



**ENOLOGY ORIGINAL RESEARCH ARTICLES**

# Use of *Oenococcus oeni* biofilm for wine malolactic fermentation management

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## ABSTRACT

Controlling the speed of malolactic fermentation in red wine is an important challenge to produce certain short-rotation wines, like *primeur* style wines, for entry-level market segments. This study shows the possibility of inducing the adhesion and biofilm formation of *Oenococcus oeni* Vitolactic F<sup>®</sup> and *Saccharomyces cerevisiae* 522D<sup>®</sup>, in a low-nutrient medium, on Nylon<sup>®</sup> carriers in a continuous flow 250 mL bioreactor. The biofilm formation medium was then replaced by fermentation media (grape must for co-alcoholic and malolactic fermentations with *O. oeni* and *S. cerevisiae* biofilms) or wine (with *O. oeni* biofilms only) and the progress of malolactic fermentation was monitored: over periods of three to four weeks under a continuous regime, stable conversion speeds for L-malic acid of 0.53 g/L/24 h (malolactic fermentation in wine medium) and of 2.04 g/L/24 h (co-fermentations fermenting grape must medium) are reached. *O. oeni* biofilms on Nylon<sup>®</sup> carriers were also transferred in wine for four successive batch fermentations: in these conditions, L-malic acid conversion speed was 0.35 g/L/24 h. These biofilm implementation systems could be the first step towards perfectly controlled industrial malolactic fermentation processes.

**KEYWORDS:** wine, biofilm, malolactic fermentation, alcoholic fermentation, continuous flow

## INTRODUCTION

Like other agricultural sectors, the wine industry must face current climatic, economic, and social upheavals. One of the multiple consequences of this context in Europe is the decline in the wine market, particularly for red wines (Ohana-Levi & Netzer, 2023). Moreover, it is difficult for winemakers to remain competitive in the entry-level segment. Among the strategies, one could be the reduction of production costs thanks to controlled and low-cost winemaking processes that guarantee consistent or even better quality for the consumer.

Alcoholic fermentation (AF) is commonly realised through seeding selected active dry *S. cerevisiae* yeasts in dedicated fermentation tanks. The operation generally lasts between one and two weeks. Malolactic fermentation (MLF) management remains a crucial challenge for its role in red wines' microbiological stability and in their organoleptic properties. The reaction provides the transformation of the L-malic acid present in the grapes into L-lactic acid thanks to the action of the lactic-acid bacterium, *O. oeni*. Although *O. oeni* has a great tolerance to the combination of ethanol and acidity, the unfavourable wine environment can still prolong MLF progress. Indeed, even when selected strains are seeded, the reaction is triggered after variable and sometimes very long latency periods (Lonvaud-Funel, 1999; Sumbly, 2019). Co-inoculation (or co-fermentation), *i.e.* simultaneous AF and MLF, has been reported to be an interesting practice for the bacteria to adapt gradually to ethanol presence and to benefit from the higher nutrient availability (Guzzon *et al.*, 2013; Muñoz *et al.*, 2014; Rosi *et al.*, 2003). In this configuration, *Lactiplantibacillus plantarum* may be used as an alternative to *O. oeni* (Pannella *et al.*, 2020; Urbina *et al.*, 2021).

Both continuous flow AF and MLF have also been studied to accelerate, secure and reduce costs of the winemaking process (Ribéreau-Gayon *et al.*, 2020). Another advantage is that continuous fermentations could follow on from thermovinification processes. Thermovinification techniques aim to disrupt the grape cell structure by heating the destemmed grapes up to 70 °C for a short time, inducing the release of polyphenols in the aqueous phase. The solid phase is thus eliminated before fermentation (Escudier *et al.*, 2008). Continuous winemaking is based on the culture, with continuous flows of active microorganisms. The aim is to reach a permanent regime during which the microbial biomass is stable and maintained in the exponential growth phase. In this situation, microbial growth rate depends on the substrate feed flow rate (Lonvaud-Funel *et al.*, 2021). When a continuous system is well managed, active microbial populations can be larger than in discontinuous culture, allowing higher fermentation rates. In the wine industry, several applications have been described since the development of these technologies in the 1950s (Ribéreau-Gayon *et al.*, 2020). Since the 1990s, the interest in continuous fermentation systems has been mainly based on the advantages of microbial immobilisation techniques.

Microorganisms' immobilisation is defined as the physical confinement of intact cells to a region of space with

conservation of biological activity. Among the different methods described, immobilisation by attachment to a surface is based on the binding of microbial cells to a carrier by covalent bonding between the cell and the support, or by adsorption/adhesion (Moreno-García *et al.*, 2018). For oenological fermentation processes, various carriers have been described, such as wood, fermentation by-products (grape stems, skins, or seeds), plastic polymers, cellulose and its derivatives, stainless steel or calcium alginate (Genisheva *et al.*, 2014; Kourkoutas *et al.*, 2004; Moreno-García *et al.*, 2018). Adsorption characteristics can be harsh to anticipate, but generally, yeasts' response is better than bacteria one (Navarro, 1980). Regarding *S. cerevisiae* for alcoholic beverages production, immobilisation techniques offer many advantages widely described and reviewed (Genisheva *et al.*, 2014; Kourkoutas *et al.*, 2004; Moreno-García *et al.*, 2018). These advantages include the cell density increase and the reduction of fermentation step as well as non-productive latency phases, the protection of the cells in a hostile environment, and the possibility of reusing cells in successive batches or continuous processes. For wine AF, the most cited applications are based on fluidised bed bioreactors, with one or several stages, used with residence times between 50 and 80 h (Genisheva *et al.*, 2014; Kassim, 2012). Regarding *O. oeni* and MLF, studies are rare but the sought interests are similar: a better process control, successive MLF batches (especially for reusing starters) and continuous flow system opportunities, and a better tolerance to the wine stressful environment (Davis *et al.*, 1985; Maicas, 2001). Among the tested applications, natural supports (grape skins and seeds, corn grains) have been successfully used for MLF in successive batches over several months (Genisheva *et al.*, 2013). Another trial of sequential continuous AF and MLF based on the immobilisation of *S. cerevisiae* and *O. oeni* on grape stems and seeds reported shorter fermentation times than the batch process configuration (Genisheva *et al.*, 2014). Previously, *O. oeni* biofilm on oak chips had also been used for a continuous MLF process with conversion rates twice as high as the ones obtained in the same conditions with free cells (Janssen, 1991).

However, today, no industrial application for winemaking, based on *O. oeni* immobilisation by attachment to a surface, has been reported. Moreover, in the laboratory-scale studies, biomass is barely characterised, and many questions remain regarding microbial population dynamics. Thus, the frontier between immobilised cells by attachment and biofilms can be tiny, and for Germec *et al.* (2015), biofilms are natural forms of cell immobilisation and can be utilised in biofilm bioreactors.

Biofilms are today defined as aggregates of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface (Vert *et al.*, 2012). The matrix is crossed by channels allowing the circulation of metabolites and nutrients (Costerton *et al.*, 1995). Biofilms are widely studied since this phenotype is the most efficient for microbial resistance to environmental stresses

(Stoodley *et al.*, 2002). When working with biofilms, the choice of an adhesion and biofilm formation medium is a matter of major interest: using nutrient-depleted media is one of the strategies. Indeed, several studies showed that biofilm formation was favoured when available nutrients were limited. This phenomenon has been highlighted in relation to carbonaceous substrates, but also to the availability of nitrogen and phosphorus (Petrova & Sauer, 2012). On the other hand, it has been demonstrated that shear forces influence the adhesion phase (Soumbo, 2019), and thus microbial biofilm strength and density (Rochex *et al.*, 2008), their structure, and their attachment and detachment rates to/from the support (Kim *et al.*, 2013).

In the field of oenology, microbial populations are part of a complex system, and biofilms have been described for various applications (Gosset *et al.*, 2022). Regarding AF applications, many studies report AF systems based on the immobilisation of *S. cerevisiae* by attachment to solid surfaces (Genisheva *et al.*, 2014; Kourkoutas *et al.*, 2004; Moreno-García *et al.*, 2018). Biofilm formation was not claimed in these studies, as they mainly focus on the fermentative capacities of the immobilised biomass, but the growth of adherent cells cannot be ruled out. In the case of *O. oeni*, the use under biofilms for MLF was described in several studies with interesting fermentative performances (Bastard *et al.*, 2016; Coelho *et al.*, 2019; Tofalo *et al.*, 2021). Finally, the ability of *S. cerevisiae* and *O. oeni* to form mixed biofilms on different surfaces found in vinification was assessed. In this recent work, mixed biofilms formed on oak surfaces allowed the efficient completion of fermentations (Palud *et al.*, 2024).

Despite the interesting results obtained due to the use of microbial biofilms for fermentations in oenology, no application has been proposed for industrialisation. This is the aim of this article: to combine the advantages of biofilm lifestyle and continuous processes to propose a continuous flow bioreactor system designed to accelerate and to secure the fermentation steps, focusing on MLF. For this purpose, bacterial biofilms were formed in a continuous mode in a nutrient-depleted medium on Nylon<sup>®</sup> membranes (a material commonly used for filtration applications during winemaking). Then, three protocols for MLF involving *O. oeni* biofilms were tested: a continuous MLF process, a protocol of successive batches, and a continuous process for co-fermentations. In the latter protocol, biofilms of *S. cerevisiae* were also involved for continuous completion of both AF and MLF.

## MATERIALS AND METHODS

### 1. Yeasts and bacterial strains

The study was conducted using *O. oeni* oenological strain Vitilactic F<sup>®</sup> (Martin Vialatte, France), a commercial strain widely distributed worldwide, with a direct seeding use recommendation. The Vitilactic F<sup>®</sup> strain was also selected for its biofilm-producing ability during a screening study at the

microplate scale (not published). Bacteria inocula were grown in MRS modified (MRS<sub>m</sub>) medium (Bastard *et al.*, 2016) containing: MRS broth (Biomérieux, ref: AEB14652, France) 55.2 g/L; L-malic acid (Sigma, Saint-Louis, USA) 4 g/L; fructose (Merck, Darmstadt, Germany) 10 g/L. The final pH was 4.8. Cultures were incubated at 28 °C for six days for growth.

The co-fermentation assays were conducted using *S. cerevisiae* strain 522D<sup>®</sup> (Lamothe-Abiet, France), a widely commercialised strain, known for its aromatic neutrality. Yeasts inocula were grown in YPD medium containing tryptone soja (Oxoid, Dardilly, France) 20 g/L, glucose (Sigma, Saint-Louis, USA) 20 g/L, yeast extract (Biomérieux, Marcy l'Étoile, France) 10 g/L. Medium final pH was 7.0. Cultures were incubated at 30 °C for 24 h for growth.

For a solid medium (used for cell counting), 15 g/L agar (Biomérieux) was added.

### 2. Biofilm formation and fermentation media

The adhesion and biofilm formation medium was a nutrient-depleted medium containing *O. oeni* essential carbon sources and growth factors, but at a minimal concentration. This medium (MRS<sub>min</sub>) was based on the MRS<sub>m</sub> composition with: peptone (Oxoid, Dardilly, France) 1 g/L; beef extract (Oxoid) 0.8 g/L; yeast extract (BD, San Jose, USA) 0.4 g/L; glucose (Sigma) 1 g/L; fructose (Merck) 1 g/L; L-malic acid (Sigma) 0.4 g/L; C<sub>6</sub>H<sub>11</sub>NO<sub>7</sub> (Sigma) 0.2 g/L; K<sub>2</sub>HPO<sub>4</sub> (Sigma) 2 g/L; C<sub>2</sub>H<sub>3</sub>NaO<sub>2</sub> · 3 H<sub>2</sub>O (Sigma) 2.4 g/L. The pH was adjusted to 3.8 (H<sub>2</sub>SO<sub>4</sub> (Sigma) concentrated solution).

The wine medium was a Syrah wine made in the Gaillac production region, from the 2019 vintage. The L-malic acid concentration was adjusted to 4.0 ± 0.3 g/L. Alcohol by Volume (ABV) was adjusted to 12 % thanks to the addition of distilled water. pH was adjusted to 3.4 (H<sub>2</sub>SO<sub>4</sub> concentrated solution).

Must medium was obtained from Gamay grapes from Gaillac, heated thanks to the thermovinification technique (30 min at 70 °C before dynamic drainage and pressing). The L-malic acid concentration was adjusted to 4.0 ± 0.3 g/L, the sugars (glucose + fructose) concentration was 183 mg/L, assimilable nitrogen concentration was 254 mg/L. pH was adjusted to 3.4 (H<sub>2</sub>SO<sub>4</sub> concentrated solution).

Liquid media were sterilised by filtration (0.2 µm cut-off), solid ones by autoclaving at 121 °C for 20 min.

### 3. Continuous biofilm formation

Twenty-two hydrophilic Nylon<sup>®</sup> membranes of 2.5 cm diameter (Millipore, reference HNWP02500) stacked on a dedicated support were placed in a 250 mL glass bioreactor. The number of membranes was determined based on the process development results obtained at the 24-well microplate scale, to conserve the ratio carrier surface/medium volume (unpublished). A bioreactor standard assembly was realised with Tygon<sup>®</sup> tubings (Figure 1). A

vent filter (0.22 µm) was placed on a dedicated connector on the top of the bioreactor. The bioreactor connected to the Tygon® assembly was autoclaved for 20 min at 121 °C for sterilisation. After autoclaving, the bioreactor was transferred into a BSC (Biological Safety Cabinet) and filled with 225 mL of sterile MRSmin and 25 mL of an *O. oeni* suspension at  $8.8 \pm 0.4$  log (CFU/mL) in sterile distilled water (SDW). *O. oeni* suspension concentration was determined thanks to absorbance measurement at 640 nm and verified by CFU counting. Then the inlet line was connected to a 1 L sterile tank containing MRSmin, and the outlet line to a 1 L empty tank (Figure 1). Bioreactor content was homogenised during two hours by recirculation at 3 mL/min thanks to a peristaltic pump LS-7523-47 (Masterflex®, Gelsenkirchen, Germany). After homogenization, a continuous flow rate of 0.12 mL/min, corresponding to a 35-hour residence time (RT), was set up thanks to a Minipulse 3 pump (Gilson, Middleton, USA). Continuous flow for biofilm formation was maintained during 10 days at  $26 \pm 1$  °C with permanent recirculation at 3 mL/min.

For *S. cerevisiae* biofilm formation, a second bioreactor was used in the same conditions except for slight modifications: 25 mL of a *S. cerevisiae* suspension at  $6.9 \pm 0.4$  log (CFU/mL) in sterile distilled water (SDW) was added (concentration adjusted according to absorbance measurement at 640 nm and verified by cell counting). For process simplification with a view to industrial application, *S. cerevisiae* biofilm formation was also performed in the MRSmin medium at  $26 \pm 1$  °C during five days at the same RT of 35 h, such that the end of biofilm formation for both microorganisms was completed at the same time.

At the end of continuous biofilm formation, the planktonic phase was sampled for cell counting for both *O. oeni* and

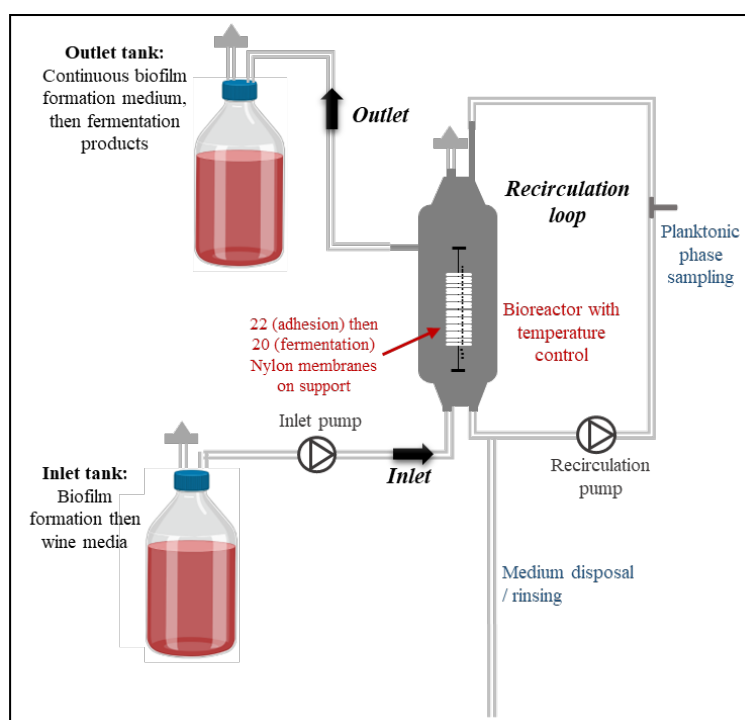
*S. cerevisiae*. Membranes were rinsed twice with 200 mL SDW and processed for scanning electron microscopy (SEM) or CFU counting.

Biofilms are then implemented throughout three fermentation process configurations (Figure 2).

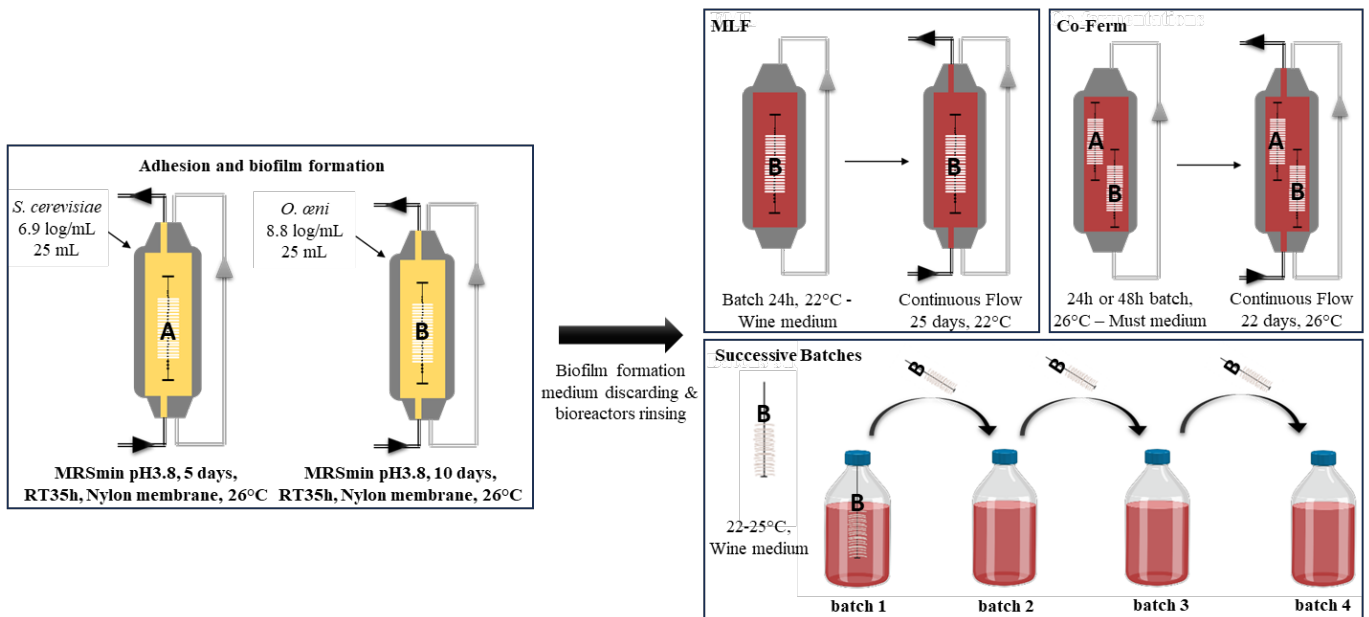
#### 4. Continuous malolactic fermentation

After medium disposal and membranes rinsing (with 2–200 mL of SDW), MRSmin was sterilely replaced in the bioreactor with 250 mL of sterile wine medium. The inlet tank was replaced with a 1 L tank containing sterile wine, and the outlet tank with a new sterile 1 L empty tank (Figure 1). The temperature was set up at  $22 \pm 1$  °C, and the continuous recirculation at 3 mL/min was initialised using the Masterflex® peristaltic pump. Conversion of L-malic acid to L-lactic acid was monitored, and the continuous flow was started once the L-malic acid concentration was less than or equal to 1 g/L, after an initial batch phase. Different flow rates were used. The corresponding RT were calculated. Planktonic phase was sampled on the recirculation loop (Figure 1) during the fermentation for MLF monitoring as well as for cell counting. At the end of each assay, the bioreactor was opened under the BSC and its content (planktonic phase) was sampled for cell counting. Wine was then discarded, and the bioreactor was rinsed twice with 200 mL SDW before membrane sampling for sessile cell population counting and SEM observation.

Membranes were discarded after 25 days of MLF; then the system was set up again at the same RT to evaluate the impact of membrane elimination on the L-malic acid conversion rate.



**FIGURE 1.** Schematic representation of the 250 mL bioreactor assembly.



**FIGURE 2.** Experimental configurations (MLF, co-ferm, and successive batches) implemented in the study. A: Nylon membrane with *S. cerevisiae* biofilm; B: Nylon membrane with *O. oeni* biofilm.

### 5. Continuous co-fermentation (AF and MLF)

After medium disposal, membranes rinsing (with  $2 \times 200$  mL of SDW) and sampling for counting and SEM, supports from each of the two bioreactors (with *S. cerevisiae* or *O. oeni* biofilms) were transferred to a unique bioreactor. MRSmin was sterilely replaced in the bioreactor with 250 mL of sterile must medium. The inlet tank was replaced by a 1 L tank containing sterile must, and the outlet tank by a new sterile 1 L empty tank (Figure 1). The temperature was set up at  $26 \pm 1$  °C, and the continuous recirculation at 3 mL/min was initialised with the Masterflex® peristaltic pump. Sugars and L-malic acid conversion were monitored, and the continuous flow was started once the L-malic acid concentration was less than or equal to 1 g/L, after an initial batch phase. Continuous feed flow rate was then adjusted to reach a stable regime with L-malic acid concentrations below 1 g/L. Corresponding RT were calculated. Fermentation monitoring and analyses at the end of assays were run the same way as previously described.

Two assays were run in parallel and named co-ferm A and co-ferm B. Membranes were discarded after three weeks of continuous fermentation.

### 6. Successive batch malolactic fermentation

The biofilm continuous formation protocol was the same as described above, with *O. oeni*, except for the number of supports: two series of 21 membranes were placed in the bioreactor before sterilisation. After the 10 days of biofilm formation, the MRSmin medium was discarded, and the membranes were rinsed and sampled for cell counting (one membrane per series). Both series of 20 membranes were then transferred to two glass bottles, each containing 500 mL of sterile wine medium. Bottles were placed at room temperature (22 to 25 °C) and the malolactic reaction was monitored.

Once the L-malic acid concentration was less than or equal to 1 g/L, membranes were transferred to two new 500 mL bottles containing sterile wine medium. The operation was repeated for four successive batches. Planktonic phases during fermentations were sampled for population and MLF monitoring. One membrane was sampled at the end of each batch. The membrane was rinsed with SDW and processed for cell number determination.

### 7. Cell counting method

Planktonic populations were sampled directly in the bioreactor or in the bottles for the successive batch experiment under BSC after homogenization, or via the sampling connector on the recirculation loop during biofilm formation and fermentations. 1 mL was transferred to 9 mL SDW.

For sessile populations counting, one membrane, previously rinsed with sterile distilled water, was transferred to a glass tube containing 5 mL sterile distilled water. The system was placed in an ultrasonic bath (USC300T, VWR, Radnor, USA) for 1 min at 45 Hz and then vortexed at maximal power for 30 s to free membrane-associated cells. 1 mL of the tube content was sampled and transferred to 9 mL SDW. For sessile cells, results are expressed per membrane surface in log (CFU/cm<sup>2</sup>). One membrane's surface is 9.82 cm<sup>2</sup> (recto verso).

In both configurations, CFU were estimated by culturing appropriate dilutions (successive 1/10 dilutions prepared in SDW) on solid MRSm at 28 °C for *O. oeni* and on solid YPD at 30 °C for *S. cerevisiae*.

When both microorganisms were present, two plate series were realised. Amphotericin B (Sigma) was added to count *O. oeni* to prevent the development of the yeast.

## 8. Malolactic fermentation monitoring

Malolactic fermentation monitoring was performed according to the manufacturer's instructions using the "K-LMAL" kit from Megazyme® (Libios, Vindry-sur-Turdine). Measures were realised with samples taken on the recirculation loop with a sterile syringe ("planktonic phase sampling", Figure 1).

## 9. Acetic acid, glucose + fructose and ABV % monitoring

Acetic acid (g/L), sugars (glucose + fructose) (g/L) and ethanol (Alcohol by Volume (ABV %)) concentrations were monitored thanks to a calibrated ATR alpha® (Bruker, Billerica, USA), based on FTIR technology.

## 10. Scanning electron microscopy

Cells were fixed on the rinsed membranes by a solution of glutaraldehyde (Sigma)/phosphate buffer (KH<sub>2</sub>PO<sub>4</sub> (Sigma), K<sub>2</sub>HPO<sub>4</sub> (Sigma), 0.4M), 30 min at room temperature. The membranes were then washed twice with a sucrose (Sigma)/phosphate buffer solution for 15 min at room temperature. Dehydration was performed by successive immersions in solutions of increasing concentrations of absolute ethanol (VWR, Radnor, USA), then of hexamethyldisilazane (HMDS) (Sigma). After the last bath in pure HMDS, samples were left under the chemical hood for 24 to 48 h to air-dry.

Dried samples were then pasted on SEM-dedicated stainless-steel supports, coated with gold for 80 s, and observed with a JEOL JSM 7100F TTLS® scanning electron microscope (Jeol Ltd., Tokyo, Japan). SEM was performed at 15 kV.

# RESULTS

## 1. Biofilm initial formation

Starting from a 7.8 log (CFU/mL) suspension of the Vitilactic F® *O. oeni* strain in the bioreactor, three assays were run (one for MLF alone (MLF) and two in parallel for co-fermentations (co-ferm A and co-ferm B)). After 10 days of biofilm formation at a continuous flow with 35 h RT, 7.5 ± 0.16 log (CFU/mL) (*n* = 3) were obtained for the planktonic phases in the bioreactors. The number of the living cells on the membranes was 7.0 ± 0.02 log (CFU/cm<sup>2</sup>) (*n* = 3),

showing that the bacteria colonised the supports placed in the bioreactor. Membranes SEM observations for *O. oeni* before MLF (Figure 3) confirmed these results.

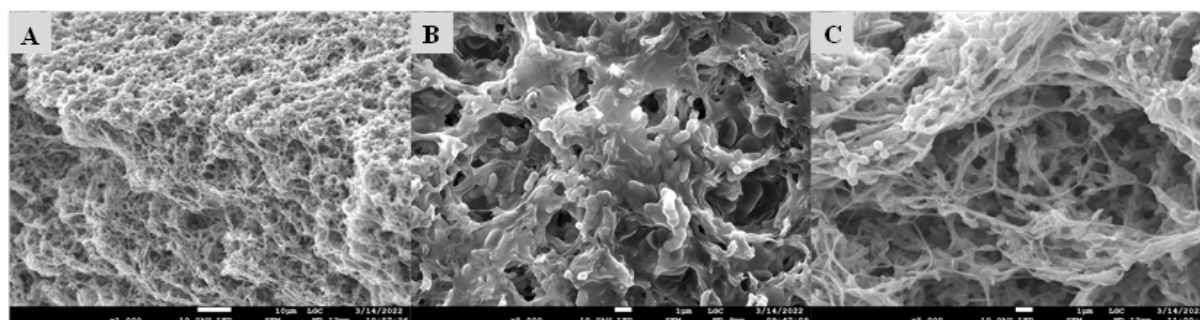
Membrane surface was colonised by *O. oeni* cells, and a substance, potentially extracellular matrix, could be observed (Figure 3B). As shown on membrane edge pictures (Figure 3C), cells also penetrated inside the membrane (total depth 175 µm), colonising the whole support. For co-ferm assays, yeasts CFU adhered to membranes after five days were also counted with a medium value of 5.2 ± 0.50 log (CFU/cm<sup>2</sup>) (*n* = 2). In the planktonic phase, 6.8 ± 0.01 log (CFU/mL) were numerated (*n* = 2). SEM observations for *S. cerevisiae* also confirmed these results, but are not shown in this article.

## 2. Continuous fermentations

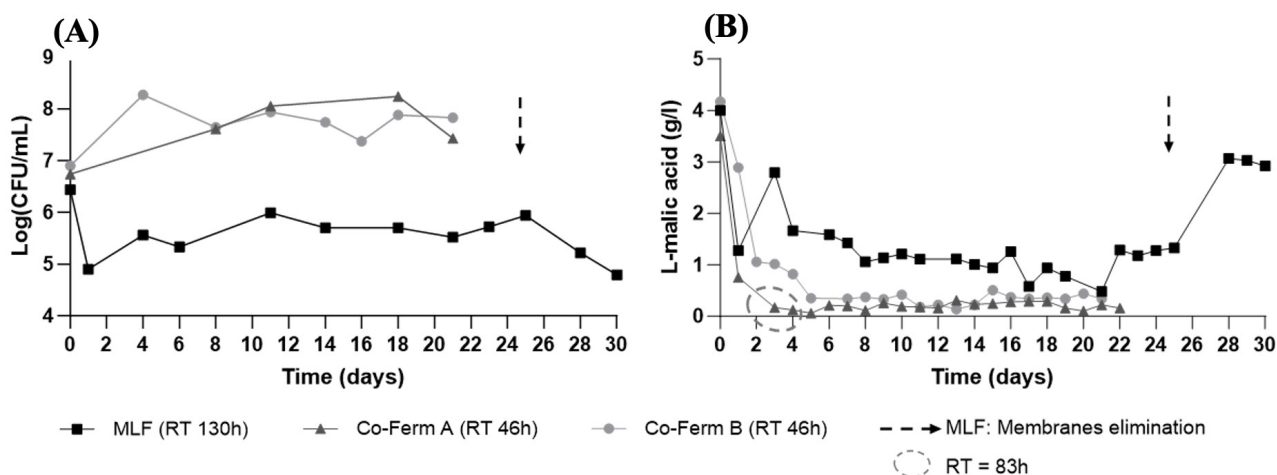
At the end of the biofilm formation phases, the MRSmin medium was replaced by the fermentation media (must for co-ferm and wine for MLF) after membrane rinsing. For co-ferm assays, yeast and bacteria biofilm supports were first transferred together in the same bioreactor. The temperature was set at 22 °C for MLF and co-ferm. For the three assays, CFU (Figure 4A) as well as L-malic acid concentrations (Figure 4B) were measured in the planktonic phase. Acetic acid concentrations are not discussed in this paper since values were stable and below 0.6 g H<sub>2</sub>SO<sub>4</sub>/L for all assays.

For the three assays, CFU on day 0 corresponds to the CFU numbered at the end of biofilm formation on 20 membranes, converted to log CFU/mL based on the bioreactor volume (250 mL). When starting the continuous flow fermentation, the feed pump flow rate was set up after 24 to 48 h of the batch phase when the L-malic acid concentration fell below 1 g/L. It was then adjusted to set up a stable regime with L-malic acid concentration below 1 g/L. During the MLF assay, the batch phase was 48 h in wine before switching to continuous flow. Membranes were discarded on day 24, but the fermentation was continued for six days. The co-ferm assays lasted 22 days after starting 24 h (co-ferm A) or 48 h (co-ferm B) batch phases in must before switching to continuous flow.

During MLF assay, medium RT was 130 h whereas it was 46 h during co-ferm assays. For co-ferm A, RT was first



**FIGURE 3.** *O. oeni* cells adhered on Nylon® membrane after 10 days of biofilm formation under continuous flow in MRSmin. A: View of a membrane, top surface and edge (×1,000). B: Cells on the membrane (×5,000). C: Cells in membrane edge (×5,000).



**FIGURE 4.** Evolution of (A) CFU (log/mL) of *O. oeni* cells in the planktonic phase, (B) L-malic acid (g/L) concentration, in the bioreactor during malolactic fermentation.

set up at 83 h from D2 to D4 and then decreased to 46 h (Figure 4B). During both co-ferm assays, AF (fermentable sugars concentration and ABV %) was also monitored: medium ABV % was stable at  $10.0 \pm 0.7$  % from D3 to the end of AF.

In these conditions, CFU values for *O. oeni* were stable for all three trials during the fermentations. However, value trends were different depending on the tested configuration. In wine (ABV 12 %, pH 3.4, no presence of yeasts), cell counts for MLF trial were stable during the continuous flow phase (D3 to D25), in the presence of membranes, with a medium value of  $5.7 \pm 0.20$  log (CFU/mL). In the co-fermentation configuration (ABV 10 %, pH 3.4, presence of yeasts), cell concentrations during continuous steady state were higher with medium values of  $7.8 \pm 0.33$  log (CFU/mL) for A (D2 to D21) and  $7.8 \pm 0.26$  for B (D3 to D21). L-malic acid concentration decreased instantly during the batch phase for the three assays, showing that bacteria were able to adapt quickly to the must or wine media, with a sufficient concentration of active cells to start substrate conversion. When switching to continuous flow, L-malic acid concentration got stable with  $1.2 \pm 0.46$  g/L for MLF trial (from day 3 to day 25 at RT 130 h). The corresponding L-malic acid conversion speed was  $0.53$  g/L/24 h. In the co-fermentation configuration, the residence time was set at 46 h: concentrations were stable and below  $0.40$  g/L after three days for A and five days for B. The calculated L-malic acid conversion speeds were  $1.7$  g/L/24 h for A and  $2.0$  g/L/24 h for B. An interesting fact was the impact of membrane removal at D25 for the MLF trial: without the sessile phase at the same flow rate, CFU tended to decrease (from  $5.9$  to  $4.8$  log (CFU/mL)), and L-malic acid concentration increased, reaching a medium concentration of  $3.0 \pm 0.06$  g/L between D28 and D30. These results confirmed the implication of *O. oeni* biofilm in the fermentation process. At the end of the fermentations (D25 for MLF and D21 for A and B), two membranes were sampled for cell counting and SEM (Figure 5).

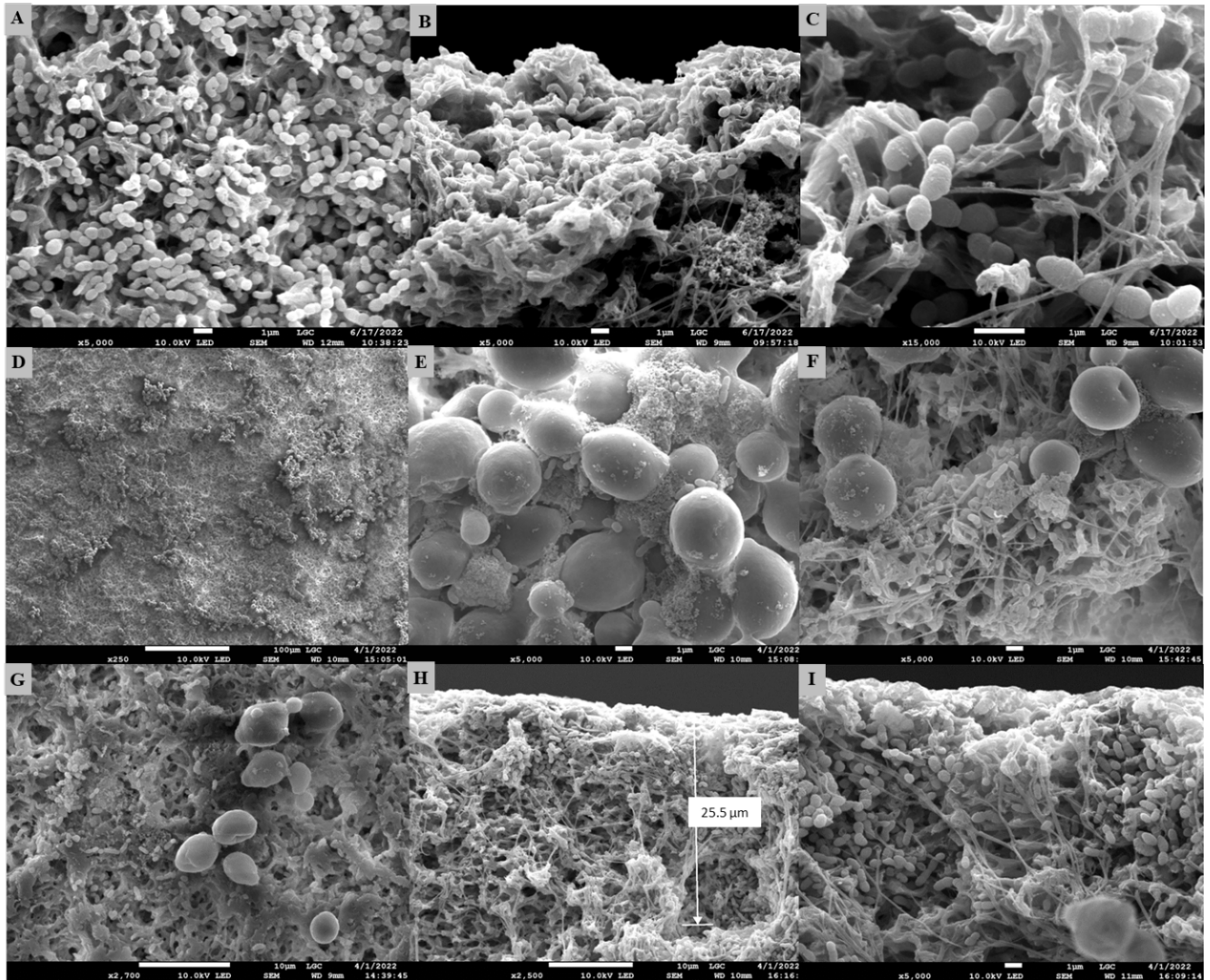
After 25 days in the bioreactor in the presence of wine media (MLF trial), *O. oeni* cells were present both on the top and

in the depth of the membranes. Bacteria formed chains, their typical multiplication mode, showing that cells were active (Figure 5, A to C). Regarding co-fermentation trials, two supports with 20 membranes were placed in the bioreactor at the beginning of fermentations: one support with *S. cerevisiae* cells only, and the other one with *O. oeni* only; trends were slightly different depending on the support. In the case of the initial adhesion of *S. cerevisiae* (Figures 5D to 5F), yeasts colonised the whole surface of the membrane. However, they are too large to penetrate it. For the *S. cerevisiae* membrane, bacteria were found both on the top and in the membrane depth, but in moderate quantities. Some of them were visible on yeasts (Figure 5E). When *O. oeni* colonised the membranes before co-fermentations (Figures 5G to 5I), an important number of cells could be observed, more in membrane depth (up to  $25.5$   $\mu\text{m}$ , Figure 5H) than on its surface. Yeasts were less numerous in this configuration. These observations were confirmed by the membrane cell counts performed at the end of fermentations for both microorganisms (Figure 6): cell counts were higher when initial biofilm formation for the concerned microorganism was realised on the numbered membrane (average difference of  $+ 1.0$  log for *O. oeni* and  $+ 0.32$  log for *S. cerevisiae*).

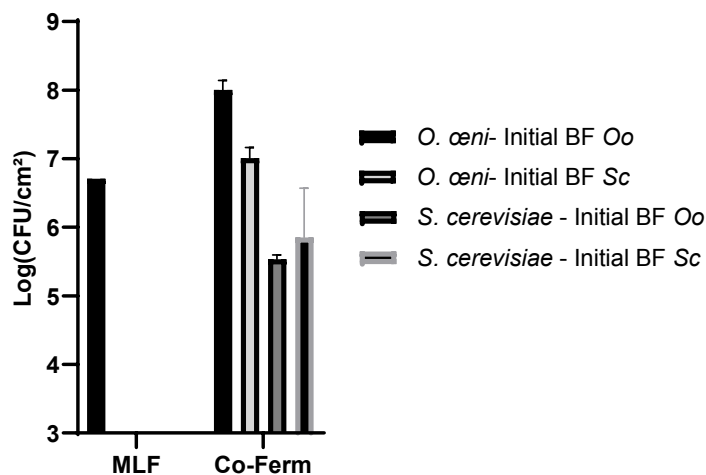
### 3. Successive batches for malolactic fermentation

In this trial, adhesion and the initial biofilm formation phase were conducted with the continuous flow protocol in MRSmin pH 3.8, with two supports of 21 membranes in the bioreactor. At the end of the 10 days at RT = 35 h,  $6.5$  log (CFU/cm<sup>2</sup>) of *O. oeni* cells were numbered on the membranes, corresponding to  $6.1$  log (CFU/mL) in each of the two bottles containing 500 mL of wine medium, used for the MLF.

When MLF started, some of the cells from the sessile phase were released into the wine medium (for example,  $5.0$  log (CFU/mL) in the wine medium for batch 1 after one day). Then, both planktonic and sessile populations were active since CFU numbered in the wine media increased during



**FIGURE 5.** Scanning Electron Microscopy (SEM) of a membrane at the end of fermentations. A to C, MLF: membrane top surface at  $\times 5,000$  (A); membrane edge at  $\times 5,000$  (B) and  $\times 15,000$  (C). D to F, co-ferm after initial colonisation by *S. cerevisiae*: membrane top surface at  $\times 250$  (D) and  $\times 5000$  (E); membrane edge at  $\times 3500$  (F). G to I, co-ferm after initial colonisation by *O. oeni*: membrane top surface at  $\times 2500$  (G); membrane edge at  $\times 2500$  (H) and  $\times 5000$  (I).



**FIGURE 6.** CFU counts (UFC/cm<sup>2</sup>) for *S. cerevisiae* and *O. oeni* on membranes at the end of fermentations. With initial *O. oeni* biofilm formation (initial BF Oo) or of *S. cerevisiae* (initial BF Sc). Co-ferm:  $n = 2 \pm \text{STD}$ , MLF:  $n = 1$ .

each batch, and cell counts on the membranes were quite stable (Figure 7). An interesting fact for batch 2 was the concentration obtained in the planktonic phase (5.8 log (CFU/mL)) three days after the support transfer from batch 2 to batch 3: this concentration was 1 log less than the last measurement realised with the membranes, underlining the role of biofilms in maintaining an active biomass reserve.

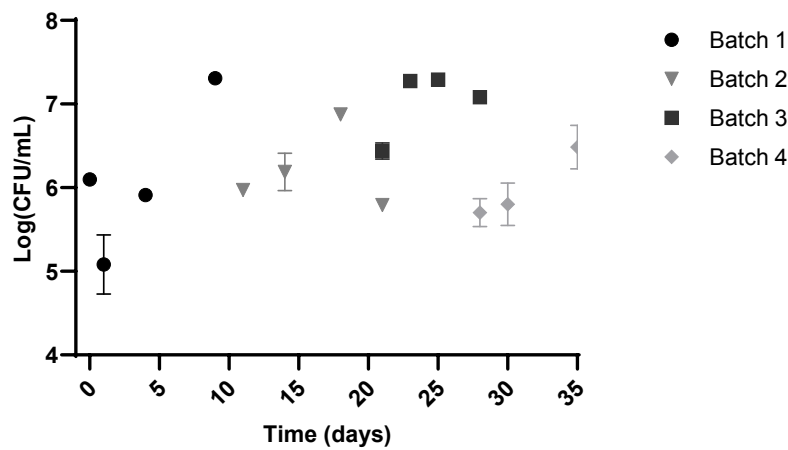
L-malic acid was consumed for batches 1, 2 and 3 in less than 10 days (Figure 8). For batch 4, the starting concentration was exceptionally higher (4.8 g/L), and the assay was stopped before the end of MLF: 2.2 g/L were converted in seven days for this batch. For batches 1 and 2, the reaction continued after the membranes were removed from the bottles, thanks to the active cells present in the wine medium. Final measured L-malic acid concentrations were below 0.5 g/L for batches 1 to 3, indicating that MLF was almost completed in treated wines. Despite slight differences, conversion speeds are close for all four batches (Table 1). These results show that the malolactic activity of sessile cells on the membranes can

be maintained during at least four successive batches, thanks to a stable reservoir of active cells.

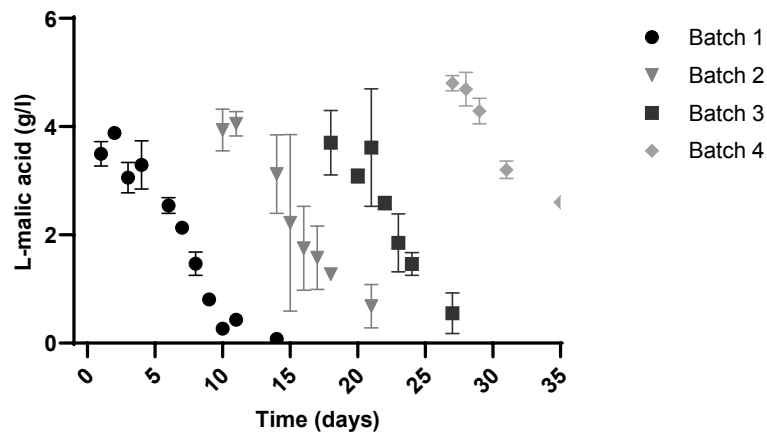
## DISCUSSION

Controlling MLF progress time is a challenge for entry-level market red wines. Continuous fermentation processes could be an interesting option for winemakers to reduce fermentation tank capacities needs, and process times, including after-harvest treatments, thanks to continuous thermovinification technologies.

In the present study, *O. oeni* biofilm formation was achieved after 10 days in a continuous flow bioreactor, in a nutrient-depleted medium on Nylon® membranes. Previous work showed the ability of the bacterium to form mature biofilms on oak chips and stainless steel coupons in an MRSm-broth (Bastard *et al.*, 2016). After two weeks, the researchers obtained concentrations of 6.3 log (CFU/cm<sup>2</sup>) on stainless steel coupons and 8.0 log (CFU/cm<sup>2</sup>) on oak chips with the *O. oeni* ATCC BAA-1163 strain. Using a commercial strain



**FIGURE 7.** Evolution of *O. oeni* CFU/mL in the planktonic phase during four successive batches in wines. Time 0 values for each batch correspond to CFU numbered on the membranes at the end of biofilm formation phase for batch 1, and previous batch for batches 2, 3 and 4, converted in log (CFU/mL of wine medium) in the bioreactor ( $n = 2 \pm \text{STD}$ ).



**FIGURE 8.** Evolution of L-malic acid concentrations (g/L) in the planktonic phase of 500 mL bottles during four successive batches ( $n = 2 \pm \text{STD}$ ).

Batch	L-malic acid conversion speed (g/L/24 h)
Batch 1	0.35 ± 0.0059
Batch 2	0.34 ± 0.0041
Batch 3	0.38 ± 0.058
Batch 4	0.34 ± 0.017

**TABLE 1.** L-malic acid average conversion speeds (g/L/24 h), calculated from linear regression equations, during the MLF of four successive batches ( $n = 2 \pm \text{STD}$ ).

(Vitalactic F<sup>®</sup>) in the described conditions, an intermediary sessile population of around 7 log (CFU/cm<sup>2</sup>) could be formed in the present work. This result demonstrates the ability of the Vitalactic F<sup>®</sup> strain to form biofilm. Moreover, this concentration is higher than 10<sup>6</sup> CFU/mL, considered to be the threshold that must be exceeded for the MLF reaction to start (Ribéreau-Gayon *et al.*, 2020).

After immersion in the must (co-ferm) or wine (MLF) fermentation media, some sessile cells were released from the carriers. During the MLF assay, 5.0 log (CFU/mL) were numbered in the planktonic phase of the bioreactor 24 h after the wine medium addition, starting from 6.9 log (CFU/mL). This phenomenon was also described by Bastard *et al.* (Bastard *et al.*, 2016), where 6.0 log (CFU/mL) were numbered in the planktonic phase during the first days of MLF, following the immersion in wine of inoculated oak chips with a 14-day *O. oeni* biofilm (initial concentration 7.7 log (CFU/mL)).

However, the stress provided by the continuous renewal of the nutrient-depleted MRSmin medium seemed to lead to active bacterial populations, adapted to the wine environment (Eze & El Zowalaty, 2019; Kim *et al.*, 2013; Petrova & Sauer, 2012; Rochex *et al.*, 2008; Soumbo, 2019). MRSmin medium was adjusted to pH 3.8, which could also contribute to microbial adaptation to the acidic must and wine media. Indeed, a previous study has highlighted that reducing the pH of a growth medium for *O. oeni* induced a higher resistance of the bacteria to ethanol presence when transferred into wine, and was favourable to MLF completion (Beltramo *et al.*, 2006).

Cell counts illustrate this adaptation of the sessile cells to the must and wine fermentation media. Indeed, for the three continuous assays, planktonic cell counts during fermentations were stable, with a higher average value for the co-ferm assays (7.8 log (CFU/mL)) than for the MLF assay (5.7 log (CFU/mL)). Successive batch trial trends are in between (average value of 6.5 log (CFU/mL)): this could be due to the experimental model, since the continuous flow induces the removal of a portion of the planktonic cells. Medium ABV % for co-ferm assays was 10 % compared to 12 % during MLF assay: it is known that AF by yeasts (ethanol production, yeast cellular proteins, nutrients

availability...) can influence *O. oeni* biomass and therefore, malolactic fermentation kinetics (Capozzi *et al.*, 2019; Lonvaud-Funel *et al.*, 2021), which could partly explain these results. A strategy to counteract this phenomenon is to co-inoculate the species in grape must. In this way, the bacteria can adapt to the progressively increasing unfavourable factors (Capozzi *et al.*, 2019; Lonvaud-Funel *et al.*, 2021) as underlined here by the co-ferm assays results. In a recent study, Palud *et al.* (2024) focused on mixed species biofilms of *S. cerevisiae* and *O. oeni* and demonstrated the possibility of completing the fermentation of a commercial grape juice (150 mL) from the co-inoculation of the biofilms (starting concentrations in juice: *O. oeni* 6.7 and *S. cerevisiae* 5.4 log (CFU/mL)) developed on oak chips. The authors highlight the fermentation performance of the mixed-species biofilms as the microbial cells were able to complete AF and MLF in 13 days, while remaining substrates were still present in the planktonic inoculation configuration (Palud *et al.*, 2024). Fermentation performances of *O. oeni* or *L. plantarum* biofilms were also demonstrated in previous studies (Bastard *et al.*, 2016; Pannella *et al.*, 2020) in batch systems. Recently, Tofalo *et al.* (2021) also showed that detached cells from *O. oeni* biofilms had a better L-malic acid conversion rate compared to the tested planktonic cells. When biofilm properties are studied, the role of the biofilm phenotype, such as extracellular matrix production as well as overexpression of stress proteins, is pointed out (Bastard *et al.*, 2016; Palud *et al.*, 2024).

In the present work, SEM observations suggest the presence of an extracellular matrix; cell counts support the good resistance of bacteria to wine stress. Moreover, biofilm fermentation performances are confirmed by L-malic acid kinetics. Average L-malic acid consumption speed was 0.53 g/L/24 h during 25 days for the MLF assay. In the co-ferm assays, the L-malic acid consumption kinetic reached a stable value of 2.0 g/L/24 h for three weeks, *i.e.* 4 g/L of L-malic acid converted in 48 h. MLF is generally considered completed when residual L-malic acid concentration is below 0.2 g/L (Lonvaud-Funel *et al.*, 2021; Ribéreau-Gayon *et al.*, 2020). In wine for the MLF trial, a stable concentration of around 1 g/L of L-malic acid was measured in the bioreactor, but the planktonic bacteria (medium value of 5.7 ± 0.20 log (CFU/mL)) could ensure the completion of the reaction in the treated

wine. In the co-ferm configuration, L-malic acid conversion was almost complete (less than 0.4 g/L in the fermented product): the reaction should end quickly thanks to the active planktonic cells ( $7.8 \pm 0.33 \log$  (CFU/mL)). The presence of a threshold quantity of L-malic acid could contribute to maintaining the active biomass required for the continuous regime; indeed, it is accepted that the malolactic activity of lactic acid bacteria increases with the concentration of the reaction substrate (Lonvaud-Funel *et al.*, 2021; Ribéreau-Gayon *et al.*, 2020).

Among the very few studies focused on continuous MLF from *O. oeni* immobilised on various carriers, the use of a biofilm bioreactor for continuous MLF was described in the PhD work of D. Janssen (Janssen, 1991). In this work, *O. oeni* biofilms on oak chips allowed L-malic acid consumption rates two to three times higher than during a batch reaction from planktonic cells, at a scale of 10 L of white wine and over three weeks. This preliminary study validated the hypothesis of a potential acceleration of the reaction under a continuous regime, although the MLF was incomplete. In another study, with *O. oeni* immobilised on grape skins in a 1.5 L packed bed bioreactor, 67 % of the 4 g/L of L-malic acid present in a wine (pH 3.1, ABV 12.2 %) was converted with an RT of 26 h and a conversion speed of 2.5 g/L/24 h (Genisheva *et al.*, 2014). The steady regime time is not specified in this study, nor are the cell counts, which compromise a precise analysis. However, the authors point out the influence of the wine parameters since, in the same conditions, but with an ABV of 12.9 % and a pH of 2.9, only 0.8 % of the L-malic acid was consumed. The impact of physicochemical parameters on MLF kinetics was previously described in the literature with an important inhibition of lactic acid bacteria cells growth when pH is below 3.0 (Ribéreau-Gayon *et al.*, 2020).

To our knowledge, no process has been described with *S. cerevisiae* and *O. oeni* co-immobilised in the same bioreactor for continuous AF and MLF. However, results for successive batch configurations for MLF reaction are reported in the literature. Thus, in the present study, and for four consecutive batches, the bacteria were able to convert 0.35 g/L/24 h, meaning that for each batch, eight days would be enough to consume 3 g/L of L-malic acid. When setting batch duration to 24 h, Maicas *et al.* (2001) obtained a conversion rate of 95 % for the first batch (starting *O. oeni* M42 cell concentration: 7.7 log (cell/mL)), and of 50 % for the three following batches. This experiment was run at a 100 mL scale, in a wine (ABV 11 %, pH 3.5; L-malic acid 3.5 g/L) and with a cellulose-based sponge as immobilisation carrier. In a similar assay, an average of 54 % of the 2.11 g/L of L-malic acid present in 400 mL of a wine media (ABV 12 %, pH 3.5) was converted within three days over 11 successive batches following the immobilisation of *O. oeni* ATCC 23279 on delignified cellulose (Agouridis *et al.*, 2008). Finally, over seven consecutive 17-day batches, the tests carried out by Genisheva *et al.* (2013) using *O. oeni* Uvaferm<sup>®</sup> Alpha (Lallemand) immobilised on grape skins enabled the conversion of between 33 % and 87 % of the 3.5 g/L of L-malic acid initially present in a white wine to be fermented

(ABV and pH unknown). The biomass is not characterised in these studies, and experimental designs differ (strain, fermentation medium parameters, etc.), but regarding L-malic acid conversion, our results are encouraging when compared with those reported in the literature. They could lead to the implementation of MLF or co-fermentation by *O. oeni* biofilm in continuous or successive batch systems in winemaking.

Whether in successive batches or in continuous trials, the importance of biofilms for the results presented in this manuscript can be emphasised. Indeed, when the supports are removed from the bioreactor, or from the 500 mL bottles in the case of batches, planktonic CFUs progressively decrease, as do L-malic acid consumption rates. Biofilms thus constitute a stable reservoir of rapidly active and cultivable biomass, a major contribution to the results obtained in this work. During the fermentations for the co-ferm assay, it was also highlighted that yeast and bacterial cells could colonise the Nylon<sup>®</sup> membranes on which biofilms of the other species were initially present. Moreover, for the three experimental systems, a part of the sessile cells was detached from the supports into the fermentation media, but then, cell counts support the hypothesis of cell proliferation in sessile form, and probably in the planktonic one. However, population dynamics are unknown, and the role of sessile and planktonic subpopulations during fermentation deserves further investigation.

The results support the interest of an approach involving biofilms for the completion of continuous MLF applied to industrial systems. The repetition of the assays varying fermentation media parameters (pH, ABV %) would be an interesting perspective before considering a potential scale-up. Also, the wine quality could be evaluated using additional chemical (volatiles, organic acids, etc.) and sensory analyses. Moreover, various strains are available for fermentations in the wine industry. They are selected according to technological properties such as aroma production, ethanol or pH tolerance. On the other hand, genotypes differ between strains regarding biofilm formation or fermentation capacities. In a study from Bastard *et al.* (2016), L-malic acid conversion rates were different after the inoculation in the same conditions of two *O. oeni* strain biofilms (ATCC BAA-1163 and Sabo 11). Other researchers studied the extracellular matrix synthesis by various *O. oeni* strains and showed that matrix properties and concentration differed depending on the tested strains and growth media (Dimopoulou *et al.*, 2012; Dimopoulou *et al.*, 2014). Finally, the existence of interactions between *S. cerevisiae* and *O. oeni* is well known (Albergaria & Arneborg, 2016; Petrucci *et al.*, 2017), and when testing other strains, the impact of these interactions would be of interest.

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