Harnessing improved understanding of *Brettanomyces bruxellensis* biology to mitigate the risk of wine spoilage

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Abstract

In the competitive global wine industry, production of wines reflective of their place of origin is critical. While the evidence is mounting that microbial populations in vineyards and wineries differ geographically and represent an important component of 'terroir', there are some microbial influences on wine style that are generally considered undesirable, regardless of whether the offending species is part of the natural winemaking microflora. *Brettanomyces* spoilage of wine remains one of the most important microbiological issues facing winemakers. While most prevalent in premium, barrel-aged reds, 'Brett' also affects sparkling wines and fortified wines. This review will cover recent advances in knowledge of the biology of this species alongside practical implications for risk management in the winery. For example, insights into genome evolution and population genetics will be discussed in the context of potential for emergence of more sulfite-tolerant strains. Strategies to reduce the risk of Brett spoilage will be updated, with discussion of alternative approaches drawn from other food and beverage sectors that may allow winemakers to reduce their reliance upon the common preservative sulfur dioxide.

Keywords: Brettanomyces, Dekkera, *spoilage, volatile phenol, wine*

Introduction

Brettanomyces bruxellensis, a yeast species first described in association with brewing (Claussen 1904), has been isolated from wine and wineries around the world since its discovery (originally as *Mycotorula intermedia*) in French wine in 1930 (Krumbholz and Tauschanoff 1933). While absent, or at least at low frequency in fresh grape musts, *B. bruxellensis* readily proliferates in wine during the maturation phase that follows the primary alcoholic fermentation undertaken by the predominant industrial yeast species *Saccharomyces cerevisiae*. This pattern of sequential growth can be observed across beer, cider and bioethanol fermentations, reflecting the fact that, despite being separated by 150 million years of evolution (Woolfit et al. 2007), both species have converged on key fermentation-relevant traits (Rozpędowska et al. 2011).

Brettanomyces bruxellensis and its closest relative *B. anomalus* have also been classified in the synonymous genus *Dekkera* (as *D. bruxellensis* and *D. anomala*) since 1964 (Van der Walt 1964) and 1984 (Smith and van Grinsen 1984), respectively, on the basis that some isolates have been observed to produce spores. Recent changes to yeast taxonomical practices mean this distinction will no longer be made and that one synonym will take priority. In the case of *Brettanomyces* versus *Dekkera*, historical significance and common usage favour the retention of *Brettanomyces* (Daniel et al. 2014). While *B. anomalus* and other members of the *Brettanomyces* genus are also intrinsically linked to human-influenced fermentation and beverage environs, *B. bruxellensis* remains the sole species in the genus unequivocally isolated from wine. Mounting evidence for hybrid lineages (Albertin et al. 2014, Borneman et al. 2014) mean that, as is the case for *S. bayanus* and *S. pastorianus* (Borneman and Pretorius 2015), *B. bruxellensis* should perhaps be considered a species complex, rather than a single species.

Noted for causing wine faults such as turbidity in unfortified table wines (Van der Walt and van Kerken 1958) and excessive volatile acidity in sherry (Ibeas et al. 1996), it is only over recent decades that the full extent of *B. bruxellensis* influence on wine style and quality has been recognised. Mousy taint (Grbin and Henschke 2000) and production of rancid 2- and 3-methylbutanoic (isovaleric) acids by some strains (Fugelsang and Zoecklein 2003, Curtin et al. 2013) can further negatively affect wine sensory properties, but the greatest impact of this species on the perceived quality of wine is through its capacity to produce 4-ethylphenol and 4-ethylguaiacol (Heresztyn 1986).

When present in wine at a concentration exceeding their combined perception threshold (425 µg/L), 4-ethylphenol and 4-ethylguaiacol impart elastoplast/bandaid, medicinal, barnyard and earthy aromas (Chatonnet et al. 1992, 1995). 4-Ethylphenol and 4-ethylguaiacol can also affect wine flavour, eliciting a metallic aftertaste in *Brettanomyces*-affected wines (Lattey et al. 2010). Together, these sensory attributes are known as 'Brett' character, and the consensus view is that *B. bruxellensis* spoils wine (Loureiro and Malfeito-Ferreira 2003). Nonetheless, there has long been a debate as to whether some degree of Brett character is appropriate and desirable to add complexity in some wine styles. While Chatonnet et al. (1992) described an 'adverse phenolic character' once ethylphenols exceeded their perception threshold, another French study found that Beaujolais wine with a relatively high concentration of 4-ethylphenol (2000 µg/L) was 'preferred' by the sensory panelists (Etiévant et al. 1989). More recent studies have shown that Australian winemaker quality scores and consumer preference ratings were generally lower for *Brettanomyces*-affected commercial wines (Lattey et al. 2010), and that Australian consumers disliked wines that were spiked with ethylphenols relative to unspiked controls (Curtin et al. 2008). The masking and accentuating effects of other volatile compounds with respect to ethlyphenols are also important to consider; short chain volatile fatty acids produced by *B. bruxellensis* were shown to increase the perception threshold of ethylphenols (Romano et al. 2009) and the degree to which Brett character impacts consumer

liking can be influenced by the level of oak volatiles (Mueller et al. 2009). Furthermore, if consumers associate 'oak' characters with high quality, they may not respond negatively to concurrent Brett characters, as observed in a recent Spanish study (Sáenz-Navajas et al. 2015). On this basis, it can be argued that in certain contexts, *B. bruxellensis* could be harnessed for bioflavouring of wine, as routinely practised in the production of various Belgian ales (Vanderhaegen et al. 2003).

Whether or not viewed solely as a spoilage yeast, *B. bruxellensis* is ubiquitous in winery environs around the world, and careful management of all winemaking processes is therefore required to mitigate the risk of deleterious impacts on perceived wine quality [see Loureiro and Malfeito-Ferreira (2006), Renouf et al. (2007a), Suárez et al. (2007), Oelofse et al. (2008), Kheir et al. (2013), Zuehlke et al. (2013)]. This review will consider how recent advances in our understanding of *B. bruxellensis* biology [see Curtin and Pretorius (2014), Schifferdecker et al. (2014), Steensels et al. (2015)] impact upon strategies that can be applied to manage the risk of Brett spoilage, providing an up-to-date summary of practical strategies to achieve this.

Flavour compound production by *Brettanomyces*

Various yeast and filamentous fungi have been characterised for their capacity to produce a range of volatile aroma compounds, or flavour compounds, that contribute to our sensory perception of fermented foods and beverages (Vandamme 2003). *Brettanomyces* species are no exception, with a broad range of esters, higher alcohols, volatile fatty acids and nitrogen-containing heterocyclic compounds described as being produced by *B. bruxellensis* (Licker et al. 1998, Grbin and Henschke 2000, Romano et al. 2008, 2009, Curtin et al. 2013, Joseph et al. 2013).

The overall 'flavour phenotype' (Cordente et al. 2012) of *B. bruxellensis* is in the savoury-leathery spectrum, and Brett-affected wines are generally not considered to exhibit primary fruit flavours. This can be partly explained by the observation that *B. bruxellensis* causes a decrease in the concentration of 'fruity' esters (Curtin et al. 2013), which is consistent with a stronger esterase activity than that observed for *S. cerevisiae* (Spaepen 1982) and the presence of three genes orthologous to isoamyl-acetate hydrolase (*IAH1*) in the *B. bruxellensis* genome (Curtin et al. 2012a). Furthermore, short chain volatile fatty acids associated with rancid aromas, such as 2- and 3-methyl butanoic (isovaleric) and butanoic (butyric) acids, are produced at relatively high concentration by *B. bruxellensis*. A positive correlation between ethylphenol concentration and these compounds was noted by Romano et al. (2009), reaffirming earlier studies that suggested they may play a role in perception of Brett character. Surprisingly though, the same study found that the perception threshold for ethylphenols was increased by an elevated concentration of isovaleric and butyric acids.

As previously described in *S. cerevisiae* [see review by Hazelwood et al. (2008)], production of Ehrlich pathway metabolites, such as higher alcohols and ethyl esters, by *B. bruxellensis* can be stimulated by addition of amino acids (Joseph et al. 2013, Parente et al. 2014). A surprising finding in the earlier study was that hydroxycinnamic acids (HCAs) affected the activity of these pathways, with the authors hypothesising a potential role of HCAs in redox balance for *B. bruxellensis* that may have broader metabolic effects. In addition, *B. bruxellensis* strains have varied capacity to produce volatile acids, higher alcohols and esters (Fugelsang and Zoecklein 2003, Curtin et al. 2013, Joseph et al. 2013), and in the absence

of ethylphenols such differences can be detected sensorially in model wine (Curtin et al. 2013). Importantly, a concentration of ethylphenols above the sensory threshold overrides other flavour differences between strains (Curtin et al. 2013), thus it is their relative capacity to grow under industrial conditions and produce ethylphenols that determine the extent to which *B. bruxellensis* strains impact on wine sensory properties.

Production of volatile phenols

Volatile phenols contribute to distinctive characters and/or off-flavours in a diverse range of foods and beverages including wine (Chatonnet et al. 1992), beer (Vanbeneden et al. 2008), cider (Le Quéré et al. 2006), distilled spirits (Jounela-Eriksson and Lehtonen 1981), olive oil (Vichi et al. 2008), soy sauce (Suezawa and Suzuki 2014) and white pepper (Steinhaus and Schieberle 2005). In contexts such as these, volatile phenols are predominantly derived from HCA precursors by microbial metabolism, a phenomena first described in the 1950s (Whiting and Carr 1957, 1959). The two-step nature of this pathway, with decarboxylation and reduction performed by different enzymes, was noted by Steinke and Paulson (1964) who observed that different organisms had one or both activities.

In grapes, free HCAs are found at their highest concentration in seeds. They are also found in a range of bound forms; grape skins contain a relatively high concentration of tartrate esters (Kammerer et al. 2004), and glucose esters have been isolated from wine (Baderschneider and Winterhalter 2001), while ethyl esters form during vinification (Hixson et al. 2012). Impure enzyme preparations containing esterase activity can increase the concentration of free HCAs through liberation from one or more of these bound forms (Dugelay et al. 1993), and some strains of *Oenococcus oeni* release HCAs from their tartrate esters during malolactic fermentation (Chescheir et al. 2015).

During alcoholic fermentation, *S. cerevisiae* is able to decarboxylate HCAs into vinylphenols, imparting what is known as 'phenolic off-flavour' (pof) in brewing literature (Thurston and Tubb 1981). In white wine, 4-vinylphenol and 4-vinylguaiacol, from coumaric and ferulic acids, respectively, are associated with medicinal aromas and flavours, nonetheless wild and commercial wine yeast strains are predominantly pof[+], meaning they produce phenolic off-flavours (Shinohara et al. 2000). Vinylphenols are found at negligible concentration in red wines because of their incorporation into pyranoanthocyanins (Morata et al. 2007). While some lactic acid bacteria possess the necessary enzymatic activities to convert HCAs and vinylphenols into the more stable and sensorially significant ethylphenols, they have minimal conversion capacity in wine (Chatonnet et al. 1995). Similarly, *Meyerozyma (Pichia) guilliermondii* can produce ethylphenols, but only during the early stages of vinification (Barata et al. 2006), and it is unclear to what degree this species contributes to Brett character.

Conversion of HCAs to ethylphenols by *Brettanomyces*

The capacity of *B. bruxellensis* and *B. anomalus* to produce ethylphenols during grape juice fermentation was first described by Heresztyn (1986), who further examined the HCA substrate range of *B. bruxellensis* in synthetic media, finding that coumaric, ferulic and sinapic acids were all metabolised, while caffeic, syringic and vanillic acids were not. Some studies have shown that 4-ethylcatechol (4-EC), derived from caffeic acid, is also found in wine (Carrillo and Tena 2007), albeit at much lower concentration than 4-ethylphenol (4-EP), derived from coumaric acid. While caffeic acid (in free and bound forms) is relatively abundant in grapes, *B. bruxellensis* metabolises this

HCA precursor less efficiently than coumaric and ferulic acids (Harris et al. 2008, Cabrita et al. 2012, Schopp et al. 2013). Furthermore, the impact of 4-EC in wine may not be as significant as that observed for 4-EP and 4-ethylguaiacol (4-EG); in cider the detection threshold of 4-EC was more than tenfold higher than that of 4-EP (Buron et al. 2012).

While *Brettanomyces* species have differing preferences for free HCAs (Harris et al. 2008), there is no evidence for variation between *B. bruxellensis* strains with respect to the final ratio of 4-EP to 4-EG in *Brettanomyces*-affected wine. The 4-EP to 4-EG ratio is known to vary in wine over a broader range (Coulter et al. 2003) than originally described by Chatonnet et al. (1992), who further noted that observed ratios 'correspond faithfully' to ratios of free acid precursors. Differences between strains in terms of their overall capacity to produce ethylphenols have been described in some studies (Fugelsang and Zoecklein 2003, Vigentini et al. 2008), but not in others (Curtin et al. 2013, Joseph et al. 2013). This discrepancy may be due to differences in biomass formation, reflecting relative capacity of strains to grow rather than relative capacity to metabolise HCAs (Vigentini et al. 2008), or may be due to differences in strain catabolism of some HCA precursors. Tartrate HCA esters were not metabolised by *B. bruxellensis* strains growing in red wines (Schopp et al. 2013) or model medium (Hixson 2012), whereas glucose esters and ethyl esters were partly metabolised to ethylphenols (Hixson 2012). Differences between strains were evident only for the ethyl esters; *B. bruxellensis* AWRI1613 was unable to catabolise ethyl coumarate or ethyl ferulate (Hixson et al. 2012).

Unravelling genetic determinants of ethylphenol production by B. bruxellensis

Genes involved in ethylphenol production by *B. bruxellensis* have recently been identified (Godoy et al. 2014, Granato et al. 2014), following earlier efforts to purify and characterise *B. anomalus* and *B. bruxellensis* proteins exhibiting phenolic acid decarboxylase (PAD) (Edlin et al. 1998, Godoy et al. 2008, Harris et al. 2009) and vinylphenol reductase (VPR) (Godoy et al. 2008, Tchobanov et al. 2008) activities. Godoy et al. (2014) utilised a degenerate primer approach to target conserved regions of phenolic acid decarboxylases from other yeast species, cloning a gene (*DbPAD*) that greatly enhanced vinylphenol production when expressed in *S. cerevisiae*. The encoded protein was predicted bioinformatically from the *B. bruxellensis* AWRI1499 genome sequence (Curtin et al. 2012a), and shares most similarity with phenolic acid decarboxylase from *M. guilliermondii* (Huang et al. 2012), the only other wine-associated yeast species known to produce ethylphenols. The *B. bruxellensis* VPR was identified by MS of trypic digests from a purified fraction containing VPR activity (Granato et al. 2014). These peptide sequences matched an open reading frame in the *B. bruxellensis* AWRI1499 genome encoding a superoxide dismutase (SOD), which appears to moonlight as a VPR while retaining its core activity. Analysis of the predicted SOD/VPR protein sequence revealed novel changes in co-factor binding regions that may explain ability of *B. bruxellensis* to produce ethylphenols. While long hypothesised to have evolved as a redox scavenging mechanism, a recent study demonstrated that the capacity of *B. bruxellensis* to produce ethylphenols may make it more attractive to fruit flies, thereby promoting long distance dissemination via the insect vector (Dweck et al. 2015). This finding parallels evidence that *S. cerevisiae* strains engineered to overproduce fruity acetate esters also attract more flies (Christiaens et al. 2014).

Brettanomyces detection and ecology

Isolation on differential media

Given their relatively slow growth rates, isolation of *Brettanomyces* yeasts from environmental samples generally requires differential medium formulations that include cyclohexamide to eliminate faster growing species (Rodrigues et al. 2001, Morneau et al. 2011). A grape juice-based enrichment medium developed to isolate *B. bruxellensis* from grape berry samples, in contrast, relied upon ethanol to select against the predominant fungal species associated with vineyards (Renouf and Lonvaud-Funel 2007).

A large number of *B. bruxellensis* isolates were obtained from Australian wine samples using Wallerstein agar medium containing 10 mg/L cycloheximide, with presumptive identification based upon slow growth (colonies visible after 7 days) and elongated, ogival cellular morphology (Curtin et al. 2008). Several other cycloheximide-resistant non-*Saccharomyces* species can grow on such media (Morneau et al. 2011), meaning that great care must be taken if the environmental sample is not finished wine. Unambiguous identification of colonies growing on differential media can be achieved using fluorescent in-situ hybridisation (FISH) with molecular probes (Stender et al. 2001), or more commonly applied DNA-typing methods such as polymerase chain reaction restriction fragment length (PCR-RFLP) of the internal transcribed spacer regions flanking the 5.8S rRNA encoding gene (Esteve-Zarzoso et al. 1999).

Direct detection using culture-independent methods

Alternatively, culture-independent methods can be used to detect *Brettanomyces* yeasts directly in environmental samples. While initially promising, enzyme-linked immunosorbent assay detection of *Brettanomyces* cells (Kuniyuki et al. 1984) has not been widely adopted. Most recent efforts to develop intact cell rapid detection methods draw upon flow cytometry, a platform that enables rapid automated counting of cells in a sample, typically augmented by non-specific fluorescent dyes that provide an indication of physiological state. More specific methods involve the use of antibodies or FISH probes, the latter recently used for detection of *B. bruxellensis* in wine at a level as low as 100 cells/mL (Serpaggi et al. 2010). More research has focused on development and refinement of methods involving DNA extraction of biomass from environmental samples, and quantitative PCR (Q-PCR) detection (Phister and Mills 2003, Andorrà et al. 2010, Willenburg and Divol 2012). Species-specific primers are utilised for Q-PCR and, for some methodologies, molecular probes to detect *Brettanomyces* DNA in an extract prepared from wine, juice or other sources. The advantages of Q-PCR are specificity and speed – with no culture period required, results are generated in a matter of hours. It has been shown, however, that Q-PCR can provide misleading results in terms of viable *Brettanomyces* populations, overestimating the number of cells in a sample that are able to divide (Willenburg and Divol 2012). This discrepancy has been attributed to *B. bruxellensis* cells entering a 'viable-but-non-culturable' (VBNC) state in response to sulfite-induced stress (Serpaggi et al. 2012), however, only actively growing populations have been shown to produce ethylphenols (Dias et al. 2003). Serpaggi et al. (2012) tested various parameters to trigger exit from the VBNC state, finding that only increasing pH (thereby reducing the concentration of molecular sulfite) enabled VBNC cells to resume growth, raising questions as to the importance of VBNC populations to eventual wine spoilage. Agents such as ethidium monoazide that bind DNA have been incorporated into sample preparation protocols prior to DNA extraction,

greatly improving the accuracy of Q-PCR enumeration of *B. bruxellensis* against reference culture-based assays (Andorrà et al. 2010). Regardless, Q-PCR methodologies require a high level of technical expertise to implement. A recent study described the specific detection of *B. bruxellensis* in DNA extracts using chemiluminescent probes fixed to optical fibre (Cecchini et al. 2012). While preliminary, this technology coupled with microfluidic DNA extraction (Chung et al. 2004) paves the way for development of sensors that could potentially provide highly specific, real-time monitoring for *B. bruxellensis* in wine undergoing maturation.

Brettanomyces bruxellensis *ecology*

Using a combination of culture-based and direct detection methods, *B. bruxellensis* has been described across all continents, in niches mostly related to industrial fermentation (Table 1), typically recovered from equipment and difficult-to-sanitise storage vessels such as oak barrels and/or fermented products exposed to them. Indeed, during wine production, the most common period during which *B. bruxellensis* proliferates and spoils wine is during barrel maturation prior to completion of malolactic fermentation (Renouf et al. 2006), although post-bottling spoilage has been reported (Renouf et al. 2007b, Coulon et al. 2010). The points of entry into industrial fermentation niches are of great interest, but remain largely obscure, with no clear association between *B. bruxellensis* and non-human-influenced environs. Outside of breweries, wineries, cider houses and soft drink manufacturing plants, *Brettanomyces* yeasts have been isolated directly from grape berries at low frequency in only one study (Guerzoni and Marchetti 1987) and after enrichment culture in another (Renouf and Lonvaud-Funel 2007). The medium used by Renouf et al. (2007b) effectively mimicked the conditions that a winery/brewery resident population would encounter when exposed to an active fermentation. Its composition is similar to that of an enrichment medium used for isolation of *S. cerevisiae* from grape berries (Mortimer and Polsinelli 1999), bark, soil, flowers, apiaries and barrels (Goddard et al. 2010). Mortimer and Polsinelli (1999) estimated that 1/1000 intact berries, and one in four damaged berries, harbour a minority (~1%) population of *S. cerevisiae*. Based upon the relative frequency of *Saccharomyces* spp. and *Brettanomyces* spp. isolation estimated by Guerzoni and Marchetti (1987), it would appear that *B. bruxellensis* may be equally rare in this niche. Despite a lack of definitive data on the occurrence of *B. bruxellensis* in the vineyard, given the ubiquitous association of this species with wine produced around the world, it is reasonable to consider it a 'natural' component of wine microflora.

Metagenomic approaches to ecology of microorganisms

Detection of rare species remains a challenge for microbial ecologists, particularly in niches where growth requirements of resident species are poorly understood. Culture-independent approaches that provide information about the broader microbial composition of an environmental niche have, until recently, relied upon low-throughput and difficult to interpret methods such as denaturing gradient gel electrophoresis (Muyzer and Smalla 1998), a method used to detect *B. bruxellensis* in enrichment cultures from grape berry samples (Renouf and Lonvaud-Funel 2007). Readily available and inexpensive high-throughput DNA sequencing technologies have caused a paradigm shift across the life sciences, and

Table 1. Examples of environmental niches from which *Brettanomyces bruxellensis* has been detected and/or isolated.

Source	Description	Country	Isolated/detected	Reference
Winemaking	Dry wine	France, South Africa, New Zealand, USA, Italy, Australia	Isolated	Krumbholz and Tauschanoff (1933), Wright and Parle (1974), Curtin et al. (2007), Agnolucci et al. (2009), Jensen et al. (2009), Coulon et al. (2010)
	Sparkling wine	Germany	Isolated	Schanderl and Draczynski (1952)
	Sherry	Spain	Detected	Ibeas et al. (1996)
	Spontaneously fermented wine	Chile	Isolated	Ganga and Martínez (2004)
	Grape berries	France, Italy	Isolated	Guerzoni and Marchetti (1987), Renouf and Lonvaud-Funel (2007), Albertin et al. (2014)
	Grape must	South Arica	Isolated	Van der Walt and van Kerken (1961)
	Winery air	USA	Isolated	(Connell et al. 2002)
	Winery equipment	South Africa	Isolated	Van der Walt and van Kerken (1961)
Brewing	English stock ale	Denmark	Isolated	Claussen (1904)
	Spoiled beer	Australia, Ireland	Isolated	Walters (1943), Gilliland (1962)
	Lambic ale	Belgium	Isolated	Verachtert et al. (1990)
	Coolship ale	USA	Detected	Bokulich et al. (2012)
	Brewery air	Belgium		Verachtert et al. (1990)
	Brewing equipment			
Other fermented foods/beverages	Cider	Ireland	Isolated	Morrissey et al. (2004)
	Kombucha (tea-beer)	Australia	Isolated	Teoh et al. (2004)
	Kefir	Taiwan	Detected	Hsieh et al. (2012)
	Sourdough bread	Germany	Detected	Meroth et al. (2003)
	Olives	Greece	Isolated	Kotzekidou (1997)
Bioethanol	Continuous sugar-cane fermentation	Brazil	Isolated	de Souza Liberal et al. (2007)
	Continuous wheat-starch fermentation	Sweden	Isolated	Passoth et al. (2007)
Non-fermentation	Soft drink	USA	Isolated	Yarrow and Ahearn (1971)

microbial ecology is no exception (Xu 2006). 'Bar code' sequencing metagenomics, whereby a region of ribosomal RNA encoding DNA is amplified from an environmental sample and then sequenced, enables simultaneous detection of hundreds to thousands of species, limited only by sequencing depth (budget). Over the recent years, multiple studies have used this technique to describe the vineyard (Bokulich et al. 2014, Pinto et al. 2014, Taylor et al. 2014, Zarraonaindia et al. 2015), winery (Bokulich et al. 2013) and wine fermentation (Bokulich et al. 2015) microbiomes. Clear geographical and cultivar influences on vineyard microbiomes (Gilbert et al. 2014), along with impacts of regional yeast upon wine composition (Knight et al. 2015), are increasingly being viewed as evidence for a microbial component to 'terroir', the concept that the characteristic properties of a wine reflect the environment in which it was produced. *Brettanomyces bruxellensis* has not been reported among the several hundred species described as members of the vineyard microbiome, whereas its niche partner *S. cerevisiae* was detected, albeit infrequently, in some must and vineyard samples (Bokulich et al. 2014, Pinto et al. 2014, Taylor et al. 2014).

Identifying the environmental niches where *B. bruxellensis* resides remains one of the foremost challenges limiting our understanding of its biology, and finding the vectors that have facilitated the global dispersion of *B. bruxellensis* may provide winemakers with the means to limit the spread of this species into new winemaking facilities. We can speculate that as the cost of sequencing decreases, and consequent depth of sequencing in metagenomic studies increases, the presence of this elusive species outside of the winery will be revealed.

Brettanomyces key physiological attributes

Attributes in common with ecological niche partner S. cerevisiae

As reviewed by Curtin and Pretorius (2014), whole genome sequencing has revealed multiple examples where evolution of fermentative physiology in *B. bruxellensis* has paralleled that in *S. cerevisiae*. A prominent case being amplification in both lineages of the alcohol dehydrogenase gene family (Curtin et al. 2012a, Piškur et al. 2012) that in *S. cerevisiae* confers greater control over alcoholic fermentation and facilitates production of Ehrlich pathway aromatic compounds.

The limitations of knowledge concerning the natural ecology of industrial yeast species is not limited to *B. bruxellensis*. Despite its status as a model organism, or perhaps because of it, little is known of *S. cerevisiae* outside of human-influenced environs, limiting our understanding of how evolution has shaped its genome and selected for traits that improve fitness in the 'wild' (Liti 2015). Traditional thinking has assumed because we predominantly find *S. cerevisiae* in fermentations of sugary substrates, this is the niche to which it has adapted; and the same could be said for *B. bruxellensis*. In support of this notion, both species converged on the 'crabtree effect', whereby a high sugar concentration stimulates fermentative metabolism over respiration in the presence of oxygen, via the same molecular mechanism of promoter rewiring (Rozpędowska et al. 2011). An interesting counter-argument to the notion that *S. cerevisiae* is a fermentation specialist draws upon the neutral theory of ecology: Goddard and Greig (2015) suggest that *S. cerevisiae* is a 'wandering nomad' that has evolved traits enabling it to persist in many different environments. If widespread field surveys proposed to refine our understanding of *S. cerevisiae* ecology (Liti 2015) could be

mirrored for *B. bruxellensis*, this might provide a stronger basis to assess the importance of fermentative adaptations in both microorganisms.

Physiological attributes that differentiate B. bruxellensis

Genomic datasets are also providing insight into evolution of traits that differentiate *B. bruxellensis* from other yeasts, such as the expansion in amino acid permeases that may facilitate survival in relatively low nutrient environments such as wine (Curtin et al. 2012a).

A distinguishing feature of *Brettanomyces* yeasts is their capacity to ferment and produce carbon dioxide from glucose more rapidly under aerobic conditions. Custers (1940) first noted this phenomenon, which was confirmed for all known members of the genus by Wikén et al. (1961) and described as a 'negative Pasteur effect'. It was renamed the 'Custers effect' by Scheffers (1966) and proposed as a diagnostic characteristic of the genus, with the exception of *B. custersianus* (Scheffers and Wikén 1969). The underlying biochemical basis for the Custers effect has not been fully elucidated, but it has been linked with the tendency of *B. bruxellensis* to accumulate a high concentration of extracellular acetate and intracellular succinate under aerobic conditions (Sanfaçon et al. 1976, Carrascosa et al. 1981) and inadequate re-oxidation of NADH via synthesis of glycerol under anaerobic conditions (Wijsman et al. 1984). Composition of growth medium or buffer used to assess fermentative behaviour experimentally influences the extent of the custers effect. *Brettanomyces* cells ferment more rapidly under aerobic conditions when the ratio of potassium to sodium is higher (Wikén et al. 1962), while the presence of nitrate in the growth medium releases inhibition of fermentation under anaerobic conditions (Galafassi et al. 2013). In continuous bioethanol fermentations, nitrate assimilation provides *B. bruxellensis* with a competitive advantage over *S. cerevisiae* (de Barros Pita et al. 2011, 2013, Galafassi et al. 2013, Neto et al. 2014).

The potential stimulatory effect of nitrate on *B. bruxellensis* growth under oxygen-limited wine-like conditions remains unexplored but is potentially of great significance. Residual ammonium post-alcoholic fermentation is scarce (Jiranek et al. 1995), while up to 53 mg/L of nitrate has been reported in California wine (Ough and Crowell 1980). The dearth of knowledge concerning nitrate concentration in wine reflects the fact that *S. cerevisiae* cannot assimilate this nitrogen source. Nitrate assimilation in yeast has been studied almost exclusively in *Ogatea polymorpha*, which carries all required genes in a single cluster (Siverio 2006), whereas in *B. bruxellensis* the structural gene cluster encoding a transporter and two reductase enzymes is separated from a second cluster of regulatory genes (Woolfit et al. 2007). Not all *B. bruxellensis* strains grow on nitrate (Conterno et al. 2006), a phenomena associated with structural gene deletion as described in two recent studies (Borneman et al. 2014, Crauwels et al. 2014).

Another feature differentiating *B. bruxellensis* from *S. cerevisiae* is its capacity to assimilate a wider range of carbon sources. For example, genome analysis revealed the presence of the entire pathway for chitin assimilation (Curtin et al. 2012a), and *B. bruxellensis* is able to grow on and ferment the cellulose breakdown disaccharide cellobiose (Blondin et al. 1982), even under oxygen-limited conditions (Reis et al. 2014), such as might be encountered during barrel maturation of wine. It is important to note that *B. bruxellensis* growth is slower on cellobiose than on simple reducing sugars, and that similar to nitrate utilisation, cellobiose assimilation varies among *B. bruxellensis* strains (Conterno et al. 2006).

Comparison of stress tolerance in B. bruxellensis *and* S. cerevisiae

Brettanomyces bruxellensis exhibits key stress tolerances enabling growth in wine, which are shared with *S. cerevisiae*. It is considered ethanol tolerant; is able to grow and produce 4-EP in synthetic medium up to 13% alcohol (v/v) (Dias et al. 2003) and in red wine at 13.4% alcohol (v/v) (Coulon et al. 2010), and has been detected in sherry (Ibeas et al. 1996), which typically has an alcohol concentration greater than 15% (v/v). There is disagreement, however, in the literature as to whether *B. bruxellensis* is more or less ethanol tolerant than *S. cerevisiae*. The growth rate of a *B. bruxellensis* strain was less affected by 12% ethanol than an *S. cerevisiae* strain (Silva et al. 2004), while in another study *B. bruxellensis* strains were shown to grow at a maximum of 15% ethanol compared with 16% for *S. cerevisiae* (Barata et al. 2008a). Rozpędowska et al. (2011) found that both species could tolerate 14% alcohol (v/v), while also reporting that *B. bruxellensis* was more acid tolerant. Indeed, 94% of *B. bruxellensis* strains from a global collection were able to grow at pH 2.0 (Conterno et al. 2006). Both species are also considered sulfite tolerant, although the use of different methods and inter-strain variation renders direct comparison difficult. Maximal sulfite concentration tolerated by *S. cerevisiae* strains varied over a tenfold range (Yuasa et al. 2004), whereas a fivefold range was observed for *B. bruxellensis* strains (Curtin et al. 2012b). Barata et al. (2008a) found the most sulfite-tolerant of 29 tested *B. bruxellensis* strains was still only able to grow at half the concentration tolerated by a commercial wine strain of *S. cerevisiae*. Sulfite tolerance in *S. cerevisiae* is conferred by *SSU1* (Park and Bakalinsky 2000), which encodes a sulfite-efflux pump. Variation in tolerance between strains has been linked to differences in gene expression for some *SSU1* alleles (Yuasa et al. 2005), an adaptive advantage converged upon in wine and vineyard yeast via two different chromosomal translocation events (Zimmer et al. 2014). Whether a similar mechanism explains variation in sulfite tolerance among *B. bruxellensis* strains remains to be elucidated.

Winemaking factors that influence *Brettanomyces* growth and spoilage potential

Growth barriers

Reinforcing experimental evidence of stress tolerance, widespread isolation from varied styles of red, white, sparkling and fortified wines suggests that, within typical ranges, the physicochemical properties of wine present no barrier to *B. bruxellensis* growth. Nevertheless, some combinations of conditions are more inhibitory than others, a concept known in predictive microbiology as the 'hurdle' (McMeekin et al. 2000). This postulates that while each hurdle may not be sufficient to inhibit growth of a food spoilage/toxigenic/pathogenic microorganism in isolation, when combined the net effect may be a synergistic complete inhibition of growth. In the context of *Brettanomyces* spoilage, a recent study highlighted interactions between the three major wine-relevant 'barriers'; pH, ethanol and free sulfite, in model wine (Sturm et al. 2014). The authors developed a mathematical model that allows calculation of the free sulfite concentration required to achieve a desired probability of 'no-growth' within a defined time period, based upon pH and ethanol concentration of the wine. Another recent study utilised response–surface methodology to investigate interactive effects of ethanol, sulfite and residual sugar on growth and ethylphenol production by *B. bruxellensis* (Chandra et al. 2014). While preliminary, and not yet validated

in real wine, mathematical models such as this are routinely used in food manufacturing to establish microbially stable formulations. Even in the absence of a fully validated model, the general principle that lower pH and/or higher ethanol concentration decreases the concentration of sulfite required to inhibit *Brettanomyces* growth can guide an approximate assessment of wine susceptibility.

Nutritional requirements of B. bruxellensis

Genomic data sets infer a scavenging lifestyle for *B. bruxellensis* (Curtin et al. 2012a), and as it exhibits minimal nutritional requirements, most wines contain sufficient carbon and nitrogen sources to support its growth. A relatively low concentration of glucose, fructose, galactose and trehalose (275 mg/L combined) is sufficient for *B. bruxellensis* to grow and produce ethylphenols above their sensory threshold level, a level of sugar that is present in even the driest of finished wines (Chatonnet et al. 1995). The relationship between residual sugar concentration, growth rate, biomass and ultimately ethylphenol production was reinforced by Barata et al. (2008b). Similarly, as little as 6 mg/L of yeast assimilable nitrogen has been shown to be sufficient for *B. bruxellensis* to achieve a population size of 10^7 cells/mL (Childs et al. 2015). Interestingly, managing fermentation through the supplementation of musts with additional nitrogen (Bell and Henschke 2005), a process that often leads to excess available nitrogen post-fermentation, does not increase the risk of *Brettanomyces* spoilage in model wine (Childs et al. 2015). As such, the benefit of enhanced alcoholic fermentation vigour leading to reduced instances of stuck or sluggish fermentation and residual sugar far outweighs the risk that residual nitrogen poses for subsequent *Bretttanomyces* growth. The potential risk that vitamin supplementation of fermentation poses with respect to *Brettanomyces* spoilage is less clear. *Brettanomyces bruxellensis* exhibits an absolute requirement for biotin and thiamin (Conterno et al. 2006), and biotin-deficient musts have been noted in the context of *S. cerevisiae* and primary fermentation (Ough et al. 1989, Hagen et al. 2008). It is possible that relatively low-biotin musts, once fermented, would yield wines constraining *B. bruxellensis* growth. Under these circumstances, vitamin supplementation during alcoholic fermentation may increase risk of *Brettanomyces* growth and spoilage.

Wine storage during maturation

How wine is stored during malolactic fermentation and maturation also plays a significant role in determining the risk of *Brettanomyces* spoilage, and those exposed to oak barrels are most at risk (Chatonnet et al. 1992, 1995). This is due, in part, to the porous nature of wood which allows microbial penetration up to 1.2 cm into staves (Swaffield et al. 1997), rendering sanitisation difficult. Also significant is the ingress of oxygen that occurs during barrel maturation, given its stimulatory effect on *Brettanomyces* growth. New oak barrels, and those made from more porous American oak, allow more atmospheric oxygen to be dosed into wine (del Alamo-Sanza and Nevares 2014). Conflicting reports describe the impact of temperature during wine maturation. In one study, optimal growth of *B. bruxellensis* was observed between 25 and 32°C, while at 15°C maximal biomass took approximately four times longer to be achieved (Brandam et al. 2008). In contrast, Barata et al. (2008b) observed that wines inoculated with *B. bruxellensis* and incubated at 15 and 25°C accumulated a similar concentration of 4-ethylphenol.

Strategies to mitigate the risk of *Brettanomyces* spoilage during wine maturation

Effective use of sulfite

Sulfite, in its free molecular form, has long been the primary agent available for preservation of wine, and is a key component of any strategy for successful control of *Brettanomyces* (Coulter et al. 2003). Taking into consideration the differences in sulfite tolerance between strains, and the way wine composition (e.g. pH and ethanol concentration) affects sulfite efficacy, prescribing the correct amount of potassium metabisulfite to add to wine to control *Brettanomyces* is fraught with difficulty. Across several studies (Table 2), additions that yield concentration ranges from 0.25 up to 1.25 mg/L of molecular sulfite have been shown to limit *B. bruxellensis* growth and/or halt production of ethylphenols. Achieving a minimum of 0.6 mg/L of molecular sulfite during barrel maturation greatly decreases the probability of wine becoming *Brettanomyces* affected. For some wine styles, it can be difficult to achieve an adequate concentration of molecular sulfite to control a *Brettanomyces* population; furthermore adverse consumer reactions and a general desire for decreased concentration of preservatives in foods and beverages have renewed emphasis on finding other ways to render wine microbiologically stable.

Chemical alternatives to sulfite

Since first being proposed in the 1970s as a wine sterilant, dimethyl dicarbonate (DMDC) has found application in the wine industry as a pre-bottling treatment (Ough 1975). While able to decrease the size of an active *Brettanomyces* population (Costa et al. 2008, Renouf and Strehaiano 2008), several factors limit its application during wine processing for *Brettanomyces* control. First, health and safety concerns necessitate specific dosing equipment, which is simplest to implement in a bottling line. In addition, DMDC has a short half-life, hydrolysing to methanol and carbon dioxide within hours. Consequently, its fungicidal impact is transient; should a handful of viable cells remain once the molecule has degraded, regrowth of the population will not be inhibited. Finally, DMDC is less effective in unfinished red wine (Renouf and Strehaiano 2008), meaning that the maximum legal dose may be insufficient to kill all *Brettanomyces* cells in an affected wine. Despite these limitations, as an adjunct to sulfite addition and filtration, pre-bottling DMDC can greatly decrease the risk of continued *Brettanomyces* spoilage post-bottling.

Other chemical treatment options during wine maturation are limited. An oenological tannin preparation was shown in one recent study to inhibit growth of *B. bruxellensis* in a short-term experiment (Portugal et al. 2013). Further work is required, however, to evaluate how various phenolic additives perform over a longer period of maturation, and to determine their relevant active components. Hydroxycinnamic acids, the precursors of ethylphenols, are themselves toxic to *Brettanomyces* yeasts at high concentration (Harris et al. 2010). Addition of weak acids for food preservation is well established, and could be considered for wine production, although these would be accompanied by the risk that if a sub-lethal dose of HCAs was used, subsequent spoilage may then be significantly worse. The use of alternate HCAs, which are not metabolised by *B. bruxellensis*, may be a strategy worth exploring.

Macromolecule alternatives to sulfite

Various macromolecules have been investigated more recently for their capacity to inhibit *B. bruxellensis* growth, with a significant focus on the potential application of killer yeasts, or their associated toxins (Comitini et al. 2004a,b, Santos et al. 2009, 2011, Comitini and Ciani 2011, Mehlomakulu et al. 2014). Given the limited range of species able to grow in wine concurrent with *B. bruxellensis*, the most likely route for killer toxin application would be via heterologous production and subsequent addition to wine; it remains to be seen whether this would be an economically viable option. Some killer yeasts, however, exert their fungicidal impact by secretion of β-glucanase (Izgue et al. 2007), an enzymatic activity already utilised in wine production for clarification. A commercial β-glucanase preparation was shown to inhibit growth of *B. bruxellensis*, and another spoilage yeast, *Zygosaccharomyces bailii*, while not affecting growth of *S. cerevisiae* (Enrique et al. 2010). The concentration required was, however, relatively high; up to 20-fold greater than that recommended by manufacturers. The same authors also found that peptides derived from bovine lactoferrin are effective in model medium and wine (Enrique et al. 2008), again only at high concentration that is likely to have a sensory impact of its own. Fungal chitosan, in contrast, shows requisite inhibition of *B. bruxellensis* within the range acceptable for oenological application (Portugal et al. 2013, Taillandier et al. 2015), but more data are required to confirm its efficacy over extended periods of wine maturation.

A highly effective strategy involves a combination of multiple chemical or macromolecular inhibitors in concert with physical removal of *Brettanomyces* cells from the wine. The variable and elongated cellular morphology of *B. bruxellensis* cells must be taken into account when considering filtration regimes, along with timing of sulfur dioxide addition. Some strains can be successfully removed from red wine using 1.2 μm membranes, while others require 0.8 μm pore size (Umiker et al. 2012). A decrease in cell size has been noted for *B. bruxellensis* post-treatment with sulfite (Agnolucci et al. 2010), and there is some evidence of *Brettanomyces* passing through 0.45 μm membranes (Millet and Lonvaud-Funel 2000), therefore the wine

Table 2. Sulfite concentration required to control *Brettanomyces bruxellensis* growth.

Sulfite dosage	How evaluated	Duration of monitoring	Reference
>0.25 mg/L mSO2	Viability and culturability in red wine	14 days	du Toit et al. (2005)
>0.4 mg/L mSO2	Viability, culturability and ethylphenol production in synthetic wine	55 days	Agnolucci et al. (2010)
>0.4 mg/L mSO2	Ethylphenol production in wine stored in bottle	130 days	Coulon et al. (2010)
>0.6 mg/L mSO2	Growth in laboratory medium with added sulfite	7 days	Curtin et al. (2012b)
>0.6 mg/L mSO2	Growth in laboratory medium with added sulfite	15 days	Vigentini et al. (2013)
1 mg/L mSO2	Culturability in red wine	35 days	Barata et al. (2008a)
>1.25 mg/L mSO2	Growth in laboratory medium with added sulfite	–	Conterno et al. (2006)
>50 mg/L Potassium metabisulfite	Culturability and ethylphenol production in wine (pH 3.8)	22 months	Portugal et al. (2013)

should ideally be filtered prior to sulfite addition, to reduce the risk of this occurrence.

Physical treatment alternatives to sulfite

Physical treatments [see review by Zuehlke et al. (2013)] can also be applied directly to wine to reduce the size of *B. bruxellensis* population. Heat treatment is surprisingly effective at a modest temperature, with 4–6 min hold time at 37.5°C sufficient to achieve a 6-log reduction in viable *B. bruxellensis* in red wine (Couto et al. 2005). A limitation of this particular study was the use of cells prepared in laboratory medium, rather than grown in wine; the authors acknowledge this may influence the physiological state of the population and affect their subsequent thermotolerance. Nonetheless, mild short-term heat treatment shows great promise as an adjunct to other *Brettanomyces* control strategies. High-powered ultrasonics [reviewed by Jiranek et al. (2008)] combined with mild heat was recently shown in a preliminary laboratory-scale study to have potential as a continuous flow treatment to reduce *B. bruxellensis* population size in wine (Gracin et al. 2015). Greater reduction in population size was achieved through exposure of wine to ultraviolet (UV) radiation by pumping through a UV-C reactor at 1000 L/min (Fredericks et al. 2011), although the long-term compositional and sensory impact on resultant wines has not been described. High-hydrostatic pressure (as reviewed by Buzrul 2012) also appears effective as a short-term antimicrobial treatment, with a hold time of ~10 min, and with some studies reporting no detectable sensory impact on the treated wine. An alternative longer term treatment involves continuous application of low-electric current (200 mA) to wine during barrel maturation, which decreases the culturable population size more slowly than traditional treatments such as sulfite but provides an ongoing barrier to further growth and ethylphenol production for extended periods of time (Lustrato et al. 2010, 2015).

Sanitation

All treatments to kill and/or remove *Brettanomyces* cells can only be effective if 'cleaned' wine is subsequently transferred into appropriately sanitised barrels or tanks. Some of the physical treatments described above have also been evaluated for their efficacy in the sanitisation of barrels. Ultraviolet irradiation was not considered as effective as ozone or steam (Guzzon et al. 2011), although all three decreased yeast populations in barrel by ~3-log CFU/cm². A critical consideration for barrel sanitation is whether a treatment is penetrative, as culturable *B. bruxellensis* has been recovered from up to 6–8 mm into staves (Barata et al. 2013). High-power ultrasonics was shown to augment standard hot-water washing, significantly reducing the *B. bruxellensis* population up to 4 mm into the oak staves (Schmid et al. 2011). Hot-water alone is, nonetheless, a highly effective barrel treatment, given sufficient time for heat to penetrate into staves (Fabrizio et al. 2015). Shaving and firing of 'old' oak barrels combines physical removal of surface layers with heat, and wine stored in such barrels contained 85% less ethylphenols than wine in untreated barrels (Pollnitz et al. 2000).

Reducing spoilage potential by removal of precursors

While greatest success in avoiding Brett spoilage is most likely to be achieved by limiting *B. bruxellensis* growth, minimising the concentration of HCA precursors in wine can reduce the degree of spoilage. Winemaking processes that maximise extraction (e.g. carbonic maceration) influence the resultant concentration of HCAs in wine (Kheir et al. 2013), thus efforts to maximise colour and tannin content in wine from red cultivars may

enhance the risk of Brett spoilage. Use of pof+ yeast strains to undertake alcoholic fermentation is one way to partly reduce the concentration of free HCAs, as described earlier. Similarly, choice of *Oenococcus oeni* strain to conduct malolactic fermentation can influence availability of free HCA precursors (Chescheir et al. 2015). Finally, while free HCAs may be adsorbed onto yeast cell walls (Salameh et al. 2007), fining-based approaches to HCA removal and avoidance of Brett spoilage have not been widely studied.

Removal of ethylphenols post-spoilage of wine

In contrast, various adsorbents have been explored for potential remediation of Brett-affected wine. For example, esterified cellulose polymers were shown to remove 20–30% of 4-EP and 4-EG in red wine, while having only a modest impact on colour intensity and concentration of anthocyanins (Larcher et al. 2012). A similar magnitude of volatile phenol removal was noted when yeast lees (in the form of active dried yeast) was added to model wine containing 4-EP and 4-EG (Chassagne et al. 2005), and there appears a scope to optimise the capacity of yeast biomass to perform this function (Pradelles et al. 2009). More aggressive adsorbents can potentially remove more phenols but at the cost of stripping wine of other aromatic compounds. Nonetheless, when combined with physical treatments such as reverse osmosis it may be possible to reduce this impact (Ugarte et al. 2005). Alternatively, more specific adsorbents such as molecularly imprinted polymers (MIPs) could be developed for removal of Brett compounds. Two recent studies describe MIPs developed against ethylphenols (Garcia et al. 2015, Teixeira et al. 2015), however, initial results indicate poor specificity when attempting to remove these compounds from spoiled wine. A preliminary study recently published inferred that some remediation is even possible post-bottling; suberin in cork closures was shown to have significant ethylphenol-binding capacity (Gallardo-Chacón and Karbowiak 2015). If combined in sequence it may be possible to decrease ethylphenol concentration in an affected wine to a concentration that is below the sensory perception threshold.

Concluding remarks

Detailed knowledge concerning the distribution of *Brettanomyces* yeasts outside of industrial fermentation niches remains one of the key factors limiting the design of strategies to eliminate *B. bruxellensis* from the winemaking process. Recent advances in understanding the biology of this species, and applied research in wine sterilisation and equipment sanitisation, have nonetheless provided winemakers with a range of approaches to manage better the impact of *Brettanomyces* on wine sensory properties.

Acknowledgements

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