Effect of abiotic and biotic factors on *Brettanomyces bruxellensis* bioadhesion properties.

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Highlights:

- pH and ethanol have negligible effects on *B. bruxellensis* bioadhesion
- Biofilms of two distinct strains are driven by the most bioadhesive one
- Mixed-species biofilms between *O. oeni* and B. *bruxellensi*s are highlighted
- Bioadhered wine bacteria reduced *B. bruxellensis* biofilms on stainless steel

Abstract:

Biofilms are central to microbial life because of the advantage conferred by these communities secreting an extracellular matrix. During the wine making process, grape must and wines host a great diversity of microorganisms able to grow in biofilm. This is the case of *Brettanomyces bruxellensis* considered the most damaging spoilage yeast, because of its negative sensory effect on wine and its ability to colonize stressful environments. In this study, the effect of different biotic and abiotic factors on *B. bruxellensis* bioadhesion and biofilm formation capacities was analyzed. Ethanol concentration and pH have negligible effect on yeast surface properties, pseudohyphae cell formation or bioadhesion, while the strain and genetic group factors highly modulate the phenotypes studied. From a biotic point of view, the presence of two distinct strains of *B. bruxellensis* does not produce any synergistic effect but a competition is observed between the strains during biofilm formation. Biofilm formation was driven by the strain with the highest bioadhesion capacity. Finally, the presence of wine bacteria reduces the bioadhesion of *B. bruxellensis*. Interactions between *O. oeni* and B. *bruxellensi*s is observed due to biofilm formation.

Keywords

Brettanomyces bruxellensis, wine, bioadhesion, mixed-species biofilms, lactic acid bacteria

1. Introduction

A large majority of microorganisms on Earth are preferentially found as communities on the surface of a support rather than as free planktonic cells in the environment (Costerton et al., 1995; Kolter and Greenberg., 2006). These communities called biofilms are characterized by a spatial organization of the microorganisms present but also by the production of extracellular matrix (Costerton et al., 1999). Biofilms are found in various environments, and as it is estimated that from 20% to 80% of terrestrial microbial biomass live in a biofilm form, these may play a crucial role in the proper functioning of most environments, anthropized or not (Richards and Melander., 2009; Flemming and Wuertz., 2019; Bridier and Briandet., 2022). In addition, the presence of biofilm can be problematic in certain fields such as medical, agri-food and maritime transport given their resistances and pathogenicity's (Hall-Stoodley et al., 2004; Piola et al., 2009; Zara et al., 2020). This resistance is mainly due to the presence of an extracellular matrix composed of polysaccharides, proteins, peptidoglycans, nucleic acids and lipids, serving as a barrier against external aggressions (Czaczyk ans Myszka., 2007; Flemming et al., 2007). However, biofilm formation is dependent on several environmental factors such as pH, temperature, carbon source concentration (Fathollahi and Coupe., 2021; Liu et al., 2023). The presence of mixtures of microorganisms genetically related or belonging to distinct species can also have a major effect on biofilm formation. Actually, it has been shown that the presence of several strains of *Escherichia coli* in the same environment induces a synergistic effect promoting the formation of biofilm (Reisner et al., 2006). On the contrary, in *Listeria monocytogenes*, biofilm formation is inhibited in the presence of *Lactiplantibacillus paraplantarum* (Winkelströter et al., 2015).

In oenology, and more particularly during the winemaking process, many microorganisms participate to the fermentations and contribute to the aromatic panel of wine, by the production of molecules of interest or wine defects (Gammacurta et al., 2017; Tempere et al., 2018; Carpena et al., 2021). Among the microorganisms producing off-flavors, *Brettanomyces bruxellensis* is the major spoilage yeast, because of the production of volatile phenols characterized by stable, horse sweat and leather odors, which mask the fruity aromas of wines (Chatonnet et al., 1992; Lattey et al., 2010). In addition, different materials are used in oenology, from terra cotta to ceramics, wood and concrete to the predominant stainless steel nowadays preferred because of its resistance to sulphites corrosion and efficient cleaning procedures (Valdez et al., 2015). Wood is mainly used for wine aging in barrels aside to concrete tanks coated in many cases with epoxy resin, which limits its porosity and improves its cleaning ability (Desenne et al., 2008).

B. bruxellensis is present throughout the winemaking process (Renouf and Lonvaud-Funel., 2007; Rubio et al., 2015). This ubiquist species is characterized by a high genetic diversity directly related to ploidy and the niche of isolation of the strain (Albertin et al., 2014; Avramova et al., 2018). Different diploid/triploid groups (2 & 4 at least respectively) have been identified (Harrouard et al., 2022). Tolerance and resistance to sulphites (SO_2), the main antimicrobial used in oenology, has been identified to be linked to the genetic group (Curtin et al., 2012; Avramova et al., 2018b). In addition, strains of *B. bruxellensis* can be found from year to year within the same winery, suggesting a high ability to persist in the winemaking environment between vintages (Cibrario et al., 2019). Indeed, *B. bruxellensis* was identified in the air, on floors, walls, winemaking vats, winemaking equipment and barrels (Fugelsang et al., 1997; Connell et al., 2002; Le Montagner et al., 2023). This persistence can be explained by the fact that *B. bruxellensis* has strong bioadhesion and biofilm formation capacities (Joseph et al., 2007; Dimopoulou et al., 2019; Lebleux et al. 2020). In addition, depending on the genetic group, differences in strain bioadhesion are observable, the "Beer -3N genetic group" being the most adhesive one (Le Montagner et al., 2023). However, the effect of biotic and abiotic factors on biofilm formation in *B. bruxellensis* has been so far poorly studied.

The first objective of this study was to evaluate the effect of two abiotic factors (pH and ethanol concentration) and materials on *B. bruxellensis* surface properties and bioadhesion ability. As other microorganisms such as *Oenococcus oeni* are known to be able to form biofilms in wine (Bastard et al., 2016), our second objective was to study the effect of biotic factors, i.e., mixed-strains and mixed-species communities on *B. bruxellensis* bioadhesion and biofilm formation.

2. Materials and methods

2.1 Abiotic factors

2.1.1 Strains and growth conditions

In order to observe the effect of abiotic factors on *B. bruxellensis* surface and bioadhesion properties, a total of 17 strains, representative of the genetic diversity of the species and presenting contrasting surface and bioadhesion phenotypes, were selected for this study (Le Montagner et al., 2023) (Table 1). These strains were isolated from different fermented matrices and belong to the CRBO collection (Microbiological Resources Center Oenology, Bordeaux, France), the AWRI collection (Australian Wine Research Institute, Adelaide, Australia), the CBS collection (Fungal Biodiversity Center, Utrecht, Netherlands), the GSP collection (Foggia University, Foggia, Italia) and the YJS collection (Laboratory for Molecular Genetics, Genomics and Microbiology, Strasbourg, France). The strains were stored at -80 °C in a mixture of YPD 70% (v/v) comprising 2% (w/v) glucose (Fisher BioReagent™), 1% (w/v) peptone (Gibco), 1% (w/v) yeast extract (Fisher BioReagent™) and glycerol 30% (v/v) before being cultured on a YPD solid medium (2% (w/v) agar (Fisher BioReagent™)) and incubated for 5 days at 25 °C.

Table 1: List of the 17 strains of *Brettanomyces bruxellensis* used to study the effect of pH and ethanol concentration. Strains belong to the Microbiological Resources Center Oenology (CRBO collection), the Australian Wine Research Institute collection (AWRI collection), the Fungal Biodiversity Center collection (CBS-KNAW collection), the Foggia University collection (GSP collection) and the

Laboratory for Molecular Genetics, Genomics and Microbiology collection (YJS collection) (*Avramova et al., 2018)

Strain	Genetic groups*	Ploidy*	Substrate
GSP 1502	Beer	3n	Beer
AWRI 1608			Red wine
YJS5400			White wine
CRBO L17118			Beer
CRBO L17119			Red wine
AWRI 1499	Wine 1	3n	Red wine
CRBO L14156			Wine
CRBO L14175			Wine
CRBO L0308	Wine 2	3n	Red wine
CRBO L1782			Wine
CBS 2499	Wine 3	2n	Red wine
CRBO L0611			Red wine
CRBO L1715			Red wine
CRBO L17102	Teq/EtOH	3n	Ethanol
CRBO L17109			Tequila
CRBO L1757	Kombucha	2n	Na
CRBO L17103			Kombucha

2.1.2 Growth and adaptation protocol to abiotic factors

All analyses of the section 2.1 were realized in Wine Like Medium (WLM) which was used for its close composition to wine (Le Montagner et al., 2023). WLM is composed of 0.05% (w/v) glucose (Fisher Bio- ReagentTM), 0.15% (w/v) fructose (Sigma Aldrich®), 0.2% (w/v) tartaric acid (Prolabo), 0.05% (w/v) citric acid (Prolabo), 0.03% (w/v) malic acid (Aldrich Chemistry), 0.25% (w/v) yeast extract (Fisher Bio- ReagentTM), 0.5% (w/v) glycerol (Sigma Aldrich®). The effect of two abiotic factors, pH and alcohol concentration, was studied. For the pH effect, 3 values were considered for WLM: 3.6, 3.8 and 4.1. The pH was adjusted with KOH 5M. For the ethanol concentration effect, 3 values were considered for WLM, 5%, 10% and 14% (v/v) (VWR Chemicals®). In order to optimize this experimentation, an experimental design was implemented (Table 2). Adaptation steps were necessary for the yeast growth in the WLM medium. Briefly, some colonies were recovered from solid medium and transferred into 10 mL of a mixture consisting of 25% (v/v) of WLM medium and 75% (v/v) of liquid YPD medium (2% (w/v) of glucose, 1% (w/v) of yeast extract and 1% (w/v) of peptone for 48 h of incubation at 25 °C under stirring at 180 rpm. This adaptation step was repeated 3 times and the proportion of WLM was gradually increased (50%, 75% and finally 90%). After 48 h of incubation (25 °C, 180 RPM), the cell suspension was collected to determine i) the surface charge ii) the surface cell hydrophobicity, iii) the pseudohyphae growth and iv) the bioadhesion capacity of each strain.

2.1.3 Cell surface charge

Cell surface charge was measured after centrifugation of the cell culture at 7000 g for 5 min at room temperature. The cell pellet was washed twice with and then resuspended in ultra-pure water with pH value defined in the experimental design. The cell suspension was filtered on nylon filter (0.45 µm) to obtain a cell suspension with a OD_{600nm} around 0.7. The measurement of the zeta potential was carried out via the Zetasizer Nano (Malvern). For each strain, three measurements were made on the same cell culture.

Table 2: Experimental design applied in the experimentation on pH and ethanol effects on *B. bruxellensis* cell surface and bioadhesion properties

Series	pH value	Ethanol concentration (% v/v)
1	4.1	5
2	3.6	14
3	3.6	10
4	3.8	5
5	4.1	10
6	3.8	10

7	4.1	14
8	3.8	14
9	3.6	5

2.1.4 Cell surface hydrophobicity

The cell hydrophobicity was determined by the MATS (Microbial Adhesion To Solvents) method which enables the determination of the hydrophilic/hydrophobic character of the surface of yeasts (Bellon-Fontaine et al., 1996). Ten milliliters of cell suspension were centrifuged for 5 min at 7000 g at room temperature; then the pellet was washed twice with distilled water and re-suspended in physiological water (NaCl 0.9%) to obtain a cell suspension with an OD_{600nm} around 0.7. A volume of cell suspension of 1.5 mL was mixed with 250 µL of either chloroform (Fisher Chemical) or hexadecane (Sigma-Aldrich). The mixture was vortexed for 2 min to create an emulsion. A rest period of 15 min allowed the separation of the 2 phases. The optical density of the cell suspension (OD_0) and the aqueous phase of the mixture was measured at 600 nm. The affinity for each solvent was calculated using the formula reported in Le Montagner et al., (2023).

2.1.5 Pseudohyphae growth

To evaluate the proportion of pseudohyphae, 1 mL of cell suspension was sampled. The sample was filtered on 0.4 µm filter (Isopore™). The filter was then placed on a pad containing a mixture of ChemSol B16 (Chemunex) buffer containing 1% (v/v) of fluorochrom V6 (Chemunex), and the pad was incubated 15 min in the dark at 30 °C. The proportion of pseudohyphae was evaluated by epifluorescence microscopy (10 fields counts).

2.1.6 Bioadhesion properties

To determine the bioadhesion capacity of the *Brettanomyces* strains, the cell suspension was centrifuged for 5 min at 7000 g at room temperature and then the cell pellet was washed twice with physiological water (NaCl 0.9%). The pellet was resuspended in a mixture WLM 90% and YPD 10% to obtain a final concentration of 10^7 cells/mL. The bioadhesion was made on 14 mm x 25 mm, 316L stainless steel coupons (Goodfellow), after a cleaning procedure as described in Le Montagner et al., (2023). The rinsed coupons were placed in 55 mm Petri dishes; ten mL of cell suspension were then added to initiate bioadhesion, which was then carried out for 3h at room temperature. A coupon washing step was then performed to remove the non-adherent cells that had sedimented. The washing step consists of 5 successive cleaning baths in sterile physiological water. The coupon was then placed in a solution of Chemsol B15 (Biomerieux) containing 1% (v/v) of 5(6)-Carboxyfluorescein Diacetate (CFDA) (Thermo Fisher Scientific) at 8 mg/mL for the detection of live cells and 0.2% (v/v) propidium iodide (PI) at 1 mg/mL for the detection of dead cells (Thermo Fisher Scientific). Cells were left 15 min at room temperature before observation to allow staining. The surface of the coupon was observed by confocal microscopy within the Bordeaux Imaging Center Bordeaux facilities of the INRAE plant pole. Observations were made using the immersion lens. Confocal acquisitions were realized using a Zeiss LSM 880 confocal laser-scanning microscope with a diving 40× objective with a numerical aperture of 1. The excitation wavelengths and emission windows were respectively 488 nm/499–553 nm and 561 nm/588–688 nm for CFDA and propidium iodide. Fluorochromes were detected sequentially line by line. The adhered dead and live cells were counted on 10 distinct fields.

2.1.7 Bioadhesion on different materials

This study was carried out on 6 strains, selected according to their contrasted bioadhesion properties (AWRI 1608, CBS 2499, YJS7820, YJS8202, YJS 8357, YJS8528) (Le Montagner et al., 2023) and 3 materials frequently encountered in oenology: a smooth 316L stainless steel (SSS) (Goodfellow), a rough 316L stainless steel (RSS) (Goodfellow) and Forepox G355 industrial food epoxy resin (Bouchillou alkya).

2.1.8 Material properties

Once the materials were cleaned, they were immersed for 3 hours at room temperature in WLM medium and then rinsed once with distilled water and dried under laminar flow host for 1 hour. Contact angle measurements (θ) were made using the sessile drop method. A drop of a test liquid was deposited on the surface of the material and the contact angle was measured using a DSA 100 goniometer (KRUSS). Measurements were made in triplicate for each material and contact angle measurements were made on at least eight positions per coupon.

2.2 Multi-strains biofilm

2.2.1 Strains and growth adaptation

Four strains of *B. bruxellensis* were selected for their bioadhesion properties described in Le Montagner et al., 2023 (Table 3). The growth conditions applied were the same as those described in section 2.1.1. The composition of the WLM medium was the same as described in section 2.1.2, with a pH value of 3.6 and an ethanol concentration of 10% (v/v). After adaptation steps described in section 2.1.2, the cell culture was collected to perform multi-strains bioadhesion competition.

Table 3: List of the 4 strains used in the mix composition according to their genetic groups (*Avramova et al., 2018) and bioadhesion properties (Le Montagner et al., 2023)

Strain	Genetic group*	Bioadhesion properties**
AWRI 1499	Wine 1	Weak

AWRI 1608	Beer	High
CBS 2499	Wine 3	High
CRBO L17109	Teq/EtOH	High Bioadhered Pseudohyphae

2.2.2 Bioadhesion

To perform the multi-strain bioadhesion, the cell culture was treated following the same protocol as in section 2.1.6. Four mixes were carried out: AWRI1499/AWRI1608 (MX1), AWRI1608/CRBOL17109 (MX2), AWRI1499/CRBOL17109 (MX3) and AWRI1608/CBS2499 (MX4). For each mix, the final concentration was 2.0 x 10^6 cell/mL (1:1). As a positive control, the bioadhesion was also carried out for the single culture of each strain. For the bioadhesion, 10 mL of mixed or single strain culture were then added to the Petri dishes containing a previously cleaned coupon of 316L stainless steel (Le Montagner et al., 2023). The bioadhesion was carried out for 3h at room temperature. Once rinsed (section 2.1.6), the coupons were placed in a 30 mL vial and 30 mL of WLM medium were added to monitor biofilm formation. The vials are then placed at 20°C until analysis. For each measurement point at 3h, 7 and 14 days, the samples were prepared in triplicate.

2.2.3 Enumeration of bioadhered cells by cultivation

The enumeration of viable cells was carried out after the 3h, 7 and 14 days of bioadhesion. The coupon was cleaned to remove non-adhered 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 then the whole suspension was placed 2 min in sonication at 47 Hz. After this sonication step, the tube was stirred at maximum vortex speed for 40 s. Dilutions series were then carried out and 100 µL of the suspension were inoculated in triplicate on YPD agar medium at 30°C. The result is then expressed as Colony Forming Unit per cm^2 (CFU/cm^2).

2.2.4 Biofilm thickness

The biofilm thickness measurement was carried out on MX1, MX2 and MX3 by confocal microscopy observations (For MX4, it was not possible to perform confocal analysis because the Bordeaux Imaging Center Bordeaux facilities was not available). After the rinsing steps described in part 2.6.2, the coupon was then placed in a solution of Chemsol B15 (Biomerieux) containing 1% (v/v) of 5(6)-carboxyfluoresceine diacetate (CFDA) (Thermo Fisher Scientific) at 8 mg/mL and 0.2% (v/v) propidium iodide (PI) at 1 mg/mL (Thermo Fisher Scientific) during 15 min. The surface of the coupon was observed by confocal microscopy within the Bordeaux Imaging Center facilities of the INRAE plant service. Observations were made using the immersion lens as described in 2.1.6. The thickness measurement was carried out by taking successive images of each focal plane with the z-stack function of the ZEN microscopy software (Zeiss). The thickness analysis was then performed on 10 biofilms areas using the ROI manager function present on the FIJI image processing software extension of the ImageJ software.

2.2.5 Strain genetic identification

In order to determine the proportion of each strain per mix, 15 yeast colonies were collected at random in each Petri dish enumerated in the section 2.2.3 (90 colonies per mix). The colonies were placed in 20µL of NaOH 20mM for cellular lysis. This mixture was incubated 10 min at 90 °C and then placed at -20 °C during 30 min. These steps were repeated 3 times. The genetic group of each colony was determined by a molecular analysis tool based on the microsatellite analysis (Typ\Brett, patent number WO2017068284, 10/2016). The results were expressed by percentage of each strain/genetic group per mix.

2.3 Pluri-species biofilm

2.3.1 Strains and growth adaptation

For the pluri-species experimentation, one strain of *B. bruxellensis* (AWRI1608) belonging to the Beer group was selected for its high bioadhesion properties. An industrial strain of *Oenococcus oeni* (Lactoenos® B7, LAB) and a strain of *Acetobacter pasteurianus* (AP001, AAB) isolated from red wine were used. The *B. bruxellensis* and AAB strains were incubated for 5 days at 25 °C. As the experimentation was conducted in red wine, adaptation steps were necessary for *B. bruxellensis* and *A. pasteurianus*. Few colonies were recovered from solid medium and transferred into 10 mL of a mixture of 25% (v/v) red wine (Graves, 12% vol, pH 3.7) and 75% (v/v) grape juice, and incubated for 48 h (25 °C, 180 RPM). The proportion of red wine was then gradually increased (50%, 75% and finally 90%). The industrial freeze-dried LAB were stored at -20 °C before utilization. LAB were inoculated at 10^8 cells/mL at 25°C in a mixture composed of 90% of red wine (v/v) and 10% of grape juice (v/v) 48h before bioadhesion.

2.3.2 Bioadhesion

To perform the pluri-species bioadhesion, 3 conditions were tested, bioadhesion Brett/LAB, bioadhesion Brett/AAB and bioadhesion Brett/LAB/AAB. The cell cultures were centrifuged for 5 min at 9000 g for bacteria and 7000 g for *B. bruxellensis* at room temperature and then the cell pellet was washed twice with physiological water (NaCl 0.09%). The pellets were the resuspended in a mixture of red wine 90% (v/v) and grape juice 10% (v/v) in order to obtain 5.0 x 10^6 cell/mL for *B. bruxellensis* and 10^6 cell/mL for bacteria. In the case of

Brett/LAB/AAB the concentration of bacteria was 1.0 x 10^6 cell/mL with a ratio of 1:1 for LAB and AAB. Bioadhesion was carried out sequentially. The bacteria were first brought into contact with the previously cleaned stainless steel (Le Montagner et al., 2023) for 48 hours. A coupon washing step was then performed to remove non-adherent bacteria as described in previous sections. *B. bruxellensis* suspension was then added for 3h at room temperature. After these 3h, another coupon washing step was performed. Once rinsed, the coupons were placed in a 30 mL vial and 30 mL a mixture of 90% (v/v) of red wine and 10% (v/v) of grape juice were added. The vials was then placed at 20°C until analysis at 3h, 7, 14 and 28 days. For each measurement point, the samples were prepared in triplicate.

2.3.3 Cultivable cells enumeration

The enumeration of viable cells was carried out after the 3h, 7 and 14 days of bioadhesion. The protocol used for this part was the same as described in section 2.2.3. For *Brettanomyces bruxellensis*, serial dilutions were spotted on YPD agar medium and incubated for 5 days at 30 °C. For LAB and AAB, the incubation medium consisted in 25% (v/v) of grape juice, 0.5% of yeast extract (Fisher BioReagent™), 2% of agar (Fisher BioReagent™) and 0.1% (w/v) of Tween 80. The pH was adjusted to 4.8 with KOH and the medium was supplemented with pimaricin at 0.1 mg/mL for LAB and with pimaricin at 0.1 mg/mL and penicillin at 12.5 µg/mL for AAB. Incubation lasted 7 days in anaerobiosis at 25 °C. The results were expressed as Colony Forming Unit per cm^2 (CFU/cm^2).

2.3.4 Scanning Electron Microscopy (SEM)

Bioadhered cells and biofilms were observed by SEM. The adhered cells were fixed on the stainless-steel coupon by a solution of 3% glutaraldehyde in 0.1 M phosphate buffer of pH 7.2 over one night at 4 ° C. The coupon was washed with 0.05 mM phosphate buffer for 10 min.

Two successive immersions were performed for dehydration for 10 min in solutions of increasing ethanol content (50, 75, 90, 100%). The coupon was placed in solution of ethanol-acetone (70/30, 50/50, 30/70, 100%) for 10 min. Next, the coupon was air-dried and stored at room temperature. The sample were coated with a thin platinum layer and then observed with a Zeiss Gemini 300 scanning electron microscope. SEM was performed using a working distance between 6.8 mm and 7.1 mm.

2.4 Statistical analysis

Kruskal-Wallis statistical test (agricolae package, R, p value < 0.05), multi-way Anova (agricolae package, R, p-value <0.05), and Principal Component Analysis (PCA) were performed using R and R-packages *agricolae* (Mendiburu, 2021).

3. Results

3.1 Effect of abiotic factors on *B. bruxellensis* on cell surface and bioadhesion properties

In our experimental conditions, the effect of 3 pH values (3.6, 3.8 and 4.1) and 3 ethanol concentrations (5%, 10%, and 14% (v/v) on surface charge (Zeta potential), surface hydrophobicity (Affinity to Chloroform and Hexadecane), pseudohyphae cells formation and finally on the bioadhesion properties of *B. bruxellensis* was investigated. The variance analysis made it possible to highlight the effect of each factor on the parameters studied (Fig. 1). The genetic group and strain factors explained more than 50% of the results obtained for all the parameters studied. The variance of the surface charge with Zeta potential analysis was 57% mediated by the genetic group followed by the 20.3% for the strain factor. No effect of pH was highlighted. Alcohol had only a weak effect with 2.6% of the variance explained. Regarding hydrophobicity, the strain effect was even higher, explaining most of the affinity to chloroform and hexadecane (62% and 65% of the total variance, respectively). The effect of alcohol and pH were again negligible as well as the combination of factors. The variance of the formation of pseudohyphae cells was also explained at 36.5% and 35.5% by the strain and the genetic group, respectively, with 5.2% variance explained by an alcohol/genetic group interaction. The variance of viable cells adhesion was explained at 37.3% by the strain and at 25.2% by the genetic group. The interaction of alcohol parameter with the genetic group and the strain explained from 5.8% to 6.3% of the total variance of bioadhesion. Finally, the concentration of bioadhered dead cells was also explained by the strain at 31.3% and at 18.2% by the genetic group. However, alcohol explained 9.9% of the bioadhesion of dead cells with interaction with the genetic group and the strain (14.2% and 18.2% of the explained variance). Indeed, with the increase in alcohol concentration, the number of dead cells increases significantly (Anova, p-value <0.05). Thus, the pH and alcohol appeared to have a limited effect on surface and bioadhesion properties of *B. bruxellensis* in our experimental conditions.

3.2 Material properties and effect on bioadhesion

In this part, different materials were studied, rough 316L stainless steel (RSS) and epoxy resin GE55 in addition to smooth 316L stainless steel (SSS). The measurement of the wettability of the different materials was carried out after cleaning the coupons and after 3 hours of immersion in WLM. The contact angle values are shown in Table 4. After cleaning, the SSS and RSS stainless steel references exhibited similar results, respectively contact angles of 104.3° and 105° showing non-wettability and therefore, a hydrophobic behavior. The epoxy resin showed a contact angle of 79.2° indicating moderate hydrophobic behavior.

After contact with WLM medium, the 2 stainless steel references showed hydrophilization and the decrease in the contact angle of the water from 104.3° to 67° and from 105° to 64.8° for SSS and RSS (similar behaviors). After immersion in the WLM medium, the epoxy resin also showed significant hydrophilization from 79.2° to 50°. The WLM medium showed a hydrophilizing action on stainless steel and Epoxy resin. No difference was observed with the apolar solvent (diiodomethane), with or without WLM immersion. These results showed that the WLM medium impacted only the hydrophilic properties of the three surfaces *ie* the polar components.

Table 4: Wettability of the different materials used in oenology

		Contact angle (θ)	
Material	Condition	Water	Diiodomethane
SSS	After cleaning	104.3	46.7
	After cleaning and immersion in WLM medium	67	46.1
RSS	After cleaning	105	64.5
	After cleaning and immersion in WLM medium	64.8	64.8
Epoxy resin	After cleaning	79.2	48
	After cleaning and immersion in WLM medium	50	48.4

The results obtained after 3H of bioadhesion on these materials are presented Fig. 2. Depending on the material, the concentration of bioadhered cells was significantly different (p-value<0.05). Bioadhesion on epoxy resin was significantly lower, with an average concentration of 6.04×10^4 cell/cm^2 against 7.56×10^5 cell/cm^2 and 1.77×10^6 cell/cm^2 for RSS and SSS, respectively. No significant differences were observed between RSS and SSS stainless steels (p-value>0.05), showing that the roughness here did not affect the bioadhesion capacity of *B. bruxellensis.*

Depending on the strain tested, the bioadhesion behavior was different depending on the material used (Fig. S1). Strains AWRI1608 and CBS2499 showed the highest bioadhesion capacity for the 3 materials tested (respectively 2.26 x 10^6 cell/cm^2 and 7.63 x 10^6 cell/cm^2 for SSS, 1.56 x 10^6 cell/cm^2 and 2.24 x 10^6 cell/cm^2 for RSS and finally 6.91 x 10^4 cell/cm^2 and 2.22 x 10^4 cell/cm^2 for Epoxy), with however significant differences between the 3 materials, bioadhesion being the most important on the SSS. For the other strains, the bioadhesion capacity was lower on the 3 materials; strain YJS8202 showed no significant difference in bioadhesion depending on the material (p-value>0.05). For the YJS8528 strain, the bioadhesion capacity was significantly higher on rough steel (p-value<0.05).

3.3 Mixed-strains biofilm

The establishment of biofilm with two genetically distinct strains of *B. bruxellensis* and contrasting bioadhesion properties was monitored over time, in order to follow biofilm formation dynamics. The MX1 composed of strains AWRI 1499 and AWRI 1608 showed a cultivable population level in the biofilm similar to that observed for AWRI 1608 strain alone, with an increase of cultivable cells during the first 7 days (from 7.43 x 10^4 CFU/cm^2 to 7.33 x 10^5 CFU/cm^2), followed by a slight decrease until day 14 (Fig 3A). Meanwhile, the AWRI 1499 strain cultivable population decreased over time. Figure 2B shows the strain relative proportion evolution. The dominant strain on day 1, 7 and 14 was AWRI 1608, and this explains why MX1 followed a behavior similar to that of AWRI 1608 alone.

The MX2 comprising strains AWRI 1608 and CRBO L17109 showed a similar trend to MX1 with populations over time comparable to that of strain AWRI 1608 alone (Fig. 3C). Monitoring the strain proportion showed that strain AWRI 1608 represented 56.8% on day 1 whereas strain CRBO L17109 represented 43.2%. However, AWRI 1608 then became dominant as it represented 98.9% and 94.3% on day 7 and 14, respectively.

The MX3 was composed of strains AWRI 1499 and CRBO 17109 (Fig. 3E). On day 1, the MX1 population level was closed to that of each strain examined alone. A decrease in population level was noticeable on day 7 for single strain biofilms (AWRI 1499 and CRBO L17109) while the concentration of adhered MX3 increased to 6.57 x 10^4 CFU/cm^2, suggesting a potential synergistic effect for biofilm establishment. However, on day 14, a strong decrease of MX3 biofilm population level to 1.33 x 10^3 CFU/cm^2 was observed, while the single strain biofilm concentration remained relatively stable. MX3 biofilm was mainly composed of CRBO L17109 with a proportion of 65.6% and 92.3% on days 1 and 7 (Fig. 3F).

Finally, the MX4, composed of strains AWRI 1608 and CBS 2499 showed a trend similar to single strains biofilms, with an increase in the biofilm population over the 14 days (Fig. 3G). The proportion in each strain in MX4 was relatively equilibrate on day 1 with 60% and 40% of AWRI 1608 and CBS 2499, respectively (Fig. 3H). During the first week, the gap between the 2 strains increased as AWRI 1608 represented 70.6% on day 7. However, on day 14, a reversal of proportion was observed; the CBS 2499 strain became dominant (71.3%).

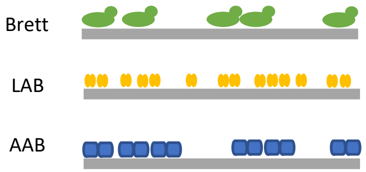
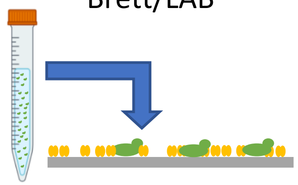
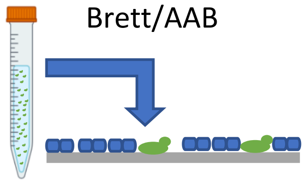
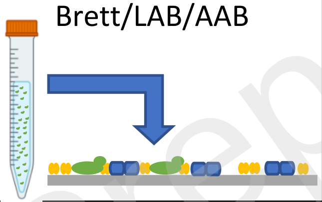
A monitoring of the mixed biofilm thickness was also carried out. Figure 4 shows the single and mixed strain biofilm thickness. The AWRI 1608 strain formed a homogeneous biofilm on stainless steel with a gradually increase in the thickness of the biofilm over time from 7.25 µm on day 1 to 12 µm and 16.7 µm on day 7 and 14, respectively. Strain CRBO L17109 has a relatively stable thickness over time from 6.11 µm to 7.65 µm between day 1 and day 14. Strain AWRI 1499 did not form a continuous biofilm on the stainless-steel coupon, but micro-colonies scattered on the surface and was not represented in Figure 4.

The MX1 and MX2 displayed similar thicknesses over time: no significant differences were observed between the 2 mixes for a given day (p-value >0.05). The thickness of these mixes increased between day 1 and day 7 and remained stable between day 7 and day 14. The MX1 and MX2 mixes were both composed by AWRI 1608 strain; the thickness of these mixes was similar to that of the AWRI 1608 single strain on day 1 and 7 (p-value >0.05), thus indicating a strong contribution of the AWRI 1608 strain during the first week of biofilm formation. In addition, on day 14, the AWRI 1608 single strain biofilm had a significantly greater thickness than that of MX1 and MX2 (p-value <0.05). Finally, MX3 composed of strains AWRI 1499 and CRBO L17109 had the lowest thickness of the 3 mixes with an increase between day 1 and day 7 from 5.14 µm to 7.26 µm, respectively. The MX3 biofilm did not differ significantly from that obtained with strain CRBO L17109 alone during the first week (p-value >0.05). On day 14, the thickness was no longer measurable because only micro-colonies were present on the surface of the stainless steel, revealing a dispersion of bioadhered cells during the second week.

Pluri-species biofilm

The study of pluri-species biofilms was carried out by associating *B. bruxellensis* either with a LAB (*O. oeni*), an AAB (*A. pasteurianus*) or both. Bioadhesion was performed sequentially as bacteria were introduced for 48 hours before *B. bruxellensis* was added. The bacteria adhered population analysis at 48 hours indicated a higher bioadhesion capacity for AAB with 8.2×10^4 CFU/cm^2 compared to LAB ($6.54. \times 10^3$ CFU/cm^2, Table 5).

Table 5 : Populations of culturable microorganisms in pluri-species biofilms in red wine.

		Bacterial bioadhesion (48 h)	*Brettanomyces* bioadhesion (3 h)	Biofilm 7 days	Biofilm 14 days	Biofilm 28 days
		Cultivable (CFU/cm^2)	Cultivable (CFU/cm^2)	Cultivable (CFU/cm^2)	Cultivable (CFU/cm^2)	Cultivable (CFU/cm^2)
Control 	Brett	/	$4.28 \times 10^5 \pm 5.01 \times 10^4$	$8.28 \times 10^4 \pm 3.00 \times 10^4$	$1.51 \times 10^5 \pm 9.81 \times 10^4$	$6.30 \times 10^4 \pm 2.21 \times 10^4$
	LAB	$6.54 \times 10^3 \pm 1.53 \times 10^2$	/	ND	ND	$1.60 \times 10^5 \pm 4.81 \times 10^4$
	AAB	$8.20 \times 10^4 \pm 5.01 \times 10^4$	/	ND	ND	ND
Brett/LAB 	Brett	/	$3.31 \times 10^3 \pm 1.34 \times 10^3$	$1.90 \times 10^3 \pm 1.15 \times 10^3$	$1.52 \times 10^3 \pm 1.62 \times 10^2$	$3.05 \times 10^3 \pm 1.9 \times 10^3$
	LAB	/	$3.87 \times 10^3 \pm 2.26 \times 10^3$	ND	ND	$7.62 \times 10^3 \pm 2.03 \times 10^3$
	AAB	/	/	/	/	/
Brett/AAB 	Brett	/	$3.73 \times 10^3 \pm 6.15 \times 10^2$	$1.52 \times 10^3 \pm 1.15 \times 10^3$	$2.48 \times 10^3 \pm 3.29 \times 10^2$	$1.71 \times 10^4 \pm 7.12 \times 10^3$
	LAB	/	/	/	/	/
	AAB	/	$8.18 \times 10^4 \pm 2.67 \times 10^4$	$9.51 \times 10^2 \pm 7.17 \times 10^2$	ND	ND
Brett/LAB/AAB 	Brett	/	$4.50 \times 10^3 \pm 4.17 \times 10^2$	$1.05 \times 10^3 \pm 6.60 \times 10^2$	$2.00 \times 10^3 \pm 7.58 \times 10^2$	$4.19 \times 10^3 \pm 1.63 \times 10^3$
	LAB	/	$5.15 \times 10^3 \pm 3.47 \times 10^3$	ND	ND	$2.49 \times 10^4 \pm 3.31 \times 10^3$
	AAB	/	$7.08 \times 10^4 \pm 2.79 \times 10^4$	$2.00 \times 10^3 \pm 2.90 \times 10^2$	ND	ND

For all assays described in this table, the *B. bruxellensis* population adhered after bacteria was lower than when *B. bruxellensis* was bioadhered alone (Fig. 5A). Indeed, after 3h, the population level of bioadhered *B. bruxellensis* were respectively 3.31 x 10^3 CFU/cm^2, 3.73 x 10^3 CFU/cm^2 and 4.50 x 10^3 CFU/cm^2 for the conditions Brett/LAB, Brett/AAB and Brett/LAB/AAB, against 4.28 x 10^5 CFU/cm^2 when *B. bruxellensis* was alone. These results indicated a significant decrease of *B. bruxellensis* bioadhesion when the bacteria were previously bioadhered (Kruskal-Wallis, p-value < 0.05) (Fig 5A). No significant adhered population evolution was observed during the first 14 days (p-value > 0.05) for *B. bruxellensis* alone. For the condition Brett/LAB, the population of *B. bruxellensis* remains stable throughout the 28 days of this study. *B. bruxellensis* populations were also stable between the day 7 and the day 14 for the Brett/AAB and Brett/LAB/AAB condition (p-value > 0.05). Moreover, for these two conditions, a significant *B. bruxellensis* population increase was observed between day 14 and day 28 (p-value < 0.05). On day 28, the *B. bruxellensis* population of the condition Brett/AAB was similar to that of the *B. bruxellensis* control, suggesting that in the long term, the presence of acid acetic bacteria does not affect the formation of biofilm in *B. bruxellensis* (p-value > 0.005). However, in the Brett/LAB/AAB and Brett/LAB at 28 days, the *B. bruxellensis* population level was significantly lower (p-value < 0.05) than when *B. bruxellensis* was the sole or with AAB (Fig 5B).

Concerning bacteria, the LABs were not detected on days 7 and 14, in control condition, but quantified at 1.6 x 10^5 CFU/cm^2 on day 28. AABs control were counted on agar medium, despite observations in Scanning Electron Microscopy (SEM) on stainless steel (data not shown), suggesting that they could be present in the Viable But Non-Cultivable form. After the 3 hours of bioadhesion of *B. bruxellensis* on the coupons previously "coated" with bacteria, the population levels of AAB and LAB were similar to the levels before the addition of *B. bruxellensis* (p-value <0.05). As for the LAB control, the LAB count revealed no presence of cultivable cells on days 7 and 14 but bacteria were visible by SEM, suggesting that the cells were in a non-culturable physiological state. In the Brett/LAB condition on day 28, 7.62 x 10^3 CFU/cm^2 of cultivable cells could be counted which is much lower than for the LAB control. For the Brett/LAB/AAB condition, the LAB population level on day 28 was higher than the Brett/LAB condition with 2.49 x 10^4 CFU/cm^2. For AABs, no count was possible for the control during the 28 days of follow-up. However, in the presence of *B. bruxellensis* and LAB, an enumeration was possible on day 7 with lower population level of 9.51 x 10^2 CFU/cm^2 and 2.0 x 10^3 CFU/cm^2 respectively for Brett/AAB and Brett/LAB/AAB conditions comparing with the control. In addition, observations by SEM could be made on days 14 and 28 (Fig.6).

Scanning Electron Microscopy observations highlighted the spatial organization of the different cells on the stainless-steel coupon surface. Fig. 6A shows an overview of the Brett/AAB status on day 14 with a x500 magnification. The microorganisms present on the surface of the coupon were randomly distributed. The presence of AAB was evident even if no culturable cells were detected after plating. A magnification x10 000 (Fig. 6B) made it possible to see with precision the organization of *B. bruxellensis* and the associated AABs. On the surface of a *B. bruxellensis* cell, an ordered agglomeration of crystals is obvious but the nature of these crystals remains unclear. AABs were also present in contact with the yeast cell. On day 28, microcolonies of LAB associated to *B. bruxellensis* were also observed in the Brett/LAB condition; Fig. 6C shows these micro-colonies at a magnification of x1000, with a complex architecture involving empty areas. A magnification x5000 (Fig. 6D) highlighted the formation of an extracellular matrix on the surface of the cells: a film covered the cells and may play a role in the biofilm structure. It was also possible to see within this biofilm the presence of LAB bound to *B. bruxellensis* cells.

4. Discussion

Brettanomyces bruxellensis was reported to be adapted to stressful environments displaying unfriendly physicochemical properties and many other microorganisms competing for nutrients (Conterno et al., 2006). In this study, the effect of abiotic factors (pH and ethanol concentration) on surface properties, pseudohyphae growth and bioadhesion was studied to see if these factors could interfere with biofilm formation in *B. bruxellensis*. In addition, synergistic or antagonist effects between distinct strains of *B. bruxellensis* or between *B. bruxellensis* and other microorganisms during bioadhesion and biofilm formation were examined.

4.1 Abiotic factors poorly modulate cell surface and bioadhesion properties

Wine is characterized by low pH (ranging from 2.9 to 4.0) and high ethanol concentration (from 12 to 16% alc vol. in average). Those two main factors have a strong effect on the growth of microorganisms. Indeed, *B. bruxellensis* was isolated from beverages such as wine, but also from beer and kombucha with acidic pH up to 2.5 for kombucha (de Miranda et al., 2022) and ethanol concentrations up to 16% (v/v) for some red wines. *B. bruxellensis* were shown to have significant strain tolerance to the acidic pH values and high ethanol concentrations (Oswald and Edwards., 2017; Cibrario et al., 2020). Both pH and ethanol were identified as having effects on the surface properties of the cells that can then directly affect the bioadhesion abilities of microorganisms. Indeed, pH changes could induce a change in cell surface charge impacting electrostatic interactions between cells and support (Boutaled et al., 2007). Ethanol has a fluidifying action of the membranes modifying their compositions and playing an important role in the secretion of adhesion proteins (Alexandre et al. 1994). However, in our experimental conditions, the pH and ethanol concentration showed a negligible effect on the surface electronegativity of *B. bruxellensis*. Results prior to this study and obtained on a different medium showed an increase in surface electronegativity along with an increase in pH value from 2 to 3.5 and then stabilization was observed for some strains according to the genetic group (Dimopoulou et al., 2019). This latter observation is congruent with our data showing that the genetic group is the most explanatory factor in the surface electronegativity which is directly influenced by the composition of membrane proteins and polysaccharides (Hong and Brown., 2010; Halder et al., 2015). The pH and ethanol concentrations also have no effect on hydrophobicity; indeed, more than 60% of the variance of this phenotype is both mediated by the strain and the genetic group. However, in *Saccharomyces cerevisiae*, hydrophobicity is greater in the presence of ethanol (Alexandre et al., 1998). In the present study, the increase in ethanol concentration from 5% to 14% results only in a slight increase in hydrophobicity showing here again that the effect of these 2 abiotic factors on surface hydrophobicity is negligible. The fact that the strain explains more than 60% of the phenotype suggest that hydrophobicity could be directly related to the presence of specific genes and/or gene expression associated with the phenotype. Indeed, in *S. cerevisiae,* hydrophobicity is impacted by the expression of genes of the *FLO* family exerting a major influence on the surface properties and bioadhesion of the species. Regarding differentiation in pseudohyphae cells, here again the abiotic factors have no effect on this phenotype being explained to more than 70% by the strain and the genetic group. This cellular morphology is mainly observed in triploid genetic groups such as the Teq/EtOH group and Beer (Le Montagner et al., 2023). However, in other species encountered in oenology such as *Hanseniaspora uvarum* and *S. cerevisiae*, an effect of ethanol and fusel alcohols such as tyrosol on invasive growth, a phenotype like pseudohyphae growth was reported (González et al., 2017, 2018). The presence of ethanol is perceived as a quorum-sensing molecule inducing filamentous growth (González et al., 2017); however, a variability of the response was observed depending on the strain and the species considered.

Finally, the effect of pH and ethanol concentration on bioadhesion of *B. bruxellensis* was examined. The initial study of Joseph et al (2007) showed a major effect of pH on bioadhesion and biofilm formation of *B. bruxellensis*. Indeed, a greater bioadhesion was observed from pH

3 and significant increase at pH 3.8 and 4 contrary to our observations showing no effect of pH on bioadhesion. This difference could be explained by the fact that the methods of quantification of bioadhesion are not the same but also that the medium used in both studies are totally different. In the case of Joseph et al (2007), a grape juice containing medium level of sugars (about 80 g/L) was used, while, in our study, a standard low sugars content wine-like medium was preferred (2 g/L). In *C. albicans*, pH also doesn't seem to impact bioadhesion; no significant differences are visible between pH 4 and pH 7 (Gonçalves et al., 2020). Vasconcellos et al (2014) show greater bioadhesion at pH 5.5 for *C. albicans* than at pH 7. However, the two studies used again different culture media thus showing the importance of this parameter to evaluate the bioadhesion capacity. In other species such as *Gardnerella vaginalis*, pH has no effect on bioadhesion (Bhat et al., 2012). *Staphylococcus epidermidis* and *Staphylococcus aureus* exhibit improved bioadhesion at basic pH and inhibition of bioadhesion at acidic pH for *S. aureus* (Memple et al., 1998; Chaieb et al., 2012). In our study, ethanol concentration explains only 2.5% the viability of bioadhered cells but however 9.9% of the bioadhered cell mortality variance. Indeed, it was observed a higher concentration of bioadhered dead cells with an ethanol concentration of 14%. In addition, it was observed that a combination of Alcohol/Strain and Alcohol/Group factors explained respectively 14.6% and 18.2% the bioadhered dead cells. This result could be explained by the ethanol tolerance that is different from one group to another. Indeed, strains of the Wine 1 group seem to be more resistant to high ethanol concentration than the other groups (Cibrario et al., 2020).

4.2 Bioadhesion of *Brettanomyces bruxellensis* is lower on epoxy resin compared to stainless steel material

The vats used during winemaking process can be shaped by different materials such as concrete, wood and stainless steel. In the case of concrete tanks, an epoxy resin coating inside the tanks is often carried out because it is easier to maintain and clean. Our study confirms the bioadhesion capacity of *B. bruxellensis* on different categories of stainless steel but also, for the first time, on epoxy resin. Thus, this species has a broad spectrum of ability to bioadhere to many materials as evidenced by previous work which reports that *B. bruxellensis* has been identified on the surface of glass, stainless steel, polystyrene and wood (Joseph et al., 2007; Oelofse et al., 2008; Kregiel et al., 2018; Lebleux et al., 2020). In addition, under our experimental conditions, differences in bioadhesion were observed between stainless steel and epoxy resin with less bioadhesion on the latter. This difference can be explained by the fact that epoxy resin has a lower surface hydrophobicity than stainless steel and is therefore rather hydrophilic (Ait Iahbib et al., 2023). This hydrophobicity plays a major role in the establishment of bioadhesion because the hydrophobic interactions established between the support and the cells are the strongest involved during bioadhesion (Urano et al., 2002; Verstrepen and Klis., 2006; Blanco et al., 2008). This decrease in epoxy resin bioadhesion could also be observed for other microorganisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* where the concentration of bioadhered cells was lower on epoxy resin than on stainless steel (Ait Iahbib et al., 2023). Nevertheless, studies on other microorganisms such as *Streptococcus mutans* and diatoms have shown that epoxy resin promotes bioadhesion (Asiry et al., 2018; Liang et al., 2019; Faria et al., 2021). The hypothesis that the roughness of the material could impact bioadhesion is also advanced in the work of Ait Iahbib (2023) who shows that the roughness of epoxy resin is less important than that of stainless steel. Roughness is known to be a factor impacting bioadhesion phenomena to trap cells and initiate bioadhesion (Yuan et al., 2017; Yang et al., 2022). In our study, the grade of stainless-steel results in a difference in roughness between the 2 references used, RSS having a significant surface roughness unlike SSS.

Bioadhesion was not significantly different on the 2 grades despite differences in roughness that could come from the fact that the 2 steels had a similar surface hydrophobicity. This observation was also reported for *Listeria monocytogenes*, *P. aeroginosa* and *Candida lipolytica* where the roughness of the support has no impact on bioadhesion (Hilbert et al., 2003; Rodriguez et al., 2008). However, studies have shown, on the contrary, that roughness plays a major role in bioadhesion (Kukhtyn et al., 2019; Tomičić and Raspor., 2017). In addition, complex surface topography with high roughness could inhibit bioadhesion due to limited contact zones with bioadhesion support (Valle et al., 2015). The roughness therefore seems a factor to be considered differently to explain the differences in bioadhesion capacity depending on the species or strain.

4.3 Effect of mixed-strain and mixed-species biofilm

During the winemaking process, it is possible to encounter an important diversity of microorganisms. Indeed, this microbial diversity strongly decreases from grape juice to wine; only species such as *B. bruxellensis*, LAB and AAB, well adapted to the "final" wine composition, persist at the end of the vinification and during the wine ageing process (Renouf et al., 2006; Camilo et al., 2022). In a given winery, several strains of *B. bruxellensis* belonging to different genetic groups can coexist simultaneously within the same wine sample (Cibrario et al., 2019). The bioadhesion and biofilm formation phenomena were so far only studied for single strain culture of *Brettanomyces bruxellensis*. Therefore, to take into account the reality of the wine microbial community, we studied the effect of the presence of 2 genetically different strains on the biofilm formation. It was thus shown that the biofilm formation is mainly driven by the strain with the highest bioadhesion capacity and that the second strain was present in small proportion. In addition, in many cases, the bioadhesion kinetics of the mixed-strain biofilm followed the bioadhesion kinetic of the dominant strain when its alone. In *Pseudomonas* *aeruginosa*, a similar observation was also reported: in a mixed-strain biofilm, one strain was present in higher concentrations than the other, thus showing some interaction and competition effect between the two strains (Oliveira et al., 2015). In addition, the authors showed that the presence of two strains of *P. aeruginosa* induced a significant increase in biofilm formation (Olivieroa et al. 2015) which is not the case in our observations where the thickness of the biofilm is greater when strain AWRI 1608 is the only one to form biofilm. In *S. cerevisiae,* adhesion is preferred between cells expressing the same surface properties to promote biofilm resistance (Mitri and Richard Foster., 2013). In *Escherichia coli*, a synergistic effect was also observed on biofilm formation during strain co-cultures. In MX4, composed of 2 strains with significant bioadhesion properties, a change in the majority strain over time was observed that could be induced by a competition between cells for nutrients (Xavier and Foster 2006). Thus, the fact that one strain moves from minority to majority can be explained by higher ability to metabolize nutrients compare to the other one. It is also conceivable that the lack of nutrients led to the death of part of the population of one of the strains, thus releasing nutrients into the environment that can be assimilated by the remaining strain. Thus, a population dynamic of *B. bruxellensis* strains was observed in the biofilm. This dynamic is also observable in the cellar where it has been shown that within the same batch of wine, the planktonic population of *B. bruxellensis* is variable over time from a genetic point of view (Cibrario et al., 2017).

In wine, other microorganisms can interact with *B. bruxellensis* such as *Oenococcus oeni* and *Acetobacter pasteurianus,* with for the latter, a strong negative effect on the sensory qualities of wine, eg production of acetic acid and ethyl acetate (du Toit and Pretorius., 2002; Zepeda-Mendoza et al., 2018). Since *O. oeni* was reported to have bioadhesion properties (Bastard et al., 2016; Coelho et al., 2019), the formation of mixed-species biofilm between *O. oeni* and *B. bruxellensis* was studied. Results showed a decrease of bioadhesion property of *B. bruxellensis* in the presence of *O. oeni*. However, it was also observed the formation of structured micro-colonies where the 2 species were organized in the form of biofilm covered with extracellular matrix. This matrix is also present in the single species biofilms of *B. bruxellensis* thus encompassing cells (Lebleux et al. 2020). The *O. oeni* enumeration on selective medium was not possible on days 7 and 14 but on days 1 and 28 indicating the presence of the bacteria, also confirmed by Scanning Electron Microscopy observations (SEM). This lack of identification can potentially be explained by the physiological state of cells in a Viable But Non Cultivable (VBNC) physiological form previously demonstrated in this species (Millet and Lovaud-Funel., 2000). A similar observation was also made in our study, where *A. pasteurianus* is no longer detected on solid medium from day 7 while cells are observed by SEM. AABs and LABs have been shown to bioadhere in contact with *B. bruxellensis*. The formation of mixed-species biofilm (yeast/bacteria) was also observed with *C. albicans* and *S. epidermidis;* cooperation was reported between these 2 species where the formation of extracellular matrix of one protects the other from specific antibiotic activity (Adam et al., 2002). In the field of fermentation, mixed-species biofilms are also observed, particularly in the case of rice wine fermentation where biofilms of *S. cerevisiae* and *Lacticaseibacillus casei* are produced; however, when they were present alone, no biofilm observations are made (Kawarai et al., 2007; Furakawa et al., 2011). In other cases, the presence of one microorganism may inhibit the formation of biofilm from another. This is the case for *Lactiplantibacillus paraplantarum* which, in the presence of *Listeria monocytogenes*, produces a bacteriocin inhibiting the formation of biofilm of the latter (Winkelströter et al., 2015; Yuan et al., 2019). Thus, the decrease in the bioadhesion of *B. bruxellensis* could be explained by a competition for nutrient or by an inhibition by metabolites (eg lactic acid) excreted by the bacteria present before *B. bruxellensis;* these metabolites could reduce its bioadhesion due to the modification of the surface physico-chemical properties of the material and/or due to the inhibition of the yeast growth.

5. Conclusion

This study was conducted on several strain representative of the genetic diversity of the species and with contrasting surface and bioadhesion properties. Our data showed that the abiotic factors such as pH and ethanol concentration have negligible effects on surface properties in our experimental conditions. An effect of ethanol was highlighted on bioadhered cell mortality probably linked to *B. bruxellensis* strains different tolerance to ethanol. The fact that the "strain" and "genetic group" factors are the most explanatory of the variance of the phenotypes studied, strongly suggests the existence of genetic determinism. In *S. cerevisiae*, hydrophobicity, pseudohyphae cell formation and bioadhesion have been shown to be directly impacted by the expression of *FLO* genes family that could be good candidates to further studied the genetic mechanisms underlying those phenotypes in *B. bruxellensis* (Smit et al., 1992; Mortensen et al., 2007; van Mulder et al., 2009; Govender et al., 2010; Zhang et al., 2021).

In the present study, we considered the diversity of microorganisms found in wine and in the cellar during the winemaking and wine ageing process. Two strains of *B. bruxellensis* can form a biofilm that is driven by the most bioadhesive one even if some competition is observed and evidenced by a lower thickness of mixed-strains biofilms compared to single strain ones. Mixed-species experiments indicate that *B. bruxellensis* biofilm can be reduced or at least delayed, but not prevented when LAB and AAB bioadhered first. Finally, the nature of the winery materials would also be a relevant parameter to consider in the prevention of *B. bruxellensis* spoilage. This emphasizes the need for implemented specific cleaning procedures.

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Legends of the figures

Figure 1: Percentage of variance explained for the different factors and each parameter analyzed (multi-way Anova, p-value <0.05)

Figure 2: Bioadhesion capacity of *B. bruxellensis* to different materials found in oenology (6 strains) in WLM medium. Epoxy: epoxy resin; RSS: rough stainless steel; SSS: smooth stainless steel. The letters indicate significant differences (Kruskall Wallis, p-value < 0.05)

Figure 3: Dynamic of mixed-strains biofilm between 2 genetically different strains of *B. bruxellensis* in WLM medium A, C, E, G represent the population level of cultivable cells of each mix and single cell biofilm. B, D, F, H represent the proportion of each strain composing the mixes over time (n=90 colonies).

Figure 4: Thickness of biofilms over time. Upper letter represents groups significantly different per day as defined by Kruskal-Wallis test (Agricolae package, R, p-value <0.05).

Figure 5: *B. bruxellensis* cultivable population in the biofilm after 3 hours (A) and 28 days of bioadhesion in red wine. Upper letter represents groups significantly different per day as defined by Kruskal-Wallis test (Agricolae package, R, p-value <0.05).

Figure 6 : Scanning electron microscopy (SEM) observation of mixed-species biofilms at different stages in red wine. A represents cells of *B. bruxellensis* and AAB (blue arrows) at day 14 with magnification x500; B is characterized by a magnification x10 000 of the Brett/AAB condition on day 14 highlighting the presence of crystals (white arrows) around the *B. bruxellensis* cell; C is an observation of a microcolony of *B. bruxellensis* and LAB on day 28 at magnification x 1000; D represents a magnification x 5000 of a microcolony with extracellular matrix (red arrows).

Figure S1: Bioadhesion capacity on different materials depending on the *B. bruxellensis* strain in WLM medium. Epoxy: epoxy resin; RSS: rough stainless steel; SSS: smooth stainless steel. The letters indicate significant differences (Kruskall Wallis, p-value < 0.05)