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ORIGINAL RESEARCH ARTICLE

Critical areas for *Brettanomyces bruxellensis* contamination and biofilm formation in the cellar: on the origin of wine spoilage

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ABSTRACT

The cellar environment harbours a consortium of microorganisms on the material surfaces and in the air. Among these microorganisms, the spoilage yeast *Brettanomyces bruxellensis* can colonise surfaces due to its specific bioadhesive properties. In this study, air and surface samples were collected in several wineries during the winter period. *B. bruxellensis* was detected in the cellar environment either in the air or on the surfaces of various materials, including in tartaric acid precipitates. Difficult-to-clean tank equipment (taps, wall angles, valves) were identified as critical areas where *B. bruxellensis* was frequently detected. To confirm that surfaces contaminated by *B. bruxellensis* could be involved in wine contamination, yeast growth and volatile phenol production were monitored in wine in contact with stainless steel harbouring biofilms. The presence of bioadherent cells and biofilms in contact with the wine resulted in significant cell release into the wine, leading to population growth and the production of volatile phenols at concentrations above the olfactory detection threshold. This study demonstrates the possibility of wine spoilage by resident and adherent populations of *B. bruxellensis* and confirms the need to pay special attention to the hygiene of hard-to-reach areas such as valves.

KEYWORDS: *Brettanomyces bruxellensis*, wine, cellar environment, biofilms, volatile phenols

INTRODUCTION

Among the types of fermentation, the winemaking process related to the transformation of grapes into wine involves a consortium of microorganisms that may originate from the grapes or the cellars (Fleet, 1993; Mortimer and Polsinelli, 1999). Fermentations could also be managed using selected starters. These microorganisms, mainly yeasts and bacteria, will influence the organoleptic properties of the finished wine, with potentially positive or negative effects. Moulds and filamentous fungi, such as *Penicillium*, *Cladosporium* and *Aspergillus* genera (Goto *et al*., 1989), are also members of the microbiota in the vineyard and cellar environments; in the vineyard, they are often responsible for the reduction of crop yield and grape berry quality (Fournier *et al* 2013; Negri *et al*., 2017). In the cellar environment, microorganisms colonise surfaces and equipment that could be reservoirs for the transfer of microbes from the winery to wine fermentations (Connell *et al*., 2002; Garijo *et al*., 2008; Ocón *et al*., 2013a; Ocón *et al*., 2013b; Bokulich *et al*., 2013). Water droplets and dust particles are also considered to be a vector of dissemination (Curiel *et al*., 2000), depending on factors such as temperature, humidity, particle size, air currents, human activity, but also the architecture of the winery (Ocón *et al*., 2013a; Ocón *et al*., 2013b). Indeed, *Saccharomyces cerevisiae* and other yeasts such as *Brettanomyces bruxellensis* have been previously identified in winery air samples (Connell *et al*., 2002). Microorganisms are also present on the surface of materials in the cellar and are likely to be sources of contamination in contact with wine (Bokulich *et al*., 2013; Tek *et al*., 2018; Abdo *et al*., 2020). Moulds are mainly found on floors and walls but also in oak barrels and can affect the sensory qualities of wine, such as the cork taint caused by the metabolism of chlorine compounds to trichloroanisole (Haas *et al*., 2010; Ciccarone *et al*., 2012). The presence of spoilage microorganisms on the surface of materials is particularly damaging when *B. bruxellensis* is detected. In fact, this yeast is considered to be the most problematic spoilage yeast in the cellar due to the production of acetic acid, volatile phenols characterised by stable, leather or solvent odours, N heterocycle responsible for the "mousy" off-flavour if curative actions are not carried out in time (Chatonnet *et al*., 1992; Grbin *et al*., 2007; Lattey *et al*., 2010; Agnolucci *et al*., 2017). *B. bruxellensis* is known to be present in the air (Beech., 1993; Connell *et al*., 2002), but also on surfaces such as floors, walls, vat rooms, winemaking equipment, pumps and pipes (Fugelsang., 1997; Cartwright *et al*., 2018; Oro *et al*., 2019; Abdo., 2020). This presence on the surface of various materials may be related to the bioadhesion properties of the species (Joseph *et al*., 2007; Lebleux *et al*., 2020; Le Montagner *et al*., 2023; Le Montagner *et al*., 2024). *B. bruxellensis* can bioadhere to materials such as stainless steel, wood, polystyrene and glass (Joseph *et al*., 2007; Oelofse *et al*., 2009; Tristezza *et al*., 2010; Kregiel *et al*., 2018; Dimopoulou *et al*., 2019). Bioadhesive cells can develop into a biofilm, a structured microbial community that, after a growth phase, produces a protective extracellular matrix. Finally, a final step is related to the dispersal of the microbial cells of the biofilm. This lifestyle provides better protection in poor and stressful environments due to a specific genomic expression profile and physiology compared to planktonic cells. In this context, the objectives of our study are i) to diagnose the presence of *B. bruxellensis* in the cellar environment in different wineries from the Bordeaux region, ii) to evaluate the impact of *B. bruxellensis* biofilms on the wine quality. The final objective of our study is to better understand the origin of wine contamination by resident *B. bruxellensis* strains in the cellar.

MATERIALS AND METHODS

1. Samplings

The present study was carried out in 3 wineries in the Bordeaux region during the period from December 2021 to February 2022. Winery CH-1 is located in the Médoc appellation, winery CH-2 in the Saint-Émilion appellation and winery CH-3 in the Pessac-Léognan appellation. Samples were taken from empty tanks after vinification.

1.1. Aerocollection

The identification of microorganisms present in the cellar air was carried out on 250 litres of air collected with an aero-collector (AirTest®, LCB Food safety). The device was placed at a height of 1 meter on a portable tripod in the centre of the different sampling areas. The air was aspirated and directed onto 90 mm Petri dishes (Grosseron) containing selective agar media. For isolation of total yeasts (TY), a YPD medium composed of 2 % glucose (Fisher Bio-Reagent™), 2 % agar (Fisher Bio-Reagent™), 1 % yeast extract (Fisher Bio-Reagent™) and 1 % peptone (Fisher Bio-Reagent™) supplemented with biphenyl (150 mg/L-Acros Organics) and chloramphenicol (150 mg/L-Fisher Chemical) was used. Finally, for the specific isolation of *B. bruxellensis* (YPD-Brett plate), the TY medium was supplemented with Actidione (500 mg/L-Thermo Scientific). The dishes were incubated at 25 °C for cultivation for 5 days for yeasts and moulds and 8 days for *B. bruxellensis*. The results are then expressed as CFU/L of air.

1.2. Surface sampling

Surface sampling was carried out inside and on the bottom of the wooden, stainless steel and concrete tanks and on the floor of the cellar. Sampling was carried out using contact dishes with a diameter of 65 mm (Grosseron) containing 18 mL of selective culture medium, poured into contact dishes to obtain a curved agar allowing application on flat surfaces, according to the compositions described in part 1.1, for total yeast and *B. bruxellensis* specific enumeration. Application to the surfaces was carried out by applying light pressure to the box for 10 seconds. The dishes were then incubated at 25 °C, face up, for 5 days for yeasts and 8 days for *B. bruxellensis*. The results are expressed in CFU/cm^2.

1.3. Swab

Swabbing was only carried out in the CH-2 and CH-3 wineries on valves, tasting valves, inside various tanks and on various winery materials. The use of swabs was carried out to collect contamination in confined spaces. The swab was applied to the surface delimited by a template of 25 cm^2 for 10 seconds and placed in a 4 mL tube of peptone buffer (Humeau) until cultured. The buffer was then diluted in series, inoculated onto total yeast medium and YPD-Brett medium and incubated at 25 °C for 5 and 8 days, respectively. Results are expressed as CFU/ cm^2.

2. Identification at species- and strain-level

To confirm that the colonies isolated on YPD-Brett medium belonged to the species *Brettanomyces bruxellensis*, identification was performed by MALDI-TOF MS Biotyper on fresh colonies less than 10 days old using protein extraction and an oenological laboratory-specific database as previously described by Windholtz *et al.,* 2021. Identification results were expressed as suggested by the manufacturer (Bruker, Karlsruhe, Germany), with scores ranging from 0 to 3. Scores > 2.3 indicate species identification, scores between 1.7 and 2.0 indicate genus identification, and a score <1.7 indicates unreliable identification. Isolates were genotyped using microsatellite marker analysis (Avramova *et al*., 2018). Briefly, DNA was extracted from fresh colonies less than 10 days old. Colonies were collected and lysed in 30 µL NaOH at 20 mM for 10 min at 99 °C. The extracted DNA was then stored at –20 °C. The PCR reaction used was that described by Avramova *et al*. (2018).

3. Bioadhesion properties of colonies isolated from surface

Bioadhesion was performed on stainless steel following the cleaning procedure described in Le Montagner *et al*., 2023. A single isolate from the surface sample was selected for bioadhesion testing. Few colonies were obtained on YPD medium and transferred to 10 mL of a mixture of 25 % (v/v) WLM medium, the composition of which is described in Le Montagner *et al*., 2023, and 75 % (v/v) YPD and incubated for 48 h (25 °C, 180 rpm). This adaptation step was repeated 3 times and the percentage of WLM was gradually increased (50 %, 75 % and finally 90 %). The bioadhesion protocol was carried out according to Le Montagner *et al*., 2023. After 3 h of bioadhesion, the surface of the coupon was observed by confocal microscopy at the Bordeaux Imaging Centre Facilities of the INRAE Plant Pole, and observations were made using an immersion lens. Confocal imaging was performed using a Zeiss LSM 880 confocal laser scanning microscope with a 40× immersion objective with a numerical aperture of 1. The excitation wavelengths and emission windows were 488 nm/499–553 nm and 561 nm/588–688 nm for CFDA and propidium iodide, respectively; fluorochromes were detected sequentially line by line. Adherent cells were counted as the mean of 10 fields of view.

4. Volatile phenols monitoring

4.1. Wine adaptation and bioadhesion on Stainless steel coupons

The red wine selected for the experiment was first analysed for the presence of *B. bruxellensis* by enumeration on YPD Brett medium and the presence of volatile phenols. Three strains belonging to the Wine 3 (CBS 2499), Wine 1 (AWRI 1499) and Beer (AWRI1608) groups were selected for this experiment. The adaptation step in wine was then carried out according to the protocol described by Le Montagner *et al*., 2023. A few colonies were obtained on YPD medium and transferred to 10 mL of a mixture of 25 % (v/v) red wine (Graves, 12.5 % vol, total $S0_2$ 23 mg/L, pH 3.7) and 75 % (v/v) grape juice and incubated for 48 h (25 °C, 180 rpm). This adaptation step was repeated 3 times and the red wine content was gradually increased (50 %, 75 % and finally 90 %).

For bioadhesion, the cell culture was centrifuged at 7000 g for 5 min at room temperature and the cell pellet was washed twice with physiological water (NaCl 0.9 %). The pellet was then resuspended in 20 mL of a mixture of 90 % wine and 10 % grape juice and adjusted to a final concentration of 10^7 cells/mL. Bioadhesion was performed on 316L stainless steel coupons measuring 14 mm × 25 mm (Goodfellow). The coupons were cleaned as previously described (Le Montagner *et al*., 2023). After rinsing, the coupons were placed in a vial containing 30 mL of the same red wine of Graves Appellation and then stored at 20 °C until analysis. For each measurement point at 3 h, 2, 4, 6, 8, 14, 21 and 28 days, samples were prepared in triplicate.

4.2. Quantification of bioadherent and planktonic cells

The viable cell count was performed by detaching cells present on the surface of the coupon. The coupon was cleaned of non-adherent cells by 5 successive washes in sterile physiological water (NaCl 0.9 %). The coupon was then placed in a 50 mL tube containing 10 mL of sterile physiological water (NaCl 0.9 %) and placed in a sonication bath at 47 Hz for 2 minutes. After this sonication step, the tube was vortexed at maximum speed for 40 seconds. Serial dilutions were then performed and 100 µL of the suspension was inoculated in triplicate on YPD agar. The result is then expressed in colony-forming units per cm^2 (CFU/cm^2).

Quantification of free cells in the wine was performed after the removal of the coupon from the vial. The vial was shaken to homogenise the wine. A series of dilutions was then made and 100 µL of the suspension was inoculated in triplicate on YPD agar. The result is then expressed in colony-forming units per millilitre (CFU/mL) of wine.

4.3. Biofilm thickness

Biofilm thickness was measured by confocal microscopy. After the rinsing steps described in part 4.2, the coupon was placed in a solution of Chemsol B15 (Biomerieux) containing 1 % (v/v) 5(6)-carboxyfluorescein diacetate (CFDA) (Thermo Fisher Scientific) at 8 mg/mL and 0.2 % (v/v) propidium iodide

(PI) at 1 mg/mL (Thermo Fisher Scientific) for 15 minutes. The surface of the coupon was observed by confocal microscopy in the facilities of the Bordeaux Imaging Centre of the INRAE Plant Service. Observations were made with the immersion objective. Confocal imaging was performed using a Zeiss LSM 880 (Zeiss) laser scanning confocal microscope with a 40× immersion objective and a numerical aperture of 1. The excitation wavelengths and emission windows were 488 nm/499–553 nm and 561 nm/588–688 nm for CFDA and propidium iodide, respectively. Fluorochromes were detected sequentially, line by line. Thickness measurement was performed by taking sequential images of each focal plane using the z-stack function of the ZEN microscopy software (Zeiss). Thickness analysis was then performed using the ROI manager function of the FIJI image processing extension of the ImageJ software.

4.4. Volatile phenols quantification

The analysis of volatile phenols was carried out on 10 mL of wine containing the coupon of bioadherent cells of *B. bruxellensis*. The wine was placed in a 25 mL vial containing 3.5 g NaCl. An internal standard consisting of deuterated 4-ethylphenol was added to the wine at a concentration of 100 µg/L. The analysis was performed by gas chromatography equipped with a solid phase microextration (SPME) autosampler coupled to a quadrupole mass spectrometer (Romano *et al*., 2008). The SPME was performed using an 85 µm polyacrylate fibre. The sample was injected into a 30 m × 0.25 mm column in splitless mode. Helium was used as carrier gas at a flow rate of 1 mL/min. The GC programme used was as follows 60 °C for 1 min, then increasing at 3 °C/min to reach 125 °C, then increasing at 5 °C/min to reach 220 °C and finally at 220 °C for 10 min. The electron impact mass spectrometer was operated in SIM mode (Selected Ion Monitoring). Volatile phenols were quantified by comparing the peak areas of specific ions (4-vinylphenol m/z 120, 4-vinylguaiacol m/z 150, 4-ethylphenol m/z 107, 4-ethylguaiacol m/z 137) with that of deuterated 4-ethylphenol (m/z 113) used as an internal standard.

5. Statistical analysis

The Kruskal–Wallis statistical test (Rstudio software, RStudio Team, 2020; p-value < 0.05) was performed using R-package agricolae (Mendiburu, 2021)

RESULTS

1. Detection of *B. bruxellensis* in the cellar environment

The presence of yeast in the air was studied over a volume of 250 litres, using specific growth media (Table 1). Nine out of ten air samples were positive for yeasts with the highest concentrations for the CH-2 cellar. Colonies growing on YPD-Brett were detected in three out of ten samples and only in two wineries (CH-1 and CH-3).

Surface analyses were carried out on various winery materials such as barrels, stainless steel tanks, concrete vats and the cellar floor (Table 2). For the vat room elements, samples were taken from the internal surfaces of the bottom and centre of empty vats ("bottom" and "inside"). 25 out of 47 surface samples were positive for the presence of yeasts at levels ranging from less than 1 to 36 CFU/cm^2. The highest number of yeast-positive samples was found in winery CH-2 (15 out of 20 samples) compared to 5 out of 11 and 5 out of 16 for wineries CH-3 and CH-1, respectively. Yeasts were detected on all types of surface materials sampled, wood, stainless steel, concrete and in various parts of the tanks (inside the tank, at the centre and bottom of the tank) and on the cellar floor. Considering positive samples for total yeasts, the sampling area (floor n = 4 and internal surface n = 21) and the type of surface material (wood n = 9, concrete n = 4 and stainless steel n = 8) have no significant effect on the total yeast count (p-value > 0.05).

For the YPD-Brett medium, 18 out of 47 samples were positive. Winery CH-1 has the highest number of positive samples (11 out of 16) compared to 2 out of 11 and 5 out of 20 for wineries CH-3 and CH-2, respectively. The CH-1 winery samples displayed also the highest cell concentration per cm^2 up to 13 CFU/cm^2 whereas the CH-2 and CH-3 winery

TABLE 1. Detection and enumeration of yeast in the air of different cellars.

			YPD-Brett		Total Yeasts	
Winery	Appellation	Number of samples	Positive sample	Enumeration* (CFU/L)	Positive sample	Enumeration* (CFU/L)
CH-1	Listrac-Médoc	2	2	4×10-3-32×10-3	2	4 × 10-3–16 × 10-3
CH-2	Saint-Émilion	4	0	/	4	12 × 10-3–68 × 10-3
CH-3	Pessac Léognan	4	1	4 × 10-3	3	0-20 × 10-3

*Enumerations are given as minimum and maximum colony counts.

TABLE 2. Detection and enumeration of yeasts by surface sampling from various materials and different wineries.

					Surface sampling			
					YPD-Brett		Total Yeasts	
Winery	Appellation	Surface	Sample areas	Number of samples	Positive sample	Enumeration* (CFU/cm²)	Positive sample	Enumeration* (CFU/cm²)
CH-1	Listrac-Médoc	Wooden tank	Bottom	8	5	1–13	3	1–2
			Inside	8	6	1–13	2	1–13
CH-2	Saint-Émilion	Stainless steel tank	Bottom	5	0	/	4	1–36
			Inside	5	0	/	4	1–36
		Wooden tank	Bottom	2	2	/	2	11–15
			Inside	2	1	/	2	11–15
		Cellar	Floor	6	2	/	3	2–4
CH-3	Pessac Léognan	Concrete tank	Bottom	5	1	/	2	1–5
			Inside	5	1	/	2	1–7
		Cellar	Floor	1	0	/	1	14

*Enumerations are given as minimum and maximum colony counts.

samples had less than 1 CFU/cm² per sample except for the cellar floor. The results showed that 14 out of 20 wooden tank surface samples were positive for *B. bruxellensis*. Considering positive samples (YPD-Brett), the sampling area does not seem to influence the number of colonies detected (p-value > 0.05) as for total yeasts.

Swabs were used to gain access to difficult sampling areas such as taps, wall angles, valves, inside pumps, pipes and water recovery nozzles (Table 3). Yeasts were detected on all surfaces sampled at population levels ranging from 8×10^{-2} to 6.4×10^{3} CFU/cm². In the CH-2 and CH-3 wineries, 25 out of 29 samples and 12 out of 17 samples respectively were positive for the presence of yeasts, indicating a low level of hygiene in these difficult-to-clean areas.

The detection of yeasts on YPD Brett medium was possible in all the areas analysed, except for the soil of the barrel cellars. The CH-2 cellar samples showed a higher proportion of positive samples than the CH-3 cellar with 14 out of 29 and 5 out of 17 positive samples respectively. However, the colony count per cm² was relatively low for the CH-2 winery samples with a maximum of 2 CFU/ cm² compared to the CH-3 winery samples where the colony count per swab ranged from 2 to 68 CFU/ cm² in the concrete vat. The swabs taken from the concrete vats in the CH-3 winery correspond mainly to samples of tartaric acid precipitation, which were found to be colonised by a high number of yeasts after optical microscopy. Samples taken from the winemaking equipment in the CH-3 winery did not show the presence of *B. bruxellensis*.

Finally, the identification of colonies isolated from the YPD Brett medium by MALDI-TOF MS confirmed that 98.1 % of the isolates belonged to *B. bruxellensis*. Using microsatellite markers, isolates were shown to belong to the Wine 3 (n = 25) and Kombucha (n = 2) genetic groups as defined by Avramova *et al*. (2018) (data not shown). Three distinct genetic profiles were highlighted in the Wine 3 group, whereas one genetic profile was identified in the Kombucha group.

2. *B. bruxellensis* biofilm and volatile phenols production in wine

One representative of each genetic profile/group was used to test bioadhesion properties. Bioadhesion was observed after 3 hours for all strains. The mean bioadhesion capacity measured was 6.6×10^{3} cell/cm². The strains isolated from winery surfaces in the present study do not show significant differences in bioadhesion capacity (p-value > 0.05) compared to spoiled wine isolates from the Wine 3 genetic group (n = 10, mean 8.0×10^{4} cell/cm²) and Kombucha genetic group (n = 4, mean 8.5×10^{3} cell/cm²) (Le Montagner *et al*., 2023).

Phenol production in wine by *B. bruxellensis* biofilms was further investigated. Growth under biofilm and planktonic life modes and the production of volatile phenols were monitored. After the wine adaptation step, bioadhesion was carried out on stainless steel coupons after 3 hours at room temperature. The rinsed coupons were then placed in red wine and several parameters were monitored for 28 days: the concentration of bioadherent and planktonic cells in the wine, the thickness of the bioadherent/biofilm cell layer and finally the production of volatile phenols. No *B. bruxellensis* was detected in the red wine selected for the experiment and the initial concentration of volatile phenols was below the analytical detection limit (3 µg/L).

TABLE 3. Detection and enumeration of yeasts by swabbing in different wineries.

					Surface sampling			
					YPD-Brett		Total Yeasts	
Winery	Appellation	Surface	Sample areas	Number of samples	Positive sample	Enumeration* (CFU/ cm^2)	Positive sample	Enumeration* (CFU/ cm^2)
CH-2	Saint-Émilion	Stainless steel tank	Valve	6	2	8×10^{-2}–12×10^{-1}	6	26–4.8×10^{3}
			Inside	6	2	4×10^{-2}–16×10^{-1}	2	1.9×10^{3}–3.6×10^{3}
			Tasting valve	6	5	4×10^{-2}–2.4×10^{-1}	6	0.9×10^{3}–6.4×10^{3}
		Wooden tank	Valve	1	0	/	1	2
			Inside	1	1	16×10^{-1}	1	3×10^{2}
			Tasting valve	1	0	/	1	4×10^{2}
		Barrel cellar	Floor	1	0	/	1	4.4×10^{3}
		Materials	Pump	3	1	12×10^{-1}	3	5.6×10^{2}–4×10^{3}
			Pipe	1	1	4×10^{-2}	1	2.8×10^{3}
			Nozzle	3	2	4×10^{-2}	3	6–18
CH-3	Pessac Léognan	Concrete tank	Valve	2	1	2	1	4
			Inside	3	2	38–68	2	12–15
			Tasting valve	2	2	4–25	1	5
		Barrel cellar	Floor	3	0	/	3	5×10^{-1}–6
		Materials	Pump	2	0	/	0	0
			Pipe	2	0	/	2	8×10^{-2}-6×10^{-1}
			Nozzle	3	0	/	3	1–10

*Enumerations are given as minimum and maximum colony counts.

2.1. Enumeration of bioadherent and planktonic cells and biofilm thickness

The enumeration of bioadherent and planktonic cells was monitored every 48 hours for 8 days and then every 7 days until day 28 for the 3 strains of *B. bruxellensis* AWRI 1499, AWRI 1608, CBS 2499 after detachment and by cell culture. Our results showed that, as expected, the initial concentration of bioadherent cells differed between strains (Figure 1A). Strains AWRI 1608 and CBS 2499 showed the highest numbers of 10^6 cells/cm^2 on the YPD medium compared to 10^3 cells/cm^2 for strain AWRI 1499. Different dynamics of bioadherent cell populations were then highlighted depending on the strain. A decrease in the level of bioadherent cells was observed for the 3 strains during the first 5 days. For the AWRI 1608 and CBS 2499 strains, this decrease was followed by a stationary phase between days 8 and 21, and then a decrease on day 28 to reach 6.3×10^4 CFU/cm^2 and 5.14×10^3 CFU/cm^2, respectively. For AWRI 1499, the stationary phase was followed by an increase in bioadherent cells from day 14 to 28, finally reaching 1.24×10^3 CFU/cm^2.

For the cultivable planktonic cells in wine, a significant unhooking was observed during the first 4 days, with the population level reaching 3.3×10^2 CFU/mL, 1.43×10^4 CFU/mL and 7.6×10^4 CFU/mL for AWRI 1499, AWRI 1608 and CBS 2499, respectively (Figure 1B). For AWRI 1608 and CBS 2499, this significant increase was followed by a stationary phase from day 6 to day 21 and a small decrease on day 28. The strain AWRI 1499 shows a stationary phase between day 4 and day 8. From day 14, as with the bioadherent cells, a significant increase in the population of wine planktonic cells was observed until the end of the monitoring, reaching the same final population levels as the AWRI 1608 strain.

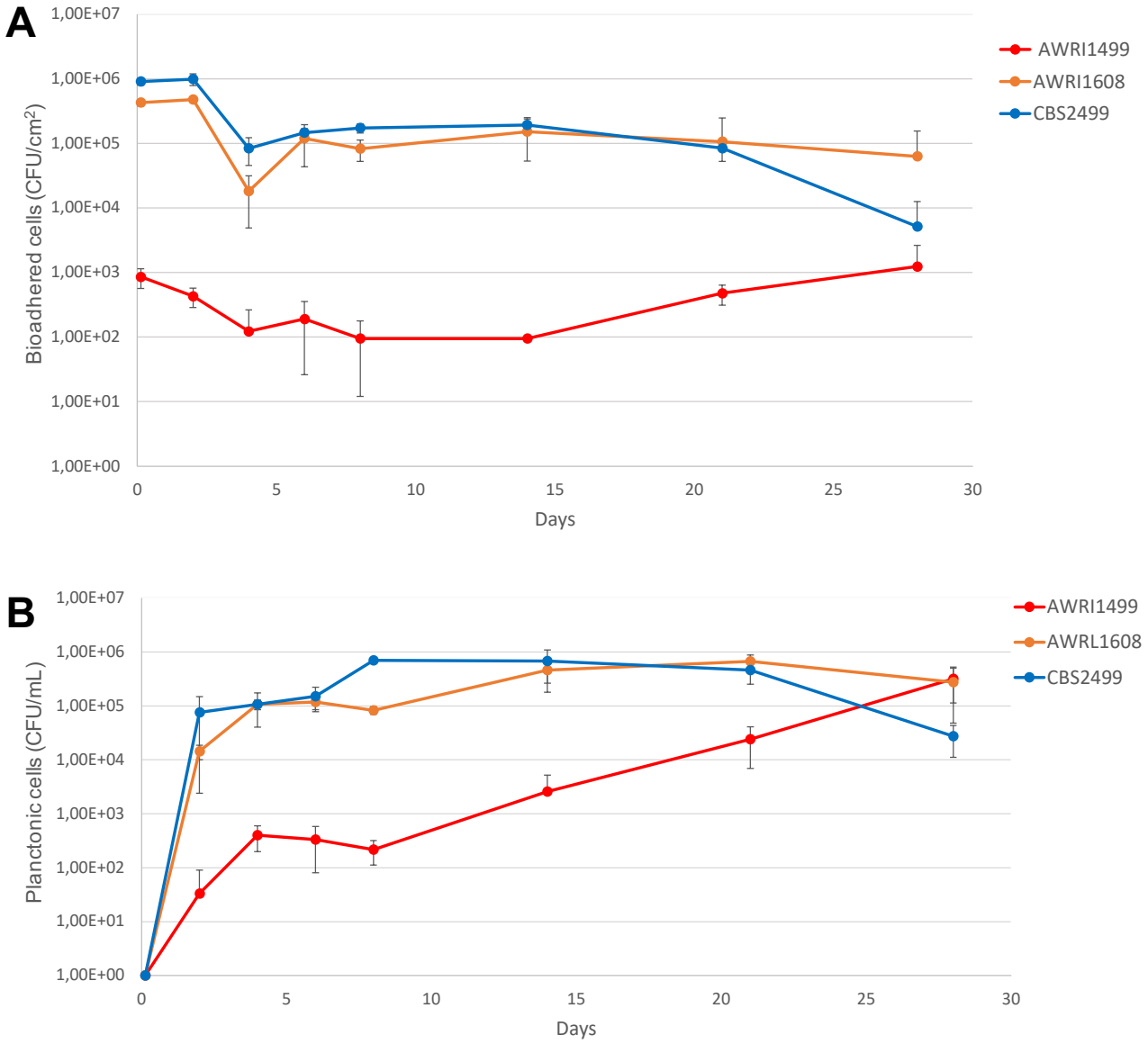

FIGURE 1. Growth monitoring of *B. bruxellensis* in wines incoculated by exposition to stainless stell coupons harbouring bioadherent cells. (A) Count of bioadherent cells (B) and planktonic cells (values are the mean of three independent analyses; the error bars indicate the standard deviation).

The thickness of the biofilm cell layer was monitored during the 28 days of the experiment. This monitoring could not be carried out on strain 1499 because it was not present homogeneously on the surface of the coupon. Therefore, this monitoring was only carried out on strains AWRI 1608 and CBS 2499 (Figure 2). The biofilm of strain AWRI 1608 was significantly thinner than that of strain CBS 2499 during the first 21 days, but a significant increase from 16 µm to 25 µm was observed on day 28. For strain CBS 2499, a significant increase was observed during the first 8 days in wine with a change from 15 µm to 24 µm, followed by a significant decrease between day 8 and day 14 and a stabilisation until the end of the monitoring.

2.2. Production of volatile phenols

The production of ethyl and vinyl phenols by *B. bruxellensis* was monitored by GC-MS quantification. The volatile phenols concentration produced by the three strains were below the detection limit during the first 2 days of monitoring. For strain CBS 2499, detection began on day 4, while for AWRI strains 1608 and AWRI 1499, detection occurred on days 6 and 21, respectively (Figure 3). The concentration of ethyl phenols was significantly higher than that of vinyl phenols for strains AWRI 1608 and CBS 2499, while for strain AWRI 1499 the concentrations of the two volatile compounds were relatively similar. The sensorial detection threshold (420 µg/L) was reached for

AWRI strains 1608 and CBS 2499 between days 8 and 14, whereas it was never reached for AWRI 1499 with a final maximum concentration of 273 µg/L on day 28. For strains CBS 2499 and AWRI 1608, the final concentrations were 1432 µg/L and 1485 µg/L, respectively.

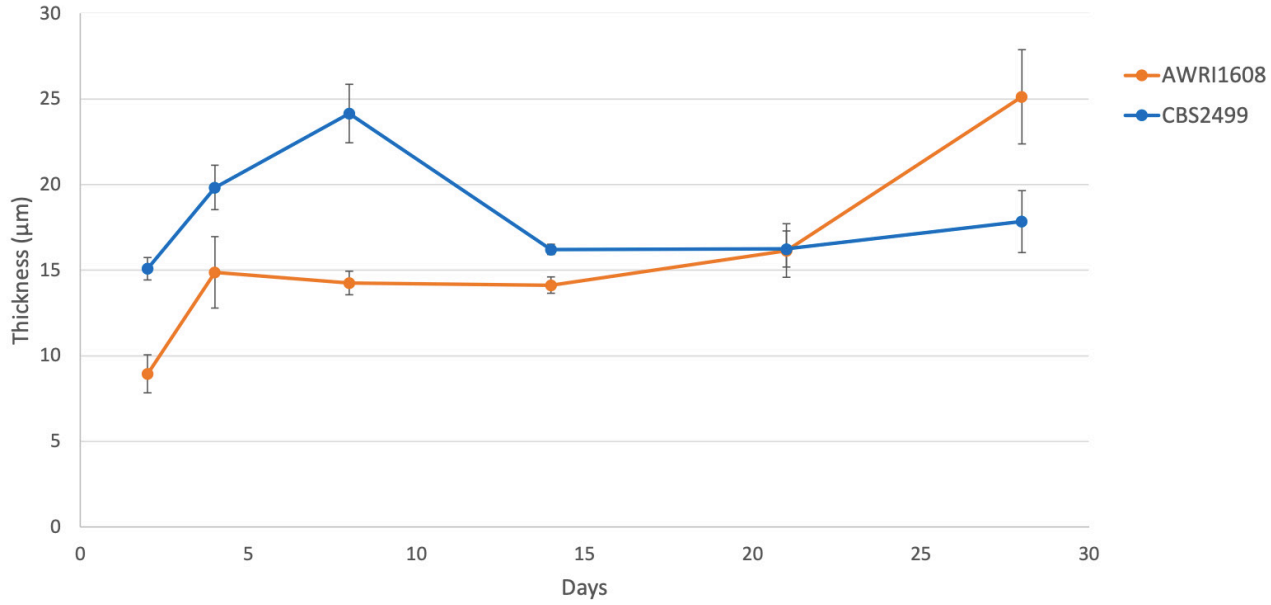

FIGURE 2. Monitoring the thickness of the biofilms on stainless steel (values are the mean of three independent analyses; the error bars indicate the standard deviation).

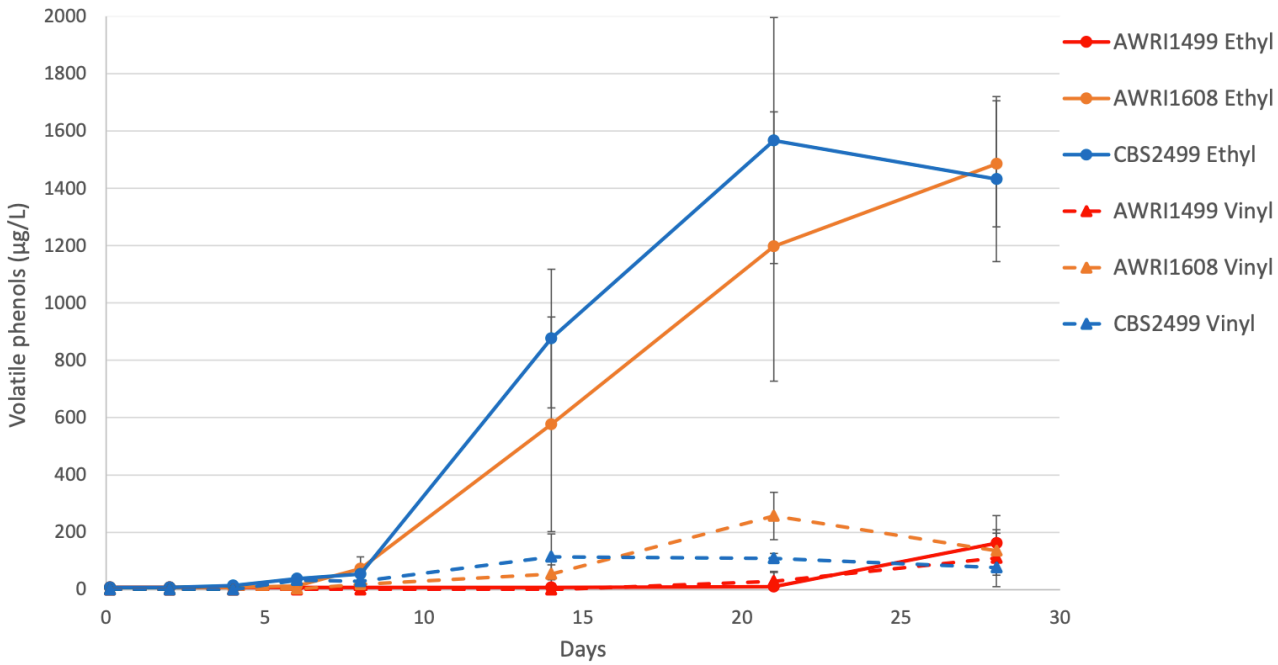

FIGURE 3. Production of volatile phenols by *B. bruxellensis* in wines inoculated by exposition to stainless steel coupons harbouring bioadherent cells (values are the mean of three independent analyses; the error bars indicate the standard deviation).

DISCUSSION

Microbial colonisation in the winery environment has been reported by several authors (Bokulich *et al.*, 2013; Abdo *et al.*, 2020). The persistence of microorganisms in cellars could be related to sites that are difficult to clean and disinfect, or to specific abilities to survive in the air or on solid surfaces (Alvarez-Ordonez *et al.*, 2019). The *B. bruxellensis* species is an example of microbes with specific adaptations to colonise harsh environments such as wine and cellars (Smith and Divol, 2016). Moreover, a recent study showed that the same *B. bruxellensis* genotype could be repeatedly isolated in wines from a given winery over decades, thus demonstrating an unsuspected persistence ability of *B. bruxellensis* that could be at the origin of recurrent wine spoilage (Cibrario *et al.*, 2019). The objectives of the present work were i) to

identify specific critical areas for *B. bruxellensis* persistence in the winery, and ii) to test whether *B. bruxellensis* biofilms could be at the origin of volatile phenol production when in contact with wines.

Air is considered in many fields as a vector for the dissemination of microorganisms and then the contamination of different matrices due to the fluxes exerted (Bryan *et al*., 2019; Sanz *et al*., 2021). This phenomenon is also observed in the winery, where the microorganisms that develop during the winemaking process can be identified in the air surrounding the vats and even in the cellar itself. Our results show the presence of yeasts in the air, in agreement with numerous studies that have identified the presence of these microorganisms (Simeray *et al*., 2001; Mandl *et al*., 2010; Ocón *et al*., 2011; Perez-Martin *et al*., 2014). This presence depends on several environmental factors such as temperature, humidity, air currents and sampling area, which can explain the differences in population levels reported in previous studies (Ocón *et al*., 2013a, Ocón *et al*., 2013b; Perez-Martin *et al*., 2014). Similar results have been observed, showing the presence of yeasts, although with variations during the winemaking season (Garijo *et al*., 2008). Some species of the genera *Penicillium*, *Aspergillus* and *Cladosporium* are responsible for the production of metabolites such as trichloroanisole, which is characterised by cork taint (Haas *et al*., 2010). Our study also shows the presence of yeasts in the air of all the wineries, with counts ranging from 4×10^{-3} CFU/L to 68×10^{-3} CFU/L of air, depending on the zone and winery considered. These concentrations, although low, are consistent with the period during which the samples were taken, namely from the end of November to the end of January, following the high microbial activity during the winemaking period. In fact, yeasts were found to be present in the air at very high population levels during the fermentation period (more than 180 MPN/cm^3 equivalent to CFU/mL). Then, a decrease is observed until low levels (less than 20 MPN/cm^3 equivalent to CFU/mL) are reached, showing variations in population levels along the season (Ocón *et al*., 2013b; Perez-Martin *et al*., 2014). In addition, a temporal succession of yeast species has been reported, with *Saccharomyces cerevisiae* being the main species found in the air, followed by non-*Saccharomyces* yeasts during the rest of the campaign (Ocón *et al*., 2010; Perez-Martin *et al*., 2014). This presence of yeasts in the air can be of interest during spontaneous fermentation, but can also be detrimental in the case of spoilage yeasts, which could contaminate the inert surface of the winery and/or the wine. Our observations confirm that *B. bruxellensis* is detected in cellar air (Beech, 1993; Connell *et al*., 2002). However, the presence of *B. bruxellensis* in the air is not confirmed in all the wineries studied and the number of colonies is low (4×10^{-3} to 32×10^{-3} CFU/L). Several factors can influence the proportion of microbes in the air and the presence of wines contaminated with *B. bruxellensis* does not necessarily mean that these yeasts are also present in the air (Perez-Martin *et al*., 2014).

Analyses of surface samples show that yeasts are present on all winemaking materials regardless of their composition (wood, stainless steel, concrete), demonstrating the significant capacity of microbes to colonise the winery environment. During the winemaking period, the cellar environment is saturated with yeasts, mainly of the *S. cerevisiae* species, which can colonise surfaces and thus persist in the cellar (Rosini, 1984; Ocón *et al*., 2010; Pretorius, 1999; Tek *et al*., 2018). Subsequently, the proportion of *S. cerevisiae* decreases, giving way to other yeasts such as *B. bruxellensis*. Our results show that *B. bruxellensis* is detected in all wineries examined, on the surface of materials such as wood, stainless steel and concrete. We show, for the first time, the detection of *B. bruxellensis* on tartaric acid precipitation collected in a concrete vat. These results are consistent with previous studies showing that *B. bruxellensis* may be present on the surface of barrels and wine materials (Fugelsang, 1997; Cartwright *et al*., 2018; Abdo, 2020). Swab samples from hard-to-reach areas showed that these areas harbour large yeast populations of up to 6.4×10^3 CFU/cm^2, particularly *B. bruxellensis*. Samples from valves showed concentrations up to 25 CFU/ cm^2. The presence of *B. bruxellensis* in valves has been reported previously (Oro *et al*., 2019). This presence can be explained by the fact that these areas are difficult to clean and that wine residues can accumulate there, facilitating the development of microorganisms.

The persistent microbial colonisation of the cellar environment could be related to the specific ability of the microbial species to bioadhere and form biofilms. To test this hypothesis, the ability of *B. bruxellensis* strains collected from surfaces and equipment to bioadhere to stainless steel coupons was evaluated. However, the bioadhesion capacity of isolates collected from surfaces was similar to that of strains collected from spoiled wine.

To date, no study has demonstrated that *B. bruxellensis* present in biofilms could be at the origin of volatile phenol production in contact with wines. Representative strains of the three main genetic groups (Avramova *et al*., 2018) were selected, for which contrasting bioadhesion properties and biofilm formation were previously established (Le Montagner *et al*., 2023; Le Montagner *et al*., 2024). The ability of the three different *B. bruxellensis* strains to survive in a biofilm on stainless steel and to produce volatile phenols in wine was monitored for 28 days. Depending on the strain, the number of bioadherent cells was different, but the behaviour was similar. We confirm the greater ability of strains AWRI 1608 and CBS 2499 to bioadhere to stainless steel (Le Montagner *et al*., 2023). In fact, during the first days of contact with the wine, the number of viable bioadherent cells decreases while that of planktonic cells increases for the three strains, suggesting a massive detachment of cells in the wine. However, during the same period, our results show an increase in the thickness of the bioadherent cell layer, suggesting that a significant proportion of bioadherent cells died or entered a Viable But Not Cultivable (VBNC) state, but remained bioadherent and served as carriers for other cells (Serpagi *et al*., 2012; Lebleux *et al*., 2020). This phenomenon was already mentioned in the growth kinetics of *B. bruxellensis* biofilm in wine (Lebleux *et al*., 2020).

The use of other methods to assess the total cell count of *B. bruxellensis*, including non-culturable cells, would have been relevant to implement in the context of our study. After 8 days in wine, the average thicknesses observed in our conditions were in the order of 14.25 µm to 24.15 µm for the 2 strains present in the form of a homogeneous layer. This thickness appears to be higher than that observed in previous work on *B. bruxellensis* after 7 days in wine (mean thickness 9.45 µm) (Lebleux *et al*., 2020). However, the composition of the wine and the strains studied were different which could, at least partially, explain these variations in thickness. Furthermore, the thickness of the *B. bruxellensis* biofilm remains low compared to that formed by *Candida albicans* (thickness between 8 and 84 µm) but remains in the same order of magnitude as that reported for *Saccharomyces cerevisiae* (thickness of 25–30 µm) (Daniels *et al*., 2013; Bojsen *et al*., 2014).

The oenological issue of the existence of bioadherent *B. bruxellensis* cells on stainless steel has been studied. Cells can detach from the biofilm and contaminate the wine by producing volatile phenols. These results are in agreement with those of Lebleux (2022). Looking forward, similar studies could be conducted on other surfaces, concrete, epoxy or oak. Our results regarding the production of vinyl phenols are congruent with the levels reported in naturally contaminated red wines (Nunes De Lima *et al*., 2021). The production of volatile phenols is related to the growth and final population level of planktonic cells, with higher production for the CBS 1499 and AWRI 1608 strains compared to the production of the CBS 1499 strains. In our conditions, the actual production of volatile phenols by bioadherent cells is impossible to determine due to the presence of planktonic cells in high concentrations. However, the results showed variable production kinetics depending on the *B. bruxellensis* strain. The production kinetics are directly related to nutrient assimilation and yeast growth, which can vary depending on the strain (Longin *et al*., 2016; Cibrario *et al*., 2020). To determine the proportion of volatile phenols strictly produced by bioadherent cells, further method development is needed. In addition, a comparative analysis of the production of volatile phenols by planktonic cells grown in liquid medium versus biofilm may be of interest.

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