevisiae* I6 – *S. cerevisiae* E4+ *S. cerevisiae* EP2 (Maurivin) + *S. cerevisiae* Suvignon (Maurivin) + mix Bretta.

In **Figure 20** we repotred counts of *B. Bruxellensis*, obtained by inoculing of different strains of *S. cerevisiae* in an artificially *B. Bruxellensis* contaminated must. At the end on alcoholic fermentation (T4), we can note a fast development of *B. bruxellensis* in every test performed, with values

between $6{,}3 \bullet 10^5$-$1{,}5 \bullet 10^6$ UFC/mL; however, as we can see in the graphic, use of different strains of *S. Cerevisiae* allowed a fast dejection of *B. bruxellensis* viable cells. Two association in particular were more effective:

- *S. cerevisiae* I6 – *S. cerevisiae* E4+ *S. cerevisiae* EP2 (Maurivin
- *S. cerevisiae* I6 – *S. cerevisiae* E4+ *S. cerevisiae* EP2 (Maurivin) + *S. cerevisiae* Sauvignon (Maurivin)

We subsequently tested the effectiveness of different *Saccharomyces*- Non-*Saccharomyces* associations, thus testing the main non-Saccharomyces strains with oenological interest:

- *C. zemplinina*
- *H. uvarum*
- *H. guilliermondii*
- *T. delbrueckii*
- *M. pulcherrima*

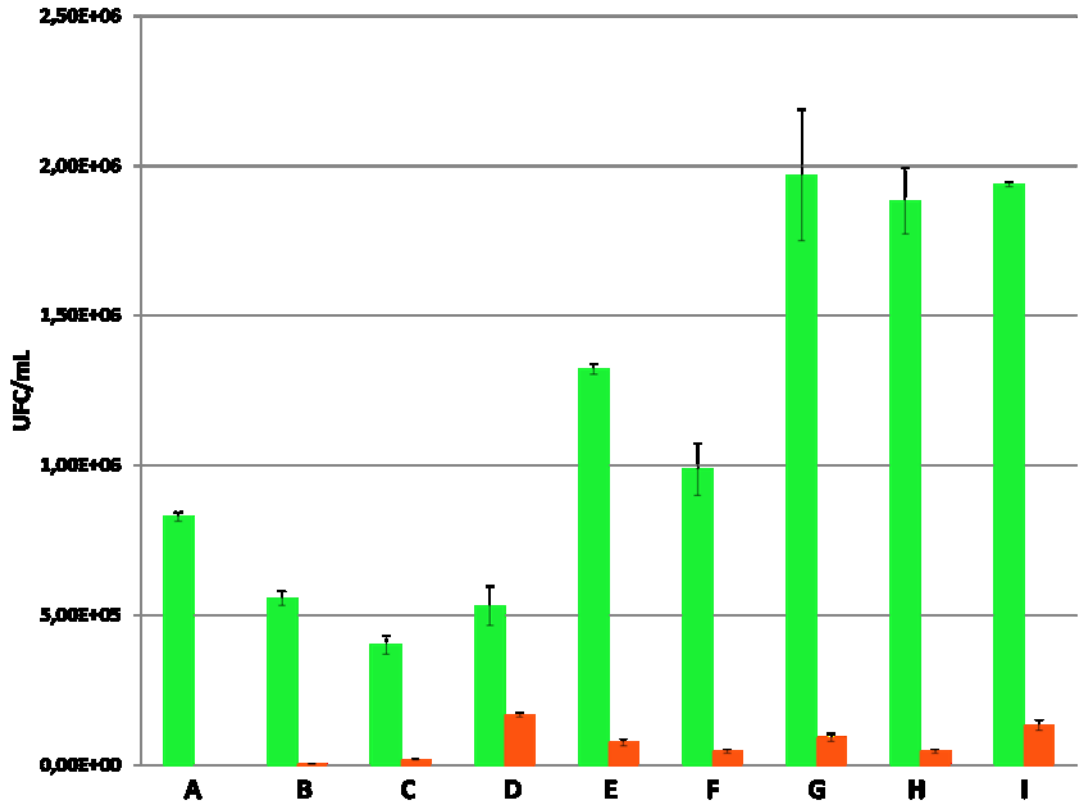


Fig 21 (UFC/mL) Count of *Brettanomyces bruxellensis*, at two different sampling moments; starting from alcoholic fermentation. T14 (green), T28 (red). A) *S. cerevisiae* I6 - *C. zemplinina* + mix Bretta, B) *S. cerevisiae* I6 – *H. uvarum* + mix Bretta, C) *S. cerevisiae* I6 – *H. guilliermondii* + mix Bretta, D) *S. cerevisiae* I6 – *T. delbrueckii* + mix Bretta, E) *S. cerevisiae* I6 – *M. pulcherrima* + mix Bretta, F) *S. cerevisiae* I6 – *C. zemplinina* - *H. uvarum* + mix Bretta, G) *S. cerevisiae* I6 – *C. zemplinina* – *H. uvarum* – *H. guilliermondii* + mix Bretta, H) *S. cerevisiae* I6 – *C. zemplinina* – *H. uvarum* – *H. guilliermondii* – *T. delbrueckii* + mix Bretta, I) *S. cerevisiae* I6 – *C. zemplinina* – *H. uvarum* – *H. guilliermondii* – *T. delbrueckii* – *M. pulcherrima* + mix Bretta.

We can note from **figure 21** that at the end of alcoholic fermentation (t14) there is a wide *B. bruxellensis* contamination with values between $4 \bullet 10^5$ and $2 \bullet 10^6$ UFC/mL. 28 days after the beginning of alcoholic fermentation, there is a sudden diminution of viable *B. bruxellensis* cells concentration, with values between 0 and $1{,}6 \bullet 10^5$ UFC/mL.

The associations proved to be most effective were the ones in which *S. Cerevisiae* autochtonous yeast has been inoculated respectively with *C. zemplinina* (A), *H. uvarum* (B) e *H. guilliermondii* (C), with concentrations of *B. bruxellensis* equal respectively to 0, $2 \bullet 10^3$ and $1{,}8 \bullet 10^4$ UFC/mL. The two other non-*Saccharomyces* yeasts tested (*T. delbrueckii* e *M. pulcherrima*) were found to be less effective. Moreover, by inoculing different non *Saccharomyces* yeast with a *S. cerevisiae* I6 the inhibiting effect obtained was lower than the one obtained with the single strain.

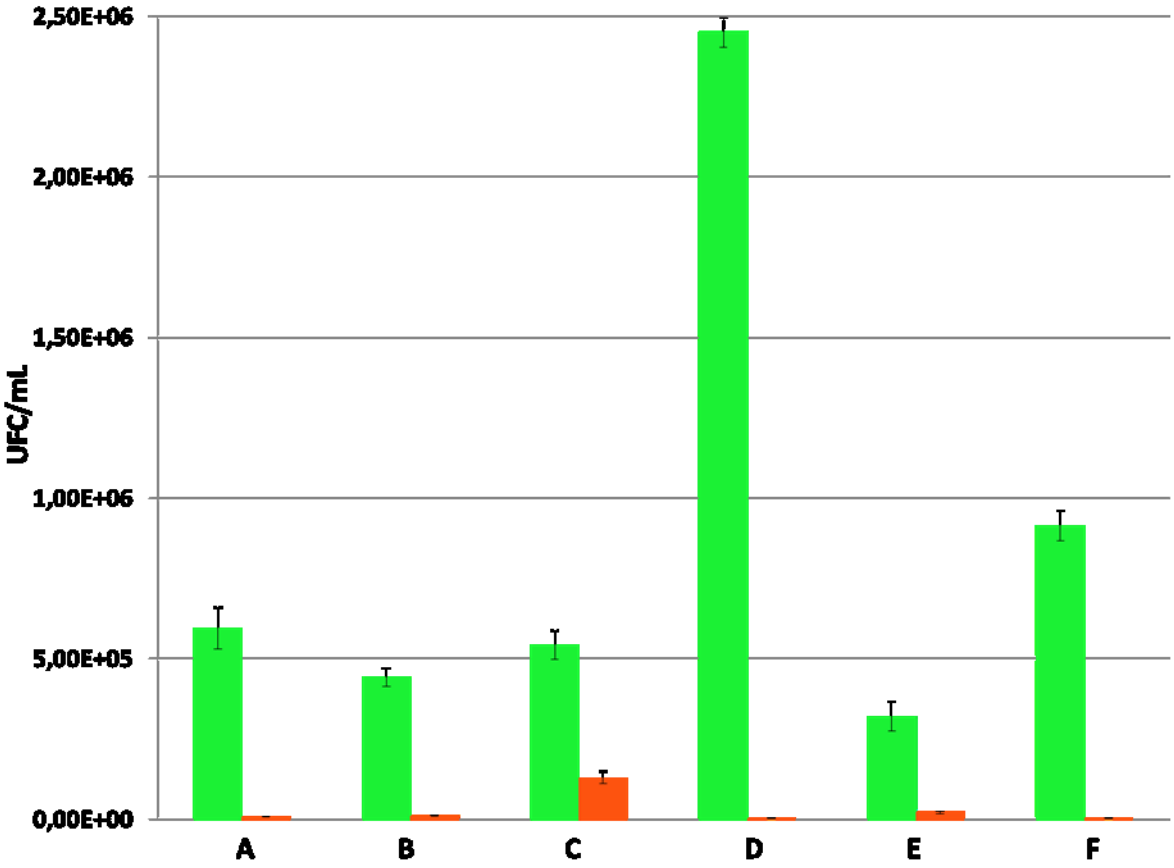


Fig 22 (UFC/mL) Count of *Brettanomyces bruxellensis*, at two different sampling moments; starting from alcoholic fermentation. T14 (green), T28 (red). A) *S. cerevisiae* I6 – *L. plantarum* UNIFG 103 + mix Bretta, B) *S. cerevisiae* I6 – *L. plantarum* UNIFG 105 + mix Bretta, C) *S. cerevisiae* I6 – *L. plantarum* UNIFG 106 + mix Bretta, D) *S. cerevisiae* I6 – *L. plantarum* UNIFG 107+ mix Bretta, E) *S. cerevisiae* I6 – *L. plantarum* UNIFG 119 + mix Bretta, F) *S. cerevisiae* I6 – *L. plantarum* UNIFG 120 + mix Bretta.

In **figure 22** we reported counts of *B. bruxellensis*, obtained by co-inoculation, at the beginning of alcoholic fermentation, of S. cerevisiae I6 and six different strains of *L. plantarum*, selected according to previous in vitro tests. Even in this case, from the graphic we can note a wide development of *B. bruxellensis* immediately after alcoholic fermentation (T14), until reaching peaks equal to $2{,}5 \bullet 10^6$ UFC/mL. Co-inoculates with *L. plantarum* strains was found to be effective in inhibiting *B. bruxellensis* development, 28 days after the beginning of alcoholic fermentation. Almost every *L. plantarum* strains tested, were found to be effective, except *L. plantarum* UNIFG 106. In fact, by co-inoculating *S. cerevisiae* I6 and *L. plantarum* UNIFG 106, after 28 days viable cells of *B. bruxellensis* have a concentration equal to $1{,}3 \bullet 10^5$.

5 CONCLUSION

The rigid-chemical environmental conditions at the end of alcoholic fermentation allow the survival of a limited number of yeast and bacteria. These conditions are determined by high concentrations of ethanol, content of fermentable sugar and acid pH. If not controlled, the metabolic activity of these microorganisms can irreparably modify the chemical composition of wine by changing the sensory properties of the final product. The risk of wine spoilage by bacteria decreased in a significant way because of the advances in wine technology and implementation of good manufacturing practices throughout winemaking process; despite this, yeast contaminations still represents a serious threat of spoilage (Agnolucci *et al*., 2009). The yeasts of the species *Brettanomyces/Dekkera bruxellensis* are the most responsible for the production of volatile phenols, causing significant economic losses in the wine industry. The development in wines of *Brettanomyces/Dekkera*, in fact, determines organoleptic changes, due to the appearance of phenolic and animals odor that in some specific conditions can remind the scents of the farm, horse sweat, medicine, animal skin and leather (Suárez *et al*., 2007). During the last period *Brettanomyces/Dekkera* has been studied by several research groups with the aim to increase knowledge and establish appropriate control measures. Investigation on genome variability among individuals represents an important tool to assess their biodiversity and to study their environmental diffusion. The safeguard of microbial strains with technological interest is becoming a strategic activity in food and wine industries.

B. bruxellensis is the only microorganism producing volatile phenols in wine at sufficient concentrations to achieve sensory thresholds (Chatonnet *et al*., 1997; Dias *et al*., 2003). As a consequence of *B. bruxellensis* negative impact on wine quality with subsequent relevant losses, advances in characterization of this yeast attract considerable attention (Zuehlke *et al*., 2013). The design of new physical/chemical/biotechnological strategies for *B. bruxellensis* control in wine,

which is crucial to avoid important economic losses, require a major comprehension of the biodiversity of this important oenological spoilage yeast.

At the best of our knowledge, the present work for the first time describes the genetic and, for some isolates, technological characterization of *B. bruxellensis* strains, collected from several wines manufactured in Apulia, one of the principal region for Italian wine production. In our work, the Sau-PCR analysis was sensitive enough to study *B. bruxellensis* strain biodiversity. Furthermore, only in one case, strains from the same cluster belong to the same geographical area. In general, our observations indicate a remarkable genetic variability of the *B. bruxellensis* identified strains, and corroborate the evidence, that, within a region, strains isolated from wines produced in the same geographical areas clustered differently, thus highlighting a complex intraspecific biodiversity in the regional wine environments. Analysis of the volatile phenol production by some representative isolates corroborates the spoilage function of *B. bruxellensis* identified in Apulian wines.

Finally, the spoilage potential of strains representative of the intraspecific biodiversity of *B. bruxellensis* within a region has been evaluated for the first time. Giving that spoilage potential reflects strains diversity, this study allows the design of new strategies for *B. bruxellensis* control in wines.

As already described in the literature (Serpaggi *et al*., 2012), spoilage yeast *Brettanomyces bruxellensis* may be in a VBNC state, a physiological condition in which the cells are unable to divide in a bacteriological medium, even if they are still alive and maintain the metabolic activities and cell functions (Agnolucci *et al*., 2010; Divol *et al*., du Toit *et al*., 2005; Kell *et al*., 1998). The entry in this state is due at the present of SO_2 (Serpaggi *et al*., 2012).

This work confirms the high level of phenotypic polymorphism existing in *B. bruxellensis* species, already evidenced at the level of sulphite sensitivity/tolerance.

Our findings indicate that VBNC comportment behaviour is a strain-dependent character in *B. bruxellensis*, and more generally in yeasts. These outcomes clearly indicate VBNC behaviour as a new key standard in the characterization of spoilage potential of *B. bruxellensis* strains. From an industrial point of view, the results are important to design physicochemical and biotechnological strategies for *B. bruxellensis* control of in wine.

This work confirmed that qPCR is a good tool to monitor and quantify the yeast *B. bruxellensis* in a fast and sensitive way.

With the use of yeast cells wall, it may be noted that these exert an adsorbing action against lipophilic species such as ethylphenol, thus favouring a partial or total reduction of molecules responsible for off-flavours. In contrast, chitosan is able to directly reduce the growth of *B. bruxellensis*.

In addition, the discovery of yeast strains capable of inhibiting the growth of *B. bruxellensis* leads us to assess the use of *M. pulcherrima* to reduce and inhibit *B. bruxellensis* in wine. Therefore, the use of yeast cells wall, the chitosan and the inhibition tests paved the way for future studies and the design of new strategies to reduce economic losses caused by *B. bruxellensis* in wine.

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7. APPENDIX

Publications

Capozzi V., Garofalo C., **Di Toro M.R**, Acconciaioco A., Massa S., Beneduce L., Spano G., (2012). Management of microbial resource for autochthonous wine grape varieties. III Convegno Nazionale Società Italiana di Microbiologia Agraria, Alimentare e Ambientale (SIMTREA), Bari, 26-28 June 2012.

C. Garofalo, M. Bely, **M.R. Di Toro**, F. Grieco, P. Lucas, G. Spano, V.Capozzi *(2013)* Autochthonous microbial resources for Apulian Nero di Troia wines. CBL 2013 -*19ème édition du Club des Bactéries Lactiques, Bordeaux October* 16-18, 2013.

Di Toro M.R., (2012) Biodiversity of *Dekkera/Brettanomyces* in Apulian wines: detection, isolation, characterization of representative biotypes and assessment of control strategies based on management biotechnological resources. XVII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of bologna, Cesena, 19- 21 September 2012.

Di Toro M.R., Salma M., Alexander H., Grieco F., Spano G., Capozzi V., (2013) Biodiversity and VBNC state in *Brettanomyces bruxellensis* isolated from Apulian wineries. International Conference on Microbial Interactions in Complex Ecosystems, Torino, 23-25 October 2013.

Di Toro, M.R., V. Capozzi, L. Beneduce, H. Alexandre, M. Tristezza, M. Durante, M. Tufariello, F. Grieco, and G. Spano. 2014. Intraspecific biodiversity and 'spoilage potential' of *Brettanomyces bruxellensis* in Apulian wines. Food Science and Technology. DOI: 10.1016/j.lwt.2014.06.059

National and international courses, congress and seminars

Course: Applied Statistic. Dr. Antonio Bevilacqua. University of Foggia, Department of food, agriculture and environmental sciences.

Seminar: Proteomica applicata ai batteri lattici e agli alimenti. Prof.ssa Maria De Angelis Università di Bari

Seminar: Il processo di produzione della birra. Prof. Stefano Buiatti - Università di Udine

Seminar: Dairy bacteriophages: How to turn a problem into a tool. Dr. Miguel Alvarez -Head of the Department of Biotechnology of Dairy Products, IPLA, Spanish National Research Council (CSIC), Oviedo (SPA)

Seminar: Nuove frontiere della scienza degli alimenti: applicazioni della spettroscopia di risonanza nucleare. Prof. F. Paolo Fanizzi - Università di Lecce

Seminar: Adaptative response of bacteria to stress: the case of *Oenococcus oeni*, the wine bacterium. Prof. Jean Guzzo - Equipe de Recherche en vigne et vin(REVV) Institut Jules Guyot Université de Bourgogne ,Dijon (FRA)

Seminar: Aspetti e problematiche della produzione di olive da tavola. Prof. Marco Poiana - Università di Reggio Calabria

Seminar: Tecniche molecolari per l'identificazione e la caratterizzazione di lieviti di interesse alimentare. Prof.ssa Angela Capece - Università della Basilicata

Seminar: *Oenococcus oeni*, the bacteria of wine malolactic fermentation. Prof. Patrick M Lucas - Università di Bordeaux (FRA)

Seminar: Workshop c/o Fiera Internazionale dell'Agricoltura, Foggia. Soluzioni innovative per lo sviluppo delle imprese pugliesi.

Seminar:“Selection of probiotic functional starter cultures for controlled food fermentation: case study on fermentation of African locust beans*”*.Dr. Irene Ouoba- London Metropolitan University**.**

I Workshop “*Collezioni microbiche e Biotecnologia”.* Prof.ssa Esperanza Garay, Direttrice della *Colección Española de Cultivos Tipos*(CECT, Valencia, Spagna), dott.ssa Cristina Varese, (Responsabile scientifico della *Mycotheca Universitatis Turinensis*-MUT, Torino).

Seminar: “*Ricerca interdisciplinare:caso o necessità*?” Prof. Claudio Tuniz ICTP Trieste.

Course: English course. Dr. Sarah Cristopher (University of Foggia).

Course: Food Packaging Dr. Amalia Conte (University of Foggia).

Workshop: XVII Workshop on the *Developments in the Italian PhD Research on Food Science Technology and Biotechnology*, University of Bologna, Cesena, 19-21 September, 2012.

Course: Laboratory safety. Dr. Roberto Di Caterina. University of the Study of Foggia. March-April 2013.

Seminar: STAR Agro Energy Scientific and Technological Advancement in Research on Agro-Energy. “The fate of tar after biomass pyrolysis: a microbiological point of view”. Dr. Lorenzo Brusetti, Faculty of Science and Technology Free University of Bozen, Bolzano. Auditorium, University of the Study of Foggia, Via Gramsci 79, Foggia, 5 April 2013

Seminar: Slow Biothecnologies slow food – “Made in Italy” landscape. “Alimenti, RandS e Territorio”. Dr. Franco Biasioli. Fondazione Edmund Mach di San Michele all’Adige. University of the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 12 June 2013

Seminar: Slow Biothecnologies slow food – “Made in Italy” landscape. “System Design: progettazione e sviluppo sostenibile”. Prof. Luigi Bistagnino. Politecnico di Torino. University of

the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 12 June 2013

Seminar: Slow Biothecnologies slow food – "Made in Italy" landscape. "Indicazioni Geografiche: buono, pulito, giusto e in fermento." Prof. Cinzia Scaffidi. Università di Scienze Gastronomiche di Pollenzo e Centro Studi Slow Food. University of the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 20 June 2013

Seminar: The New Frontier of Consumer Research Welfare Effects of Europe's Nutrition and Health Claims Regulation: the Italian Yogurt Market. Alessandro Bonanno (Wageningen University). University of the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 1 July 2013

Seminar: The New Frontier of Consumer Research Consumer Perception of Health Claims: an application of the Protection Motivation Theory Giuseppe Nocella (Reading University). University of the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 1 July 2013

Seminar: The New Frontier of Consumer Research Consumers' Attitudes and Acceptance of new products: the new frontier of research methodologies Rosalba Riolo (Hypatia Marketing and Digital Research). University of the Study of Foggia, Aula Magna, Department of Agriculture, Food and Environment Sciences, Via Napoli 25, Foggia, 1 July 2013

Visiting PhD student

IUVV (Institut Universitaire de la Vigne et du Vin) de l'Université de Bourgogne, (Dijon, France), on VBNC state of *Brettanomyces bruxellensis* strains isolated from apulian wine. Oenology Research unit, Prof. Hervè Alexandre

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Intraspecific biodiversity and 'spoilage potential' of *Brettanomyces bruxellensis* in Apulian wines

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ABSTRACT

The yeast *Brettanomyces bruxellensis*, generally considered the main oenological spoilage microbe, is able to survive during the winemaking process and it confers off-odors to wine, in reason of its ability to produce considerable amounts of volatile phenols. Forty-eight isolates of *B. bruxellensis*, obtained from several wines collected in Apulia (Southern Italy), were genetically characterized using an integrated approach, including a strain biodiversity analysis by *Sau*-PCR. Furthermore, the production of volatile phenols was assessed in wine and in synthetic medium, confirming the oenological spoilage potential of the analysed strains. Our findings indicate a remarkable genetic variability of the *B. bruxellensis* identified strains and corroborate the evidence of a high level of genotypic and phenotypic polymorphism within *B. bruxellensis* species. Moreover, the observation reported suggest that strains from wines produced in the same geographical areas often clustered differently, indicating a complex intraspecific biodiversity in the regional wine environments. Diversity in volatile phenol production reflects intraspecific biodiversity highlighted by *Sau*-PCR. Strains diversity linked to differences in 'spoilage potential' increase the industrial relevance of this study, allowing the design of new strategies for *B. bruxellensis* control in wines.

1. Introduction

The alcoholic or primary fermentation in wine is a natural process performed by native yeasts, mainly belonging to *Saccharomyces* spp., which considerably contributes to the chemical and organoleptic properties of the final product. Additionally, most red and some white grape wines undergo a secondary fermentation denoted as malolactic fermentation (MLF) (Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985). The MLF is carried out by wine lactic acid bacteria (LAB) such as *Oenococcus oeni*, a dominant bacterial species which is well adapted to the harsh wine conditions and confers the finest oenological malolactic characteristics (Alexandre, Costello, Remize, Guzzo, & Guilloux-Benatier, 2004; Fugelsang & Edwards, 2007; Massera Soria, Catania, Krieger, & Combina, 2009). However, other microbial species may occur in the complex consortia established in the must/wine environment, they are able to compromise the quality of wine. Yeasts belonging to the genus *Brettanomyces/Dekkera* are generally considered the main oenological spoilage microbes (Wedral, Shewfelt, & Frank, 2010). Currently, five species are recognized as members of this genus: *Brettanomyces bruxellensis*, *Brettanomyces anomalus*, *Brettanomyces custersianus*, *Brettanomyces naardenensis* and *Brettanomyces nanus* (Zuehlke, Petrova, & Edwards, 2013). Currently, only strains of *B. bruxellensis* were found able to produce several compounds that are detrimental for the organoleptic quality of the wine, including some classes of volatile phenols which derive from the sequential conversion of specific hydroxycinnamic acids such as ferulate and p-coumarate (ferulic and p-coumaric acids). Hydroxycinnamate decarboxylase first transforms these hydroxycinnamic acids into vinylphenols, which are then respectively reduced to 4-ethylphenol and 4-ethylguaiacol by the vinylphenol reductase (Dias, Pereira-da-Silva, Tavares, Malfeito-Ferreira, & Loureiro, 2003; Edlin, Narbad, Gasson, Dickinson, & Lloyd, 1998; Suárez, Suárez-Lepe, Morata, & Calderón, 2006). Volatile phenols greatly influence the aroma of

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wine. In fact, elevated concentrations of 4-ethylphenol are associated with unpleasant aromas described as “phenolic”, “horse sweat”, “stable” or “leather” (Suárez et al., 2006). The sensory threshold of these compounds is 230 μg/L for 4-ethylphenol and 47 μg/L for 4-ethylguaiacol (Chatonnet, Boidron, & Pons, 1990; Suárez et al., 2006). Analytical methods have been developed either to monitor metabolites responsible for wine depreciation (Pollnitz, Pardon, & Sefton, 2000) or to detect and estimate the population of these spoilage yeasts (Benito, Palomero, Morata, Calderon, & Suarez-Lepe, 2009). The isolation and identification of *Brettanomyces* species in wine may be performed by selective media (Suárez et al., 2006), PCR specific amplifications with subsequent enzymatic digestion (Cocolin, Rantsiou, Iacumin, Zironi, & Comi, 2004) or real-time PCR approach, which allows a fast determination and enumeration of *Brettanomyces* directly in wine (Phister & Mills, 2003; Tofalo, Scirone, Corsetti, & Suzzi, 2012). Moreover, several techniques, such as nested PCR (Ibeas, Lozano, Perdigones, & Jimenez, 1996), restriction fragment length polymorphism analysis (PCR-RFLP) (Mitrakul, Henick-Kling, & Egli, 1999), PCR-denaturing gradient gel electrophoresis (DGGE; Renouf, Claisse, & Lonvaud-Funel, 2006), infrared spectroscopy (Oelofse, Malherbe, Pretorius, & Du Toit, 2010), and macrorestriction analysis of the chromosomal DNA by pulsed-field gel electrophoresis (REA-PFGE; Miot-Sertier & Lonvaud-Funel, 2007) have been also used to study intraspecific differences among *B. bruxellensis* strains. Recently, a *Sau*-PCR method was also successfully employed to study *B. bruxellensis* biodiversity in a given geographic area (Campolongo, Rantsiou, Giordano, Gerbi, & Cocolin, 2010).

In this work, we report the first survey on the characterization of yeasts belonging to the genus *Brettanomyces* in Apulian wines, a South Italian region representing about the 13.2% of Italian wine production (Confagricoltura Puglia, 2013). The genetic variability among the identified strains has been assessed. Moreover, the ability of selected *B. bruxellensis* strains to produce volatile phenols was analysed.

2. Materials and methods

2.1. Sampling and growth conditions

Samples of this study were chosen to have representative data covering the main Apulian wine production areas (Bari, Foggia, Lecce, Barletta and Taranto). Samples were collected from fermenting musts, wines, ageing wines (tanks and barrels) in wineries located in the following seven different areas of Apulia region producing wine with Denomination of Controlled Origin: San Severo, Tavoliere, Cacc'e Mmitte, Rosso Barletta, Castel del Monte, Salice Salentino, Primitivo di Manduria. The *Brettanomyces* strains were isolated from wines using a modified WLN agar medium (Wallerstein Laboratory Nutrient media 60 g/L, sorbic acid 0.25 g/L, trehalose 5 g/L, p-coumaric acid 100 mg/L, agar 15 g/L, cycloheximide 30 mg/L and pH 5.5). WLN plates were incubated over a period of 5–11 days at 30 °C (Oelofse, Lonvaud-Funel, & du Toit, 2009). One colony was randomly selected from each contaminated sample and analysed.

2.2. Strains identification

Brettanomyces spp. strains were identified following two different PCR methods, which respectively employed the ITS1/ITS4 and DB90/DB394 primer pairs. The first primer set, ITS1/ITS4, was used for RFLP analysis of 5.8S rRNA gene and the two ribosomal internal transcribed spacers, according to Pham et al. (2011). The amplification products were digested with the restriction enzymes *Hae III*, *Hinf I*, *Cfo I* (M-Medical, Italy) and then analysed by agarose gel electrophoresis.

The yeasts RFLP profiles were identified using the yeast-ID database (CECT-IATA, Spanish Type Culture Collection, Universitat de València, València, Spain). The PCR method with the DB90/DB394 primer pair was performed as described by Cocolin et al. (2004). In order to distinguish between *B. bruxellensis* and *B. anomalus* strains, a restriction analysis with the endonuclease *DdeI* was also performed. The *Brettanomyces*-specific PCR product was subjected to restriction analysis as described by Cocolin et al. (2004). The amplicons corresponding to three different strains (Unifg 14, Unifg 47, Unifg 39) were also sequenced (PRIMM, Italy) to confirm the assignment of the yeast species.

2.3. Sau-PCR analysis

Genomic DNA (200 ng) extracted from strains identified as *B. bruxellensis* was digested using *Sau3AI* restriction endonuclease, as previously described (Corich, Mattiazzi, Soldati, Carraro, & Giacomini, 2005). DNA was digested overnight at 37 °C and 1 μL of the digestion mixture was used as template for the PCR amplification with primer SAG1 (Campolongo et al., 2010). PCR products were separated on a 2% agarose gel in TBE (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, for 120 min at 120 V. Electrophoresis gels were scanned with Versadoc system (Bio-Rad, Richmond, CA, USA), converted to TIFF images, compared and analysed with FPQuestTM software (BioRad Laboratories, Richmond, USA); then, the *Sau*-PCR electrophoretic patterns were grouped, and analysed for the similarity and through the Dice coefficient. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA).

2.4. Volatile phenol production

Seven representative strains of *B. bruxellensis* were cultivated in a media described by Oelofse et al. (2009). The base media was composed of YNB (Yeast Nitrogen Base) without amino acids 6.7 g/L (Sigma), casamino acids 3.0 g/L, biotin 0.6 g/L, thiamine 1.6 g/L and hydroxycinnamic acid precursors 100 mg/L [p-coumaric acid (p-CA) or ferulic acid (FA)]. The base medium was evaluated with different carbon source compositions. The capital letters symbolise the carbon source type: A, glucose 20 g/L and B, glucose 2 g/L and ethanol 8% (v/v). The roman numbering indicates the specific precursor added: **I**, p-CA; **II**, FA; **III**, p-CA and FA. The pH value was adjusted to 3.6 using o-phosphoric acid. One hundred milliliter of medium were inoculated with 100 μL of each *B. bruxellensis* strains at OD_{600}nm = 1.0. Cultures were incubated at 25 °C for 7 days until late exponential/beginning stationary phase was reached. Volatile phenols production was evaluated in red wine with alcohol 13%, pH 3.8 and sterilised by filtration (0.45 μm; Millipore, France). Before direct inoculation in wine, strains were pre-conditioned in YNB media A (without p-CA and FA) added with progressive incremental amounts of sterile red wine. Briefly, 9 mL of each *B. bruxellensis* strains at OD_{600}nm = 1.0 were suspended in a medium base: red wine mixture in proportion 9:1. The ratio among inoculated base medium and red wine ratio was progressively adjusted to 7:3, 1:1 and 3:7 over a 2 days period, by incremental addition of sterile red wine.

Prior to direct inoculation in wine, cells were centrifuged at 4000 rpm and suspended in the base media to give a final OD_{600} = 0.6. Then, 1 mL of this suspension was used to inoculate 80 mL of sterile red wine added with p-CA and FA at 100 mg/L each. Incubation was carried out at 25 °C without agitation and samples were taken for volatile phenol analysis after 7 and 30 days.

2.5. Chromatographic analysis of hydroxycinnamic acids

Synthetic wine samples were extracted by liquid–liquid extraction with diethyl ether. Briefly, 5 mL of wine were adjusted to pH 2 and then extracted twice with 5 mL of diethyl ether by mixing the mixture for 10 min. The organic phase was dried over Na_2SO_4 and then the solvent was evaporated under nitrogen. The dry residue obtained was dissolved in 0.5 mL of 1:1 methanol–water and 20 μL of each extract were analysed by HPLC (Cabrita, Palma, Patao, & Freitas, 2011). The HPLC apparatus consisted of an Agilent 1100 equipped with a photodiode array detector. The wavelength used for quantification of hydroxycinnamic acids were 280 and 320 nm. Separation was achieved according to Li, Shewry, and Ward (2008) with the following modifications: a Phenomenex-Luna column (5 μ C18; 100 Å; 250 × 4.6 mm) was used and the temperature of the column was set at 30 °C. A gradient elution program was utilized with a mobile phase consisting of acetonitrile (solution A) and 1% (v/v) H_3PO_4 in water (solution B) as follows: isocratic elution, 100% B, 0–30 min; linear gradient from 100% B to 85% B, 30–55 min; linear gradient from 85% B to 50% B, 55–80 min; linear gradient from 50% B to 30% B, 80–82 min; post time, 10 min before the next injection. The flow rate of the mobile phase was 1.0 mL/min, and the injection volume was 20 μL. All hydroxycinnamic acids were quantified using a calibration curves produced using commercial standards (Sigma–Aldrich, USA).

2.6. Chromatographic analysis of volatile phenols

Extraction of free volatile phenols in wine was carried out by means of liquid–liquid extraction according to Cabrita et al. (2011). An Agilent gas chromatograph model 6890N coupled to a mass selective detector model 5973 inert was used. An amount of 1 μL of extract was injected in splitless mode on a DB-Wax capillary column (60 m × 0.25 mm i.d.; 0.25 μm film thickness). Oven temperature program was: 90 °C (5 min) – 3 °C/min – 230 °C (25 min). Injector temperature was 250 °C. Mass detector conditions were: electronic impact (EI) mode at 70 eV; scanning rate: 1 scan/s; mass acquisition range: 40–450 amu. Helium was used as carrier gas under a flow rate of 1 mL/min. Peak identifications were based on comparison of their mass fragmentation with those of pure standards and/or with those reported in the commercial library Nist98 (Agilent, USA). The quantitative analysis of positively identified compounds was performed by total ion current using the calibration curves proposed for these compounds.

2.7. Bioinformatic tools and GenBank accession numbers

Analyses of DNA was carried out using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and MEGA 4 software (Tamura, Dudley, Nei, & Kumar, 2007). The accession numbers of the D1/D2 26S rDNA sequence of the *B. bruxellensis* Unifg 14, Unifg 47, Unifg 39 strains are KF841628, KF841629 and KF841630, respectively.

3. Results and discussion

3.1. Isolation of Brettanomyces yeasts in Apulian wines

A screening procedure was carried out for *Brettanomyces/Dekkera* spp. isolation from post-alcoholic fermentation of red wine. The presence of volatile phenols in samples aseptically taken from the tanks and barrels of different wineries, was undetectable (data not shown). Wine samples were directly applied onto agar plates containing modified WLN medium (Oelofse et al., 2009). Forty-eight colonies were then isolated and putative *Brettanomyces* clones were identified by PCR- RFLP analysis of the ITS1/ITS4 rDNA fragment. The results obtained indicated that all the isolates belonged to the genus *Brettanomyces* (Esteve-Zarzoso, Belloch, Uruburu, & Querol, 1999; Pham et al., 2011). Restriction with either *HaeIII* or *HinfI* generated a couple of fragments with a length of 350 and 100 bp, or 250 and 200 bp, respectively (data not shown). Interestingly, the restriction analysis with *CfoI* (data not shown) gave two different restriction profiles, the former was composed of 3 fragments (230, 130 and 80 bp) while the latter consisted of 4 fragments (240, 120, 80 and 50 bp). These findings are in accordance with those previously reported by Pham et al. (2011).

The restriction analysis of the amplicons obtained by the PCR assay with the specific primer pair DB90F/DB394R was performed to confirm whether the identified strains belonged to either *B. bruxellensis* or *B. anomalus* species. As reported in Fig. 1, the restriction pattern expected for *B. bruxellensis* using *DdeI* as restriction enzyme digestion, consisting in two different restriction fragments of about 154 bp and 129 bp (Fig. 1), thus indicating that all the isolated strains belonged to *B. bruxellensis*. To further verify such identification, the sequences of DB90F/DB394R fragments were determined and resulted to be identical to those from *B. bruxellensis* type strain (Sturm et al., 2014), thus confirming that the isolates identified in Apulian wines belonged to *B. bruxellensis* species. Our findings confirmed that *B. bruxellensis* is the only species belonging to the *Brettanomyces* genus associated with the wine environment.

3.2. Biodiversity within the isolated B. bruxellensis strains

In the present study, a *Sau*-PCR approach was employed to assess the biodiversity of *B. bruxellensis* strains isolated from Apulian wines. The number of bands generated by *Sau*-PCR was in the range 9–16, with an average of 11 band per strain. The majority of the 48 strains showed unique *Sau*-PCR pattern, with only six exceptions in which identical pattern were generated (Fig. 2). As shown by the dendrogram obtained by analysing the *Sau*-PCR patterns (Fig. 2), seven clusters could be differentiated at a similarity level of 70%. Two groups included 9 and 13 isolates (clusters 1 and 4, respectively). Additionally, smaller groups, which include from three to seven isolates, were observed. Among the seven clusters, only one (cluster 2, with 6 isolates) was composed of strains isolated only from cellars of the same geographical area within the Apulian region (San Severo, Foggia, Italy). Indeed, apart from such cluster, the isolates from wines produced in the same

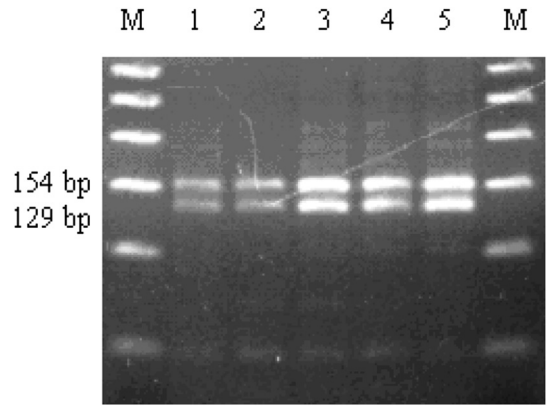
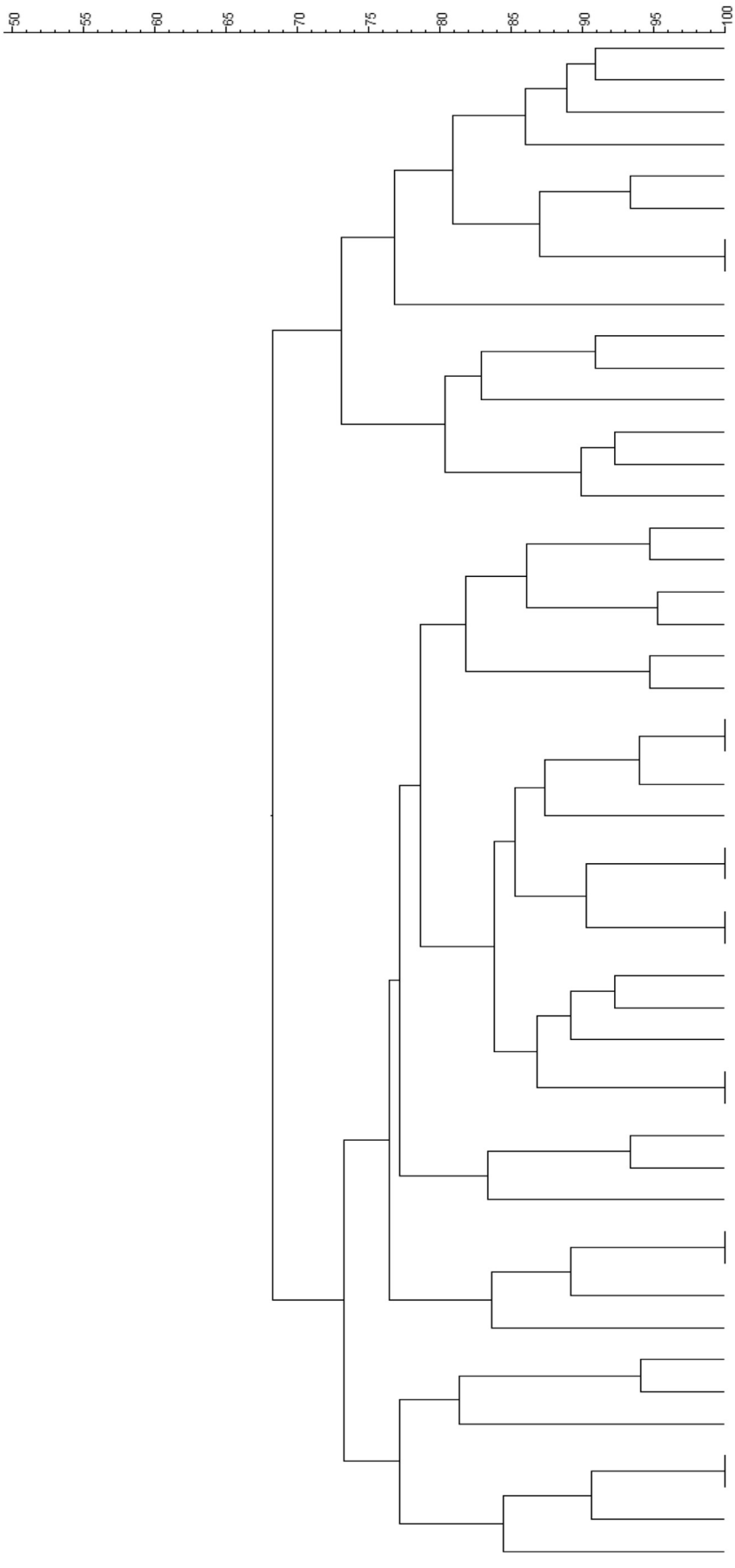


Fig. 1. Examples of restriction patterns of the PCR products obtained with specific primers DB90F and DB394R produced by digestion with *DdeI*. Line 1, Unifg8; Line 2, Unifg14; Line 3, Unifg23; Line 4, Unifg29; Line 5, Unifg37. M, marker 50 bp (Promega).



Strain	Origin	Source
Ufg 9	Bari	Barrel
Ufg 44	Lecce	Barrel
Ufg 17	Foggia	Tank
Ufg 46	Barletta	Tank
Ufg 37	Foggia	Tank
Ufg 38	Lecce	Tank
Ufg 25	Taranto	Barrel
Ufg 45	Taranto	Tank
Ufg 48	Barletta	Tank
Ufg 11	Foggia	Tank
Ufg 1	Foggia	Barrel
Ufg 2	Foggia	Barrel
Ufg 10	Foggia	Tank
Ufg 16	Foggia	Ferm. Must
Ufg 39	Foggia	Tank
Ufg 19	Foggia	Tank
Ufg 43	Taranto	Barrel
Ufg 8	Lecce	Barrel
Ufg 35	Lecce	Tank
Ufg 34	Taranto	Barrel
Ufg 7	Bari	Barrel
Ufg 13	Barletta	Tank
Ufg 23	Barletta	Barrel
Ufg 33	Taranto	Barrel
Ufg 32	Taranto	Barrel
Ufg 21	Lecce	Barrel
Ufg 40	Lecce	Barrel
Ufg 27	Foggia	Tank
Ufg 30	Foggia	Tank
Ufg 12	Barletta	Tank
Ufg 26	Bari	Tank
Ufg 41	Bari	Barrel
Ufg 4	Taranto	Tank
Ufg 15	Taranto	Ferm. Must
Ufg 5	Taranto	Tank
Ufg 6	Bari	Barrel
Ufg 29	Barletta	Tank
Ufg 36	Barletta	Tank
Ufg 24	Barletta	Barrel
Ufg 47	Foggia	Tank
Ufg 3	Bari	Tank
Ufg20	Taranto	Tank
Ufg 31	Lecce	Barrel
Ufg 28	Foggia	Tank
Ufg 18	Foggia	Tank
Ufg 42	Bari	Barrel
Ufg 14	Taranto	Tank
Ufg 22	Bari	Barrel

Fig. 2. Dendrogram from *Sau*-PCR profiles. The relationship between the isolated strains of *B. bruxellensis*is is reported. The strains used for further analysis are in bold and underlined.

cellar clustered differently, thus highlighting a complex intraspecific biodiversity in the wine environments. *Sau*-PCR fingerprinting method (Corich et al., 2005), has been already used to define the intraspecific diversity of *B. bruxellensis* strains in wines from the North-western of Italy (Campolongo et al., 2010) and our results corroborate the trends reported in that study. Indeed, Campolongo et al. (2010) differentiated 12 clusters and 10 individual strains (with a 70% coefficient of similarity). Nine out of the 12 clusters identified, consisted of strains isolated from the same region. *B. bruxellensis* biogeography in winemaking areas has been receiving increasing attention, often with controversial results. In cross-national studies, Conterno, Joseph, Arvik, Henick-Kling, and Bisson (2006) found a certain linkage between clusters and geography of isolation, while Vigentini et al. (2012) detected a poor correlation of clusters and the geographic location of isolation. Curtin, Bellon, Henschke, Godden, and de Barros Lopes (2007), studying biodiversity in 31 winemaking regions of Australia, highlighted the presence of three genotypes found across multiple winemaking regions. At the regional level, Campolongo et al. (2010), highlighted a good correlation of genetic clusters with region of isolation (Piedmont/Liguria, Italy). Studying intraspecific biodiversity at level of winemaking areas, we found, according to Campolongo et al. (2010), that strains isolated from the same area could be grouped into different clusters (with one exception), suggesting a high biodiversity between strains of *B. bruxellensis*. As observed by Curtin et al. (2007), these controversial findings might be explained by potential vectors (e.g. wine transported between wineries located in different geographic regions, insects), but also by adaptation phenomena to the wine environment in multiple location.

3.3. Volatile phenol production

With the aim to analyse the ability of the identified isolates to produce volatile phenols, seven strains, representative of each of the clusters obtained by *Sau*-PCR analysis were chosen: Unifg 8 = strain A, cluster 3; Unifg 14 = strain B, cluster 7; Unifg 39 = strain C, cluster 2; Unifg 47 = strain D, cluster 6; Unifg 29 = strain E, cluster 5; Unifg 27 = strain F, cluster 4; Unifg 46 = strain G, cluster 1. Strains A, B, C, D, E, F and G of *B. bruxellensis* were inoculated both in wine and in synthetic media and samples were analyzed for volatile phenol content after 7 (synthetic media and wine) and 30 days (wine) (Fig. 3). After seven days, in the (negative) controls, we found a 4-etylphenol (4-EP)/4-ethylguaicol (4-EG) content of 120/100 μg/L (synthetic medium AI), 100/83 μg/L (synthetic medium AII), 100/86 μg/L (synthetic medium AIII), 11/100 μg/L (synthetic medium BI), 100/110 μg/L (synthetic medium BII), 100/88 μg/L (synthetic medium BIII), and 78/11 μg/L (wine). In the basic medium supplied with glucose (20 g/L) and p-coumaric acid (p-CA, 100 mg/L), all the analysed strains produced 4-etylphenol in a range of 1410 μg/L and 1900 μg/L, after seven days of incubation. Strain E produced the greatest amounts of ethyl phenols (1900 μg/L). All strains were able to produce 4-ethylphenol and to transform the added p-coumaric acid (Fig. 3A). Strain B was able to transform all the added p-coumaric acid into 4-ethylphenol. In synthetic medium BI (containing 2 g/L glucose, 8% (v/v) ethanol, and 100 mg/L p-coumaric acid) the production of 4-ethylphenol was lower than in media A, although, even in these conditions, all strains were able to produce 4-ethylphenols but not all the p-coumaric acid was transformed. This finding is in agreement with Dias et al. (2003) and Oelofse et al. (2009), who both showed that the maximum conversion rates of p-coumaric acid to 4-ethylphenol is lower in the ethanol-containing media. In contrast, when the precursor of volatile phenol was ferulic acid, the production of 4-ethylguaiacol in media A was not different from that observed in media B. Indeed, in some cases, such as for strain C, the production of 4-ethylguaiacol increased in presence of ethanol, i.e. 2750 μg/L and 3760 μg/L in media A and B, respectively (Fig. 3A). It was interesting to observe that the analysed strains produced different levels of volatile phenols and transformed different amounts of their precursor. Such variability was much more evident when both precursor of volatile phenol, i.e. (p-coumaric acid and ferulic acid, were simultaneously added to the media). In this case, the production of volatile phenols ranged from 1140 μg/L to 3030 μg/L. Growth curves performed in synthetic media for the seven strains analysed, highlighted no clear correlation with spoilage potential (data not shown), in accordance with the findings previously reported by Oelofse et al. (2009).

The wines inoculated with strains D and F showed a similar volatile phenol profile seven days after inoculation, with a production of 4-ethylphenol of 100 μg/L and 114 μg/L, respectively (Fig. 3B). In contrast, 4-ethylguaiacol was undetectable seven days after inoculation. When incubation was extended to 30 days, a significantly increased production of 4-ethylphenol was observed. (Fig. 3C). For instance, when testing strain D, a production of 100 μg/L and 1200 μg/L 4-ethylphenol was observed after seven and thirty days from inoculation, respectively. The same trend was recognised for strain A, with a production of 540 μg/L 4-ethylphenol seven days after inoculation, while an increased level of 4-ethylphenol (1539 μg/L) was measured thirty days after inoculation. Strain A produced greater amounts of 4-ethylphenols compared to the other strains, thus overcoming the detection threshold of 425 μg/L, as stated by Chatonnet, Viala, and Dubourdieu (1995). Indeed, strain A was likely to reproduce the situation that can be usually found in a wine that is naturally contaminated by *Brettanomyces*. Concerning minor differences in spoilage potential, as reported in Fig. 3B and C, strains D and F produced similar amounts of 4-ethylphenol after 7 days whereas after 30 days, strain D produced significant higher amount of 4-ethylphenol compare with strain F. These differences in spoilage potential were observed also with strains A and B. In the case of the strain B and E, after 30 days, we registered an increase in the production of 4-ethylphenol as reported in Fig. 3C. In contrast to 4-ethylphenol, low concentrations of 4-ethylguaiacol were produced by all the analysed strains. Very low concentrations of 4-ethylguaiacol were produced by all the analysed strains, even after 30 days of incubation. Overall, even if with tangible differences, all the analysed strains displayed a considerable 'spoilage potential' in reason of their 4-etylphenol/4-ethylguaiacol production.

4. Conclusions

B. bruxellensis is the only microorganism producing volatile phenols in wine at sufficient concentrations to achieve sensory thresholds (Chatonnet, Viala, & Dubourdieu, 1997; Dias et al., 2003). As a consequence of *B. bruxellensis* negative impact on wine quality with subsequent relevant losses, advances in characterization of this yeast attract considerable attention (Zuehlke et al., 2013). The design of new physical/chemical/biotechnological strategies for *B. bruxellensis* control in wine, which is crucial to avoid important economic losses, require a major comprehension of the biodiversity of this important oenological spoilage yeast.

At the best of our knowledge, the present work for the first time describes the genetic and, for some isolates, technological characterization of *B. bruxellensis* strains, collected from several wines manufactured in Apulia, one of the principal region for Italian wine production. In our work, the *Sau*-PCR analysis was sensitive enough to study *B. bruxellensis* strain biodiversity. Furthermore, only in one

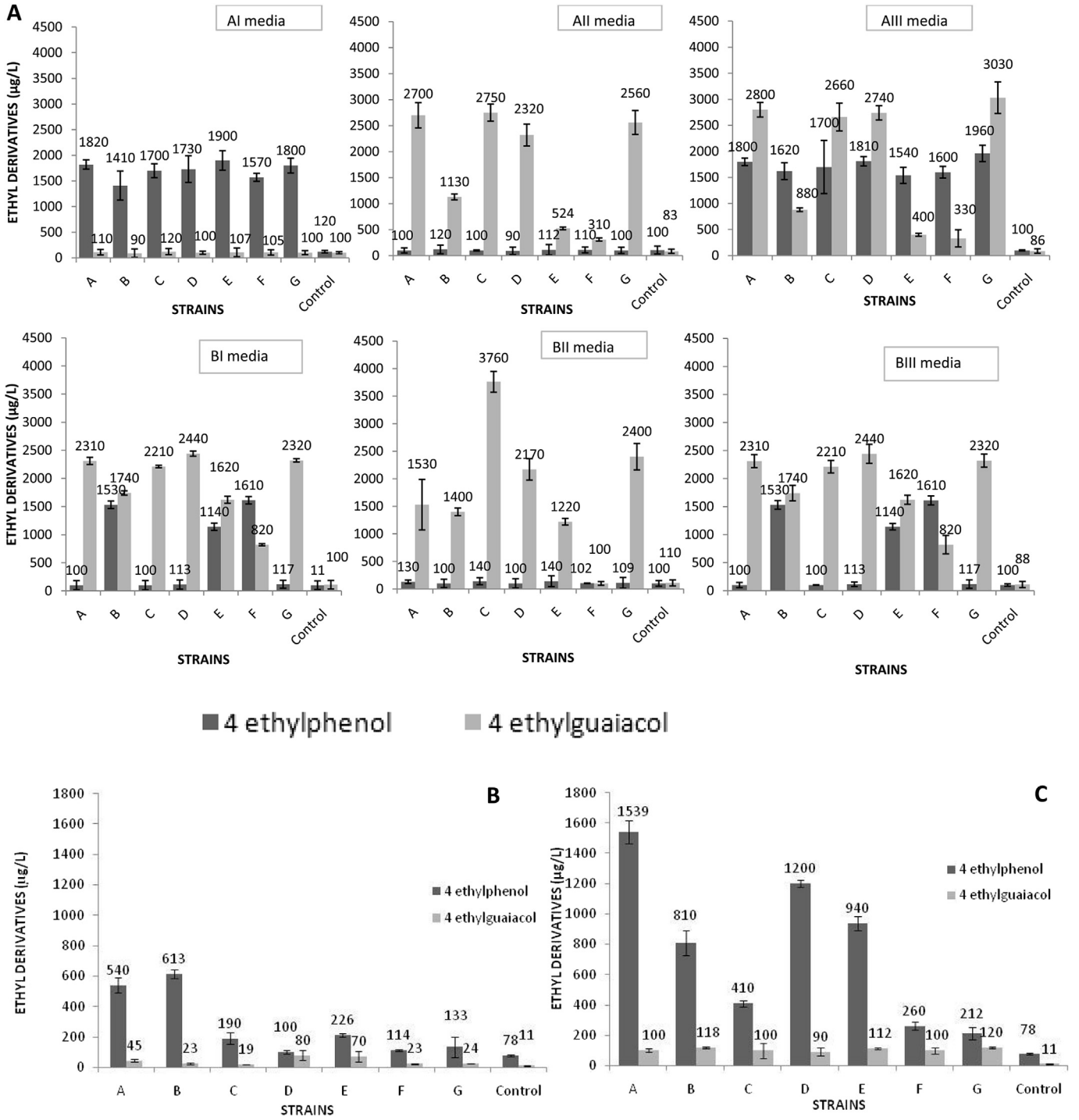


Fig. 3. (A) Volatile phenol analysis of *B. bruxellensis* strains in AI medium (containing glucose and 100 mg/L p-coumaric acid), AII medium (containing glucose and 100 mg/L ferulic acid), AIII medium (containing glucose and 200 mg/L of p-coumaric acid and ferulic acid together); BI medium (containing glucose-ethanol and 100 mg/L p-coumaric acid), BII medium (containing glucose-ethanol and 100 mg/L ferulic acid) and BIII medium (containing glucose-ethanol and 200 mg/L of p-coumaric acid and ferulic acid). (B) Volatile phenol analysis of *B. bruxellensis* strains in wine after 7 days and (C) after 30 days. The values are average of three independent replicates.

case, strains from the same cluster belong to the same geographical area. In general, our observations indicate a remarkable genetic variability of the *B. bruxellensis* identified strains, and corroborate the evidence, that, within a region, strains isolated from wines produced in the same geographical areas clustered differently, thus highlighting a complex intraspecific biodiversity in the regional wine environments. Analysis of the volatile phenol production by some representative isolates corroborates the spoilage function of *B. bruxellensis* identified in Apulian wines.

Finally, the spoilage potential of strains representative of the intraspecific biodiversity of *B. bruxellensis* within a region has been evaluated for the first time. Giving that spoilage potential reflects strains diversity, this study allows the design of new strategies for *B. bruxellensis* control in wines.

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