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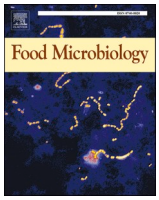

Effect of abiotic and biotic factors on *Brettanomyces bruxellensis* bioadhesion properties

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ABSTRACT

Biofilms are central to microbial life because of the advantage that this mode of life provides, whereas the planktonic form is considered to be transient in the environment. During the winemaking process, grape must and wines host a wide diversity of microorganisms able to grow in biofilm. This is the case of *Brettanomyces bruxellensis* considered the most harmful spoilage yeast, due to its negative sensory effect on wine and its ability to colonise stressful environments. In this study, the effect of different biotic and abiotic factors on the bioadhesion and biofilm formation capacities of *B. bruxellensis* was analyzed. Ethanol concentration and pH had negligible effect on yeast surface properties, pseudohyphal cell formation or bioadhesion, while the strain and genetic group factors strongly modulated the phenotypes studied. From a biotic point of view, the presence of two different strains of *B. bruxellensis* did not lead to a synergistic effect. A competition between the strains was rather observed during biofilm formation which seemed to be driven by the strain with the highest bioadhesion capacity. Finally, the presence of wine bacteria reduced the bioadhesion of *B. bruxellensis*. Due to biofilm formation, *O. oeni* cells were observed attached to *B. bruxellensis* as well as extracellular matrix on the surface of the cells.

1. Introduction

The vast majority of microorganisms on Earth are preferentially found as communities on the surface of a support matrix rather than as free planktonic cells in the environment (Costerton et al., 1995; Kolter and Greenberg., 2006). These communities called biofilms are characterised by a spatial organization of the microorganisms present but also by the production of extracellular matrix (Costerton et al., 1999). Biofilms are found in a wide variety of environments, and as it is estimated that between 20% and 80% of terrestrial microbial biomass lives in a biofilm form, they may play a crucial role in the proper functioning of most environments, anthropised or not (Richards and Melander., 2009; Flemming and Wuertz., 2019; Bridier and Briandet., 2022). Moreover, the presence of biofilms can be problematic in certain sectors such as medical, agri-food and maritime transport, due to their resistances to biocide exposure and pathogenicities (Hall-Stoodley et al., 2004; Piola et al., 2009; Zara et al., 2020). These adaptative strategies are mainly due to the presence of an extracellular matrix composed of polysaccharides, proteins, peptidoglycans, nucleic acids and lipids, which act as a barrier against external aggressions (Czaczyk and Myszka, 2007; Flemming et al., 2007). However, biofilm formation is dependent on several environmental factors such as pH, temperature, and nutrient concentration (Fathollahi and Coupe., 2021; Liu et al., 2023). The presence of mixtures of microorganisms that are genetically related or belong to different species can also have a significant effect on biofilm formation. Indeed, it has been shown that the presence of several strains of *Escherichia coli* in the same environment induces a synergistic effect that promotes biofilm formation (Reisner et al., 2006). Conversely, in *Listeria monocytogenes*, biofilm formation is inhibited in the presence of *Lactiplantibacillus paraplantarum* (Winkelströter et al., 2015).

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In oenology, and in particular during the winemaking process, many microorganisms take part during fermentation and contribute to the wine aroma panel, by producing molecules of interest or wine defects (Gammacurta et al., 2017; Tempère et al., 2018; Carpena et al., 2021). Among the microorganisms that produce off-flavors, *Brettanomyces bruxellensis* is the main spoilage yeast, due to the production of volatile phenols characterised by stable, horse sweat and leather odours that mask the fruity aromas of wines (Chatonnet et al., 1992; Lattey et al., 2010). Different materials are used in oenology for fermentation and wine storage: terracotta, ceramics, wood, concrete, stainless steel, the latter now preferred because of its resistance to sulphite corrosion and efficient cleaning procedures (Valdez et al., 2015). Wood is mainly used for ageing wine in barrels. Concrete tanks are often coated with epoxy resin, to limit their porosity and make them easier to clean (Desenne et al., 2008).

B. bruxellensis is present throughout the winemaking process (Renouf and Lonvaud-Funel., 2007; Rubio et al., 2015). This ubiquitous species is characterised by a high genetic diversity which is directly related to the ploidy and the isolation niche of the strain (Albertin et al., 2014; Avramova et al., 2018a). At least, two different diploid and four different triploid groups have been identified (Harrouard et al., 2022). Tolerance and resistance to sulphites (SO_2), the main antimicrobial used in oenology, have been found to be linked to the genetic group (Curtin et al., 2012; Avramova et al., 2018b). Strains of *B. bruxellensis* can be found from year to year within the same winery, suggesting a high capacity to persist in the winemaking environment between vintages (Cibrario et al., 2019). Indeed, *B. bruxellensis* has been identified in the air, on floors, walls, winemaking vats, winemaking equipment and barrels (Fugelsang, 1997; Connell et al., 2002; Le Montagner et al., 2023). This persistence can be explained by the fact that *B. bruxellensis* has bioadhesion and biofilm formation capacities (Joseph et al., 2007; Dimopoulou et al., 2019; Lebleux et al., 2020). Furthermore, differences in strain bioadhesion are observe depending on the genetic group, with the "Beer -3 N 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 surface materials on the surface properties and bioadhesion ability of *B. bruxellensis*. Since 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 investigate the effect of biotic factors, namely 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 the surface and bioadhesion properties of *B. bruxellensis*, a total of 17 strains representative of the genetic diversity of the species and with 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, Italy) 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) containing 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 solid YPD medium (2% (w/v) agar (Fisher BioReagent™) and incubated at 25 °C for 5 days.

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., 2018a).

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 in section 2.1 were performed in Wine Like Medium (WLM), which was used because of its similarity to wine (Le Montagner et al., 2023). WLM is composed of 0.05% (w/v) glucose (Fisher BioReagent™), 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 BioReagent™), 0.5% (w/v) glycerol (Sigma Aldrich®). The effect of two abiotic factors, pH and ethanol concentration, was investigated. For the effect of pH, 3 values were considered for WLM: 3.6, 3.8 and 4.1. The pH was adjusted with KOH 5 M. For the ethanol concentration effect, 3 values were considered for WLM, 5%, 10% and 14% (v/v) (VWR Chemicals®). Adaptation steps were necessary for the yeast growth in the WLM medium displaying different pH and ethanol concentrations. Briefly, some colonies were recovered from the solid medium and transferred to 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 with stirring at 180 rpm. This adaptation step was repeated 3 times with the proportion of WLM gradually increased (50%, 75% and finally 90%). After 48 h of incubation (25 °C, 180 RPM in a 50 mL Falcon™ tube), the cell suspension was collected to determine i) surface charge ii) cell surface hydrophobicity, iii) the pseudohyphae growth and iv) the bioadhesion capacity of each strain on 316 L stainless steel coupons.

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 ultrapure water and then resuspended in ultrapure water at the pH defined in the experimental design. The cell suspension was filtered on a nylon filter (0.45 µm) to obtain a cell suspension with an OD_{600nm} around 0.7. The zeta potential was measured using the Zetasizer Nano (Malvern). For each strain, three measurements (technical replicates) were performed on the same cell culture.

2.1.4. Cell surface hydrophobicity

Cell hydrophobicity was determined using the MATS (Microbial Adhesion To Solvents) method which allows the determination of the hydrophilic/hydrophobic character of the surface of yeasts (Bellon-Fontaine et al., 1996). Ten milliliters of the cell suspension were centrifuged for 5 min at 7000 g at room temperature; the pellet was then

washed twice with distilled water and resuspended in sodium chloride solution (NaCl 0.9%) to obtain a cell suspension with an OD_{600nm} of around 0.7. A volume of 1.5 mL of cell suspension was mixed with 250 μL of either chloroform (Fisher Chemical) or hexadecane (Sigma-Aldrich). The mixture was vortexed for 2 min to produce an emulsion. A resting period of 15 min allowed the separation of the 2 phases. The optical density of the cell suspension (OD_0) and of the aqueous phase of the mixture was measured at 600 nm. The affinity for each solvent was calculated using the formula reported by Le Montagner et al. (2023).

2.1.5. Pseudohyphae growth

To assess the proportion of pseudohyphae, 1 mL of the cell suspension collected at the end of the adaptation protocol in WLM was sampled. The sample was filtered through a 0.4 μm filter (Isopore™). The filter was then placed on a pad containing a mixture of ChemSol B16 buffer (Chemunex) with 1% (v/v) of Fluorochrom V6 (Chemunex), and the pad was incubated for 15 min at 30 °C in the dark. The proportion of pseudohyphae was assessed by epifluorescence microscopy (10 field counts) and expressed as a percentage.

2.1.6. Bioadhesion properties

To determine the bioadhesion capacity of the *Brettanomyces* strains, the cell suspension was centrifuged at 7000 g for 5 min at room temperature and then the cell pellet was washed twice with sodium chloride solution (NaCl 0.9%). The pellet was resuspended in a mixture of WLM 90% and YPD 10% to give a final concentration of 10^7 cells/mL. Bioadhesion was measured on 14 mm × 25 mm, 316 L 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 was then added to initiate bioadhesion, which was then carried out for 3 h at room temperature. A coupon washing step was then performed to remove the non-adherent cells that had sedimented. The wash step consisted of 5 consecutive cleaning baths in sterile sodium chloride solution. The coupon was then placed in a solution of Chemsol B15 (Biomerieux) containing 1% (v/v) 5 (6)-Carboxyfluorescein Diacetate (CFDA) (Thermo Fisher Scientific) at 8 mg/mL to detect of live cells and 0.2% (v/v) propidium iodide (PI) at 1 mg/mL to detect of dead cells (Thermo Fisher Scientific). Cells were left at room temperature for 15 min before observation to allow staining. The surface of the coupon was observed by confocal microscopy in the facilities of Bordeaux Imaging Center Bordeaux of the INRAE Plant Pole. Observations were made with the immersion lens. Confocal acquisitions were performed 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, respectively. The fluorochromes were detected sequentially, line by line. The adherent dead and live cells were counted on 10 different fields.

2.1.7. Bioadhesion on different materials

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

2.1.8. Material properties

After cleaning, the materials were immersed in WLM medium for 3 h at room temperature and then rinsed once with distilled water and dried under laminar flow hood, for 1 h. Contact angle (θ) measurements 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 a minimum 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 as described in Le Montagner et al. (2023) (Table 2). 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 a multi-strains bioadhesion competition.

2.2.2. Bioadhesion and biofilm formation

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

2.2.3. Enumeration of bioadhered cells by cultivation

Viable cell counts were performed after the 3 h, 7 days and 14 days of bioadhesion and biofilm formation. The coupon was cleaned to remove non-adhered cells by 5 consecutive washes in sterile sodium chloride solution (NaCl 0.9%). The coupon was then placed in a 50 mL tube containing 10 mL of sterile sodium chloride solution (NaCl 0.9%) and then the entire suspension was sonicated at 47 Hz for 2 min. After this sonication step, the tube was vortexed at maximum speed for 40 s. Dilution series were then performed and 100 μL of the suspension was inoculated in triplicate on YPD agar medium at 30 °C. The result is then expressed as Colony Forming Units per cm^2 (CFU/cm^2).

2.2.4. Biofilm thickness

Biofilm thickness was measured on MX1, MX2 and MX3 by confocal microscopy observations (confocal analysis could not be performed on MX4 due to lack of facilities at the Bordeaux Imaging Center). 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) 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 min. The surface of the coupon was observed by confocal microscopy in the facilities of the Bordeaux Imaging Center of INRAE Plant Pole. Observations were made with the immersion lens as described in 2.1.6. The thickness was measured out by taking successive images of each focal plane using the z-stack function of the ZEN

Table 2
List of the 4 strains used in the mix composition according to their genetic groups (*Avramova et al., 2018a) 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 Bioadherent Pseudohyphae

microscopy software (Zeiss). Thickness analysis was then performed on 10 biofilm areas of the ROI manager function present on the FIJI image processing software extension of the ImageJ software.

2.2.5. Strain genetic identification

To determine the proportion of each strain per mix, 15 yeast colonies were randomly collected in two Petri dishes per mix at the same dilution (see in section 2.2.3) in triplicate (90 colonies per mix). Each colony was placed in 20 µL of NaOH 20 mM for cell lysis. This mixture was incubated at 90 °C for 10 min and then placed at −20 °C for 30 min. This was 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 as the 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. As the experimentation was conducted in red wine, adaptation steps were necessary for *B. bruxellensis* and *A. pasteurianus*. From 2 to 3 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) 48 h before bioadhesion.

2.3.2. Bioadhesion and biofilm formation

In wine, *B. bruxellensis* shares the same niche as lactic acid and acetic acid bacteria. The aim of this experiment was to evaluate the impact of the presence of bioadherent bacteria on the stainless steel coupon on the subsequent bioadhesion and biofilm formation of *B. bruxellensis*. Therefore, 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 sodium chloride solution (NaCl 0.09%). The pellets were then the resuspended in a mixture of red wine 90% (v/v) and grape juice 10% (v/v) to give 5.0×10^6 cells/mL for *B. bruxellensis* and 10^6 cells/mL for bacteria. In the case of Brett/LAB/AAB, the concentration of bacteria was 1.0×10^6 cell/mL with a ratio of 1:1 for LAB and AAB. Bioadhesion was performed sequentially. Bacteria were first contacted with previously cleaned stainless steel coupon for 48 h (Le Montagner et al., 2023). A coupon washing step was then performed to remove non-adherent bacteria as described in the previous sections. *B. bruxellensis* suspension was then added for 3 h at room temperature. After these 3 h, the coupons were washed again. After rinsing, 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 was added. The vials were then placed at 20 °C until analysis at 3 h, 7, 14 and 28 days. Samples were prepared in triplicate for each measurement point.

2.3.3. Culturable cells enumeration

The enumeration of viable cells was carried out after the 3 h, 7 days and 14 days of bioadhesion and biofilm formation. The protocol used for this part was the same as described in section 2.2.3. For *Brettanomyces bruxellensis*, serial dilutions were plated on YPD agar medium and incubated at 30 °C for 5 days. 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 in anaerobiosis at 25 °C was performed for 7 days. Results were expressed as Colony Forming Units per cm^2 (CFU/cm^2).

2.3.4. Scanning electron microscopy (SEM)

Bioadherent cells and biofilms were observed by SEM. The adherent cells were fixed on the stainless steel coupon with a solution of 3% glutaraldehyde in 0.1 M phosphate buffer of pH 7.2 overnight at 4 °C. The coupon was washed with 0.05 mM phosphate buffer for 10 min. For dehydration, two successive immersions in solutions of increasing ethanol content (50, 75, 90, 100%) were carried out for 10 min. The coupon was immersed in solution of ethanol-acetone solution (70/30, 50/50, 30/70, 100%) for 10 min. The coupon was then air dried and stored at room temperature. The samples were coated with a thin layer of platinum and then observed using a Zeiss Gemini 300 scanning electron microscope. SEM was performed with 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 the surface charge (Zeta potential), surface hydrophobicity (affinity to chloroform and hexadecane), pseudohyphae cell 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 found. Alcohol had only a weak effect with 2.6% of the variance explained. For 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 effects of alcohol and pH were again negligible as was the combination of factors. The variance of pseudohyphae cells formation was also explained by strain and genetic group at 36.5% and 35.5%, respectively, with 5.2% variance explained by an alcohol/genetic group interaction. The variance of viable cell adhesion was explained by the strain (37.3%) and genetic group (25.2%). 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 by the genetic group at 18.2%. 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, the number of dead cells increases significantly with increasing in alcohol concentration (Anova, p-value <0.05). Thus, pH and alcohol appeared to have a limited effect on the surface and bioadhesion properties of *B. bruxellensis* under our experimental conditions.

3.2. Material properties and effect on bioadhesion

Different materials were studied, rough 316 L stainless steel (RSS)

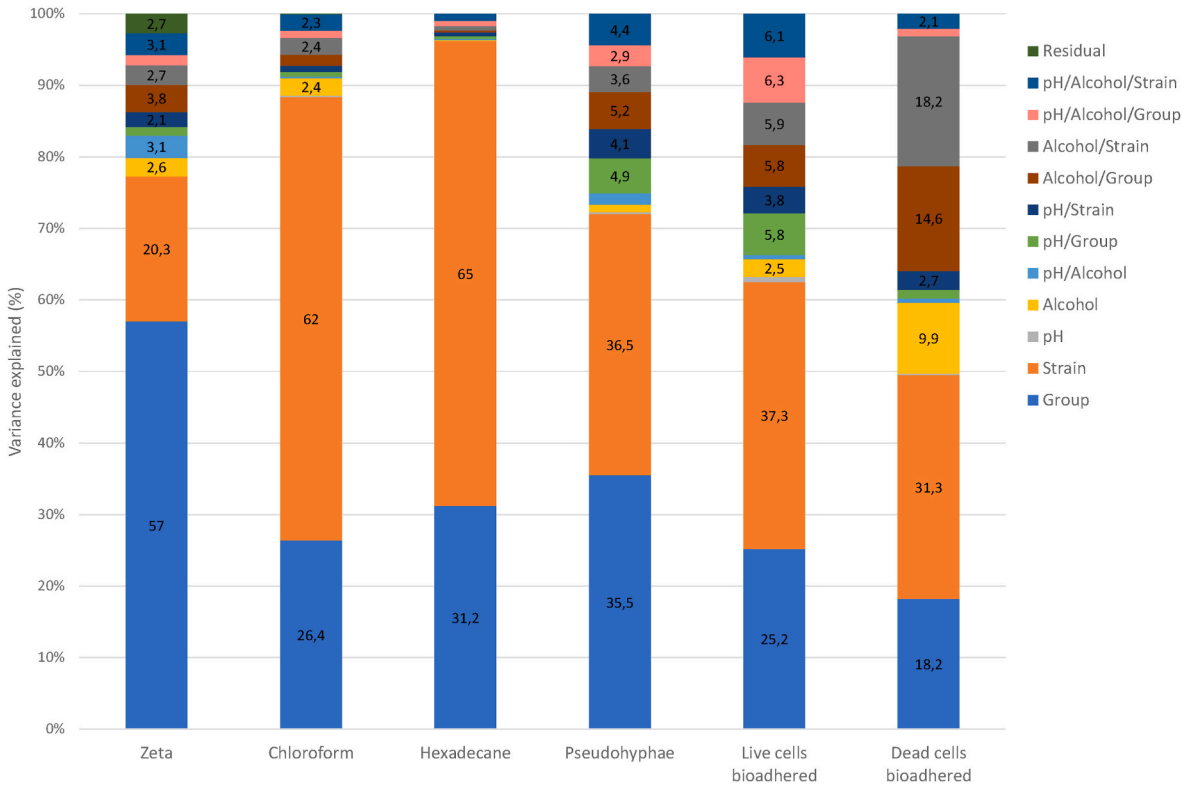

Fig. 1. Percentage of variance explained for the different factors and each parameter analyzed (multi-way Anova, p-value <0.05).

and epoxy resin GE55 in addition to smooth 316 L stainless steel (SSS). The wettability of the different materials was measured after cleaning the coupons and after immersion in WLM 3 h. The contact angle values are given in Table 3. After cleaning, the SSS and RSS stainless steel references showed similar results, with contact angles of 104.3° and 105°, respectively indicating 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, respectively (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 affected only the hydrophilic properties of the three surfaces *ie* the polar components.

The results obtained after 3 h of bioadhesion on these materials are shown in Fig. 2. Depending on the material, the concentration of bioadherent cells was significantly different (p-value<0.05). Bioadhesion

Table 3
Wettability of the different materials used in oenology.

Material	Condition	Contact angle (θ)	
		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

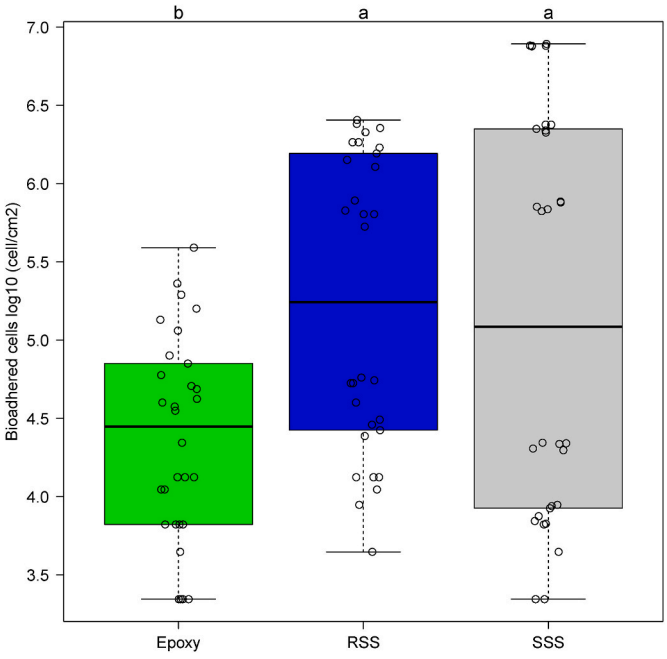

Fig. 2. Bioadhesion capacity of *B. bruxellensis* to different materials found in oenology (6 strains) in WLM medium. Epoxy: epoxy resin; RSS: rough stainless steel; SSS: smooth stainless steel. The letters indicate significant differences (Kruskall Wallis, p-value <0.05).

to epoxy resin was significantly lower, with an average concentration of 6.04×10^4 cell/cm^2 versus 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), indicating 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 (2.26×10^6 cell/cm^2 and 7.63×10^6 cell/cm^2 for SSS, 1.56×10^6 cell/cm^2 and 2.24×10^6 cell/cm^2 for RSS and finally 6.91×10^4 cell/cm^2 and 2.22×10^4 cell/cm^2 for epoxy), although there were significant differences between the 3 materials, with bioadhesion on SSS being the most important. 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 strain YJS8528, 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* with contrasting bioadhesion properties was monitored over time, in order to follow the dynamics of biofilm formation. MX1, composed of strains AWRI 1499 and AWRI 1608 showed a level of culturable population in the biofilm similar to that observed for strain

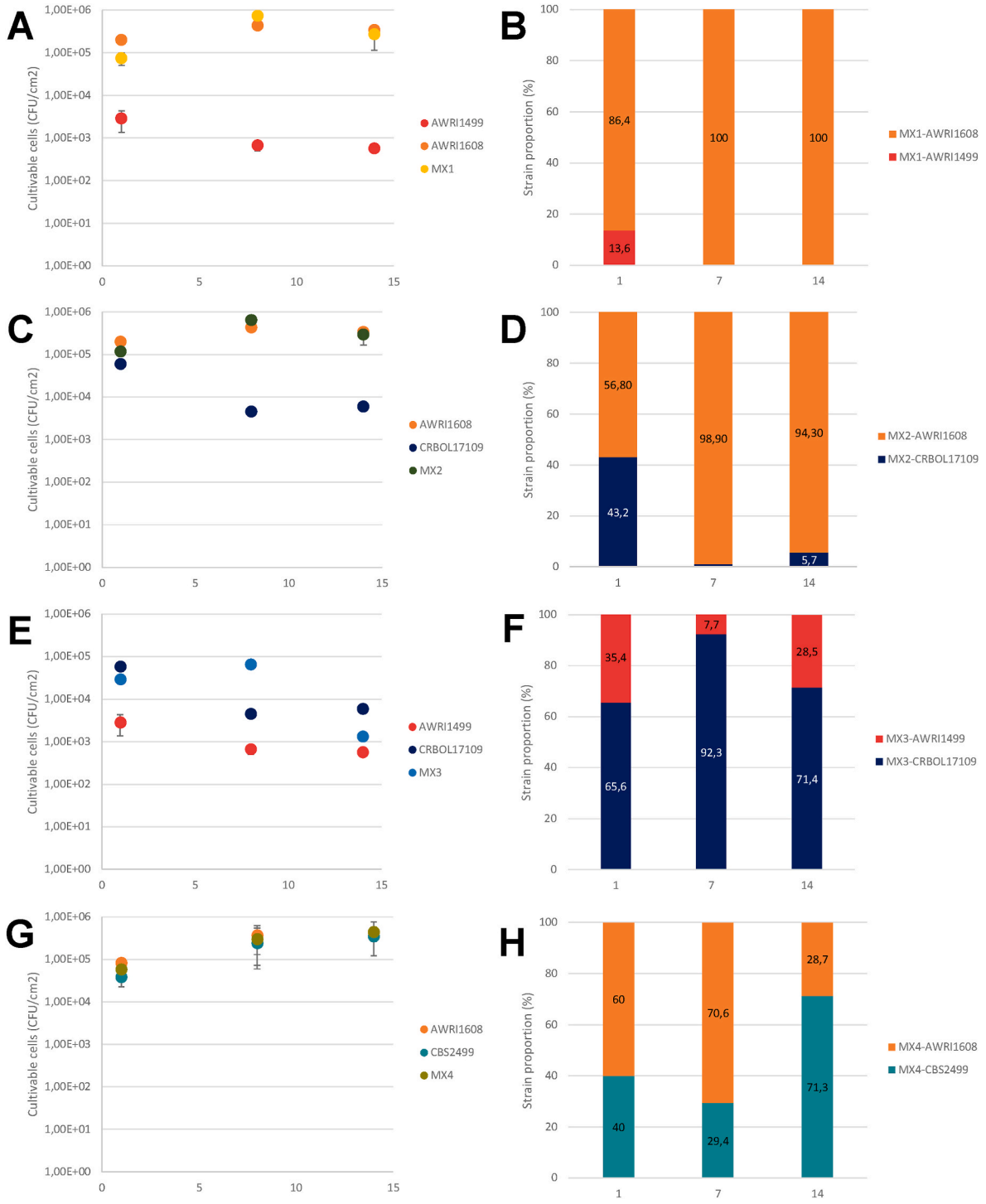

Fig. 3. Dynamic of mixed-strains biofilm between 2 genetically different strains of *B. bruxellensis* in WLM medium A, C, E, G represent the population level of culturable cells of each mix and single cell biofilm. B, D, F, H represent the proportion of each strain composing the mixes over time (n =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

AWRI 1608 alone, with an increase in culturable cells during the first 7 days (from 7.43 × 10^4 CFU/cm^2 to 7.33 × 10^5 CFU/cm^2), followed by a slight decrease until day 14 (Fig. 3A). Meanwhile, the culturable population of the strain AWRI 1499 decreased over time. Fig. 3B shows the evolution of the relative proportions of the strains. The dominant strain on days 1, 7 and 14 was AWRI 1608. This explains why MX1 behaved similarly to AWRI 1608 alone.

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 while strain CRBO L17109 represented 43.2%. However, AWRI 1608 then became dominant as it representing 98.9% and 94.3% on days 7 and 14, respectively.

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

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

The thickness of the mixed biofilm was also monitored. Fig. 4 shows the single and mixed strain biofilm thickness. Strain AWRI 1608 formed a homogeneous biofilm on stainless steel with a gradual increase in biofilm thickness over time from 7.25 μm on day 1–12 μm and 16.7 μm on days 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 scattered micro-colonies on the surface; it was not shown in Fig. 4.

MX1 and MX2 showed similar thicknesses over time: no significant differences were observed between the 2 mixes on any given day (p-value >0.05). The thickness of the biofilm formed by 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 of the AWRI 1608 strain; the thickness of these mixes was similar to that of the AWRI 1608 single strain on days 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 single strain AWRI 1608 biofilm had a significantly greater thickness than that of MX1 and MX2 (p-value <0.05). Finally, MX3 consisting of the AWRI 1499 and CRBO L17109 strains had the lowest biofilm thickness, which increased from 5.14 μm to 7.26 μm, between day 1 and day 7. The MX3 biofilm was not significantly different 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 stainless steel surface, revealing a dispersion of bioadherent cells during the second week.

3.4. Pluri-species biofilm

The study of pluri-species biofilms was carried out by associating *B. bruxellensis* with either a LAB (*O. oeni*), an AAB (*A. pasteurianus*) or both. Bioadhesion was performed sequentially, with bacteria introduced for 48 h before *B. bruxellensis* was added.

In all assays with mixtures of species, the population of adherent *B. bruxellensis* was lower than when *B. bruxellensis* was assayed alone (Fig. 5A, Table S1). Indeed, after 3 h, the population level of bioadhered *B. bruxellensis* were respectively 3.31 × 10^3 CFU/cm^2, 3.73 × 10^3 CFU/cm^2 and 4.50 × 10^3 CFU/cm^2 for the Brett/LAB, Brett/AAB and Brett/LAB/AAB conditions, respectively compared to 4.28 × 10^5 CFU/cm^2 when *B. bruxellensis* was alone. These results indicated a significant decrease in *B. bruxellensis* bioadhesion when the bacteria were previously bioadhered (Kruskal-Wallis, p-value <0.05) (Fig. 5A). For *B. bruxellensis* alone, no significant evolution of the adhered population was observed during the first 14 days (p-value >0.05) (Table S1). For the Brett/LAB condition, the *B. bruxellensis* population remained stable throughout the 28 days of this study. 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 acetic acid bacteria does not affect *B. bruxellensis* biofilm formation (p-value >0.005). However, in the Brett/LAB/AAB and Brett/LAB after 28

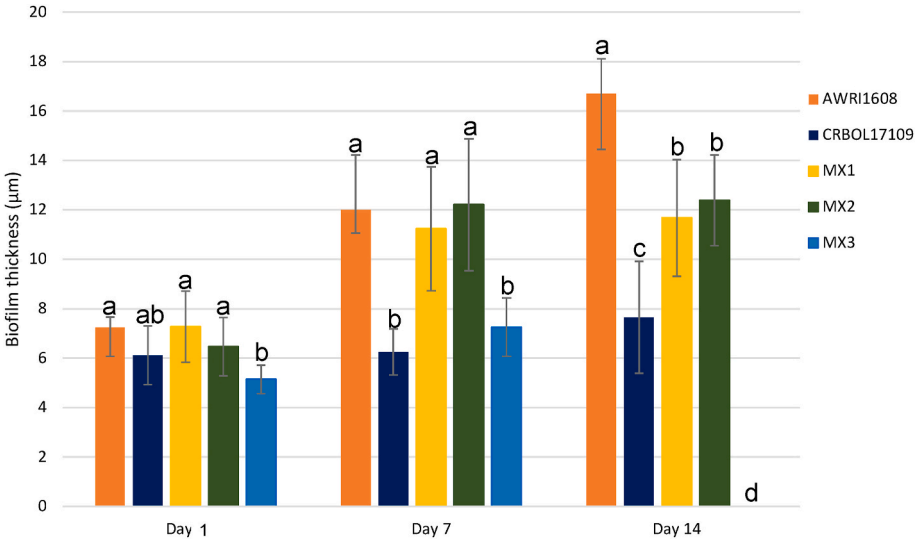

Fig. 4. Thickness of biofilms over time. Upper letter represents groups significantly different per day as defined by Kruskal-Wallis test (Agricolae package, R, p-value <0.05).

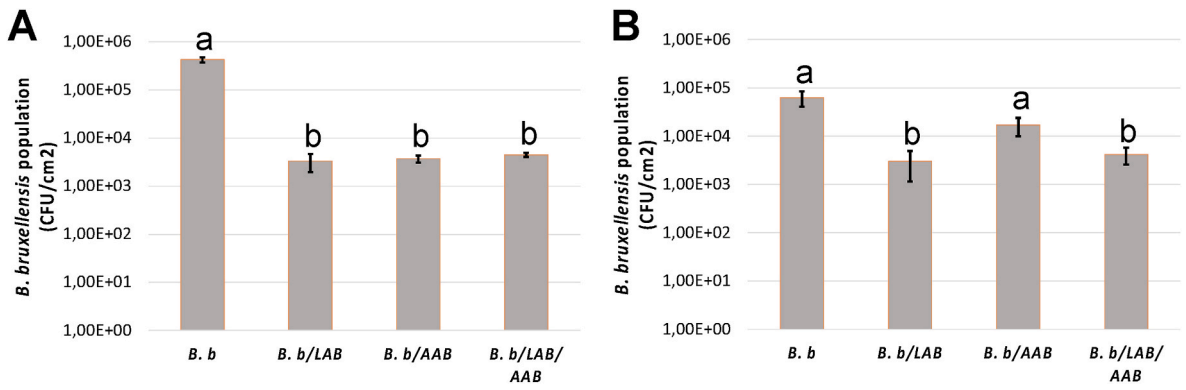

Fig. 5. *B. bruxellensis* culturable population in the biofilm after 3 h (A) and 28 days of bioadhesion in red wine. Upper letter represents groups significantly different per day as defined by Kruskal-Wallis test (Agricolae package, R, p-value <0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

days, the *B. bruxellensis* population level was significantly lower (p-value <0.05) than when *B. bruxellensis* was present alone or with AAB (Fig. 5B).

After the 3 h 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) (Table S1). As for the LAB control, the LAB count in the presence of *B. bruxellensis* on days 7 and 14 showed no presence of culturable cells but bacteria were visible by SEM, suggesting that the cells were in a non-culturable physiological state. 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 (9.51×10^2 CFU/cm^2 and 2.0×10^3 CFU/cm^2 for Brett/AAB and Brett/LAB/AAB, respectively). In addition, SEM observations 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 at a magnification of ×5000. The microorganisms present on the surface of the coupon were randomly distributed. The presence of AAB was evident

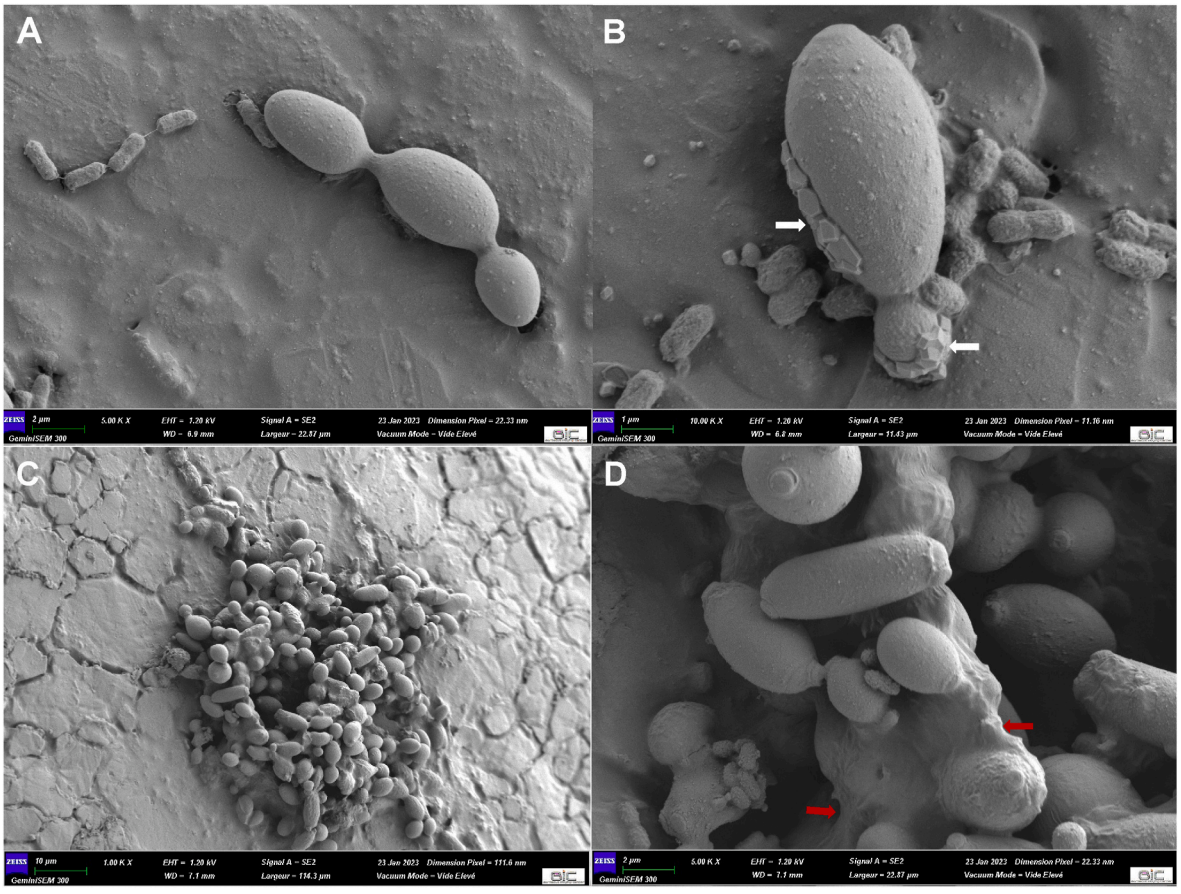

Fig. 6. Scanning electron microscopy (SEM) observation of bioadhesion at different stages. A represents cells of *B. bruxellensis* and AAB at day 14 with magnification ×5000; B is characterised by a magnification ×10 000 of the Brett/AAB condition on day 14 highlighting the presence of crystals (white arrows) around the *B. bruxellensis* cell; C is an observation of a microcolony of *B. bruxellensis* and LAB on day 28 at magnification ×1000; D represents a magnification ×5000 of a microcolony with extracellular matrix (red arrows).

even when no culturable cells were detected after plating. At a magnification of ×10, 000 (Fig. 6B), the organization of *B. bruxellensis* and the associated AABs were clearly visible. On the surface of a *B. bruxellensis* cell, an ordered agglomeration of crystals is evident 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 with *B. bruxellensis* were also observed in the Brett/LAB condition; Fig. 6C shows these micro-colonies at a magnification of ×1000, with a complex architecture including empty areas. At a magnification of ×5000 (Fig. 6D), the formation of an extracellular matrix on the surface of the cells was highlighted: a film covered the cells and may play a role in the biofilm structure. Within this biofilm, the presence of LAB bound to *B. bruxellensis* cells was also observed.

4. Discussion

Brettanomyces bruxellensis has been reported to be adapted to stressful environments with hostile physico-chemical properties and to compete successfully for nutrients with many other microorganisms (Conterno et al., 2006). In this study, the effect of abiotic factors (pH and ethanol concentration) on surface properties, pseudohyphae growth and bioadhesion was investigated to see if these factors could interfere with biofilm formation in *B. bruxellensis*. In addition, synergistic or antagonistic effects between different strains of *B. bruxellensis* or between *B. bruxellensis* and other microorganisms were investigated during bioadhesion and biofilm formation.

4.1. Abiotic factors poorly modulate cell surface and bioadhesion properties

Ethanol and pH have a strong influence on the growth of microorganisms. *B. bruxellensis* has been isolated from wine, but also from beer and kombucha (de Miranda et al., 2022). The pH of kombucha is up to 2.5 and the ethanol concentration of wine is on average between 12 and 16% alcohol by volume. *B. bruxellensis* have been shown to be tolerant to low pH and high ethanol concentrations (Oswald and Edwards., 2017; Cibrario et al., 2020). Both pH and ethanol have been shown to affect the surface properties of the cells, which can then directly affect the bioadhesion abilities of microorganisms. Indeed, pH changes could induce a change in cell surface charge, which may modulate electrostatic interactions between cells and the support (Boutaleb et al., 2008). Ethanol has a fluidifying action on membranes, modifying their composition 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 obtained in different media prior to this study, showed an increase in surface electronegativity along with an increase in pH value from 2 to 3.5, followed by a stabilization for some strains depending on the genetic group (Dimopoulou et al., 2019). This latter observation is consistent with our data showing that the genetic group is the most explanatory factor for the surface electronegativity, which is directly influenced by the composition of membrane proteins and polysaccharides (Hong and Brown., 2010; Halder et al., 2015). Neither pH nor ethanol concentration has any effect on hydrophobicity; indeed, more than 60% of the variance in this phenotype is mediated both by the strain and the genetic group. In *Saccharomyces cerevisiae* however, hydrophobicity is greater in the presence of ethanol (Alexandre et al., 1998). In the present study, increasing the ethanol concentration from 5% to 14% results in only a slight increase in hydrophobicity, again showing that the effect of these two abiotic factors on surface hydrophobicity is negligible. The fact that the strain explains more than 60% of the phenotype suggests 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 influenced by the expression of genes of the *FLO* family which have a major impact on the surface properties and bioadhesion of the species. Regarding the differentiation into pseudohyphae cells, here again the abiotic factors have no effect on this phenotype, which is 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 has been reported (González et al., 2017, 2018). Ethanol can be perceived as a quorum-sensing molecule that induces 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 does not seem to affect 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* (Mempel et al., 1998; Chaieb et al., 2007). In our study, ethanol concentration explains only 2.5% of the viability of bioadherent cells but 9.9% of the bioadherent cell mortality variance. Indeed, a higher concentration of bioadherent dead cells was observed at an ethanol concentration of 14%. In addition, it was observed that a combination of Alcohol/Strain and Alcohol/Group factors explained 14.6% and 18.2% of the bioadherent dead cells, respectively. This result could be explained by the differences in ethanol tolerance between the groups. Indeed, the strains of the Wine 1 group seem to be more resistant to high ethanol concentrations than the other groups (Cibrario et al., 2020).

4.2. The bioadhesion of Brettanomyces bruxellensis is lower on epoxy resin than on stainless steel material

The vats used in the winemaking process can be made from a variety of materials including concrete, wood and stainless steel. In the case of concrete tanks, an epoxy resin coating is often applied to the inside the tank 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 bioadhesion capacity on many materials, as evidenced by previous studies reporting *B. bruxellensis* 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). Furthermore, 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 more hydrophilic (Ait lahbib et al., 2023). This hydrophobicity plays an important role in the establishment of bioadhesion because the hydrophobic interactions established between the support and the cells are the strongest involved in bioadhesion (Urano et al., 2002; Verstrepen and Klis., 2006; Blanco et al., 2008). This decrease in bioadhesion to epoxy resin has also been observed for other microorganisms such as

Pseudomonas aeruginosa and *Staphylococcus aureus* where the concentration of bioadherent cells was lower on epoxy resin than on stainless steel (Ait lahbib 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 material roughness could impact bioadhesion is also mentioned 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 that affects bioadhesion phenomena to trap cells and initiate bioadhesion (Yuan et al., 2017; Yang et al., 2022). In our study, the type of stainless-steel leads to a difference in roughness between the two references used, with RSS having a significant surface roughness in contrast to SSS. Bioadhesion was not significantly different on the 2 grades despite the differences in roughness, which could be due to 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 support roughness has no impact on bioadhesion (Hilbert et al., 2003; Rodriguez et al., 2008). However, on the contrary, studies have shown that roughness plays an important 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). Therefore, roughness seems to be a factor that should 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 decreases sharply from grape juice to wine; only species such as *B. bruxellensis*, LAB and AAB, which are 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 phenomena of bioadhesion and biofilm formation have only been studied for single strain cultures of *Brettanomyces bruxellensis*. Therefore, to take into account the reality of the wine microbial community, we studied the effect of the presence of two genetically different strains on the bioadhesion and biofilm formation. In many cases, the bioadhesion kinetics of the mixed-strain followed the bioadhesion kinetics of the dominant strain when it was alone. Biofilm formation was mainly driven by the strain with the highest bioadhesion capacity. A similar observation was also reported in *Pseudomonas aeruginosa*, where one strain was present in higher concentrations than the other in mixed-strain biofilms, indicating some interaction and competitive effects between the two strains (Oliveira et al., 2015). Furthermore, the authors showed that the presence of two strains of *P. aeruginosa* induced a significant increase in biofilm formation (Oliveira et al., 2015). This is not the case in our observations: the thickness of the biofilm is greater when strain AWRI 1608 is the only one to form biofilm. Similar results have been reported for other microorganisms. Actually, the presence of two strains of *Streptococcus pneumoniae* leads to a reduction in biofilm size (Valente et al., 2021). The reduction in biofilm thickness is more frequently observed when the biofilm is composed of multiple species compared to a single-species biofilm (Chen et al., 2019). Indeed, competition between different microorganisms affects biofilm formation, and different behaviours such as neutralism and amensalism have been identified (Molin et al., 2004; Habimana et al., 2011). In *S. cerevisiae,* adhesion between cells expressing the same surface properties is favored to promote biofilm resistance (Mitri and Richard Foster., 2013). In *Escherichia coli*, a synergistic effect on biofilm formation was also observed during strain co-cultures. In MX4, composed of 2 strains with significant bioadhesion properties, a change in the dominant strain over time was observed and could be induced by a competition between cells for nutrients (Xavier and Foster, 2007). Thus, the fact that one strain moves from minority to majority could be explained by greater ability to metabolize nutrients. It is also possible that the nutrient deprivation led to the death of part of the population of one of the strains, releasing into the environment nutrients that could be assimilated by the remaining strain. A population dynamic of *B. bruxellensis* strains is thus observed during bioadhesion and biofilm formation. This dynamic is also observed in the cellar where it has been shown that within the same batch of wine, the planktonic population of *B. bruxellensis* is genetically variable over time (Cibrario et al., 2017).

In wine, other microorganisms can interact with *B. bruxellensis* such as *Oenococcus oeni* and *Acetobacter pasteurianus,* with the latter having a strong negative effect on the sensory properties of the wine, with the production of acetic acid and ethyl acetate (Du Toit and Pretorius, 2002; Zepeda-Mendoza et al., 2018). Since *O. oeni* has been reported to have bioadhesion properties (Bastard et al., 2016; Coelho et al., 2019; Tofalo et al., 2021), we investigated the formation of mixed-species biofilms between *O. oeni* and *B. bruxellensis*. The results showed a decrease of bioadhesion property of *B. bruxellensis* in the presence of *O. oeni* and *A. pasteurianus*. However, after biofilm settlement, *O. oeni* and *A. pasteurianus* were no longer detected though plating on solid medium while cells were observed by SEM. This may be due to Viable But Non Culturable (VBNC) physiological forms previously demonstrated for *O. oeni* (Millet and Lonvaud-Funel, 2000). The formation of structured micro-colonies was observed where the 2 species were organized in the form of biofilm covered with extracellular matrix. This matrix encompassing cells was also reported in the single species biofilms of *B. bruxellensis* (Lebleux et al., 2020). The formation of mixed-species biofilms (yeast/bacteria) has been observed with *C. albicans* and *S. epidermidis;* cooperation between these 2 species has been reported, with the formation of extracellular matrix by one species protecting the other from specific antibiotic activity (Adam et al., 2002). In the field of food 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 are grown alone, no biofilm observations are made (Kawarai et al., 2007; FURUKAWA et al., 2011). In other cases, the presence of one microorganism can inhibit biofilm formation by another. This is the case for *Lactiplantibacillus paraplantarum* which, in the presence of *Listeria monocytogenes*, produces a bacteriocin that inhibits biofilm formation of the latter (Winkelströter et al., 2015; Yuan et al., 2020). Thus, the decrease in the bioadhesion of *B. bruxellensis* could be explained by a competition for nutrients or by an inhibition by metabolites (eg lactic acid) excreted by the bacteria present in front of *B. bruxellensis;* these metabolites could reduce its bioadhesion capacity by modifying the surface physico-chemical properties of the material and/or by inhibiting the yeast growth.

5. Conclusion

This study was carried out on several strains representative of the genetic diversity of the species and with contrasting surface and bioadhesion properties. Our data show that the abiotic factors such as pH and ethanol concentration have negligible effects on surface properties under our experimental conditions. An effect of ethanol on the mortality of bioadherent cells is highlighted, and probably related to the different tolerance of *B. bruxellensis* strains. The fact that the "strain" and the "genetic group" explain most of the variance of the phenotypes studied strongly suggests the existence of a genetic determinism. In *S. cerevisiae*, hydrophobicity, pseudohyphae cell formation and bioadhesion have been shown to be directly influenced by the expression of *FLO* genes family, which could be good candidates to further study the genetic mechanisms underlying these phenotypes in *B. bruxellensis* (Smit et al., 1992; Mortensen et al., 2007; Van Mulders et al., 2009; Govender et al., 2010; Zhang et al., 2021).

In the present study, we also 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 through the impulsion of the most bioadhesive strain. However, some competition is observed, evidenced by a lower thickness of mixed-strains biofilms compared to single-strain ones. Interestingly, mixed-species experiments indicate that the bioadhesion of *B. bruxellensis* can be reduced by the presence of LAB and AAB. However biofilm formation of *B. bruxellensis* is not prevented. The nature of the winery materials would also be a relevant parameter to consider in the prevention of *B. bruxellensis* bioadhesion and spoilage, with the need to implement appropriate cleaning procedures.

CRediT authorship contribution statement

Paul Le Montagner: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yacine Bakhtiar:** Methodology, Investigation, Formal analysis. **Cecile Miot-Sertier:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Morgan Guilbaud:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Warren Albertin:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Virginie Moine:** Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Marguerite Dols-Lafargue:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Isabelle Masneuf-Pomarède:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Paul Le Montagner reports financial support was provided by BioLaffort. Virginie Moine reports a relationship with BioLaffort that includes: employment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2024.104480.

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