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Yeast derivatives: an innovative approach to produce *Oenococcus oeni* under biofilm form?

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Abstract. The malolactic fermentation can occur naturally or be induced by inoculation of selected bacterial strains, most commonly of *Oenococcus oeni*. However, due to climate change, practices are evolving to adapt to more challenging conditions, especially the increased alcohol content in wine which is particularly harmful to the bacteria. Winemakers are deploying various strategies, including the use of newly selected resistant lactic acid bacteria or the addition of yeast derived nutrients to promote the malolactic fermentation. More recently the use of bacterial biofilms has been described and investigated. A biofilm is a living community of one or more microbial species adhering to a surface and embedded in a self-produced polymeric matrix that confers new properties and resistance to the bacteria. However, the use of starters in biofilm form represents a major challenge, particularly due to the use of -not always authorized- abiotic supports (polystyrene, stainless steel, or wood). The present work proposes the development of innovative biofilms of *O. oeni* on different yeast derived biotic supports and the investigation of the ability of these new formulations on malolactic fermentation.

1. Introduction

Bacterial biofilms are generally defined as aggregates of bacterial cells attached to a surface and embedded in a polymeric matrix [1-4]. The biofilm is crossed by channels allowing the circulation of nutrients and metabolites [5-6]. Bacteria can adhere equally well to a biotic surface (e.g. mucosal cells) or an abiotic surface (e.g. floor or equipment on the farm or in the processing plant). Biofilm formation occurs in several stages according to a well-established pattern (Figure 1): adhesion to the surface, growth, biofilm maturation, and detachment [7].

Bacterial biofilms isolated from various environments share common features [8-10]: (i) bacterial cells are held together by a polymeric matrix composed of exopolysaccharides, proteins and nucleic acids; (ii) biofilm development occurs in response to extracellular signals, either present in the environment or produced by bacterial cells; (iii) the biofilm protects bacteria from the host immune system, desiccation and biocides.

Biofilms appear to facilitate the survival of bacteria in the environment and in their hosts, because biofilm-forming bacteria have different characteristics to planktonic bacteria. Under this lifestyle, bacteria are more resistant to chemical (acids, alcohol, detergents, etc.) or physical (temperature, pH, etc.) stresses [1, 11].

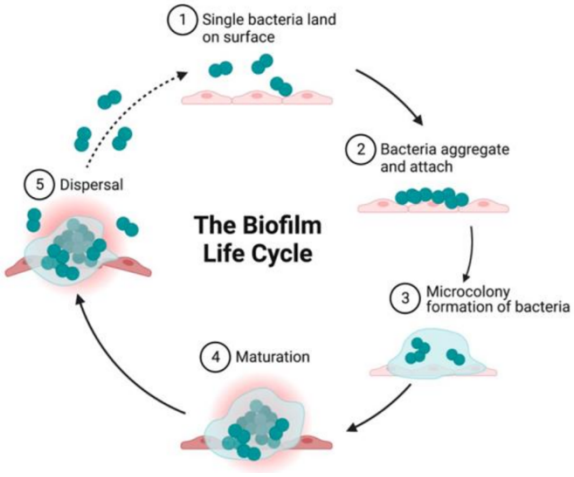

Figure 1. Steps of biofilm development (Sharma *et al.*, 2024)

Malolactic fermentation (MLF), the second fermentation of wine, generally takes place after alcoholic fermentation, once all the sugars have been consumed. It enables the enzymatic conversion of L-malic acid (diacid)

into L-lactic acid (monoacid), while releasing CO_2 [12]; thanks to the malolactic enzyme [13]. This fermentation will have an impact on the wine via (i) the deacidification of the wine by 0.1 to 0.3 pH units [14], (ii) the microbiological stability of the wine [15], and (iii) the contribution to the aromatic richness of the wine [16-17]. It is therefore important to know how to control MLF.

This fermentation is initiated in wine by lactic acid bacteria, mainly by *Oenococcus oeni* or, more rarely, by *Lactiplantibacillus plantarum*. For MLF to take place efficiently, the bacteria must be able to survive the stresses of the wine in which they are inoculated at a rate of at least 10^6 CFU/mL [14].

O. oeni is a lactic acid bacterium that has developed various mechanisms of resistance to the harsh environmental conditions of wine, such as the production of stress proteins, the activation of metabolisms to regulate pH, the modification of membrane fluidity and composition, or the ability to form biofilms [18-23]. Thus, a strategy of choice is to grow these cells in biofilms to improve their resistance to stress.

Among the various methods used to inoculate must, the use of biofilm cells has recently been described [24-25]. The ability of *O. oeni* to form biofilms has been studied on winemaking equipment such as stainless steel and oak chips [24]. Results indicate that biofilm confers increased wine stress tolerance, efficient malolactic activities and modification of wood volatile composition. In addition, fermentation occurs faster and with better reproducibility compared to planktonic lifestyles [26]. Analyses of volatile and non-volatile components revealed chemical differences, particularly when bacterial biofilms were present at the wood interface. Recently, a study showed that detached biofilm cells had better malic acid degradation kinetics and influenced the aromatic composition of wines [27].

In this context, the aim of this work is to select an *O. oeni* strain with a high capacity to grow biofilm on a biotic support (in this case yeast derivatives), while at the same time offering the best performance in terms of malolactic fermentation. To reach this objective, the study was divided into 3 phases: (i) characterization of 10 strains on criteria of biofilm formation and MLF capacity; (ii) development of a protocol for biofilm development on biotic supports; (iii) validation of biofilm protypes in applicative tests.

2. Material & Methods

2.1. Strains and growth media

This study was conducted using 11 indigenous strains of *O.oeni* and two commercial strains. They were grown in MRS modified medium containing MRS Broth Low pH Condalab® (Madrid, Spain) 50 g/L; fructose 10 g/L; L-malic acid 4 g/L. The pH was adjusted to 4.8 (NaOH concentrated solution). 20 g/L agar was added to solid MRSm medium. Cultures were incubated at 28 °C. All the assays were performed in triplicate.

2.2. Bacterial cultures preparation

Five ml of MRSm are inoculated with 2 loops of each cryotube, then incubated at 28°C for 72h (Culture C1). A second preculture, made by transferring 5ml of C1 into 10ml of MRSm, is incubated at 28°C for 24h (Culture C2).

Fifty mL of MRSm medium are inoculated with C2 to obtain an OD_{600nm} of 0.05, corresponding to a bacterial concentration of around 10^6 CFU/ml (Culture C3). To monitor C3 growth, OD_{600nm} and plate count on MRSm agar medium are performed every 3h.

For this enumeration, successive 1/10 dilutions are made in physiological water (9 g/L NaCl) down to dilution 10^{-5}. Dilutions 10^{-1} to 10^{-5} are inoculated onto agar media. Plates are incubated for 7 days at 28°C in anaerobic jars (GasPak EZ).

2.3. Biofilm development on abiotic media

The ability of the bacterial strains to grow on a biofilm form on abiotic supports was assessed as described in a previous study [24].

Cells are then counted on MRSm agar medium. A biological triplicate is performed for each strain.

2.4. Biofilm development on biotic supports

The aim is to develop *O. oeni* biofilms on different yeast derivatives such as yeast hulls, inactivated yeasts and yeast autolysates.

2.4.1. Biofilm development according to bacterial pitching rate

Among all the yeast derivatives available, the choice of an optimal support for biofilm development had to be made using strain 4.1, which had previously demonstrated its capabilities of biofilm development.

In a 24-well polystyrene plate, each well is seeded with 100 mg of yeast derivatives and 1ml of MRSm pH 4.8. The plate is left overnight at room temperature to allow the yeast derivatives to settle to the bottom of the wells. The plate is then inoculated with different concentrations of strain 4.1: 10^5-10^6-10^7-10^8 CFU/mL (in triplicate) and incubated at 28°C for 3 or 6 days. One plate per yeast derivative is produced.

After incubation (3 and 6 days), the supernatant is removed as far as possible to recover only the yeast derivatives and the biofilm-bound strain. A 2-minute sonication cycle is then used to count the number of bacteria adhering to and developing in biofilm on the various biotic supports.

2.4.2. Optimising conditions for biofilm development

To optimize the bacteria-yeast derivative system for the analysis of developed biofilms, the volume of supernatant

was optimized. The aim is to inoculate each yeast derivative with 10^7 CFU/ml of strain 4.1, without the presence of supernatant. After 3- and 6-days incubation at 28°C, counts were taken to assess biofilm formation for each yeast derivative tested.

Scanning electron microscopy (SEM) observations enabled the protocol to be readjusted to optimize the ratio of yeast derivatives to bacteria and to maintain optimum system humidity. A final biofilm development protocol was thus defined:

8 to 10 C2 cultures are produced (the quantity depends on the number of wells to be seeded for biofilms). After 24h at 28°C, the C2 cultures are centrifuged (5 minutes at 4000rpm), the supernatants removed, and the pellets collected in approximately 3mL of MRSm medium to obtain a concentrated culture (Culture C3).

One mL of Culture C3 is collected, diluted 100-fold in MRSm and the OD_{600nm} is read. The OD - CFU/ml correspondence established above is used to obtain the bacterial concentration.

Depending on the OD obtained, the bacterial concentration is adjusted by adding MRSm medium to obtain $1.2.10^5$ CFU/µL (Culture C4). Culture C4 is used to seed a polystyrene microplate for biofilms.

Each well is filled with 40 mg of the yeast derivative studied and 85µl of bacterial culture (C4), corresponding to a final quantity of 10^7 CFU per well. The wells at the edges of the plates are filled with water to prevent dehydration of the samples.

The plate is placed in a rack filled with water to approximately 1/3 of the plate height, then incubated at 28°C for 6 days. After 3 days, 30µl of MRSm medium is added to each well to keep the system moist and provide nutrients.

After 6 days, (a) the biofilm developed in 1 well is counted (biological triplicate) and (b) the biofilm developed in 1 other well is completely removed to perform MLF. The biofilm is harvested and transferred to 50mL or 5ml of wine (target inoculation: 5.106 CFU/mL) for MLF. L-malic acid consumption is monitored by robot-assisted enzymatic assay (Y15). 40 mg of derivatives are added to each volume of wine. Thus, in 50 mL of wine, the concentration of derivatives is 0.8 mg/mL of wine; it is 8 mg/mL of wine when only 5 mL of wine is inoculated.

2.5. Microscopic observations

2.5.1. Optical microscopy

10µl of biofilm are deposited in a drop of water between slide and coverslip. An observation is then made at the x100 objective.

2.5.2. Scanning electron microscopy (SEM)

The biofilm sample is rinsed with physiological water, then fixed with a fixation solution (sodium phosphate buffer 0.1mol/L, 2.5% glutaraldehyde) for 1h at 4°C. The fixed sample is then washed 3x10 minutes in 50mM sodium phosphate buffer pH 7.2, then dehydrated as follows:

- Ethanol 70% 3x10 minutes
- Ethanol 90% 3x10 minutes
- Ethanol 100% 3x10 minutes
- Ethanol absolute / acetone (70/30) 10 minutes
- Ethanol absolute / acetone (50/50) 10 minutes
- Ethanol absolute / acetone (30/70) 10 minutes
- Acetone 100% 10 minutes

The samples are air-dried and then fixed on a wooden coupon before being introduced into the microscope.

2.6. Study of wine stress resistance – triggering malolactic fermentation

2.6.1. Wine preparation

Twenty ml of YPD medium are inoculated with 2 loops from a yeast cryotube (Fermentis SafOEno™ BC S103) and incubated 24h at 28°C. A second preculture is made by subculturing 50% in fresh YPD medium and incubated 24h at 28°C. From this last culture, 5 must (white or red grape juice), were inoculated at 10^8 CFU/mL. The inoculated juice is homogenized and incubated for one week at 28°C. Glucose/fructose consumption is monitored using the OEnofoss until a concentration of less than 1g/L is reached. The fermented juice is then filtered over a 0.22µm membrane to remove wine lees. The fermented juice is adjusted to the defined modalities (Table 1) with tartaric acid and malic acid.

Table 1. Characteristics of the wines used to perform MLF

Wines n°	Origin	Ethanol %	pH	Malic acid (g/L)
1	Syrah	15	3.5	2
2	Chardonnay	13	3.1	2.5
3	Syrah	15	3.4	2.2
4	Chardonnay	14	3.4	2.5

2.6.2 Adaptation of planktonic lactic acid bacteria strains to wine

From cryotubes stored at -80°C, C1 and C2 precultures are grown at OD_{600nm}=0.05 in MRSm medium. At the end of the exponential phase, 50% of the culture is mixed with 50% of the wine and incubated for 72 h at 28°C. The operation is repeated once, then the wines are inoculated at 5.10^6 CFU/mL, homogenized and incubated at 20°C. The corresponding OD-UFC/mL allows inoculation to be carried out based on the OD_{600nm} measurement taken on the last culture.

2.6.2. Monitoring malolactic fermentation

L-malic acid consumption is monitored over time by enzymatic assay (Food Quality Biosystems) assisted by the Y15 (Biosystems).

A population count is carried out at T0 and then regularly during MLF.

3. Results

3.1. Biofilm-formation capacity of *O. oeni* strains

Steel coupons are traditionally used to assess the ability of bacterial strains to grow biofilms. The biofilm growth results for each strain in CFU/cm² are shown in Figure 2.

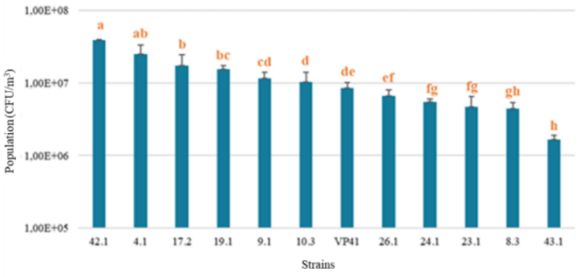

Figure 2. Ability of strains to develop as a biofilm on a steel coupon.

Kruskal-Wallis statistical test: Letter difference between times for one condition

All strains are capable of biofilm growth, with cell numbers ranging from $3.87.10^7$ CFU/cm² to $1.65.10^6$ CFU/cm². In this case, the Kruskal-Wallis statistical test reveals 8 statistically different groups (p-value < 0.001). This time, strains 42.1 and 4.1 were the best performers. It is notable that the quantities of biofilm cells counted are equal to or greater than what had been counted for other *O. oeni* strains on identical supports.

3.2. Resistance to wine stresses - Performing malolactic fermentations

In parallel, this panel of strains were evaluated for their ability to withstand wine-induced stress and perform MLF, small scales trials were carried out. For this purpose, strains were pre-adapted using the "pied de cuve" method before being inoculated into red or white wines at a population of around 5.10^6 CFU/mL (Figures 3 and 4).

In the Syrah at 2 g/L of L-malic acid (Figures 3), 3 groups of strains were identified: (i) 1 group not carrying out MLF (strains 23.1 and 17.2), (ii) 1 group consuming L-malic acid up to around 1 g/L (strains 42. 1, 24.1, commercial 1 and 9.1) and (iii) 1 group degrading L-malic acid to final concentrations of between 0.59 and 0.05 g/L (strains 4.1, 8.3, 10.3, 19.1, 26.1, commercial 2 and 43.1).

In the Chardonnay at 2.5 g/L of L-malic acid (Figure 4), MLF was not carried out for some of the strains (24.1, 23.1, 26.1, 17.2, 42.1, 43.1 and commercial 2). For the others, they seem to have started consuming L-malic acid up to around 1.3 g/L (strains 9.1 and 19.1) or up to around 0.9 g/L (strains 10.3, 8.3, 4.1 and commercial 1), only to stabilize and not complete MLF.

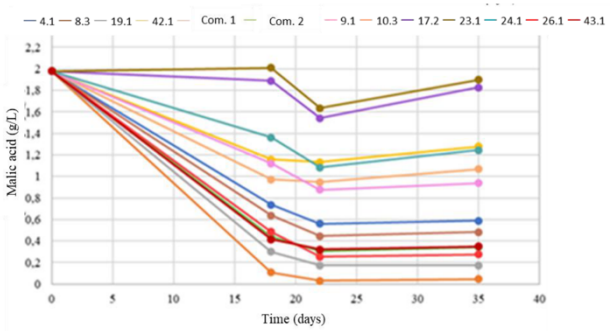

Figure 3. Malic acid consumption in red wine inoculated with adapted planktonic bacteria

Syrah wine: ABV 15% v/v, pH 3.5, L-malic acid 2g/L

The best-performing strains for the parameters studied (ability to trigger MLF, generation time, biofilm growth on an abiotic support) were strains 4.1, 8.3, 10.3 and 19.1. The rest of the study will be carried out with the strain 4.1.

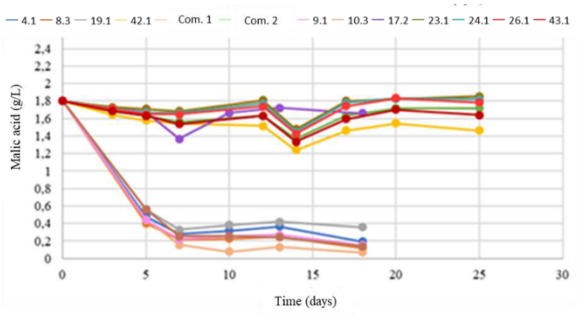

Figure 4. Malic acid consumption in white wine inoculated with adapted planktonic bacteria

Chardonnay wine: ABV 13% v/v, pH 3.1, L-malic acid 1.8g/L

3.3. Development of biofilms on biotic supports

One of the major objectives of this project is to develop biofilms on yeast derivatives. An innovative protocol has been developed to produce biofilms on this type of support, meeting the technical constraints of (i) separating planktonic and biofilm bacteria and (ii) recovering the biofilm. To do this, the starting inoculum and the quantity of support to be used had to be calibrated, and numerous microscopic observations (optical, epifluorescence and electronic) were carried out to visualise the bacteria in biofilms and identify the most suitable support(s).

3.3.1. Definition of the bacteria-yeast derivative ratio

Biofilm formation tests on a biotic support were carried out in 96-well polystyrene microplates. For the first biofilm formation tests, 10^5 to 10^8 CFU/mL were added to 100 mg of yeast derivatives for 3 to 6 days. Optical microscopy observations (Figure 5) revealed bacteria bound to the support, but in very low concentrations.

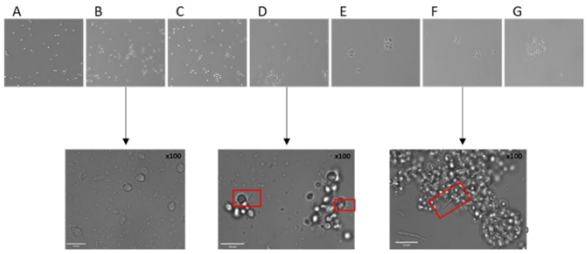

Figure 5. Observation of yeast derivatives by optical microscopy

A: Inactivated yeast 2; B: Inactivated yeasts 1; C: Yeast autolysate 2; D Yeast autolysate 1; E: Yeast hulls 3; F: Yeast hulls 1; G: Yeast hulls 2.

Red frames indicate bacterial chains linked to yeast derivatives.

It was therefore necessary to adapt the process by (i) removing the supernatant from the biofilm production system and (ii) modifying the ratio of bacteria to yeast derivatives, to optimise adhesion between the yeast derivatives and the bacteria. The protocol now in place uses 40 mg of yeast derivatives in the presence of 10^5, 10^7 or 10^9 CFU. The microplate is placed in a container of water to maintain humidity in the system and prevent the biofilms in the wells from drying out. In addition, 30µl of MRSm medium is added to each well every 3 days.

3.3.2. Observation of bacteria in biofilms

To observe cells in biofilms and identify characteristic structures, different microscopy techniques were tested: techniques using fluorescent markers and electron microscopy techniques.

The environmental SEM images clearly demonstrate the ability of bacteria to develop biofilms on these biotic supports (Figure 6a, b). After 12 days, the images show fewer cells embedded in the matrix, perhaps suggesting partial degradation of the biofilm. Nevertheless, the protocol developed enables us to obtain a mature bacterial biofilm with the presence of a matrix encompassing the bacteria-yeast derivative system (Figure 6b).

3.3.3. Optimising conditions for biofilm development

To develop the protocol for biofilm formation on a biotic support, various parameters had to be tested. Firstly, the level of inoculation was assessed using strain 4.1 inoculated at 10^5, 10^7 or 10^9 CFU on inactivated yeasts and yeast hulls support (Figure 7). On inactivated yeasts, the seeding rate did not change the number of cells counted over time, except at 3 days (Figure 7a). However, the differences observed were less than 1 log. On yeast hulls support, significant differences were observed as a function of the pitching rate at 3, 10 and 15 days (Figure 7b), underlining the importance of the pitching rate in this case.

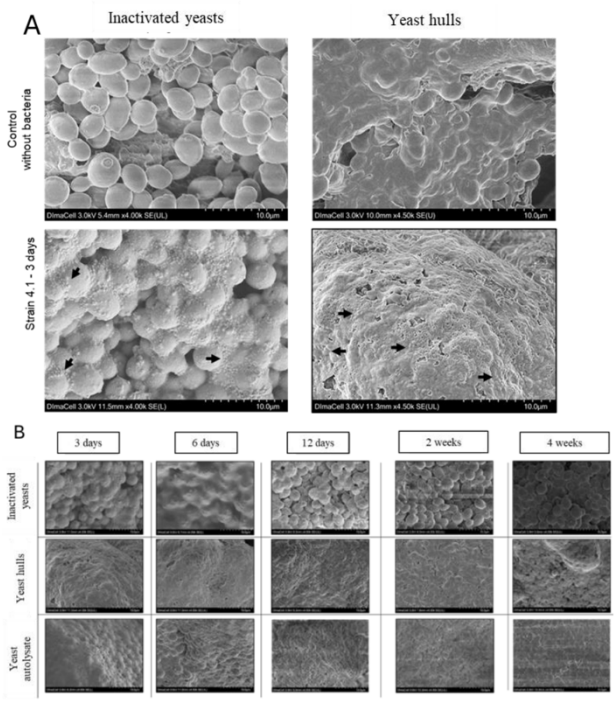

Figure 6. Observation by environmental SEM of yeast derivatives without (A) or with (B) strain 4.1 developed as a biofilm on inactivated yeast, yeast hull and yeast autolysate supports over time (3 days to 4 weeks of development).

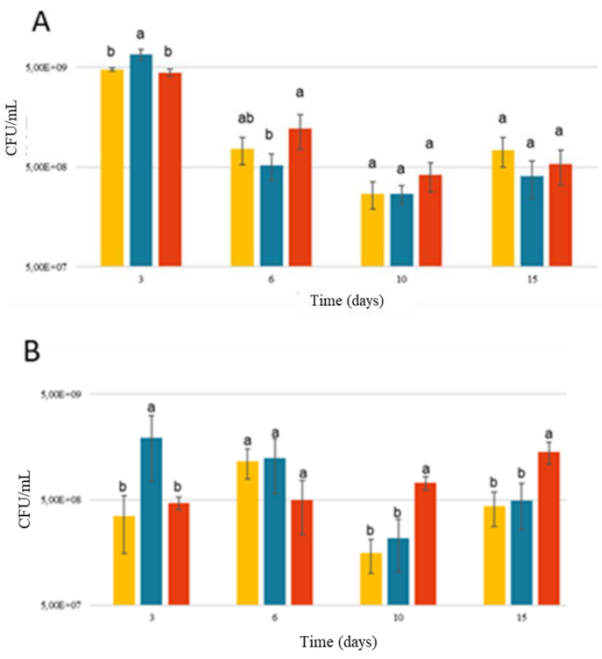

Figure 7. Enumeration of strain 4.1 developed in biofilm as a function of the quantity of bacteria inoculated: 10^5 CFU (yellow), 10^7 CFU (blue) or 10^9 CFU (red) on inactivated yeast support (A) and on yeast hull support (B).

Kruskal-Wallis statistical test: Letter difference between times for one condition.

Secondly, it was necessary to assess the importance of the biofilm washing step before use. The unwashed population was 1 log higher than the washed population for both media tested (data not shown).

3.4. Inducing MLF with *O. oeni* biofilms

MLF triggered by strain 4.1 grown in biofilms for 3, 6 or 10 days on 3 types of support inactivated yeast, yeast hulls and yeast autolysate) was monitored in red and white wines (wines n° 3 and 4). Only the results obtained with the 6-day biofilms will be presented, as the 3-day and 10-day biofilms did not give conclusive results.

The results clearly show the importance of the pitching rate. For example, in red wine (wine n° 3), in the 'biofilms 6 days' condition on yeast hull support, in 5 ml of wine, MLF was much faster than in the 'adapted planktonic bacteria' condition (5 days compared with 17 days for 0.4 g/L malic acid). In the '6-day biofilm' condition in 50 mL of wine, MLF was achieved, albeit later than with the adapted planktonic bacteria (Figure 8).

In the white wine (wine n° 4), the 'biofilms 6 days' condition on yeast hull support in 5 mL of wine showed much faster MLF than for the adapted planktonic bacteria (7 days compared with 18 for 0.3 g/L malic acid). In the 'biofilms 6 days' condition in 50 ml of wine, MLF started late only for the yeast hull and yeast autolysate supports (Figure 9).

In addition, the counts show a loss of viability of between ½ and 1 log over time for biotic biofilms, unlike adapted planktonic bacteria, which remain stable over time.

However, for all the trials, the standard deviations indicate considerable variability. Further development will be required to optimise the system and achieve systematic MLF. Nevertheless, strain 4.1 and the yeast hulls support appear to be the pair of choice for producing MLF in biofilm form.

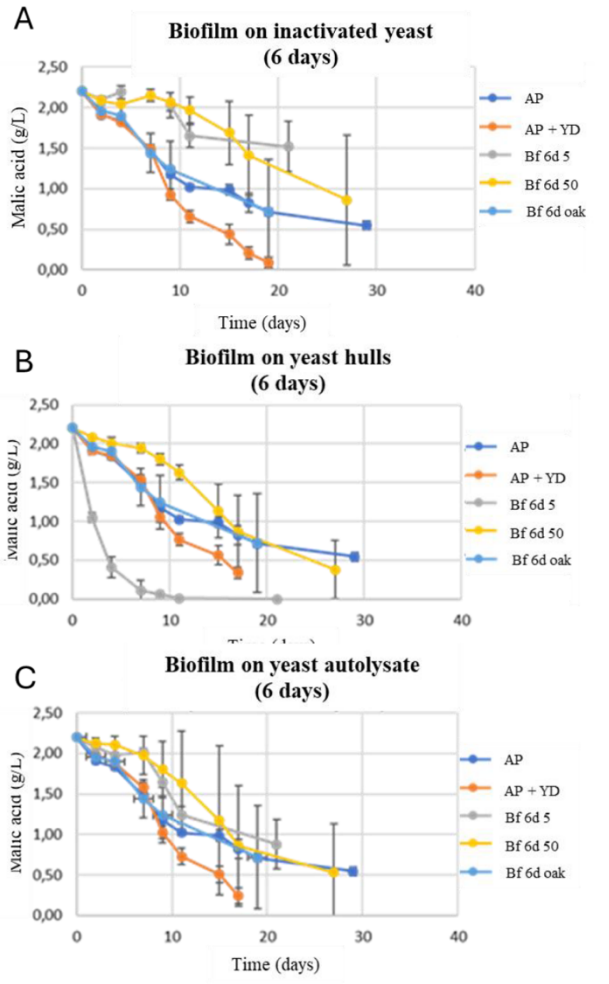

Figure 8. MLF on red wine using biofilm on inactivated yeasts (A), yeast hulls (B) or yeast autolysate (C) supports and planktonic adapted bacteria with or without yeast derivatives.

Syrah wine: ABV 15% v/v, pH 3.4, L-malic acid 2.2 g/L

AP: adapted planktonic bacteria, AP+ YD: adapted planktonic bacteria + yeast derivative, Bf 6d 5: biofilm on yeast derivative for 6 days with 5 mL inoculum, Bf 6d 50: biofilm on yeast derivative for 6 days with 50 mL inoculum, Bf 6d oak: biofilm on oak for 6 days

4. Discussion

Biofilm lifestyle is well known to protect bacteria from harsh environmental conditions. In previous study, cells from *O. oeni* biofilms were much more resistant than planktonic ones [24]. In this context, a protocol for the development of biofilms on biotic supports (yeast derivatives) was developed and validated. Various electron microscopy observations confirmed the biofilm development of *O. oeni* strains. This study made it possible to identify preferred bacterial/yeast derivative pairs for biofilm development, resistance to wine matrix stress and MLF. In the course of this work, we also validated that the use of bacteria in biofilms developed at a rate of 10^7 to 10^8 CFU/mL on biotic supports enables optimal initiation of MLF. These new fermentation formulations were used to initiate and carry out malolactic fermentation, while influencing the metabolomic compounds of the resulting wines, with moderate to significant effects depending on the type of support used.

Although the use of starters containing *O. oeni* alone or in combination with yeasts has been extensively studied and is commonly used by winemakers [28-29], few studies have focused on the use of these starters in biofilm form [24-27]. It is currently known that *O. oeni* biofilm cells improve fermentation capacity and the production of desired volatile compounds [24-27]. However, the use of starters in biofilm form represents a major challenge, particularly due to the use of abiotic supports (polystyrene, stainless steel or wood), which are not always authorised for use in winemaking. So, the use of bacterial biofilms developed on a biotic support (in this case yeast derivatives) appears to be an advantageous approach.

However, this work is only a first approach to the development of bacterial biofilms on biotic supports. Optimisations of the biofilm production protocol are underway and will, in time, lead to better-performing adapted biofilms.

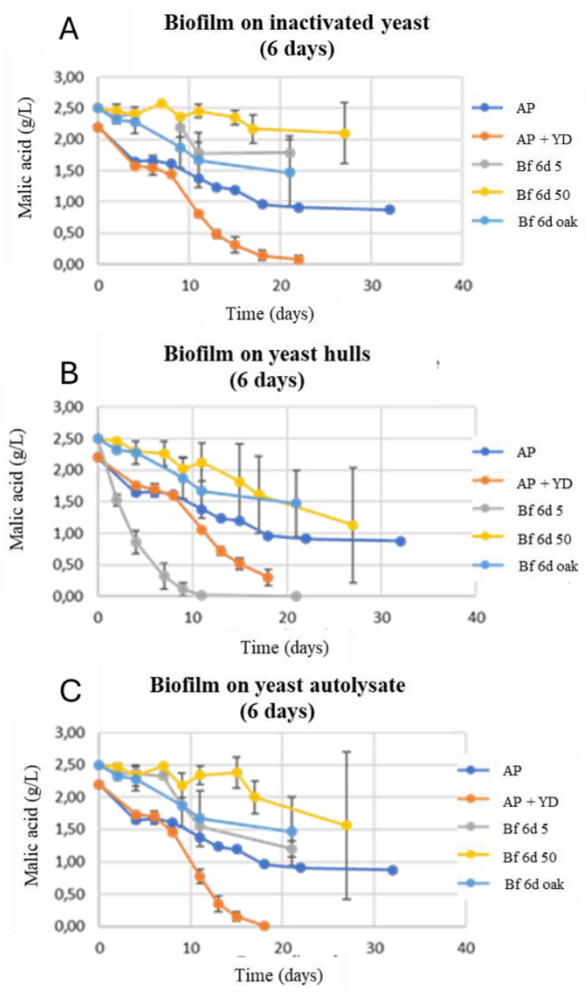

Figure 9. MLF on white wine using biofilm on inactivated yeasts (A), yeast hulls (B) or yeast autolysate (C) supports and planktonic adapted bacteria with or without yeast derivatives.

Chardonnay wine: ABV 14% v/v, pH 3.4, L-malic acid 2.5g/L

AP: adapted planktonic bacteria, AP+ YD: adapted planktonic bacteria + yeast derivative, Bf 6d 5: biofilm on yeast derivative for 6 days with 5 mL inoculum, Bf 6d 50: biofilm on yeast derivative for 6 days with 50 mL inoculum, Bf 6d oak: biofilm on oak for 6 days

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