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REVIEW ARTICLE

Microbial biofilms in oenology

Marianne Gosset[1,2], Christine Roques[1] and Patricia Taillandier[1*]

[1]LGC, Laboratoire de Génie Chimique, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France
[2]AB7 Industries, Chemin des Monges, BP9, 31450 Deyme, France

*correspondence:
patricia.taillandier@ensiacet.fr

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ABSTRACT

Biofilms are sessile microbial communities whose lifestyle confers specific properties. In recent years, they have attracted great interest in many research fields. Biofilms are indeed the cause of recurrent industrial problems, while microbial contaminations can finally impact the quality of a finished product or human health. However, these same properties can be interesting for several applications. Oenology is no exception, whether for potential damages to wine quality caused by biofilms or their beneficial effects through winemaking steps, from grape to bottle. This paper reviews yeast and bacterial biofilms in oenology, their nature, their negative effects, as well as the applications linked to their presence in wine and the mechanisms involved in their formation.

KEYWORDS: Biofilms, yeast, bacteria, wine, velum flor, extracellular matrix, adhesins

INTRODUCTION

Biofilms are defined by Costerton *et al*. (1999) as "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface" (Costerton *et al*., 1999). Several definitions have been given since, but most authors agree on three essential elements for the existence and development of a biofilm: microbes, extracellular polymeric matrix and surface (Dunne, 2002). Biofilm formation is a complex process which can be summarised in four steps: the first step of cell attachment or adhesion to a surface, a growth phase during which microcolonies are formed, a maturation phase with the production of a protective extracellular matrix (ECM) and a fourth step called dispersion with microbial cells being removed from the biofilm (Achinas *et al*., 2019; Costerton, 1999; Flemming and Wingender, 2010; O'Toole *et al*., 2000). The genomic expression profile and physiology of a microorganism organised in a biofilm differ from those of the same microorganism in its planktonic form. It does confer better protection to biofilms against unfavourable environmental conditions, including antimicrobial treatments and changes in metabolic pathways. Biofilms are thus the most frequent and efficient defensive way of life for microorganisms. In hostile environments (temperature, pH, etc.), where individual cells could be easily killed, the sessile mode of growth is inherently safest (Stoodley *et al*., 2002). Agro-industry has a strong interest in both the negative and positive effects of biofilms. On the one hand, the challenge of controlling microbial contaminants organised in biofilms is the subject of several studies (Achinas *et al*., 2019; Kumar and Anand, 1998). On the other hand, biofilms are natural forms of cell immobilisation that can be utilised in bioreactors, their related properties being used for maintaining the metabolic activity of microorganisms of interest in stressful environments and under continuous processes for various applications (Cheng *et al*., 2010; Germec *et al*., 2015).

Immobilisation is defined as the physical confinement of intact cells to a region of space with conservation of biological activity (Moreno-García *et al*., 2018a). Microorganism immobilisation techniques have been used in the agro-industry since the 1970s. These processes enable an accumulation of biomass and thus increase cell densities (Kourkoutas *et al*., 2004; Moreno-García *et al*., 2018a; Nedović *et al*., 2015). In the food industry especially, immobilisation facilitates continuous production systems and limits the need for separation techniques, such as centrifugation or filtration, which are usually necessary to remove the biomass (Verstrepen and Klis, 2006). Immobilisation techniques can be differentiated based on two principles: (1) the attachment of cells via chemical and physical bonds, either on solid surfaces or by interactions between cells, and (2) the trapping of cells within a support, the latter constituting a barrier limiting the development of microorganisms in suspension in the medium (Genisheva *et al*., 2014a; Kourkoutas *et al*., 2004). Immobilized microorganisms offer many advantages for industrial processes in the production of alcoholic beverages (García-Martínez *et al*., 2015; Groboillot *et al*., 1994; Moreno-García *et al*., 2018a; Nedović *et al*., 2015; Puig-Pujol *et al*., 2013). The use of continuous processes based on immobilisation methods is also described for the production of acetic acid or vinegar (de Ory *et al*., 2004), as well as for processes involving lactic acid bacteria such as yoghurt or cheese production (Champagne *et al*., 1994).

In the field of oenology, microbial populations are part of a complex system where every species interact with others and the environment. Microbial diversity in grapes is high, including fungi, yeasts and bacteria. Some of these microorganisms, called the wine microbial consortium, can survive and grow in wines. This consortium includes yeast species, lactic acid bacteria and acetic acid bacteria (Barata *et al*., 2012). Among them, some species are of oenological interest contributing to the sought organoleptic properties of the final product. Undesirable species responsible for potential defects are also present and need to be controlled, either preventively by optimising the different stages of the winemaking process to avoid their multiplication or curatively with various types of antimicrobial treatments (Lonvaud-Funel *et al*., 2021). The microbial ecosystem from grape to wine evolves spontaneously and continuously during the winemaking process in relation to its technological characteristics (environmental parameters associated with the actions taken by the winemaker) and the interactions between the different present species (Renouf, 2006). On the first side, the winemaker has to make choices at each stage (inoculation for the alcoholic fermentation, for the malolactic fermentation, method of protection of musts and wines, characteristics of maturation…), leading to the predominance of certain species. Naturally, the most resistant species will be selected along the process with increasing stressful conditions, i.e., ethanol concentrations rising to around 12 % combined with an acidic pH (3.0 to 3.8) (Renouf, 2006). On the other side, the biochemical activities of the present microorganisms contribute to the interactions responsible for the wine microbial consortium balance, finally modulating wine aroma and flavour (Fleet, 2003). These interactions between some species have been widely studied using various methods (Albergaria and Arneborg, 2016; Alexandre *et al*., 2004, Petruzzi *et al*., 2017), but economic players in the oenology sector are working to understand this ecosystem better.

The complex environment of wine is therefore not immune to the opportunities and problems described in relation to biofilms. Thus, microbial biofilms in wine have been the subject of several studies since the 1990s, as they can present interests or disadvantages for the winemaker. As summarised in Figure 1, this review aims to look at microbial (bacterial or fungal) biofilms in wine, their nature, as well as the mechanisms of formation and regulation described, and whether they are of oenological interest (positive biofilms) or a potential source of wine defects or wineries contamination (negative biofilms).

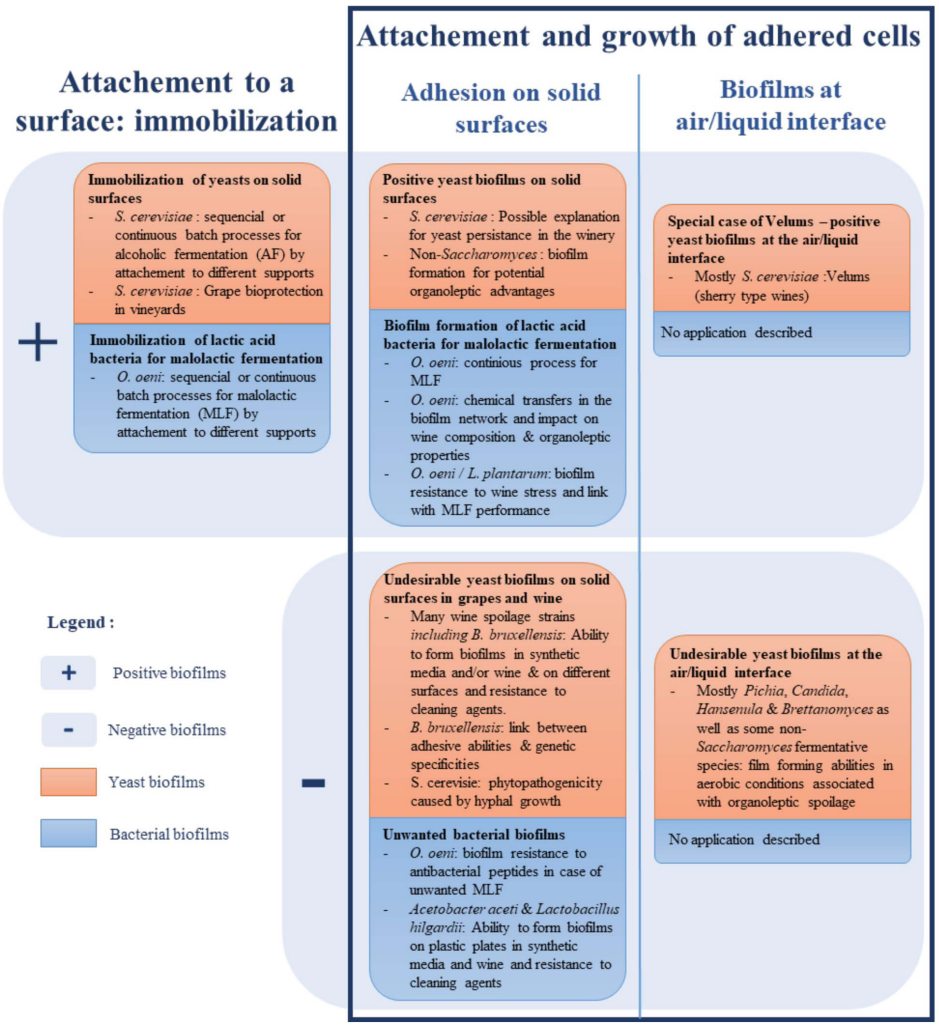

FIGURE 1. Microbial biofilms in oenology. Biofilms are classified here depending on the type of adhesion (immobilisation by attachment or claimed biofilm) the microorganism involved (yeasts or bacteria), the type of adhesion surface (liquid or solid) and the impact on wine properties (Personal diagram: detailed references for each application are available in the review body).

When it comes to validating the existence of a biofilm, there is a lack of consensus among the diversity of techniques used. Many methods are available and developed to characterise adhesion strength and extent, biofilm biomass and viability, or its matrix composition (Azeredo *et al*., 2017). These methods do not give the same information, and some can be quite imprecise. Wine biofilms are no exception, and as shown by the articles gathered here, a multidisciplinary approach appears to be needed.

YEAST BIOFILMS IN OENOLOGY

The first studies on yeast biofilms, in any application, were devoted to the human pathogens, such as *Candida* species (Douglas, 2003), and today, they still represent a large part of the literature dedicated to fungal biofilms. The first work demonstrating the ability of *S. cerevisiae* to form biofilms was published in 2001. The authors showed that biofilm formation, defined as the aggregation of microorganisms into multicellular structures capable of adhering to surfaces, is favoured in a low-glucose medium on plastic surfaces (Reynolds and Fink, 2001). *S. cerevisiae* also formed mat complexes on semi-solid medium, i.e. multicellular structures composed of a network of cables organised as a central hub from which emanate multiple radial spokes (Martineau *et al*., 2007; Reynolds and Fink, 2001). Biofilm or mat formation is made possible by a group of cell wall-related proteins called adhesins. They are considered specific for some bindings involving different biotic or abiotic surfaces, even if some of them are ubiquitous. Their expression is controlled by several regulatory pathways for which the knowledge is progressively increasing (Brückner and Mösch, 2012; Guo *et al*., 2000; Lipke, 2018; Verstrepen and Klis, 2006). In wine, different types of yeast biofilms and associated mechanisms are described. Fungal biofilms found in wine, as well as related species, will be detailed in the next paragraph; a focus will be made on

S. cerevisiae for the mechanisms involved as it is the most extensively studied.

1. Different types of yeast biofilms

1.1. Immobilisation by attachment on solid surfaces

Many studies report alcoholic fermentation systems based on the immobilisation of *S. cerevisiae* by attachment to solid surfaces. A wide variety of supports are tested, from winemaking by-products (grape stalks, seeds, skins), fruits, cereals, plastic polymers, ceramics, etc. Reactors (essentially fixed-bed type) produce ethanol in continuous or repeated batch processes. The performances of these devices are generally higher than those obtained via conventional inoculation, but implementation on an industrial scale is limited by practical difficulties, especially the compatibility of the supports with the regulations in force and the risks of contamination. The reviews from Genisheva *et al*. (2014b), Kourkoutas *et al*. (2004) and more recently Moreno-García *et al*. (2018a) summarise the works carried out and the results obtained when *S. cerevisiae* was immobilised by attachment to a surface for the alcoholic fermentation of grape must. Biofilm formation was not claimed in these studies, as the expected properties of the immobilised biomass are known fermentative capacities but not the concept of modified capabilities when *S. cerevisiae* was under a biofilm.

Recent applications aiming to reduce synthetic crop protection products allow the use of microorganisms as biocontrol agents. The objective is to colonise the environment to prevent certain pathogens from taking hold. The *Candida sake* CPA-1 strain was used to effectively control the development of *Botrytis cinerea*, a deleterious fungus for grape rots. Carbó *et al*. works led to the development of a dry yeast formulation, more resistant to stress conditions, allowing the formation of protective films on the grapes. The yeast was dried in the presence of biodegradable supports before being sprayed on the vines (Carbó *et al*., 2017; Carbó *et al*., 2019). This innovation could refer to biocontrol agents, immobilised on surfaces with no claim of growth under a biofilm, here protecting grapes and even musts. Their protection properties are conferred by the formulation and the spraying method.

1.2. Positive yeast biofilms on solid surfaces

From the grape to the bottle, the interest in yeast biofilms depends on the nature of the use of wine microorganisms. Here, we mean by 'positive', a biofilm that can have a beneficial effect on the sanitary quality of grapes, the fermentative management, and the organoleptic properties of the wine, but also a biofilm whose study allows a better understanding of the winery ecosystem regarding yeasts of oenological interest.

Concerning this point, the formation of *S. cerevisiae* biofilms on solid surfaces has been studied to explain yeast persistence in the vineyards as well as in the wineries. Sidari *et al*. (2014) evaluated the impact of polyphenols, antioxidant compounds that play an important role in the organoleptic properties of wine and are in variable quantities depending on the grape variety and the winemaking process, on the ability of *S. cerevisiae* strains to form biofilms on plastic surfaces, to grow as mat colonies, to invade media, and to display filamentous growth. The tests were carried out using strains isolated from wines in synthetic media containing various concentrations of carbon and nitrogen sources. Indeed, microscopic observations showed that the addition of polyphenols to these media induced different responses of yeast strains with respect to cellular morphologies and biofilm formation. The authors suggested a possible implication in the various persistence of strains in the microbial ecosystems of grapes and wine (Sidari *et al*., 2014). Tek *et al*. studied the ability of commercial strains of *S. cerevisiae* to form biofilms on grapes and on different plastic materials (polystyrene and polyvinyl chloride, the material used in pipes for transferring wines in the winery). They showed that the adhesion of yeasts to surfaces and the resulting formation of biofilms was significant, whether in a sugar-rich or sugar-depleted medium. Formed biofilms resulted from several ways of life (replicative or not) and cell growth, including invasive growth, budding, sporulation, or a mat-sectoring-like phenotype. On grape pulp, invasive growth was important and predominated over the other ways of life, whatever the strain, whereas on plastic, adhesion was strain-dependent. It suggests that commercial yeasts can colonise and survive in the winery and the vineyard environment. Consequently, the persistence of commercial strains may impact the indigenous microbial population in the winery, which constitutes a potentially troublesome phenomenon in the case of winemaking itineraries with spontaneous fermentation (Tek *et al*., 2018).

Although *S. cerevisia*e is the most widely used yeast for alcoholic fermentation in wine, several non-*Saccharomyces* yeasts, such as *Candida zemplinina,* also named *Starmarella bacillaris* (Russo *et al*., 2020), are being evaluated for various oenological interests (aroma production or positive organoleptic impact, bioprotection role against some microbial contaminants). Very recently, the ability of some *C. zemplinina* strains to adhere to polystyrene, wood and stainless steel surfaces in must has been evaluated by CFU counting (Perpetuini *et al*., 2021a). The sessile growth was strain and support dependent, with the highest cell quantities counted on oak chips and stainless steel coupons. On polystyrene plates, some sessile cells were counted, but growth was favoured at the planktonic state with a log more of CFU/ml counted. This work also focused on assessing the impact that biofilms formed on wood chips have on the fermentation kinetics and volatile profile of the Montepulciano d'Abruzzo wines studied. Differences in the amount of glycerol, ethanol and some esters were highlighted, suggesting a potential positive role of the different biofilms in the production of new wine profiles.

Few studies were conducted on biofilm formation on solid surfaces by yeasts of oenological interest. *S. cerevisiae* is considered a good candidate for studying yeast biofilms (Bojsen *et al*., 2012; Reynolds and Fink, 2001; Verstrepen and Klis, 2006), but no real interest was taken in the

alcoholic fermentation of grape by biofilms, perhaps because AF step is not limiting in current oenological practices. Nevertheless, the wide range of known species and metabolic pathways suggest an increasing interest in yeast biofilm regarding their resistance to stressful conditions and thus large potential applications.

1.3. Special case of Velums—positive yeast biofilms at the air–liquid interface

Sherry-type wines are white wines which can be dry or sweet, with organoleptic properties associated with the formation of a biofilm at the air–liquid interface by floating cells attached to each other (Martínez *et al*., 1995; Zara *et al*., 2005). Such biofilms are named velums and they prevent the oxidation of the wine during its ageing in barrels. It is formed at the end of alcoholic fermentation and ensures that the yeasts have access to oxygen and develop according to a specific respiratory metabolism in an a priori non-fermentable environment allowing the wines to acquire their typical oxidative aromas. Spanish sherry produced in the Xeres and Montilla-Moriles regions is the most studied sherry-type wine to date. Other wines that use this technique: in France, Vin Jaune is produced in the Jura area; in Hungary, Szamorodni wines belonging to the Tokay family; in Italy, Vernaccia di Oristano is made in Sardinia. The yeast involved in velums is mainly *S. cerevisiae*, although the presence of several other species has also been described, such as *Brettanomyces bruxellensis*, isolated from Spanish sherry (Esteve-Zarzoso *et al*., 2001; Ibeas *et al*., 1996) as well as *Pichia* sp., *Hansenula* sp. and *Candida* sp. (Lepe and Leal, 2004). Velums and their characteristics have been reviewed several times from different angles (Alexandre, 2013; Benítez *et al*., 2011; Legras *et al*., 2016; Pozo-Bayón and Moreno-Arribas, 2011). Benítez *et al*. focus on sherry, its manufacturing process and chemical composition (Benítez *et al*., 2011), whereas the review proposed by Pozo-Bayón and Moreno-Arribas provides an extensive description of the role of the FLO11 gene in yeast biofilm formation involved in this ageing method (Pozo-Bayón and Moreno-Arribas, 2011). The work of Alexandre broadens the field to include other velum wines and sheds light on the ecology of the yeast flora encountered, as well as on its genetic and metabolic properties (Alexandre, 2013). Finally, based on recent studies, Legras *et al*. complete the elements that allow understanding of the metabolic properties of yeasts under this specific biofilm type compared to alcoholic fermentation. In this sense, it introduces the latest work carried out using proteomics and metabolomics. These techniques also allow us to elucidate the link with the organoleptic properties and deepen our knowledge of the aromatic molecules of velum wines (Legras *et al*., 2016).

By using molecular and physiological analysis techniques, monitoring of populations involved in the successive stages of sherry winemaking highlighted the evolution of the yeast flora as the technical itinerary progressed (Esteve-Zarzoso *et al*., 2001). This new aspect of velums was recently further explored by focusing on diversity and dynamics during wine ageing. Microscopy analysis of surface biofilms formed on several Vin Jaunes at different stages of their ageing spotlighted, for the first time, great variability in biofilm structure and velum morphology despite the quasi-exclusive implication of *S. cerevisiae*. Scanning electron microscopy images also confirmed the presence of an ECM (Figure 2). The genotyping of the flor yeasts present

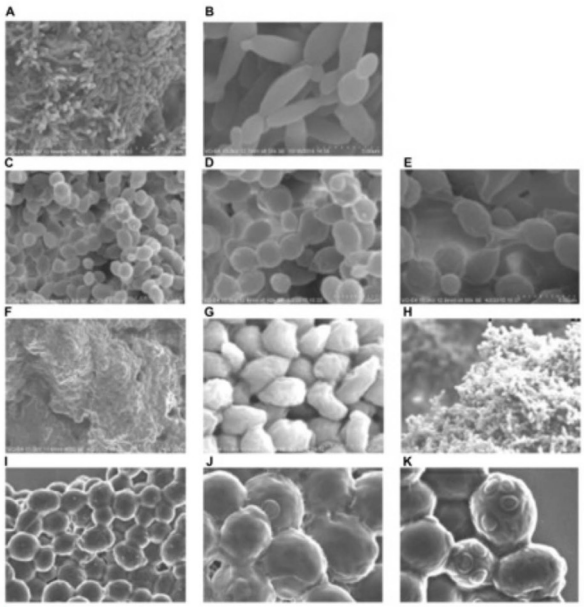

FIGURE 2. Scanning electron microscopy of velum: (A) ×2,000 (B) ×8,500 ovoid yeasts and elongated pseudomycelium yeasts; (C) ×3,000 (D) ×6,000 (E) ×8,000 yeast biofilm where yeasts are present in short chains of several cells; (F) ×650 (G) ×7,000 (I) ×5,000 (J,K) ×10,000 very dense biofilm with network of yeasts, with all cells embedded in an extracellular matrix; (H) ×1,000 velum in 3D like structure is formed by a sequence of ovoid cells attached together by their pole in an apparently disorganised manner (David-Vaizant and Alexandre, 2018).

within the same velum underlined yeast strains succession during the ageing of the wine, which goes in the direction of an adaptation of the flor to the environment. Moreover, none of the flor strains involved were isolated in the wine at the end of the alcoholic fermentation, suggesting they could originate from the cellar environment. A matrix effect, combined with an adaptation to the environment, is proposed to explain why the velums formed by the same strain can vary from one wine to another (David-Vaizant and Alexandre, 2018).

1.4. Undesirable yeast biofilms at the air/liquid interface

Yeasts can also generate organoleptic defects at different stages of wine production and even after bottling. Research on this topic consists in characterising involved species and the subsequent biofilms to inhibit their development effectively.

In the case of significant air exposure, undesirable yeast species can grow at the air-wine interface and form film layers. Responsible species are generally aerobic, highly oxygen-demanding ascomycetes yeasts, mostly belonging to the genera *Pichia*, *Hansenula*, *Candida* and *Brettanomyces*. For example, different species of *Pichia* can be responsible for chalky film layers inducing high concentrations of acetaldehyde. *Candida*, via ethanol oxidation, also induces high concentrations of acetaldehyde, as well as esters and volatile acids. *Metschnikowia pulcherrima* has also been identified in films responsible for similar organoleptic spoilage due to the production of acetaldehyde and ethylacetate (reviewed by: Du Toit and Pretorius, 2000; Loureiro and Malfeito-Ferreira, 2003). The study by Volleková *et al*. identified strains at the species level, including *Candida humicola* and *Apiotrichum humicola*, from a spoiled red wine (Vollekova *et al*., 1996). Other fermentative yeast species have a film-forming ability in wine (*Saccharomycodes ludwigii*, *Zygosaccharomyces bailii* and *Schizosaccharomyces pombe*); their development induces various defects such as gas or sediment production or a haziness appearance (David-Vaizant and Alexandre, 2018).

1.5. Undesirable yeast biofilms on solid surfaces in grapes and wine

Some studies focus on the capacity of yeasts identified as wine contaminants to form biofilms, with the aim of limiting their multiplication and colonisation by improving cleaning and disinfection strategies. Perpetuini *et al*. (2018) focused on the microorganisms present in a contaminated certified organic wine with a dominance of strains belonging to the *Pichia* genus, especially the species *Pichia manshurica*. The ability of the strains to form biofilms in YPD medium and in grape was evaluated. All the tested strains were capable of forming biofilms on polystyrene plates (Perpetuini *et al*., 2018). A similar approach was extended to 40 species isolated from the membrane filter dedicated to the quality control of bottled wines. The species identified belonged to the genera *Pichia*, *Candida*, *Sporopachydermia*, *Lodderomyces* and *Clavispora*. The adhesion properties were further explored and differed according to the species and the nature of the supports involved. Adhesion is shown by CFU-counting on both materials, with the planktonic state being preferred in contact with polystyrene supports (Perpetuini *et al*., 2021b). The resistance to some cleaning agents and potassium metabisulphite was also evaluated in these two studies but unfortunately only on planktonic cells by MICs determination. Additionally, Tristezza *et al*. (2010) evaluated the cleaning/disinfectant activity of six different agents on biofilms from six yeast species and compared their activity on planktonic cells. The study showed that four of the six yeast strains studied (*B. bruxellensis*, *S. cerevisiae*, *Saccharomycodes ludwigii* and *Schizosaccharomyces pombe*) formed biofilms in both wine and synthetic medium. *Zygosaccharomyces bailii* formed biofilms in wine only, whereas *Pichia guilliermondii* only in the synthetic medium. As expected, biofilms showed better tolerance to cleaning and disinfecting agents than planktonic cells, with chlorine-based alkaline solutions appearing to be the most active against biofilms. In contrast, soda-based detergents and peracetic acid-based disinfectants were effective on planktonic cells but had to be ten times more concentrated to impact most biofilms (Tristezza *et al*., 2010).

One of the most common wine defects is related to the presence of *Brettanomyces bruxellensis*, a spoilage yeast producing volatile phenols responsible for so-called stable odours in wine. In 2007, Joseph *et al*. isolated several strains of *B. bruxellensis* from various geographical origins. Most of the studied strains rapidly adhered to a polystyrene surface in a medium with low sugar concentrations. Some of them could form mature biofilms over relatively long incubation periods (Joseph *et al*., 2007). However, this first study did not consider the genetic diversity of the species. Kregiel *et al*. (2018) confirmed these adhesion abilities by pinpointing nutritional environment influence. Indeed, better adhesion results were obtained in a minimal culture medium (2 % glucose or 2 % sucrose (w/v), 0.1 % $(NH_4)_2SO_4$ (w/v), 0.3 % KH_2PO_4 (w/v), 0.2 % $MgSO_4$, $7H_2O$ (w/v), 0.05 % yeast extract (w/v)), whatever the carbon source (Kregiel *et al*., 2018). More recently, a study combined different methods trying to correlate the microbiological (thanks to biofilm biomass quantification by optical density measurement), physiochemical (determination of electrophoretic mobility and microbial adhesion to hydrocarbon), and biochemical (cell lipid extraction, fatty acid determination and exopolysaccharides quantification) properties of eight different strains representative of the genetic diversity, and their ability to form biofilms. The characteristics of the adhesion step and then of biofilm formation (appearance and size of the mat depending on the medium, ECM composition, surface charge, etc.) proved to be dependent on the genetic group to which the strains tested belong. Thus, using various methods to detect and identify the strains present in the cellars could make it possible to predict their persistence (Dimopoulou *et al*., 2019). The link between the morphology of *B. bruxellensis* biofilm and genetic specificities was confirmed by the work of Lebleux and her colleagues (Lebleux *et al*., 2020). The three microscopy techniques (Confocal Laser Scanning, Scanning Electron and

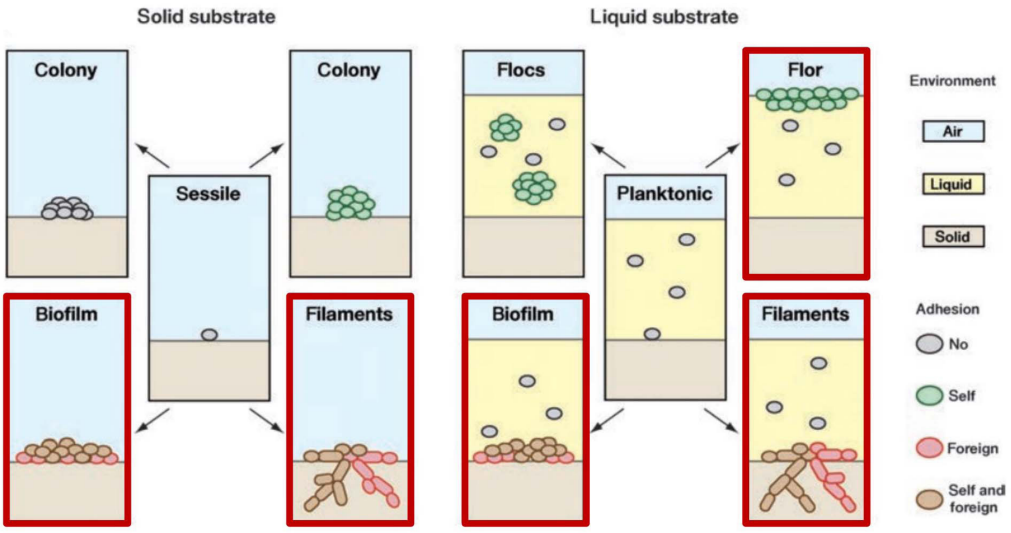

FIGURE 3. *S. cerevisiae* ways of life. Diagram showing the development of colonies, biofilms, filaments (filamentous, invasive or pseudohyphal growth), flocs and velums, thanks to cell-to-cell adhesion or cell-to-substrate adhesion, from planktonic or sessile cells, in a liquid or solid (including agar) medium. Cell adhesion properties, as well as physical environment properties are explained in the legend on the right of the diagram. In the context of this review, we focus on the lifestyles involving mechanisms conferring foreign adhesive properties i.e. velum, filaments and what is called here biofilms formation (red boxes) (adapted from Brückner and Mösch, 2012).

Epifluorescence) used in this study allowed a better characterisation of the structure of the cell showing a multi-layered morphology. In addition, the ability of two strains to form microcolonies on stainless steel coupons in wine was demonstrated, with the stressful properties of the wine inducing a significant release of cells from the mature biofilm formed in a synthetic medium. The growth of the released cells resumed after a few days, which suggests, as already proposed for other microorganisms, increased persistence properties in wine conferred by the biofilm lifestyle. In the same way, the resistance to two cleaning agents (a 5 % lactic acid solution and a solution of foaming caustic soda and oxygen peroxide at 5 % as a reference widely used in wineries) of 14-day-old biofilms of *B. bruxellensis* formed on stainless steel coupons in YPD medium was very recently evaluated (Deluchat *et al*., 2021). The test was carried out on five *B. bruxellensis* strains from different origins: culturable populations were evaluated by CFU counting on agar media and viable ones by flow cytometry counting methods. The biofilm population reached about 6 log/cm² for each strain with both counting methods. After 15 minutes of contact with the cleaning solutions, the residual biomass was quantified. The reference solution eliminated the entire population for three of the five strains. Viable but non-culturable cells were detected by flow cytometry for the other two strains. The lactic acid test solution could not eliminate the entire culturable population, which was intact for one of the five strains. The effectiveness of the agents was so strain-dependent, the presence of cells in a viable but non-cultivable state being a possible explanation for the persistence of *B. bruxellensis* populations in the winery from one year to the next. Finally, the genes involved in the biofilm phenotype are still unknown. By cloning plasmids containing or not two loci (*CEN1* and *CEN2*), Ishchuk *et al*. (2016) tried to understand better the high variability of karyotypes and ploidy in *B. bruxellensis*. After demonstrating that *CEN1* and *CEN2* were functional centromeres, the study showed the implication of the two loci in ploidy shifts and karyotype changes in the yeast. Moreover, replicative transformants carrying both *CEN1* or *CEN2* formed more biofilm, these results suggesting that *CEN1* and *CEN2* could be involved in *B. bruxellensis* adaptation to new environments (Ishchuk *et al*., 2016).

Outside the winery, *S. cerevisiae* can also be a plant pathogen of grapevines that can cause stunted growth and even plant death. *S. cerevisiae* multiplies essentially by budding, but under certain conditions, it can produce hyphae inducing invasive colonisation (Figure 3). This mode of filamentous vegetative growth seemed to be responsible for phytopathogenicity and the mechanisms observed could explain the presence of yeasts over very long periods in the vineyard (Gognies *et al*., 2006; Gognies and Belarbi, 2002). Yeast biofilms can include hyphal elements (Baillie and Douglas, 1999; Palková and Váchová, 2016), and although invasive growth phenotypes can be considered different from biofilm development ones, many of the factors regulating their formation can be expected to be shared (Bojsen *et al*., 2012).

2. Formation mechanisms of *Saccharomyces cerevisiae* biofilms

2.1. General mechanisms described for *S. cerevisiae* biofilm formation

Beyond its common use, the remarkable ability of *S. cerevisiae* to adapt its lifestyle to the fluctuating

environment makes it a model of choice for studying fungal biofilm formation (Bojsen *et al*., 2012; Reynolds and Fink, 2001; Verstrepen and Klis, 2006). Indeed, depending on the properties of the strains (production or not of adhesins…), their status (vegetative or sexual growth) and the media in which they multiply, which can impair previous properties, different adhesion lifestyles may be observed, inducing morphological changes. In wine, budding cells producing proteins involved in cell-to-cell adhesion can flocculate, i.e. form aggregates together which, beyond a certain size, can sediment to the bottom of the container. In the case of the expression of genes conferring cell-to-substrate adhesion properties, adhesion and its subsequent growth can happen at the interface between wine and air or on solid surfaces. Though cell-to-cell adhesion mechanisms are fundamental for biofilm formation and cohesion, in the context of yeast biofilms in wine, we focus on the foreign adhesion, i.e., cell-to-surface adhesion lifestyles highlighted in Figure 3 (Brückner and Mösch, 2012). The real distinction between adherent cells to a surface and their subsequent growth and filaments formation can be difficult: the mechanisms conferring adhesive properties are, in fact, quite similar and delimit here the studied perimeter. However, it has been shown that filament-to-yeast ratios in biofilms can impact their structure, such as cohesion properties or compression strength (Baillie and Douglas, 1999; Lebleux *et al*., 2020; Paramonova *et al*., 2009).

Biofilm formation is first of all conditioned by cell–cell adhesion and cell–surface adhesion. This attachment step is made possible by a family of cell wall proteins, called adhesins, through hydrophobic or amyloid-like interactions (Bojsen *et al*., 2012; Bouyx *et al*., 2021; Lipke, 2018). Adhesins called flocculins are glycosylphosphatidylinositol-anchored cell wall proteins (GPI-CWPs) composed of 1100-1500 amino acids and encoded by the FLO gene family (*FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*). These five flocculins have a molecular architecture with an N-terminal domain flanked by a secretory signal sequence, a large middle region containing serine/threonine-rich repeats highly glycosylated, and a C-terminal part with a (GPI)-anchor site to link the flocculin to the cell wall (Bouyx *et al*., 2021; Verstrepen and Klis, 2006).

Based on the phenotypic properties of the N-terminal part, they can be classified into two groups. The first type includes the Flo1, Flo5, Flo9 and Flo10 proteins involved in cell-cell adhesion and floc formation. The second group is represented by the Flo11p protein, involved in several phenotypes believed to depend on hydrophobic interactions, including biofilm formation on solid or semi-solid agar and plastic surfaces and at the air–liquid interface, as well as filamentous growth (Brückner and Mösch, 2012; Guo *et al*., 2000; Verstrepen and Klis, 2006; Zara *et al*., 2020).

Recently, the role of another gene, MIG1 (encoding a transcription factor), in biofilm formation and the ethanol resistance of these cells was investigated. The gene appeared to be involved in *FLO1*, *5* and *9* regulation as well as in ethanol tolerance of immobilised yeasts involved in ethanol production processes. The results also suggest a possible contribution of these FLO genes, regulated by *MIG1*, alongside *FLO11* in biofilm formation (Yang *et al*., 2018).

The regulatory pathways of adhesion involving the *FLO11* gene have been particularly studied and are of high interest in the context of this review. Transcription of the gene is triggered in response to certain stress factors, including the limitation of access to some nutrients. Among the described pathways, cAMP-protein kinase A (PKA) and SNF1 pathways are linked to glucose availability (Bojsen *et al*., 2012; Livas *et al*., 2011). The Cyc8p/Tup1p complex prevents *FLO11* repression, Cyc8p being the repressor and Tup1p blocking repression when complexing with Cyc8p; free representatives of Tup1p are also involved in the repression of putative extracellular protease that degrades Flo11p (Nguyen *et al*., 2018). Glucose presence also seems to impact this pathway by increasing Cyc8p levels and thus, limiting cell adhesion (Nguyen *et al*., 2020). Finally, by switching on the Mitogen-Activated Protein Kinase-dependent (MAPK) pathway and inactivating the TOR pathway, nitrogen starvation favours *FLO11* gene expression and biofilm formation (Zara *et al*., 2011). Some of these pathways can be interconnected. Indeed, under nitrogen starvation, an integrative network of the cAMP-PKA, MAPK and TOR pathways has been shown to be involved in regulating filamentous growth in *S. cerevisiae* by modulating *FLO11* expression (Vinod *et al*., 2008).

Once the first adhesion step is initiated, cells proliferate, producing an ECM commonly composed of carbohydrates, proteins, lipids and nucleic acids (Zara *et al*., 2020). It appears that within the mature biofilm of *S. cerevisiae* the Flo11p protein can be excreted from cells and also be integrated into the ECM regulating biofilm expansion and architecture (Karunanithi *et al*., 2010). The matrix confers a number of advantages to yeasts organised in biofilms, such as mechanical properties, adhesion and cohesion between cells, including communication and genetic exchanges; it is also a source of nutrients, enzymatic activity and physical protection against the environment (Zara *et al*., 2020).

The role of Quorum Sensing as an intrinsic factor in yeast biofilm formation has been shown for several fungal species (Albuquerque and Casadevall, 2012; Ramage *et al*., 2002; Wongsuk *et al*., 2016). In *S. cerevisiae*, nitric oxide has been recently identified as a Quorum Sensing Molecule (QSM) stimulating biofilm formation above a certain concentration (Yang *et al*., 2019). The production of another QSM, the 2-phenylethanol, also appeared to be correlated with the expression of *FLO11* and thus with biofilm formation (Zhang *et al*., 2021).

Taken together, these works pinpoint the complexity of the mechanisms involving several FLO genes but also other molecules that allow cells to display a wide variety of adhesive properties. Some of these, as well as specific ones, have been studied in the case of velum formation.

2.2. Specific mechanisms of velum formation

The state of knowledge allows us to understand the mechanisms of velum formation in sherry-type wines, mostly involving *S. cerevisiae*. At the end of alcoholic fermentation, available nitrogen and carbon are limited, and ethanol concentration is important. Oxidative stress increases and in this stressful changing environment, *S. cerevisiae* must adapt by triggering specific metabolic pathways during the diauxic transition. The activation of these regulatory pathways, pinpointed in the context of biofilm formation by *S. cerevisiae,* allows the transcription of the *FLO11* gene, which is essential for velum formation (Legras *et al*., 2016).

Figure 4 schematises a model for velum formation based on *FLO11* gene expression which encodes the Flo11p hydrophobic protein, thereby increasing the cell surface hydrophobicity (Ishigami *et al*., 2006; Zara *et al*., 2005). This phenomenon leads to the formation of aggregates trapping CO_2 bubbles and rising the wine surface (cell buoyancy). The production of Flo11p causes a drop in the inositol intracellular concentration. Indeed, this sugar is used for the glycosylphosphatidyl-inositol anchoring of Flo11p. In contact with air, the presence of oxygen, associated with this phenomenon, induces lipid synthesis via the activation of the expression of genes regulated by inositol-chorin (ACC1) responsive elements and increases cell budding.

Expansion of minisatellites in the central domain of the *FLO11* gene was also shown to increase protein glycosylation as well as the hydrophobicity of the Flo11p glycoprotein of velum yeasts. Such a biofilm formation and the propensity of yeasts to float are thus influenced by the copy number of the *FLO11* gene but also by its transcription level (Zara *et al*., 2009).

Nevertheless, the model based on *FLO11* expression and cell-surface hydrophobicity increase following the diauxic shift (Zara *et al*., 2005) might not be a general one. Indeed, all flor strains present specific restriction patterns. In a recent study, a PCR ITS analysis of a large yeast sample isolated after alcoholic fermentation in the velum of Savagnin Jura wines showed that none of the *S. cerevisiae* strains isolated at the end of alcoholic fermentation were flor strains (David-Vaizant and Alexandre, 2018). Similar conclusions were made following a study dedicated to the yeast population evolution during sherry winemaking (Esteve-Zarzoso *et al*., 2001).

Beyond the predominant role of *FLO11* in velum formation, other genes such as *HSP12*, *NRG1, CCW14* and *YGP1* are also involved. Indeed, a mutation or the deletion of *HSP12*, encoding a heat-shock protein found to be active during the stationary phase in cells depleted for glucose or metabolising ethanol and fatty acids, leads to the inability of yeast to form a biofilm (Zara *et al*., 2002). Furthermore, when the C-terminal part of Nrg1p is deleted, the truncated form of the protein has a negative effect on *FLO11* repression and film formation is promoted via the expression of the gene (Ishigami *et al*., 2004). Finally, *CCW14* and *YGP1*, coding for two cell surface glycoproteins, seem to have an impact on film formation. Indeed, both gene deletion induces a decrease in the weight of flor biofilm and the deletion of *CCW14* reduces cell adherence to polystyrene (Moreno-García *et al*., 2018b).

In addition to the mechanisms allowing them to float and form these films, velum yeasts are extremely tolerant to ethanol and acetaldehyde, which is not the case for *S. cerevisiae*

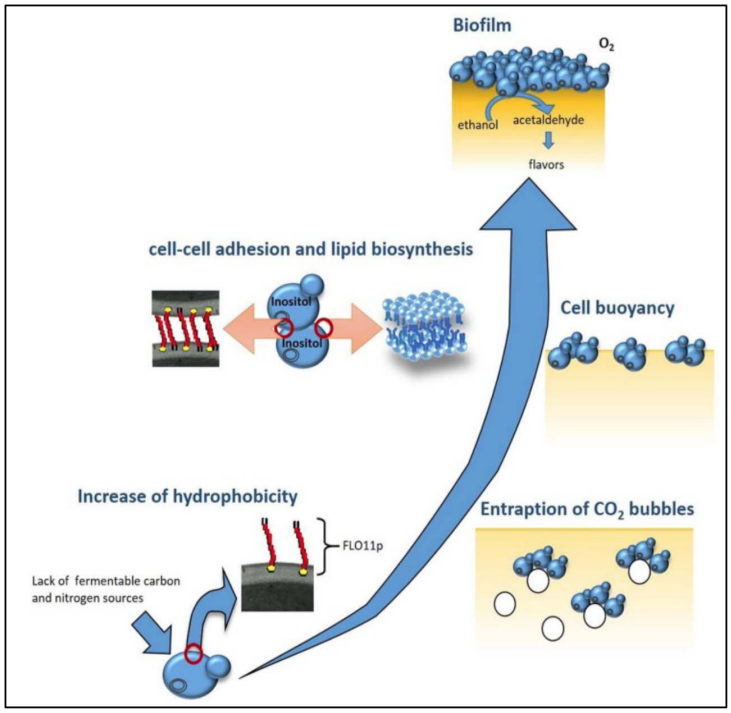

FIGURE 4. Yeast velum formation (Legras *et al.*, 2016).

strains involved in alcoholic fermentation; thus, confirming the involvement of specific strains and a specific evolution among flora. The correlation between this resistance and the induction of the HSP genes (*HSP12*, *HSP82*, *HSP104* and *HSP26*) was highlighted. On the other hand, the genes coding for aldehyde dehydrogenase (ALD genes) are not expressed in the same way in velum yeasts compared to fermentative yeasts; aldehyde dehydrogenase enzymatic activity is thus more important for velum yeasts in relation to their increased use of ethanol as the main carbon source. Finally, resistance to ethanol as well as to oxidative stress induced by the aerobic environment are key factors in the formation of velums and the biomass evolution and survival: a number of genes involved have been highlighted, but their importance has not yet been fully characterised (reviewed by Alexandre (2013)). Among them, the *BNT2* gene, encoding a v-snare interacting protein involved in intracellular protein trafficking, has been shown to be linked both to velum formation and ethanol tolerance. Indeed, gene deletion affects biofilm formation and decreases flor resistance to high ethanol concentrations (Espinazo-Romeu *et al*., 2008).

Altogether, these elements, and surely other mechanisms still not highlighted, are responsible for the constitution of a multilayer floating fungal biofilm protecting the wine from direct exposure to oxygen and contributing to the changes in its composition, giving it particular organoleptic properties.

BACTERIAL BIOFILMS IN OENOLOGY

Although bacterial biofilms are the most documented in the literature, specific works in oenology are pretty recent and rare. Bacteria in wine are essentially of two types, acetic and lactic. The formers are problematic because they are responsible for organoleptic defects in wine. Biofilms of acetic bacteria at the air-liquid interface are known and used for producing acetic acid, the only industrial processing application in the food industry to date (Halan *et al*., 2012; Maksimova, 2014). However, they are not widely explored in oenology, apart from rare cases of contamination mainly due to *Acetobacter* strains (Bartowsky and Henschke, 2008). Lactic acid bacteria in wine mostly belong to *Lactobacillus*, *Pediococcus* and Œnococcus genus (Lonvaud-Funel, 1999). They can be responsible for wine diseases but are also involved in malolactic fermentation (MLF), which consists of the enzymatic conversion of L-malic acid to L-lactic acid and CO_2. This MLF step is the most studied in terms of the possibilities of bacteria immobilisation. The control of this reaction, historically often spontaneous, is more and more sought after because of the properties it confers to the wine (decrease of acidity, contribution of certain interesting aromas). It is nevertheless tedious and the duration of the reaction can be more than 6 weeks, with sometimes long latency periods before starting, because of the stress undergone by the bacteria in the wine. This phenomenon is restrictive for fast-moving markets and implies maintaining the temperature in the cellars around 20 °C until the end of MLF, with the associated energy costs. However, MLF is not always desired, and winemakers may have to fight the presence of the lactic acid bacteria responsible.

We will present here different works involving bacterial biofilms in wine, whether they are of oenological interest or undesirable because they are responsible for cellar contamination or diseases. The mechanisms described so far are devoted to the study of the resistance to wine stress conditions of Œnococcus œni biofilm. This bacterium is mainly responsible for MLF, so that the following chapters will focus on it.

1. Different types of bacterial biofilms

1.1. Immobilisation of lactic acid bacteria for malolactic fermentation

As for *S. cerevisiae*, several studies aiming to use the properties of immobilisation for production purposes (improvement of fermentation rates, development of continuous processes...) are reported before the development of works dedicated to lactic acid bacteria biofilms. Nevertheless, most of the studies are based on inclusion immobilisation techniques, such as a recent paper from Ruipérez *et al*. dedicated to continuous malolactic fermentation of red wine thanks to encapsulated *O. œni* (Ruipérez *et al*., 2022).

The processes involving simple attachment to a surface are more limited. Maicas *et al*. achieved high L-malic acid conversion by immobilising *O. œni* on positively charged cellulose sponges for sequential batch systems for red wine fermentation (Maicas *et al*., 2001). Delignified cellulosic material (DCM), an immobilisation polymer obtained from sawdust, allowed MLF to be conducted with incomplete L-malic acid conversion in the case of a post-alcoholic fermentation inoculation (Agouridis *et al*., 2008) and with a complete process after 5 days at 10 °C in the case of simultaneous inoculation with *S. cerevisiae* (Servetas *et al*., 2013). Immobilisation trials by attachment of *O. œni* to grape skins or seeds have also been conducted for the realisation of white wine MLF in repeated batches or the sequential realisation of both AF and MLF steps in a continuous process way (Genisheva *et al*., 2013; Genisheva *et al*., 2014b). The malolactic reaction was successfully conducted over several weeks; nevertheless, MLF performance was highly influenced by pH variations in the medium.

1.2. Biofilm formation of lactic acid bacteria for malolactic fermentation

The use of biofilms for MLF was first described in the PhD work of D. Janssen (Janssen, 1991). His continuous reactor system, based on *O. œni* biofilms on oak chips, allowed malic acid consumption rates two to three times higher than during a "normal" reaction, at a scale of 10 litres of white wine and over 3 weeks. This preliminary work validated the hypothesis of a potential acceleration of the reaction under a continuous regime, although the MLF was incomplete.

After showing the ability of the bacterium to form mature biofilms on polystyrene (24-well microplates), oak chips and stainless steel coupons (Figure 5) in an MRSm-broth

(MRS modified with 4 g/l L-malic acid and 10 g/l fructose), Bastard *et al*. went further in the characterisation of the *O. œni* biofilm especially its impact on wine-wood interactions (Bastard, 2015; Bastard *et al*., 2016). For this purpose, the MLF of a white wine in a barrel was reconstituted in the laboratory and the wood aromatic compounds of the wine were studied in connection with the enzymatic activity of the bacteria and the covering effect of the biofilm (Figure 5). The authors demonstrated that the biofilm could be able to modulate the organoleptic profile of the wine. Indeed, some aromatic compounds brought by the oak, such as cis-whisky lactone, vanillin, eugenol, guaiacol and furfural, revealed lower concentrations in a white wine with MLF carried out in barrel by the *O. œni* biofilm in comparison to planktonic cells. In the same wine, the concentration of another compound, the trans-whisky lactone, increased. The hypothesis of interactions between elements of the ECM and the compounds of the wood and the wine is a possible explanation (Bastard *et al*., 2016). The hypothesis of modified chemical transfers linked to *O. œni* biofilm was verified and extended to grape (polyphenols) and fermentative (higher alcohols) compounds to evaluate the impact of bacterial biofilms on the chemical composition of wine and consequently on its tasting properties. Indeed, wines showed differences in chemical composition, depending on the sessile or planktonic lifestyle of the bacteria, as biofilms seemed to non-specifically trap aromatic compounds from wood and grapes, regardless of the oenological conditions (Coelho *et al*., 2019). Beyond these results, interesting fermentative performances were obtained for *O. œni* biofilm, the biofilms inducing a complete, faster and more reproducible malolactic reaction in comparison with planktonic cells (Bastard *et al*., 2016; Coelho *et al*., 2019).

The biofilm lifestyle for MLF completion was also studied on 68 strains of *Lactobacillus plantarum* by Pannella *et al*. (2020). The ability of the tested strains to form biofilms on oak chips as well as on polystyrene microplates (MRS broth) was strain-dependent. Among the biofilm-forming strains, the *L. plantarum* Lpls22 strain was selected for an assay in synthetic wines containing 12 % ethanol with varying pH (3.2 or 3.5). The biofilms formed on oak chips and the planktonic cells were evaluated for their comparative resistance to wine stress conditions and their ability to convert L-malic acid. The biofilms, as well as the cells released from biofilms, were much more resistant than planktonic cell cultures at all pH levels and allowed a rapid and complete MLF reaction (Pannella *et al*., 2020).

1.3. Unwanted bacterial biofilms

As MLF is not always desired, the presence of *O. œni* is sometimes undesirable: it is in this context that Nel *et al*. (2002) set up *O. œni* biofilms on stainless steel coupons in an acidic grape medium, stainless steel being a material widely encountered in wineries, to evaluate the antibacterial activity of three bacteriocins (Pediocin PD-1, Plantaricin 423 and Nisin). Suspensions of the bacteriocins were added to the medium containing 9-day-old biofilms with final activity levels between 100 and 3000 AU (Activity Units). After periods of 1 to 5 hours of contact with the bacteriocins, stainless steel coupons were removed, stained with Live/Dead BacLight viability probes and observed under an epifluorescence microscope. A viable cell population was determined and compared to a control with no proteins, mature viable biofilms in controls covering 75 to 86 % of the coupons. All the attached viable cells were killed after 5 hours of contact with the three peptides at a 3000 AU/ml concentration. PD-1 appeared to be the most efficient, being the only bacteriocin removing all the cells from the coupon. It was then successfully tested on a biofilm formed in a Chardonnay must in the same conditions. Thus, the PD-1

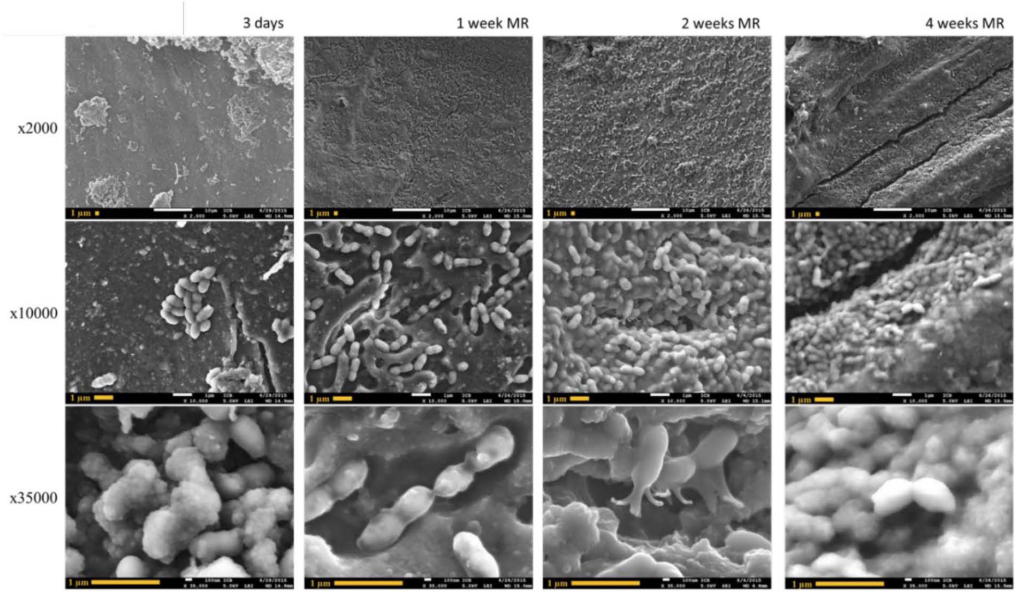

FIGURE 5. Scanning electron microscopy at x2000 x10000 and x35000 of *O. œni* ATCC BAA-1163 biofilm formation on oak chips after 3 days 1, 2, et 4 weeks (Bastard *et al.*, 2016).

peptide could be evaluated as an efficient sanitation method (Nel *et al*., 2002).

Apart from the control of MLF, rare studies are related to negative bacterial biofilms in wine. In 2010, Tristezza *et al*. (2010) evaluated the ability of yeasts but also of two bacterial species to form biofilm on plastic plates. The acetic acid bacterium, *Acetobacter aceti*, was able to form biofilms in wine and synthetic media; the other species tested, *Lactobacillus hilgardii*, only formed biofilms in synthetic media. The findings on resistance to cleaning and disinfecting agents tested were consistent with those reported for yeasts, namely an increased resistance of cells under biofilms compared to cells in suspension (Tristezza *et al*., 2010).

2. Mechanisms involved in the stress resistance of *Œnococcus œni* biofilms

Beyond the available data on bacterial biofilms in the food industry, various studies on *O. œni* performed before the description of its biofilm have led us to consider this lifestyle as a protective way against wine stress.

Several studies focus on the stress response mechanisms of *O. œni*. This response can materialise through membrane modifications allowing to maintain its integrity (Grandvalet *et al*., 2008; Maitre *et al*., 2014), the activation of H+-ATPases and MLF to regulate the internal pH of bacteria (Galland *et al*., 2003; Salema *et al*., 1996), or the expression of stress response proteins such as the HSPs (Heat Shock Proteins). The different stress marker genes, as well as their products, are gathered in the review by Guzzo (2011). In parallel, there is also a focus on synthesising an extracellular matrix composed of Extracellular Polymeric Substances (EPS) by *O. œni* and the role of this structure in improving the bacterium's survival in wine (Dimopoulou *et al*., 2016). This structure is similar to the ECM described for yeast biofilms but named EPS in the works reported in this chapter.

Strains isolated from different wines were highlighted to produce EPS. Polysaccharide (PS) chemical structure analysis suggested that most strains produced a mixture of EPS, independent of the gtf phenotype associated with β-glucane PS synthesis, confirming the implication in strain survival in wine as well as in the colloidal balance of the wine (Ciezack *et al*., 2010). Subsequently, several genes involved in the production of EPS and distributed in the bacterial genome were identified, with most of the tested strains having genes encoding for homo- and heteropolysaccharides synthesis. The nature, as well as the quantity of EPS, were variable depending on the culture medium and the strains, but all of them had genes dedicated to the metabolism of this structure. It suggests a role for EPS in the adaptation of the bacteria to its environment and potentially in the technological performances of malolactic starters (Dimopoulou *et al*., 2012; Dimopoulou *et al*., 2014). Additional work has shown that some strains possess polysaccharide capsules, but the protective potential of these EPS has not been directly assessed in wine (Dimopoulou *et al*., 2016).

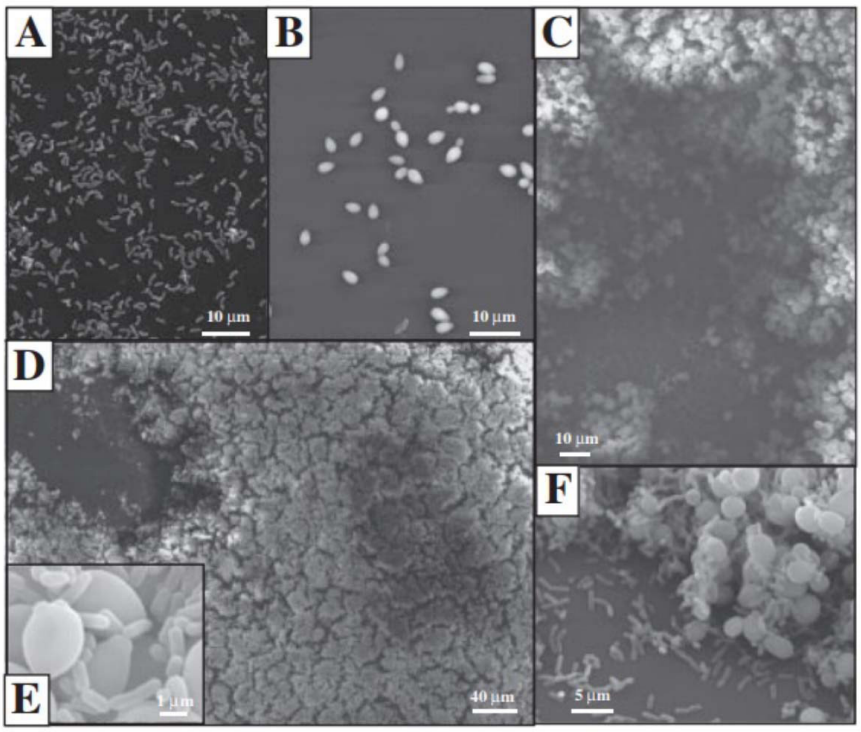

FIGURE 6. Scanning electron microscopy images of biofilms on glass plates. A- Attached cells of *L. plantarum* ML11-11. B- Attached cells of *S. cerevisiae* Y11-43. C- Mixed biofilm of ML11-11 and *S. cerevisiae* X2180-1A. D- Mixed biofilm of ML11-11 and Y11-43. E- Magnification of ML11-11 and Y11-43 biofilm. F- Outer part of the mixed biofilm of ML11-11 and *S. cerevisiae* BY4741 (Furukawa *et al.*, 2010).

Regarding *O. œni* biofilm, A. Bastard and his team observed a better resistance of cells detached from a 14 days biofilm grown in an MRSm broth compared to "classical" planktonic cells grown in the same medium after inoculation in wine. Going further, they showed that the stress marker genes (*cfa*, *clpL1* and *hsp18*) were less expressed in the absence of stress in sessile than in planktonic bacteria. Moreover, these genes were overexpressed when the biofilm was immersed in wine. Furthermore, when immersed in wine, genes involved in EPS synthesis (*levO*, *wobB*, *wobO*, *dsrO*) were also overexpressed, suggesting that the matrix (mainly polysaccharidic in nature) was enhanced upon contact with the wine, inducing a protective effect for the biofilm cells. Thus, adaptive responses to wine stress were induced within the *O. œni* biofilm: these responses could explain the enhanced resistance of the *O. œni* biofilm in wine (Bastard, 2015; Bastard *et al*., 2016). The nature of the EPS produced according to bacterial lifestyles was also evaluated: differences were highlighted via ATR-FTIR spectral analysis, but further studies would allow going further in the composition characteristics. Finally, the study of the expression of the malolactic enzyme (MleA) by *O. œni* biofilm, evaluated in the same work by RT-qPCR, evidenced the metabolic reactivity of the biofilm, the synthesis of the enzyme by the biofilm being correlated with the concentration of malic acid in the medium.

MIXED SPECIES BIOFILMS

As already underlined, wine is a complex microbial environment dominated by *S. cerevisiae*, with many interactions which can have harmful or beneficial effects on the winemaking process (Albergaria and Arneborg, 2016). A better understanding of microbial interactions is crucial from technological, microbiological and environmental perspectives, but their complexity hinders progress on the subject (Senne de Oliveira Lino *et al*., 2021). In wine, the number of microbial interactions concerning planktonic populations has been described (Albergaria and Arneborg, 2016; Ciani *et al*., 2016), but no work has been published on biofilms. Nevertheless, mixed species biofilms found in other beverages are the subject of a few studies.

In the context of soft beverages contamination, Kregiel *et al*. (2018) suggested that the acetic acid bacterium *Asiaia* spp. was responsible for forming hydrophobic flocs, whose structures would be reinforced in the presence of yeast cells including *B. bruxellensis* (Kregiel *et al*., 2018).

Then the possibility of forming mixed yeast-lactic acid bacteria biofilms, for example, in *S. cerevisiae* and *L. plantarum,* has been demonstrated on several occasions in fermented beverages such as rice vinegar (Abe *et al*., 2013; Furukawa *et al*., 2010, 2011; Kawarai *et al*., 2007). SEM images of biofilms obtained by Furukawa *et al*. (2010) are shown in Figure 6. The nature of the interactions between species as well as the mechanisms involved are still unknown. To the best of our knowledge, such studies have not been carried out with microorganisms isolated from wine.

Understanding yeast-lactic acid bacteria interactions is a very important topic in winemaking, with *S. cerevisiae* and *O. œni* being the most commonly used wine microorganisms. The possible existence and influence of wine mixed species biofilms involving both species have not been studied yet, but new perspectives are offered by recent technical advances. For example, Bartle *et al*. analysed recently the interactions of *O. œni* and *S. cerevisiae* thanks to a quantitative trait loci (QTL) mapping whose objective was to evaluate how the genetic variability of 67 yeast strains might affect the progress of MLF after co-inoculation with a single commercial strain of *O. œni* (Bartle *et al*., 2021). This technique used for the first time to characterise yeast-bacteria interactions could be adapted to explore wine biofilms further.

CONCLUSION

Although the existence of biofilms in oenology has been known for a long time through yeast velums, their study is still limited considering a large number of potential applications.

To date, we can classify the work dedicated to bacterial or fungal wine biofilms into three major families of applications: (1) Concerning the microorganisms used in winemaking: understanding the implantation of biofilms in the environment and the mechanisms involved in their development, to better control the winemaking processes and to improve the quality of the wine; (2) studying the propensity of cellar and wine contaminants to form biofilms and their resistance to conventional cleaning agents to adapt cleaning procedures and improve fighting methods against these species; (3) in the vineyard, the possibility of using biofilms as biocontrol agents to limit the use of phytosanitary products.

The first family is the subject of a certain number of studies, but only a few of them go in the direction of developing processes involving biofilms despite the promising results obtained. In addition to potential organoleptic benefits, these types of processes could optimise production costs for some market segments by reducing fermentation times. As for the other two families, the first studies have been published really recently. Thus, tons of opportunities are offered by biofilm behaviour exploration, including mixed species biofilms, and the growing number of studies dedicated to them in the food industry suggests studying wine biofilms is a hot topic still in an early stage.

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