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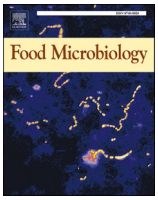

High intraspecific variation of the cell surface physico-chemical and bioadhesion properties in *Brettanomyces bruxellensis*

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ABSTRACT

Brettanomyces bruxellensis is the most damaging spoilage yeast in the wine industry because of its negative impact on the wine organoleptic qualities. The strain persistence in cellars over several years associated with recurrent wine contamination suggest specific properties to persist and survive in the environment through bioadhesion phenomena. In this work, the physico-chemical surface properties, morphology and ability to adhere to stainless steel were studied both on synthetic medium and on wine. More than 50 strains representative of the genetic diversity of the species were considered. Microscopy techniques made it possible to highlight a high morphological diversity of the cells with the presence of pseudohyphae forms for some genetic groups. Analysis of the physico-chemical properties of the cell surface reveals contrasting behaviors: most of the strains display a negative surface charge and hydrophilic behavior while the Beer 1 genetic group has a hydrophobic behavior. All strains showed bioadhesion abilities on stainless steel after only 3 h with differences in the concentration of bioadhered cells ranging from 2.2×10^2 cell/cm^2 to 7.6×10^6 cell/cm^2. Finally, our results show high variability of the bioadhesion properties, the first step in the biofilm formation, according to the genetic group with the most marked bioadhesion capacity for the beer group.

1. Introduction

The transformation of fruits into beverages involves fermentation processes. Traditionally, these fermentations are carried out by microorganisms naturally present in the environment and enable the development of the organoleptic properties and the preservation of the initial product (Liu et al., 2017). In wine, several microorganisms such as yeasts and bacteria are involved in the fermentation process. They contribute to the development of the aromatic panel by producing molecules of interest or wine defects (Gammacurta et al., 2017; Miljić et al., 2017; Tempère et al., 2018; Carpena et al., 2021). The presence of spoilage microorganisms is a real challenge for the sector, and their presence is carefully monitored (Lonvaud-Funel, 2016). *Brettanomyces bruxellensis* is one of the most studied spoilage yeasts. Its presence is synonymous with the production of volatile phenols and more particularly 4-ethylphenol (4 EP) characterized by stable odors, horse sweat which cause consumer rejection (Chatonnet et al., 1992; Lattey et al., 2010 Agnolucci et al., 2017). The presence of *B. bruxellensis* is reported throughout the winemaking process, from grapes to the finished wine, due to mechanisms allowing it to grow under stressful conditions (Renouf and Lonvaud-Funel., 2007; Rubio et al., 2015; Avramova et al., 2018a). Genetic typing of 1488 isolates via the analysis of microsatellite markers revealed 6 different clusters according to the ploidy, the niche of origin of the isolates and geographical origin of the strains (Albertin et al., 2014; Avramova et al., 2018b). At a large scale, a diploid-triploid complex for *Brettanomyces bruxellensis* was reported with 57.8% of the

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isolates showing more than 2 alleles for at least one locus. Later, genomes sequence analysis of *B. bruxellensis* validating previously defined populations (Borneman et al., 2014; Gounot et al., 2020; Eberlein et al., 2021); six sub-groups can be considered, two diploids and four triploids (Harrouard et al., 2022). Indeed, among diploids clusters, one group is composed of isolates from wine and the other is associated with kombucha, beer and wine isolates. For triploids clusters, one group gathered beer and wine isolates, one group bioethanol and tequila isolates and the two other groups are strongly associated with winemaking. In addition, a link was highlighted between sulphite tolerance (SO_2) and triploid wine groups indicating adaptation of specific branches *B. bruxellensis* species to the environmental conditions and human activity (Curtin et al., 2012; Avramova et al., 2018a; Harrouard et al., 2022). This may be the result of the recurrent use of high doses of sulphur dioxide to control the population of *B. bruxellensis* thus resulting in the selection of resistant/tolerant strains to the applied doses (Curtin et al., 2012). Ecological studies have shown that *B. bruxellensis* can be present throughout the winemaking campaign from year to year, thus showing a persistence in the cellar (Bokulich et al., 2013; Cibrario et al., 2019). In a given winery, some strains have been isolated from the wines over 80 vintages, suggesting that cellar could be the first source of contamination (Cibrario et al., 2019). Indeed, many studies have highlighted the presence of *B. bruxellensis* in the cellar. Its presence was reported on the surface of the walls and in the air but also on the small materials and equipment's of the winery, the tanks, pumps and wastewater then showing the possible dispersal of *B. bruxellensis* in the winemaking environment (Fugelsang, 1997; Connell et al., 2002). The recurrent presence of *B. bruxellensis* in the cellar and equipment suggest specific properties of the species to persist and survive in this environment. The ability to bioadhere and to form biofilms has been previously cited as a key property for the persistent colonization of food processing environment and materials (Bridier et al., 2015). Indeed, yeasts can adhere to different surfaces and initiate biofilm formation (Verstrepen and Klis., 2006). The biofilm lifestyle is the way of life preferred by microorganisms in natural environment (Davey and O'Toole, 2000; Tek et al., 2018). Biofilm is defined as a structured community of microorganisms adhered to a surface and producing an extracellular matrix. This lifestyle brings several advantages to the microorganisms such as a higher resistance to environmental conditions (temperature, pH, alcohol, humidity …) but also to the treatments that can be applied to it (antibiotics, chemicals, heat …) (Davey and O'Toole, 2000; Bastard et al., 2016). The formation of a biofilm involves several key steps such as bioadhesion, maturation and dispersion. The bioadhesion is the first and more important step in the formation of a biofilm and it is closely related to the hydrophobic and van der Waals interactions that will be established between the yeast and the surface of the material (Blanco et al., 2010). These interactions are defined according to the physico-chemical surface properties resulting from the membrane composition of the cell. These surface properties are also mediated by gene expression, particularly those of the *FLO* family (van Mulders et al., 2009). These genes in *Saccharomyces cerevisiae* encode surface adhesins and flocculins that impact surface physico-chemical properties and are regulated by environmental factors (Guo et al., 2000; van Holle., 2012; Lenhart et al., 2019). In yeasts, the ability to form biofilms is proven in several areas, either in the medical field (*Candida albicans*) (Beckwith et al., 2022) or to produce biofuel (*Saccharomyces cerevisiae*) (Belini et al., 2020) with in most cases, negative impacts. In the food industry, and more particularly in the winemaking process, it is possible to find several biofilm-forming spoilage yeasts such as *Schizosaccharomyces pombe* or *Zygosaccharomyces bailii* (Zara et al., 2020). Concerning *B. bruxellensis*, little work has been done till now on the characterization of surface physico-chemical properties as well as on bioadhesion and the ability to form biofilm (Joseph et al., 2007; Tristezza et al., 2010; Ishchuk et al., 2016; Dimopoulou et al., 2019, 2021; Lebleux et al., 2020). In the previous cited studies, all the authors agreed that *B. bruxellensis* can adhere to different materials. The term biofilm was first mentioned in *B. bruxellensis* by Joseph et al. (2007), who showed the ability to develop biofilm on polystyrene surfaces after a long incubation period without visual observations of biofilm organization. Lebleux et al. (2020) were the first to report the formation of biofilm in culture medium and wine in *B. bruxellensis* with the formation of cells with specific morphologies, namely chlamydospores. However, all the mentioned studies were conducted on a small number of strains with limited representation of genetic diversity. Therefore, our objective is to identify the variability of physico-chemical surface and bioadhesion properties of *Brettanomyces bruxellensis* on stainless steel. Considering the importance of *Saccharomyces cerevisiae* in the fermented food and wine industry, this species was included in our study as an outgroup.

2. Materials and methods

2.1. Strains and growth conditions

A total of 54 strains of *B. bruxellensis* representative of the genetic diversity of the species were used in this study (Avramova et al., 2018a) (Table S1). These strains originated from different geographical areas. They were also isolated from different substrates and are part of the CRBO collection (Microbiological Ressources Center Oenology, Bordeaux, France), the UWOPS collection (Culture collection of the University of Western Ontario, London, Canada), the AWRI collection (Australian Wine Research Institute, Adelaide, Australia), the GSP collection (Foggia University), the YJS collection (Laboratory for Molecular Genetics, Genomics and Microbiology, Strasbourg University) and CBS-KNAW collection (Fungal Biodiversity Center, Utrecht, Netherlands). In the present study, the beer group was separated according to the strain's substrate of origins, wine (Beer 1) and beer (Beer 2). Furthermore, 5 industrial strains of *Saccharomyces cerevisiae* (IOC 18 2007, ACTIFLORE® BO213, ZYMAFLORE® XPURE, ACTIFLORE® F33 and ZYMAFLORE® VL2) were used as an outgroup to compare the bioadhesion properties. The *B. bruxellensis* strains were stored at −80 °C in a mixture of YPD 70% comprising 2% (w/v) glucose (Fisher BioReagent™), 1% (w/v) yeast extract (Fisher BioReagent™), 1% (w/v) peptone (Gibco) and glycerol 30% (v/v) before being cultured on a YPD solid medium (2% (w/v) agar Fisher BioReagent™) and are incubated for 5 days at 25 °C.

2.2. Growth and adaptation protocol

A standard liquid wine medium called Wine Like Medium (WLM) that mimics wine composition was used for the yeast growth (standard protocol). This medium was chosen because it supports the growth of a collection of 39 *B. bruxellensis* strains better than YPD medium (see below). 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®) and 10% (v/v) absolute ethanol (VWR Chemicals®). The pH is adjusted to 3.6 with KOH. Adaptation steps are necessary in order to adapt yeasts to the WLM medium. Briefly, few 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 culture was collected to perform filamentous growth, surface charge and bioadhesion analysis. For growth in red wine, adaptation steps were also necessary. Few colonies were recovered from solid medium and transferred into 10 mL of a mixture consisting of 25% (v/v) of red wine (Graves, 12.5% vol, pH 3.7) and 75% (v/v) of grape juice, and incubated for 48 h (25 °C, 180 RPM). This adaptation step was repeated 3 times

and the proportion of red wine was gradually increased (50%, 75% and finally 90%).

2.3. Filamentous growth (pseudohyphae)

The cell culture was sampled (1 mL) to evaluate the proportion of pseudohyphae. 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 30 min in the dark at 30 °C. The proportion of pseudohyphae was evaluated by epifluorescence microscopy (10 fields of vision counts).

An observation by confocal microscopy was also performed to see the cell morphology (Bordeaux Imaging Center Bordeaux platform of the INRAe plant pole). The dyes used were 5(6)-Carboxyfluorescein Diacetate (CFDA) (Thermo Fisher Scientific) for cell viability and 4',6-Diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) for nucleus coloration. Observations were made using the immersion lens. Confocal acquisitions were realized using a Zeiss LSM 880 confocal laser-scanning microscope with a diving 63× objective with a numerical aperture of 1. The excitation wavelengths and emission windows were 488 nm/450-490 nm and 488 nm/588-669 nm, for DAPI and CFDA respectively.

2.4. Physico-chemical properties of stainless steel

As a model material currently used in the wine industry, stainless steel 316 L (SS) (Goodfellow) was chosen for the bioadhesion assays.

2.4.1. Cleaning procedure of the coupons

The coupons were placed for 5 min in a bath of water and acetone (50:50) to remove adhesive and then they were placed for 5 min in a bath of 70% ethanol for the disinfection. The coupons were then rinsed with distilled water and dried with optical paper.

2.4.2. Wettability of the coupons

After cleaning, some SS coupons were immersed 3 h at room temperature in WLM or Bordeaux red wine (Graves appellation, 2019), rinsed once in distilled water then dried 1 h under a flow cabinet. Contact angle measurements (θ) were performed using sessile drop method by deposition of a test liquid droplet onto the dry samples and measurements were carried out under atmospheric condition at room temperature using a goniometer DSA 100 (KRUSS, France). At least three samples were used and contact angle measurements were performed on at least eight positions on each sample.

2.4.3. Surface free energy

Surface free energy of stainless steel (SS) was determined from contact angles (θ) of water (Milli-Q-Millipore) and diiodomethane (Sigma, France) using sessile drop technique combined with the Young-Owens et Wendt equation. In this approach, the pure liquid (L) contact angle (θ) is expressed as follows:

$$\cos\theta = -1 + \frac{2\left(\gamma_S^D + \gamma_L^D\right)^{\frac{1}{2}}}{\gamma_{LV}} + \frac{2\left(\gamma_S^P \gamma_L^P\right)^{\frac{1}{2}}}{\gamma_{LV}} \quad (1)$$

where γ^D denotes the Dispersive-Lifshitz–van der Waals component of surface free energy, γ^P the polar components of surface free energy. The total surface free energy (γ) was defined, respectively, by:

$$\gamma = \gamma^D + \gamma^P \quad (2)$$

2.5. Analyses of the physico-chemical properties of the cells surfaces

2.5.1. Surface charge determination

The measurement of the surface charge was carried out by centrifuging the cell culture at 7000 g for 5 min at room temperature and then the cell pellet was washed twice in ultra-pure water at pH 3.6. Cell pellet is then resuspended in ultra-pure water at pH 3.6 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.

2.5.2. Microbial adhesion to solvents (MATS)

This technique makes it possible to determine the hydrophilic/hydrophobic character present on 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 were resuspended in physiological water (NaCl 0.9%) to obtain a cell suspension with an OD_{600nm} around 0.7.1.5 mL of cell suspension were mixed with 250 μL of two solvents, chloroform (Fisher Chemical) or hexadecane (Sigma-Aldrich). The mixture was vortexed for 2 min to create an emulsion. Then 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 (DO) were measured at 600 nm. The affinity for each solvent was calculated using the following formula:

$$\%\ affinity = \left(1 - \frac{OD}{OD_0}\right) \times 100$$

For each strain and solvent, three independent measurements were carried out on the same cell culture.

2.6. Bioadhesion

2.6.1. Confocal microscopy

To perform the bioadhesion, the cell culture 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 then resuspended in 20 mL of a mixture WLM 90% and YPD 10% and adjusted to obtain a final concentration of 10^7 cells/mL. The bioadhesion was made on 316 L stainless steel coupons of dimension 14 mm × 25 mm (Goodfellow). The coupons were cleaned according to the procedure described in 2.4.1 section. The rinsed coupons were placed in Petri dishes with a diameter of 55 mm 10 mL of cell suspension was then added to the Petri dishes to initiate bioadhesion. The bioadhesion was carried out for 3 h at room temperature.

The quantification of bioadhesion was performed by confocal microscopy. After the 3 h of bioadhesion, a coupon washing step was performed to remove the non-adherent cells that had sedimented. This step consists of 5 successive cleaning baths in a sterile container containing physiological water. The coupon was then placed in a solution of Chemsol B15 (Biomérieux) containing 1% (v/v) of 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) with a standby time of 15 min. The surface of the coupon was observed by confocal microscopy within the Bordeaux Imaging Center Bordeaux platform 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 cells were counted by the mean of 10 fields of vision counts.

2.6.2. Enumeration of bioadhered cells by cultivation

The enumeration of viable cells was carried out after the 3 h of bioadhesion (see bioadhesion protocol in part 2.6.1). The coupon was cleaned from the no adhered cells by 5 successive washes in sterile physiological water (NaCl 0.9%). The coupon was then placed in a 50

mL tube containing 20 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 speed for 40 s. Dilutions series were then carried out and 100 μL of the suspension were inoculated in triplicata on YPD agar medium. The result is then expressed as Colony Forming Unit per cm^2 (CFU/cm2).

2.6.3. Scanning electron microscopy (SEM)

Brettanomyces bruxellensis cells were adhered on stainless steel coupon from WLM culture at 10^7 cell/mL for 3 h. 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 7.9 mm and 8.0 mm.

2.7. Statistical analysis

Kruskal-Wallis statistical test (agricolae package, R, p value < 0.05), Anova 1-way (p-value <0.05), Student *t*-test (p-value <0.05), Spearman test (p-value <0.05) and Principal Component Analysis (PCA) were performed using R and R-packages *agricolae*(Mendiburu, 2021), *ade4* (Dray and Dufour., 2007).

3. Results

3.1. Validation of the experimental protocols

3.1.1. 316 L stainless steel properties

After a 3 h-fouling of red wine or WLM, stainless steel coupons were dried, and their wettability was determined. Contact angle measurements and surface free energies calculations showed that cleaned SS coupons exhibited hydrophobic and apolar properties (Table S2) illustrated by a high contact angle (104.3°) and very low polar components (0.01). After contact with red wine or WLM, surfaces exhibited *i)* an hydrophilisation illustrated by the decrease of the water contact angles and *ii)* polar properties with polar components of 7.6 and 9.9, for SS fouled with red wine and WLM, respectively. Hence, red wine and WLM appeared to influence similarly the physico-chemical properties of stainless-steel coupons. Moreover, surface tension of both liquids was found similar, *ie* 46.1 and 50.3 mN m^{-1} respectively for red wine and WLM (data not shown). All together, these results showed that WLM is a relevant model medium for red wine in bioadhesion assays, causing similar surface properties after fouling of stainless steel.

3.1.2. Repeatability of physico-surface properties, bioadhesion and pseudohyphae assays

For each series of analysis, the CBS2499 strain was used as control strain to evaluate the repeatability of the analysis. Eight independent series of triplicate were performed. The standard deviations obtained for the MATS and zeta potential analysis are low showing robustness and repeatability of the method (Table S3). For both bioadhesion and cultivability, the standard deviation obtained was one 10-fold lower than the bioadhesion value showing good repeatability.

3.2. Filamentous growth (pseudohyphae)

In our experimental conditions, the presence of specific filamentous growth was observed for 65% of *B. bruxellensis* isolates. These cells are characterized by the absence of separation between the mother and daughter cells, which generates a filament of cells. The observation of branching on filamentous cells shows the ability of the cells to initiate several axes of budding (Fig. 1A). Observation by confocal microscopy using DAPI that binds region rich in nucleic acids confirms that each cell has its own nucleus, thus suggesting that filamentous cells of *B. bruxellensis* are indeed true pseudohyphae cells (Fig. 1B). The proportion of pseudohyphae cells was determined by epifluorescence microscopy on the 54 strains analyzed after 48 h of incubation at 25 °C under stirring at 180 rpm. The values obtained showed high variability depending on the strains tested, ranging from 0 to 38.3% (Fig. 2A) and 35 strains of *B. bruxellensis* (65% of the strains) showed specific filamentous growth. Indeed, the proportion of pseudohyphae varied significantly according to genetic groups and ploidy level. Ploidy level significantly impacts the proportion of pseudohyphae, with 4.74% and 1.92% for the triploid and diploid group, respectively (Kruskal-Wallis, p-value <0.05) (Fig. 2B). The group with the highest proportions was the triploid Teq/EtOH group with 16.1% in average of pseudohyphae cells. The highest proportions of pseudohyphae cells (up to 38% of the strain culture) was obtained for the strain CRBOL17109 belonging to the Teq/EtOH group (Fig. 2A). The size of the pseudohyphae varied from one filament to another; however, 30 μm seems to be an average length for the majority of producing strains. The genetic groups Wine 1 and Beer 2 have low pseudohyphae cell proportions but frequencies as important as the Teq/EtOH and Beer 1 groups. In addition, the Wine 2 and Wine 3 groups showed the lowest frequencies with 1.4% and 0.02% in average of pseudohyphae cells, respectively. Of the 10 strains of the Wine 3 group tested, only 3 strains presented pseudohyphae cells with rather low proportions. Regarding the 5 strains of *S. cerevisiae* tested during this study, none of them showed an ability to produce filamentous growth (Fig. 2A).

3.3. Surface properties

The surface charge of the 54 *B. bruxellensis* strains and 5 *S. cerevisiae* strains was measured on the same cell suspension after adaptation steps at a pH close to wine (pH 3.6) and alcohol content of 10% (WLM medium). The surface charge was evaluated through the zeta potential. In our experimental conditions, *S. cerevisiae* showed a significant (Student-test, p-value <0.05) higher negative surface charge (-32.1 ± 2.74 mV) compared to *B. bruxellensis* (-24.47 ± 6.21 mV). The values obtained revealed a negative surface charge for all the *B. bruxellensis* strains tested, with significant variations depending on the genetic groups (Fig. 3). Wine 1 group had the highest electronegativities (-33 ± 2.2 mV) with a maximum value reaching at -36 mV for strains CRBOL14190 and CRBOL14156 similar to *S. cerevisiae*. The Beer 1 group also had significant electronegativity values (-27 ± 4.3 mV). The Wine 2, Teq/EtOH and Kombucha groups had similar values with a surface charge of -22 ± 2.0; -22 ± 2.1, -21 ± 1.2 mV, respectively. The Beer 2 group had the lowest electronegativities ($-15{,}8 \pm 4.4$ mV).

Using the MATS method, the hydrophilic/hydrophobic character of all strains was also tested through their affinity to non-polar solvents, chloroform and hexadecane, respectively. In view of the results obtained, the affinity of *B. bruxellensis* strains for solvents varies from 0 to 99%, again reflecting high intraspecific variability. The general behavior of the strains was similar between the two solvents: a significant plot correlation has been shown between hexadecane and chloroform data (Spearman test, p-value $<2.2e\text{-}16$, $r = 0.848$). For *S. cerevisiae,* the affinity varied from 0 to 21%. Forty-four yeast strains studied had an affinity to chloroform lower than 50% (Fig. 4A). In addition, strains belonging to the Beer 1 group showed higher chloroform affinities of up to 90% affinity for CRBOL1120 reflecting basic polar cell surface. The Wine 2, Beer 2, Kombucha and Wine 3 groups exhibit low affinity to chloroform apart from strain CBS2499 which has an affinity of 93%.

For hexadecane, 10 strains had an affinity higher than 30%. The affinities of the Wine 1 and Beer 1 groups are lower except for strain CRBOL1120 which has an affinity to hexadecane of 99% (Fig. 4B). The CBS2499 strain of the Wine 3 group also has a high affinity (95%)

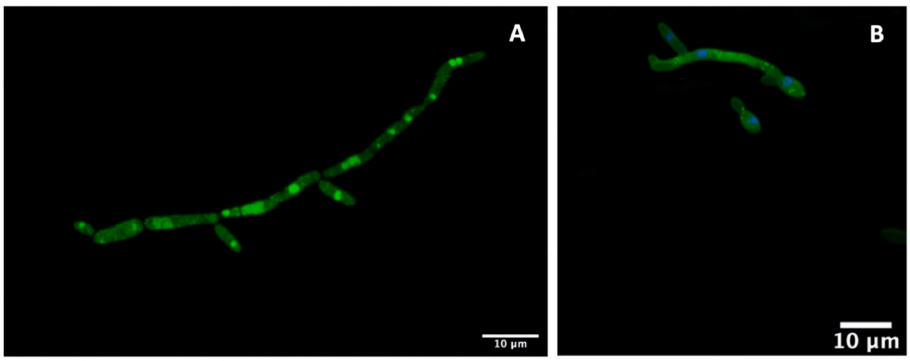

Fig. 1. Pseudohyphae cells observed by confocal microcopy with dye viablility (A); Pseudohyphae cell observed by confocal microscopy with different dyes (DAPI for nucleus and CFDA for live cells) (B).

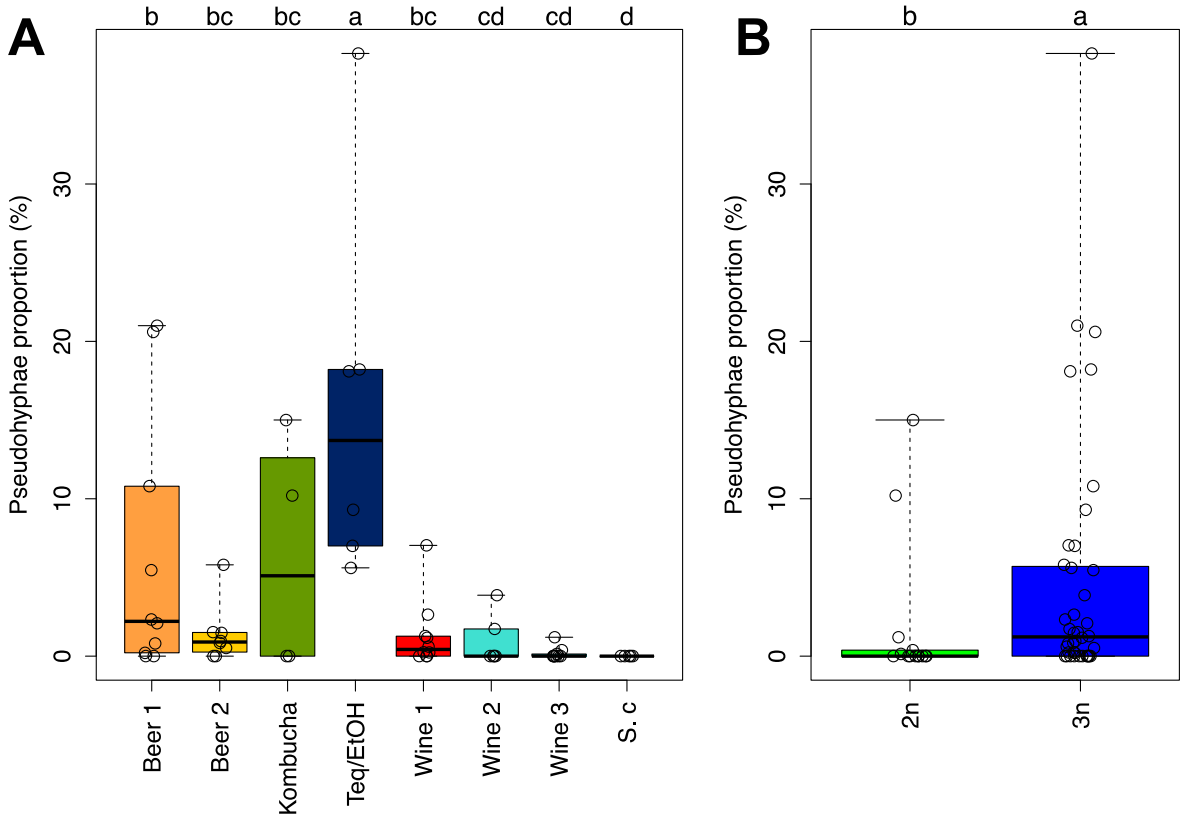

Fig. 2. Proportion of pseudohyphae cells for the 54 strains. The colors represent the genetic groups of Brettanomyces bruxellensis (A). Pseudohyphae proportion according to the ploidy of B. bruxellensis (B). Upper letter represents groups significantly different as defined by Kruskal-Wallis statistical test (Agricolae package, R, p value < 0.05).

indicating hydrophobic behavior (outlier). For the 2 solvents, no significant differences are observed between *B. bruxellensis* and *S. cerevisiae* at the species level. Overall, the tested strains of *B. bruxellensis* and *Saccharomyces cerevisiae* therefore exhibit a predominantly hydrophilic behavior.

3.4. Bioadhesion

Observations of bioadhered cells by confocal microscopy on stainless steel 316 L after 3 h have shown high viability of the yeast cells and variable bioadhesion abilities depending on the genetic group. Morphological variability within bioadhered cells is also revealed. Indeed, *B. bruxellensis* has different morphologies depending on the strain studied. Spherical, ovoid cells were observed (Fig. 5A) and even pseudohyphae cells (Fig. 5B), thus demonstrating their capacity to bioadhere to stainless steel material. This morphological variability was also observed in Scanning Electron Microscopy where observations of bioadhered pseudohyphae were made (Fig. 6A). SEM observation also showed that, after 3 h of bioadhesion, interactions are established by physical contact with stainless steel and between cells. It is also possible to see structures established between cells and with support (white arrows Fig. 6). There is therefore in addition to cell-support adhesion a cell-cell adhesion (Fig. 6B). After 3 h of bioadhesion on stainless steel, the minimum adhered population density was 2.2×10^2 cell/cm^2 for the CRBOL1781 strain of the Wine 2 group while the maximum was 7.6×10^6 cell/cm^2 for the YJS5400 strain of the Beer 1 group (Fig. 7A). The most bioadhesive genetic groups were the Beer 1 and Beer 2 groups ($3{,}2 \times 10^6$ cell/cm^2 and $9{,}16 \times 10^5$ cell/cm^2, respectively), in which all

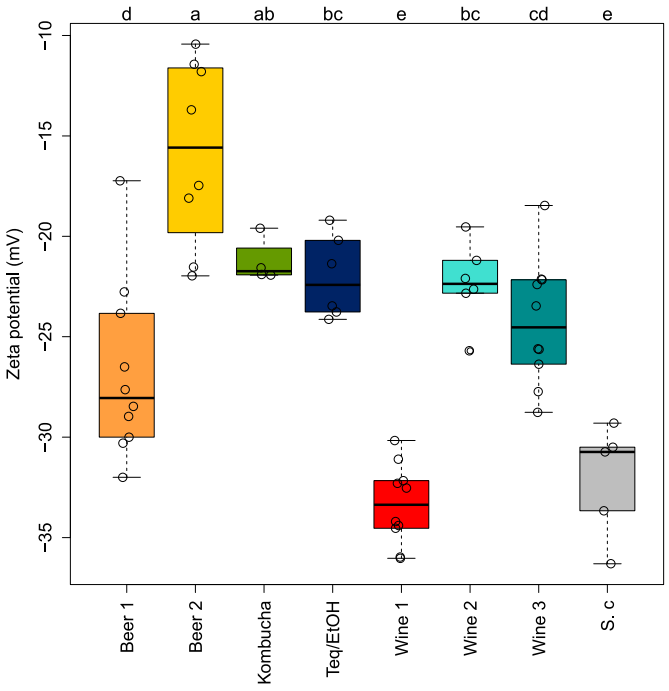

Fig. 3. Zeta potential of the 54 strains of Brettanomyces bruxellensis. The different colors represent genetic groups. Upper letter represents groups significantly different as defined by Kruskal-Wallis statistical test (Agricolae package, R, p value < 0.05).

strains produced adhered population densities higher than 10^4 cell/cm^2. On the other hand, the Wine 2, Kombucha and Wine 3 genetic groups displayed the weakest bioadhesion capacity. A significant difference between triploid and diploid strains was shown (p-value >0.05) (Fig. S1A) with higher capacity for triploid strains to bioadhere compared to diploid strains. At the species level, bioadhesion observation did not show significant differences between *B. bruxellensis* and *S. cerevisiae* (p-value >0.05). Indeed, *S. cerevisiae* exhibits a bioadhesion behavior like the Wine 1 and Teq/EtOH and Beer 2 groups.

The population of cultivable cells liberated from the coupon by sonication was maximal for the Beer groups (Beer 1 and Beer 2) with a maximum for the CRBOL1120 strain with 1.2×10^6 CFU/cm^2 (Fig. 7B). Similar genetic group ranking was obtained whether the criterion was enumeration by cultivation after sonication or direct cells count by confocal microscopy. The ploidy effect is also present for cultivable cells with a higher concentration for triploid strains due to a higher concentration of bioadhered cells (Fig. S1B). Furthermore, cultivable observation did show significant differences between *B. bruxellensis* and *S. cerevisiae* (p-value >0.05). In addition, *S. cerevisiae* exhibits similar enumeration counts of bioadhered cells than the Beer groups (data not show).

3.5. PCA of the combined data

Principal component analysis was performed to establish correlations between surface properties, bioadhesion and pseudohyphae growth. Principal component analysis of the combined data is given in Fig. 8. The data obtained for the WLM matrix are given in Table S3. In PCA, the dimension 1 represents 49.7% of the total variation from the original data set and mainly explains the affinity to solvent, bioadhesion and cultivable capacity. The dimension 2, which represents 18% of the total variation from the original data set, mainly explained the production of pseudohyphae cells. Bioadhesion and cultivable capacities are correlated with high affinity to solvent and hydrophobic behavior. Some genetic groups like Kombucha, Wine 2, Beer 2 and Teq/EtOH are negatively correlated to solvent affinity, bioadhesion and cultivable and show similar behaviors. A separation of the strains of the Beer group depending on the substrate of origin is also noticeable; indeed, the 2 subgroups are separated along the dimension 1, with strains (n = 8) of the Beer 2 group closely gathered and negatively correlated with affinity to solvent and bioadhesion whereas strains (n = 7) from Beer 1 group showing the higher variability between its individuals with a greater

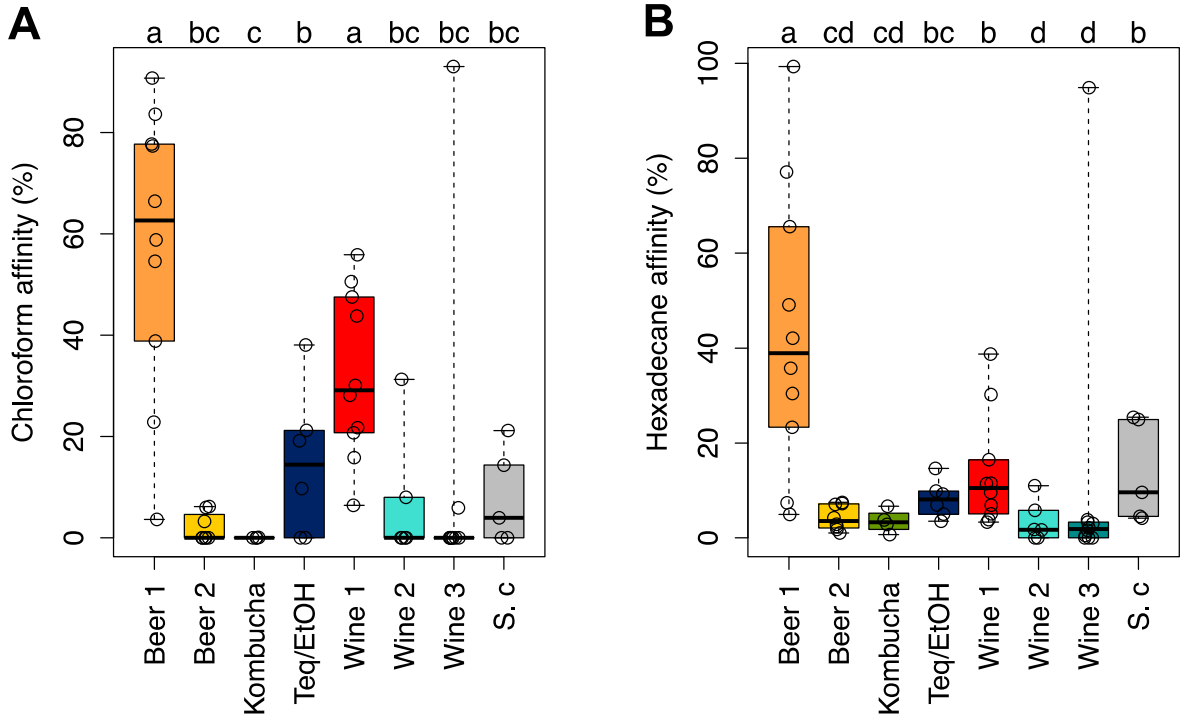

Fig. 4. Percentage of affinity to the chloroform and hexadecane used in the MATS analysis for the 54 strains studied (A) Chloroform and (B) Hexadecane. Upper letter represents groups significantly different as defined by Kruskal-Wallis statistical test (Agricolae package, R, p-value <0.05).

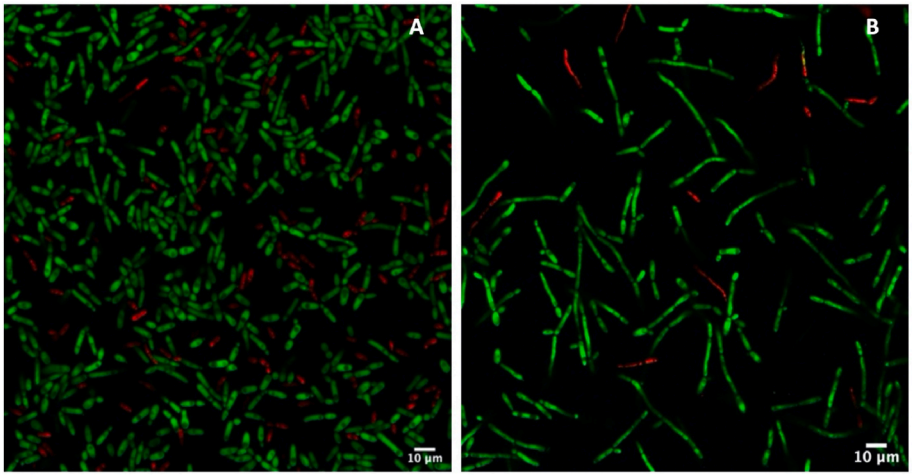

Fig. 5. Confocal microscopy observations after 3 h of bioadhesion of cells on SS. Image A represent ovoid cells and image B represent pseudohyphae cells. Different dyes were used, PI for dead cells (red) and CFDA for live cells (green).

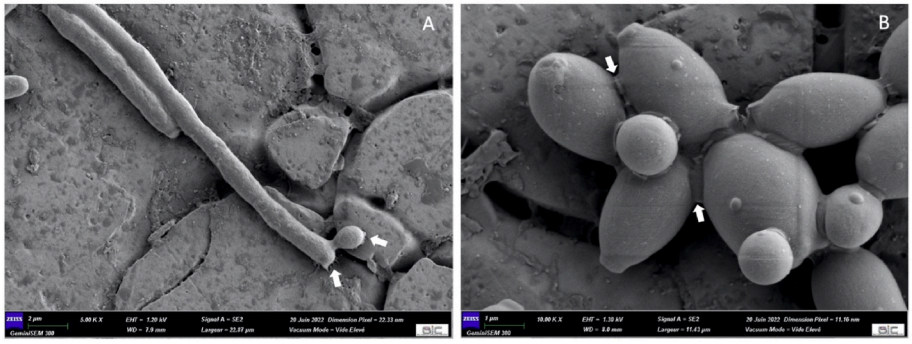

Fig. 6. SEM observation of 3 h-aged cells adhered on stainless steel chips in WLM. Magnifications were performed at 5000× for pseudohyphae observation(A) and 10000x for ovoid cells (B), White arrows represent hypothetical EPS.

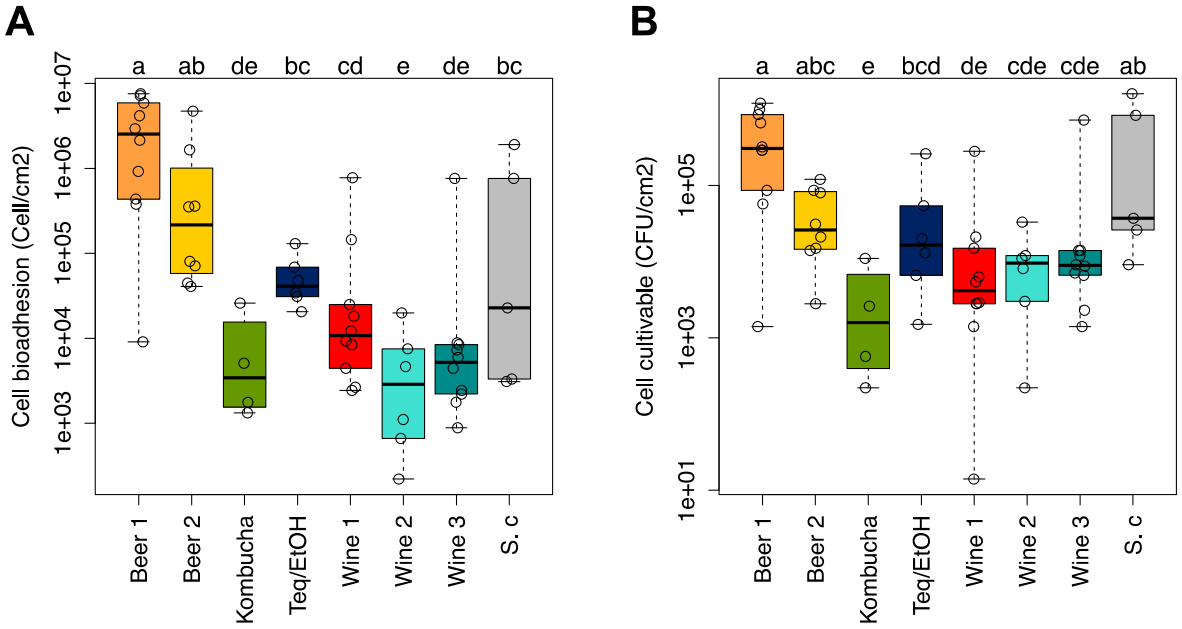

Fig. 7. Bioadhesion ability (A) and Cultivable capacity after bioadhesion (B) of the 54 strains after 3 h on SS. The color represents the genetic groups of the strains. Upper letter represents groups significantly different as defined by Kruskal-Wallis statistical test (agricolae package, R, p value < 0.05).

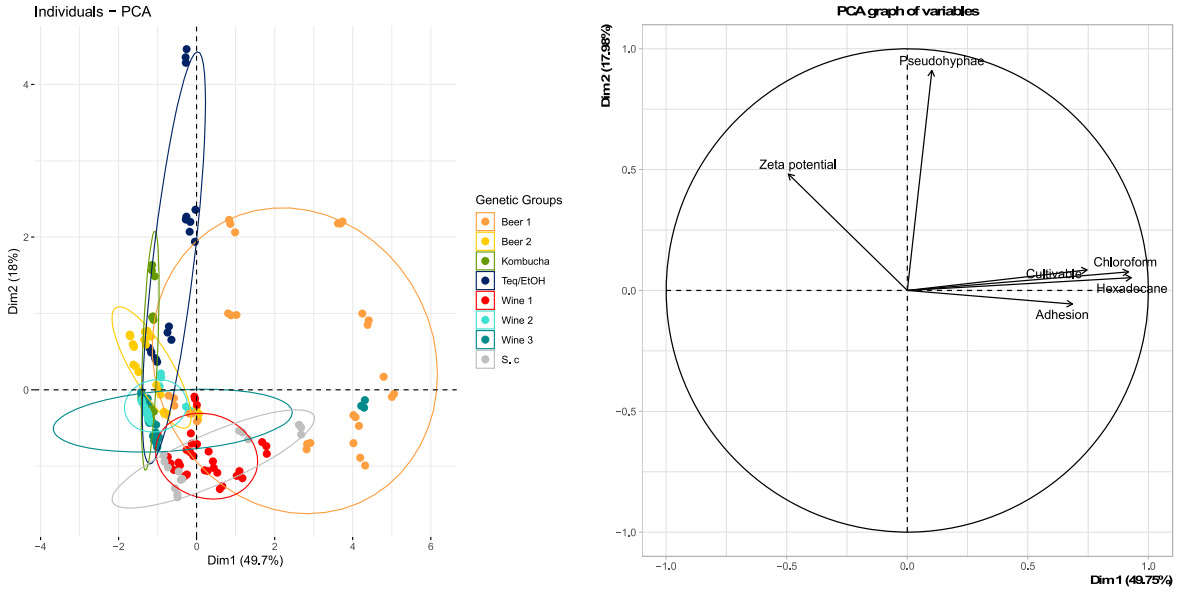

Fig. 8. Principal component analysis of the combined data (zeta potential, pseudohyphae, MATS, bioadhesion and Cultivable).

distribution and for some isolates (AWRI1608; AWRI1677; YJS5400; CRBOL1749 and ISA1700) a positive correlation with solvent affinity, bioadhesion and cultivable capacity. The CBS2499 strain of the Wine 3 group show a similar behavior to the strains of the Beer 1 group. Regarding *S. cerevisiae*, it is possible to see a behavior like the Wine 1 group but with 2 strains (F33 and IOC 18 2007) with significant bioadhesion and hydrophobic behavior.

3.6. *Validation of physico-chemical properties of surface, bioadhesion and filamentous growth on a red wine matrix*

The analysis of the surface physico-chemical properties and bioadhesion abilities within a wine matrix was carried out on a subgroup of 14 strains that had been previously analyzed on the WLM matrix. The analyses performed were the same as for the WLM matrix in order to be able to compare the 2 matrices. The data obtained for the wine matrix are given in Table S4. Principal component analysis of combined data of wine and WLM experimentations is shown in Fig. 9. In PCA, the dimension 1 represents 52.5% of the total variation from the original data set and mainly explains the affinity to solvent, bioadhesion and cultivable counts. The dimension 2, which represents 18.1% of the total variation from the original data set, mainly explained the production of pseudohyphae cells. Bioadhesion and cultivable capacities are correlated with high affinity to solvent and hydrophobic behavior. Globally, combined data related to zeta potential, affinity to solvents, bioadhesion and cultivable counts in wine and in WLM are overlaid in PCA with the exception for Pseudohyphae. The combination of data obtained during experiments conducted on WLM or wine matrix shows that for the Beer, Wine 3 and Wine 1 groups the phenotypes measured are similar with superposed confidence ellipses (95%). Only the Teq/EtOH group shows different phenotypes between the 2 matrices due to the fact that no pseudohyphae form was observed in wine for all the strains tested. The

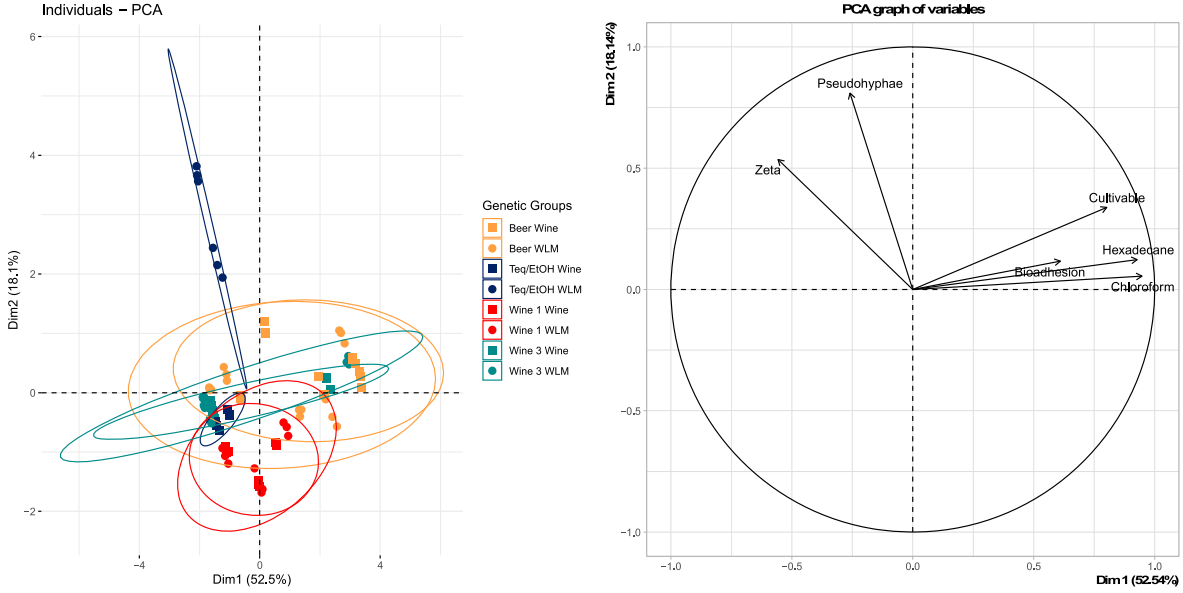

Fig. 9. Principal component analysis (PCA) of the combined data for wine and WLM experimentations (zeta potential, pseudohyphae, MATS and bioadhesion and cultivable).

cells observed in wine show ovoidal budding shapes. No differentiation into filamentous cells was observed in wine showing an impact of the medium on cell physiology. These results showed that WLM is a relevant model medium for red wine to assess surface and bioadhesion properties of *B. bruxellensis* in a context of the wine industry.

4. Discussion

In this study, different protocols were used to describe the physico-chemical, bioadhesion properties and filamentous growth of a subset of *B bruxellensis* strains representative of the species genetic diversity (Avramova et al., 2018a, 2018b). We focused on bioadhesion phenomenon since it represents a crucial step in the biofilm formation. Indeed, the ability of *B bruxellensis* to bioadhere and then form biofilm could be a potential resistance strategy (Lebleu et al., 2019) and could play a major role in the persistence of *Brettanomyces bruxellensis* in the cellar environment, and then recurrent wine contamination.

4.1. The ability to form pseudohyphae is a common feature in Brettanomyces bruxellensis

The pseudohyphae form is a cellular dimorphism described in several yeasts such as *Saccharomyces cerevisiae*, *Candida albicans*, *Hanseniaspora uvarum* (Lambrechts et al., 1996; Belini. V., 2021; Sudbery. P., 2001 and González et al., 2018) and is known in *S. cerevisiae* to be induced by environmental signals activating MAP-kinase and cAMP-dependent pathways regulating Flo11p (Rupp et al., 1999; Guo et al., 2000; Gancedo. J., 2001; Lenhart et al., 2019).

In our experimental conditions, no strains of *S. cerevisiae* produced pseudohyphae while 65% of strain of *B. bruxellensis* were able to form pseudohyphae cells with frequency depending on the genetic group and the ploidy level. These red wines spoilage species display a great adaptability to the stressful environmental conditions (Conterno et al., 2006). The formation of pseudohyphae cells could form part of the adaptation strategy of *Brettanomyces bruxellensis* but knowledge related to this cell morphology and the factors that induce it are still limited. Presence of sulfites in the culture medium can affect the presence of pseudohyphae cells in *B. bruxellensis* (Louw et al., 2016). Our results showed that triploid strains have higher ability to induce pseudohyphal cells than diploid strains. Similar observations have been reported in other yeast like *S. cerevisiae* where the ploidy affects the filamentous growth (Cullen and Sprague., 2012; Gancedo J., 2001). Indeed, according to the ploidy, different cell differentiations were observed in *S. cerevisiae*. In the case of diploid cells, in the presence of nitrogen starvation, pseudohyphal growth is considered while for haploid cells, a deficiency in carbon source will induce invasive growth (Cullen and Sprague., 2000, 2012; Lenhart et al., 2019). These facts would therefore explain the absence of pseudohyphal cells in *S. cerevisiae* under our conditions in view of the rich composition of the culture medium used. In our study, pseudohyphal cells was mainly observed for the Teq/EtOH genetic group. In Bioethanol process fermentations, an increase in the proportion of pseudohyphal cells was observed during the stationary phase, in connection with low ethanol production and a significant presence of residual sugars (Reis et al., 2013; Belini et al., 2020). In addition, the absence of pseudohyphae form in the wine matrix shows the impact of the medium on the phenotype. However, observations of pseudohyphal cells have already been made on wine matrix by Lebleux et al. (2020) with a longer contact time and on different strains. The presence of Quorum-Sensing molecules such as 2-phenylethanol could also explain the presence of these specific forms as they are known to induce pseudohyphal growth in some yeasts (González et al., 2018; Zhang et al., 2021).

4.2. Physico chemicals properties of B. bruxellensis reveal a negative charge surface and contrasting solvent affinity

The negative surface charge measured for the *B. bruxellensis* strains studied could result from the cell wall composition. Indeed, the wall charge is directly influenced by the presence of wall proteins and polysaccharides (Hong and Brown, 2010; Halder et al., 2015; Dimopoulou et al., 2019; Lavaisse et al., 2019). The cell wall constitutes 10–25% of the cell volume, which has a major influence on cellular behavior (Klis et al., 2006; Vichi et al., 2010). In this study, all strains showed a negative surface charge the most negatively charged being the strains in Wine 1 group. Previous works in a rich medium has also shown that strains belonging to the Wine 1 genetic group were the most negatively charged while strains of the Beer groups were the least charged (Dimopoulou et al., 2019). In our case, related to the Beer group, the strains isolated from wine (Beer 1) have a negative charge close to that of the Wine 1 group while the strains isolated in beer (Beer 2) have the least negative surface charge. These results are correlated with other observations showing a negative yeast surface charge due to a high proportion of carboxylic and phosphodiester grouping in cell wall but also because the culture medium is acidic inducing protonation of amino groups (Tazhibaeva et al., 2003). These results indicate that depending on the genetic group and the origin of the isolates, the composition of the cell wall may differ and could impact the surface properties as well as the ability to colonize surfaces, bioadhere and induce growth in the form of biofilm (Sheppard and Howell., 2016). However, in our experimental conditions, no correlation was highlighted between the negative charge of the cells and the ability to bioadhere to stainless steel material.

Regarding to hydrophobicity, only the Beer 1 group has a significant affinity to the solvent and therefore a predominantly hydrophobic behavior. Conversely, the other genetic groups exhibit hydrophilic behavior. This trend has already been observed before in a rich culture medium, even for the orange group, which exhibited hydrophilic behavior (Dimopoulou et al., 2019). This difference could be explained by the fact that culture media are different between the two experiments. Natural adaptations by the cells are initiated to face up to culture conditions (Chrzanowski et al., 2008) and modifications in wall composition could explain the changes in surface charge for the Beer group and then hydrophobicity (Suzzi et al., 1994). In *S. cerevisiae*, the expression of genes of the *FLO* family and *FLO11* impacts on hydrophobicity (Mortensen et al., 2007; Govender et al., 2010, 2010van Mulders et al., 2009). The genes of the *FLO* family have a major influence on cellular metabolism or surface properties. Yeasts with hydrophobic behavior have been shown to be able to adhere to negatively charged substrates such as plastic (Amaral et al., 2006; Blanco et al., 2008).

4.3. The ability of Brettanomyces bruxellensis to bioadhere to stainless steel surface is link to its hydrophobicity

Bioadhesion is the first mechanism of biofilm formation initiated by microorganisms in order to set up microorganism/support interactions. These interactions are directly related to the surface physico-chemical properties of the microorganism and of the support. Surface properties influence bioadhesion behavior because they are responsible for the first interactions between the cell and the surface (Verstrepen and Klis., 2006). In *Brettanomyces bruxellensis*, these bioadhesion abilities have already been demonstrated without giving precise information on morphology and arrangement (Dimopoulou et al., 2019; Ishchuk et al., 2016; Joseph et al., 2007). By using confocal and scanning electron microscopy, the number of viable bioadhered cells per cm^2 and their morphology on stainless steel chips after 3 h were obtained. The ability to bioadhere on stainless steel surface was reported for all the strains tested with first evidence for strains belonging to the Kombucha, Teq/EtOH and Wine 2 genetic groups. Significant differences between

the genetic groups ranging from 2×10^2 cell/cm^2 to 7.6×10^6 cell/cm^2 with a maximal average thickness of 8.61 μm were demonstrated after 3 h of bioadhesion. Similar observations on polystyrene plate also showed bioadhesion after 7 days for 12 strains tested with an average thickness of 9.2 μm and stationary bioadhered cell concentrations over 14 days (Lebleux et al., 2020). Indeed, the Beer group has the most important ability to bioadhere but also the most marked cellular surface hydrophobicity. Stainless steel is also hydrophobic even if the WLM medium reduces this hydrophobicity. Then, in our experimental conditions, the surface properties of stainless steel and the high cell surface hydrophobicity of the Beer group could explain its high ability to bioadhere. Our results suggest that in *B. bruxellensis*, hydrophobic interactions play a major role in bioadhesion as it has been previously reported for other species like *Candida albicans* (Blanco et al., 2010).

In *Saccharomyces cerevisiae*, bioadhered cells were also observed for all the strains tested. This ability has already been demonstrated in previous works with a correlation between hydrophobicity and abilities to colonize immediately charged surfaces (Urano et al., 2002; Verstrepen and Klis., 2006, van Mulders et al., 2009). Morphological diversity was observed with the presence of bioadhered pseudohyphae cells mainly for the Teq/EtOH group. This presence of bioadhered pseudohyphae cells would probably have an impact on bioadhesion and biofilm formation. Work on biofilm formation in *B. bruxellensis* has shown that the presence of pseudohyphae cells is identified at the base of the biofilm, showing that these specific forms play a major role during bioadhesion (Lebleux et al., 2020). Indeed, it has been shown that the presence of pseudohyphae forms in species of the genus *Candida* had an impact on the architecture during the formation of biofilm but also on the bioadhesion of other cells (Park et al., 2017; Kumari et al., 2018). In scanning electron microscopy, the nature of the structures that developed between cells and with stainless steel is currently unknown, but a hypothesis may be that these are made of exopolysaccharides as these polymers are reported to be involved in the consolidation of bioadhesion and biofilm formation (Czaczyk and Myszka, 2007). From a general point of view, bioadhesion is mediated via the expression of genes of the *FLO* family involved in several cellular mechanisms such as cell differentiation (pseudohyphal growth, invasive growth), flocculation and bioadhesion (Smit et al., 1992; Zhang et al., 2021). This gene family encodes membrane proteins with similarities to pathogenic fungi adhesins (Guo et al., 2000). The *FLO11* gene plays a central role in bioadhesion phenomena by encoding adhesins that will interact through hydrophobic interactions with the surface of materials allowing the establishment of bioadhesion (Reynolds and Fink., 2001, 2001van Mulders et al., 2009). Once the cells have reached a surface, flocculins encoded by *FLO* genes will come into play and allow cell-cell adhesion that can thus initiate the formation of biofilm (Guo et al., 2000). The recent publication of the genomes sequence of *B. bruxellensis* (Gounot et al., 2020; Eberlein et al., 2021) opens the way to elucidate the underlying molecular mechanisms associated with pseudohyphal growth, bioadhesion and biofilm formation phenotypes in the species, in relation with ploidy level.

5. Conclusion

On a subset of strains representative of the genetic diversity of the species, this study highlighted different behaviors according to the genetic group indicating a high phenotypic variability whether at the morphological, cell surface properties or bioadhesion level. The surface properties defined by the composition of the cell play a central role in bioadhesion ability because they define the interactions that will take place between the cell and the surface. Differences in cell surface properties will therefore induce different bioadhesion and biofilm formation behaviors depending on the strain but also the genetic group. The results obtained show that the Beer group has the most marked bioadhesion capacity. This could imply a major risk of persistence in the cellar of these particularly bioadhesive strains at the origin of recurrent wine spoilage. The specific detection of this genetic group in the cellar would be of importance for winemakers in order to adapt their cleaning protocols. Further research work on the impact of abiotic parameters and materials on bioadhesion properties would allow to better understand the factors favoring the persistence of *B. bruxellensis* in the wine environment.

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 supports were provided by National Association of Technical Research and Biolaffort company.

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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.2023.104217.

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