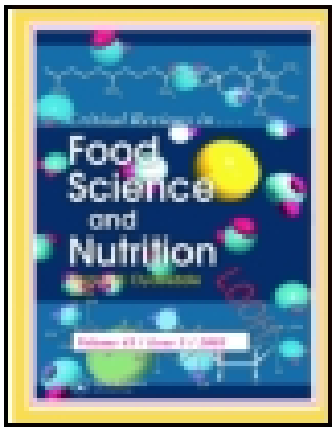




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# Wine microbiome, a dynamic world of microbial interactions

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**Wine microbiome, a dynamic world of microbial interactions**

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Summary

Most fermented products are generated by a mixture of microbes. These microbial consortia possess various biological activities responsible for the nutritional, hygienic, and aromatic qualities of the product. Wine is no exception. Substantial yeast and bacterial biodiversity is observed on grapes, and in both must and wine.

The diverse microorganisms present interact throughout the winemaking process. The interactions modulate the hygienic and sensorial properties of the wine. Many studies have been conducted to elucidate the nature of these interactions, with the aim of establishing better control of the two fermentations occurring during wine processing. However, wine is a very complex medium making such studies difficult. In this review, we present the current state of research on

microbial interactions in wines. We consider the different kinds of interactions between different microorganisms together with the consequences of these interactions. We underline the major challenges to obtaining a better understanding of how microbes interact. Finally, strategies and methodologies that may help unravel microbe interactions in wine are suggested.

Keywords: wine, yeast, bacteria, interactions, fermentation, co-culture

## 1. Introduction

Microbes coexist and interact in many environments, and this is of practical relevance in various fields (Ivey et al., 2013). Indeed, microbial interactions occur in bioremediation of pollutants, agriculture, forestry, environmental protection, food processing, biotechnology, medicine, and dentistry (Frey-Klett et al., 2011). There have been numerous studies documenting the range of effects exhibited during microbial interactions; however, knowledge of the molecular mechanisms responsible for these effects is scant. Wine constitutes a particularly interesting model to study interactions between microorganisms. The first relevant complex interactions between microorganisms are on the surface of the grapes in the vineyard. Interactions continue throughout the alcoholic fermentation (AF) by yeast (Ciani et al., 2010) and the malolactic fermentation (MLF) by lactic acid bacteria (LAB) (Alexandre et al., 2004).

Grape must and wine thus constitute a complex microbial ecosystem containing a mixture of different species and strains (Barata et al., 2012a). Consequently, individual microorganisms interact, and the types of interaction found in mixed populations of microorganisms are generally classified as direct or indirect (Ivey et al., 2013). Competition, commensalism, mutualism, amensalism (or antagonism) and neutralism are considered to be indirect interactions; direct interactions, for example parasitism, may also occur during fermentation. This paper presents current knowledge of microbial interactions in wine.

These interactions have a tremendous impact on the quality and other characteristics of wines. Indeed, hygienic and organoleptic qualities of wines are results of the metabolic activity of a succession of different microorganisms. Metabolite production by microorganisms can be substantially modified depending on the presence or absence of other microbes. Also, many

microbes use extracellular signals to transmit information about population density and environmental conditions, and thereby interact. A particular aim of this review is to provide an overview of what is known about cell-signalling and quorum-sensing molecules in wine. Interaction studies are difficult to conduct. Indeed, the dynamics of the biochemical activities, growth, survival and death of microorganisms during AF are the results of interactions between microorganisms of the microbial consortium and between microbes and their environment: this environment clearly changes during the fermentation process. Although microbial growth dynamics during natural fermentations have received extensive attention (Zott et al., 2011; Barata et al., 2012), the reports are mainly descriptive and do not give very much insight into the mechanisms of interaction. This lack of information is a major hindrance for progress with, and control of, natural fermentations or fermentations conducted using multi-starter cultures. The growth of indigenous yeasts or bacteria can prevent the development of starter cultures and thus limit the impact of the selected yeasts or bacteria, and thereby affect the functionality of the product (Smid & Lacroix, 2013). Determining the aromatic profile of a wine using selected mixed-starter cultures of yeast or bacteria cannot be effective without understanding how microbes interact with each other.

In this review we will also consider various strategies that could be used to unravel the molecular details of the mechanisms underlying interactions between microbes in the wine environment.

## 2. Microbial ecology of grapes and must

What is the best way to define the microbiome present on grape berries? Microbial ecosystems initially depend on the health quality of the harvest, and many biotic and abiotic factors . In

addition, the analytical techniques used to inventory microbial consortia have significant consequences for the description obtained for these communities. Indeed, traditional microbiological methods involving isolation coupled with enumeration of microorganisms in selective nutritive media can lead to biased results. Minority colonies constituting less than 1% of the total population cannot be detected (Fleet et al., 2002, David et al., 2014), and these methods fail to detect viable but non-culturable organisms (Davey & Kell, 1996; Quiros et al., 2009; Salma et al., 2013). The development of molecular methods (Doaré-Lebrun et al., 2006; Renouf et al., 2007; Laforgue et al., 2009; Zott et al., 2010), independent of the microbial species cultivability and gene expression, associated with selective flow cytometric methods of enumeration currently allow a more comprehensive vision of microbial biodiversity. These methods are also powerful tools for monitoring microbial consortia from grape harvest to wine storage.

*2.1 Yeast community*

Bunches of grapes are the main natural reservoir of indigenous wine yeasts. Yeasts are spatially distributed over the grape berries and grape bunches. Ninety-three different yeast species belonging to 30 different genera, isolated from 49 different grape varieties growing in 22 countries have been reported in the literature (Barata et al., 2008; 2012a; Bisson & Joseph, 2009). Renouf et al. (2007) identified 47 yeast species belonging to 22 different genera using PCR-DGGE (Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis): *Aureobasidium*, *Auriculibuller*, *Brettanomyces*, *Bulleromyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Issatchenkia*, *Kluyveromyces*, *Lipomyces*, *Metschnikowia*,

*Pichia*, *Rhodosporidium*, *Rhodotorula*, *Saccharomyces*, *Sporidiobolus*, *Sporobolomyces*, *Torulaspora*, *Yarrowia*, *Zygoascus*, and *Zygosaccharomyces*. These yeasts were isolated from the surface of grape berries of six different varieties.

Although large numbers of yeast species are identified on grape berries, the population densities are low. Indeed, yeast populations on immature grapes are low ($10^1$ to $10^3$ CFU/g) but increase (to $10^3$-$10^6$ UFC/g) at harvest time (Jolly et al., 2003; Prakitchaiwattana et al., 2004; Combina et al., 2005; Renouf et al., 2005; Raspor et al., 2006; Barata et al., 2012b; Setati et al., 2012). The population dynamics of yeasts may be related to the increased surface area of each berry and to the availability of nutrients: during maturation, the berries grow larger, more nutrients are available on the surface of the berries, the sugar concentration increases and the acidity decreases (Combina et al., 2005; Cadez et al., 2010).

Other factors can modify the species balance directly or indirectly by affecting grape skin integrity. Several studies report that yeast diversity is dependent on climatic and microclimatic conditions, but the detailed results are contradictory. Higher yeast counts have been described for vintages with high rainfall (Longo et al., 1991; De la Torre et al., 1999; Combina et al., 2005; Cadez et al., 2010), probably due to substantial fungal proliferation. However, the opposite is reported by Rementeria et al. (2003). Other studies, and particularly for large scale investigations, do not provide evidence for any relationship between climatic conditions and yeast biodiversity (Barata et al., 2012a). Vineyard factors such as grape variety and berry color are often described as factors influencing diversity (Guerzoni & Marchetti, 1987; De La Torre et al., 1999; Sabate et al., 2002; Renouf et al., 2005; Nisiotou et al., 2007). For example, in similar

soil and climatic conditions, *Cryptococcus* was the genera most frequently isolated (90% of all isolates) from Grenache grapes whereas *Hanseniaspora* was the genus most frequently isolated from Carignan (75%) (Sabate et al., 2002).

The health status of berries can also affect the diversity of yeasts. For example, the *Botrytis cinerea*, being able to penetrate the surface and release nutrients, may influence the microbial flora present on the grape surface (Nisiotou & Nychas, 2007; Barata et al., 2008). Indeed, Sipiczki (2006) reported the development of the genus *Metchnikowia* on berries affected by *Botrytis cinerea*. Members of the genus *Metschnikowia* seems to have an inhibitory effect on other yeasts, filamentous fungi and bacteria, through a mechanism of iron sequestration (Sipiczki, 2006). The relationship between yeast and some animals may also contribute to the variability of yeast populations on berries: there is some evidence from vineyards indicating associations between yeasts and insects, particularly bees, social waps and *Drosophila* (Stevic et al., 1962; Fermaud et al., 2000; Stefanini et al., 2012). Francesca et al. (2010) suggest that migratory birds may serve as vectors of *S. cerevisiae* cells.

Differences in yeast populations associated with grapes obtained from organic and conventional vineyards have been reported (Comitini & Ciani, 2008; Cadez et al., 2010; Tofalo et al., 2011; Cordero-Bueso et al., 2011; Schmid et al., 2011;Tello et al., 2012; Milanovic et al., 2013; Martins et al., 2014). These various studies were carried out in different vineyards in different countries (Austria, France, Italy, Spain and Slovenia) subject to different climates and pesticides, and different regulatory constraints: these differences may explain the contradictory results.

Generally, many of these variables (for example climatic conditions or cultivar) are not independent and may be clustered into broad groups of effects. Bokulich et al. (2013) concluded that grape-associated microbial biogeography is non-randomly associated with regional, varietal and climatic factors across multiscale viticultural zones. According to Setati et al. (2012), yeast species distribution is subject to significant intra-vineyard spatial fluctuations; also, the frequently reported heterogeneity of grape samples harvested from single vineyards at the same stage of ripeness might therefore, at least in part, be due to differing microbiota in different sections of the vineyard.

The various biotic and abiotic factors have influences on the diversity of yeasts present on berries. In addition, the interactions between resident populations may also affect this diversity. Few data are available clearly to describe these interactions. Castoria et al. (2001) have suggested that the yeast-like fungus *Aureobasidium pullulans* is able to reduce basidiomycete diversity. More generally, further studies are required.

### *2.2 Bacterial community*

The review by Barata et al. (2012a) lists over 50 bacterial species that have been identified on grape berries. The species isolated mostly belong to two groups: Firmicutes and Proteobacteria. Firmicutes present include the gram-positive Lactobacillaceae (*Lactobacillus* and *Pediococcus*), Leuconostocaceae (*Leuconostoc, Weiseilla* and *Oenococcus*), Bacillaceae (*Bacillus*) and Enterococcaceae (*Enterococcus faecium, E. durans, E.avium, E. hermaniensis*). Except for *Bacillus* and *Enterococcus* spp., these species belong to the technological group of lactic acid bacteria (LAB), characterized by a low GC-content and a tolerance to acidity. *Lactobacilli* are

divided into facultative (*Lactobacillus plantarum, L. casei*) and obligatory (*L. hilgardii, L. brevis, L. fructivorans, L. sanfranciscensis*) heterofermentative species (Lonvaud, 1999; Renouf et al., 2007). Group I Lactobacilli (homofermentative species including *L. mali* or *L. acidophilus*) were rarely detected on grapes (Renouf et al., 2007; Kačániová et al., 2012). By contrast, there are numerous reports of the homofermentative cocci *Pediococcus damnosus, P. pentosaceus, P. parvulus* and *P. acidilactici* on grapes or in musts. Similarly, the heterofermentative cocci *Leuconostoc mesenteroides, Weisella parameenteroides* and *Oenococcus oeni* (*O. oeni*) are frequently found. Gram-negative Proteobacteria, in particular β-Proteobacteria (*Pseudomonas jesseni, Burkholderia vietnamiensis)* and γ-Proteobacteria (*Serratia rubidae, Serratia marcescens, Enterobacter gergovia, Enterobacter ludwigii, Klebsiella oxytoca, Citrobacter freundii*) are not often listed among oenological microbial flora (Renouf et al., 2007; Nisiotou et al., 2011). However, α-Proteobacteria (*Acetobacter* spp., *Gluconobacter oxydans, Gl. cerinus, Gl. hansenii, Gl. saccharivorans, Gl. intermedius* and *Asaia krungthepensis*) are frequently included among oenological flora (Barata et al., 2012ab; Ultee et al., 2012). These strictly aerobic bacteria are also known as acetic acid bacteria (AAB).

While literature is well documented on the factors affecting the biodiversity of yeasts on grapes, only few data are available concerning the influence of environmental factors on the bacterial community. Analyses of grape berry bacterial microbiota revealed changes in the size and structure of the population during the berry ripening process, with levels rising gradually and reaching their highest value when the berries were overripe. As the season progressed to maturity, gram-negative bacterial communities declined whereas gram-positive communities increased (Martins et al., 2012). Moreover, the farming system can impact the bacterial

community structure. For example, a negative correlation between copper concentrations and bacterial cell densities has been observed (Martins et al., 2012). At harvest time, averages of the different microbial populations were around $10^3$ CFU/berry for gram-negative aerobic or anaerobic bacteria and $10^4$ CFU/berry for gram-positive anaerobic bacteria (Renouf et al., 2005). Levels of the different bacterial populations of grapes are also dependent on the health quality of the harvest (Renouf et al., 2005; Kačániová et al., 2012).

According to Barata et al. (2012a), most LAB (mostly *Lactobacillus* spp. and *Pediococcus* spp.) are detected on sound grapes, with maximal populations around $10^2$ CFU/g. These observations agreed with those of Lonvaud (1999) which were that LAB densities in crushed grapes were about $10^2$ CFU/mL to $10^4$ CFU/mL, depending on climatic conditions during the final days of grape maturation, and inversely correlating with must acidity. It can be also underlined that botrytized grapes can constitute rich reservoirs for LAB (Barbe et al., 2001). The frequency of detection of *O. oeni* on grapes is much lower and requires adequate methods to promote the development and allow detection of minority populations (Renouf et al., 2005; 2007). The microbial species identified included LAB, some of which, like *P. parvulus* (Llaubères et al., 1990), *L. sanfranciscensis* (Korakli et al., 2003), *Leuconostoc mesenteroides* (Richard et al., 2005) and the gram-negative bacterium *Burkholderia vietnamiensis* (Gaur & Wilkinson, 1996), produce large amounts of exopolysaccharides. These macromolecules can constitute a biofilm able to protect bacterial cells against environmental aggression and allowing anaerobic bacteria to survive on the grape berry surface (Renouf et al., 2005). It has been suggested that there is a link between the application of anti-fungal treatments on the vineyard (use of sulfur- and copper-based products) and the induction of biofilm formation.

AAB, frequently *Gluconobacter* spp., are often detected on healthy grapes (Renouf et al., 2005; 2007; Ultee et al., 2013). AAB populations are stimulated by berry damage, and grow to around $10^6$ CFU/g on rotten grapes (Barbe et al., 2001; Barata et al., 2012b). The conditions of winemaking result in loss of these strictly aerobic bacteria, although they can survive in the absence of oxygen (Bartowsky & Henschke, 2008). An illustration is the case of *Gluconobacter cerinus* detected on Riesling must and isolated throughout the fermentation period (Ultee et al., 2013). The populations of the other gram-negative bacteria also decline or disappear during the first days of AF, presumably because these species are not acidophilic.

### *2.3 Other microorganisms*

The microbial community on grapes contains other microorganisms, generally considered to act as spoilage agents. They include filamentous fungi of the genera *Aspergillus* and *Penicillium*, which may greatly influence the hygienic characteristics or sensory quality of wine through the production of mycotoxins (aflatoxins, ochratoxin A and others) or off-flavors (such as geosmin, IPMP and 2-MIB), respectively (Steel et al., 2013; Rousseaux et al., 2014). Other microorganisms may also be present and responsible for diseases, such as downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*) and gray mold (*Botrytis cinerea*, which also generates off-flavors) (Kassemeyer & Berkelmann-Löhnertz, 2009; Steel et al., 2013).

## 3. Interactions in wine

Wine is a complex microbial ecosystem containing mixtures of diverse microorganisms favoring interactions: there are presumably yeast-yeast interactions, bacteria-yeast interactions, bacteria-

bacteria interactions and filamentous fungi-yeast interactions. Physical contact between microorganisms, quorum sensing, predation, parasitism, symbiosis and inhibition are all direct interactions; indirect interactions are due to the presence of extracellular metabolites and include neutralism, mutualism, commensalism, amensalism and competition (Verachtert et al., 1990; Nissen et al., 2003) (Figure 1). There may also be horizontal gene transfer and DNA exchange between two microbes may benefit one of the two partners.

Filamentous fungi are present in the consortia and can interact with each other or with other microorganisms; however, they grow poorly during the fermentation process, and consequently, we will not discuss filamentous fungi-yeast interactions. Note that various strains of yeast have been reported to produce compounds inhibiting filamentous fungi (Fleet, 2003; Bleve et al., 2006; Ponsone et al., 2011; Cubaiu et al., 2012; Kapetanakou et al., 2012).

### *3.1 Yeast-yeast interactions*

Fermentations involving added or natural complex yeast consortia exhibit numerous kinds of interactions (Frey-Klett et al., 2011). Some yeasts develop simultaneously during AF, and physiological and metabolic interactions are established in most cases. For winemaking, the effects of these interactions are characterized as being positive, negative or neutral (Sieuwerts et al., 2008).

#### *3.1.1 Negative interactions*

Ethanol produced notably by *S. cerevisiae* is the major compound that influences diversity of yeasts during AF, especially non-*Saccharomyces* species (Heard & Fleet, 1988). Indeed, several

studies have demonstrated that the accumulation of ethanol during AF leads to a biodiversity declin (Constanti et al., 1997; Beltran et al., 2002; Combina et al., 2005). This decrease is owing to a low ethanol tolerance of most of the non-*Saccharomyces* yeast (Fleet et al., 1984; Heard & Fleet, 1985; Fleet, 1990; Pina et al., 2004; Jolly et al., 2005). Even if ethanol tolerance within a specific species could vary greatly (Caridi & Ramondino, 1999), most of indigenous yeast species (*Hanseniaspora, Candida, Pichia, Kluyveromyces, Metschnikowia* and *Issatchenkia*) usually do not survive above ethanol concentration ranging from 3 to 10% (v/v) (Jolly et al., 2014). However, some non-*Saccharomyces* species can survive until the end of the AF due to their high resistance to ethanol (Pina et al., 2004; Combina et al., 2005): *Torulaspora delbrueckii, Candida zemplinina, Zygosaccharomyces bailii, Schizosaccharomyces pombe* and *Pichia* spp. (Ciani & Ferraro, 1998; Santos et al., 2008; Jolly et al., 2014).

One of the most famous examples of negative interaction is the amensalism (the growth of one strain is restrained by the coexistence of another and by the secretion of metabolites). The most extreme amensalism described is the killer phenomenon, discovered 50 years ago (Bevan & Makover, 1963): the production of specific extracellular proteins and glycoproteins by certain yeast strains (killer yeasts) that kill other strains (sensitive yeasts). There is an extensive literature describing this phenomenon for *S. cerevisiae* strains and detailing the nature of these proteins (Young, 1987; van Vuuren & Jacobs, 1992; Shimizu, 1993; Musmanno et al., 1999; Gutierrez et al., 2001). The killer phenomenon contributes to the succession of different yeast strains during fermentation. Perez et al. (2001) observed that, added to sterile filtered must, an initial proportion of 2-6% of killer yeasts was responsible for protracted fermentation and suppression of isogenic sensitive strains. Pommier et al. (2005) reported the interactions between

two strains of *S. cerevisiae* (a killer strain and a sensitive strain) in co-cultures using a specific membrane bioreactor. Killer strains of *S. cerevisiae* sometimes predominate at the completion of fermentation, suggesting that they have asserted their killer property and taken over the fermentation (Fleet, 2003). However, it has been difficult to assess if the killer phenomenon was involved in the premature disappearance of non-*Saccharomyces* yeasts during the early stages of fermentation because the killer toxins produced by *S. cerevisiae* are active only against strains of the same species. Recently, however, Albergaria et al. (2010) found that the 2-10kDa protein fraction of *S. cerevisiae* CCMI 885 supernatants expresses a fungistatic effect on *Kluyveromyces marxianus, K. thermotolerans, Torulaspora delbrueckii* and *Hanseniaspora guilliermondii* and a fungicidal effect on *K. marxianus*. Branco et al. (2014) using mass spectrometry identified peptides derived from the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in this fraction.

Some non-*Saccharomyces* yeasts have been reported to present a killer character. For example, *K. phaffii* produces a killer toxin (zymocin KpKt) against yeasts including those of genus *Hanseniaspora* (Ciani & Fatichenti, 2001). Comitini et al. (2004) found that *Pichia anomala* and *K. wickerhamii* can secrete two toxins (mycocins) KwKt and PIKT, active against spoilage yeast of the *Brettanomyces* genus. Santos et al. (2009) described a toxin (PMKT2) produced by *Pichia membranifaciens* active against *B. bruxellensis*. Farris et al. (1991) and Lopes & Sangorrin (2010) found that *Metschnikowia pulcherrima* exhibited killer activity. Thus, killer interactions may determine species and strain populations during fermentation.

Other compounds formed during fermentation may also affect cell growth or death. Short fatty acids, medium-chain fatty acids, acetic acid (including acetic, hexanoic, octanoic and decanoic acids) and acetaldehyde produced by different yeast species have all been shown to play antagonistic roles against each other (Bisson, 1999; Fleet, 2003; Giannattasio et al., 2005; Ivey et al., 2013).

An antimicrobial activity of strains of *Metschnikowia pulcherrima* against various non-*Saccharomyces* yeasts has been demonstrated. These strains expressed a broad and effective antimicrobial action against undesired wild spoilage yeasts, including those of the *Brettanomyces*/*Dekkera*, *Hanseniaspora* and *Pichia* genera (Oro et al., 2014). The antimicrobial activity of *Metschnikowia pulcherrima* seems to come from the pulcherriminic acid (the precursor of pulcherrimin pigment), which depletes the medium of iron, making it unavailable to the other yeasts (Sipiczki, 2006; Türkel & Ener, 2009; Oro et al., 2014).

Competition for nutrients and other compounds can modulate the population of yeast during fermentation. Some non-*Saccharomyces* yeasts found in grape must and during fermentation are described as being aerobic such as *Pichia* spp., *Debaryomyces* spp., *Rhodotorula* spp., *Candida* spp. and *Cryptococcus albidus* (Combina et al., 2005; Jolly et al., 2014). In winemaking conditions, low available oxygen levels during fermentation promotes the growth of species that grow in anaerobic conditions, such as *S. cerevisiae* (Holm Hansen et al., 2001). The removal of residual oxygen from fermenting must can contribute to the early death of non-*Saccharomyces* species. Non-*Saccharomyces* yeasts with an oxidative and weakly fermentative metabolism

appear to be less tolerant to low oxygen availability than *S. cerevisiae* (Holm Hansen et al., 2001).

In fermenting wine musts, assimilable nitrogen and vitamins may be rapidly depleted if the initial nutrient content of the grape juice is poor. Competition for assimilable nitrogen is a determinant factor for the behavior of strains during fermentation. Taillandier et al. (2014) reported that *S. cerevisiae* was not able to develop because of nitrogen exhaustion by *Torulaspora delbrueckii* growth during the first 48h, leading to sluggish fermentation. In wine fermentations where the initial microflora is mainly composed of non-*Saccharomyces* species, amino acid and vitamin consumption during the first days of fermentation can severely impede the subsequent growth of *S. cerevisiae* strains (Fleet, 2003). Medina et al. (2012) reported that the competitive advantage usually observed for *S. cerevisiae* in mixed cultures is limited by reduced nutrient (nitrogen, vitamins) availability caused by their retention or removal from the medium by non-*Saccharomyces* strains (*Hanseniaspora vineae* and *Metschkowia pulcherrima*). Mortimer (2000) observed that the growth of *S. cerevisiae* is affected by thiamine limitation due to the presence of a *Kloeckera apiculata* strain.

*3.1.2 Positive interactions*

Most of the synergistic interactions between yeasts observed are between non-*Saccharomyces* and *S. cerevisiae*. For example, in a *Kloecker aapiculata /S. cerevisiae* co-culture, the apiculate cells remained viable for longer than in pure culture (Mendoza et al., 2007).

Commensalism between non-*Saccharomyces* and *S. cerevisiae* has been also evidenced. The high extracellular proteolytic activity of some non-*Saccharomyces* yeasts (Charoenchai et al.,

1997; Dizy & Bisson, 2000) causes the release of amino acids from proteins present in the medium, and these amino acids are then used by *S. cerevisiae* (Fleet, 2003). The early death of non-*Saccharomyces* yeasts after the early stages of AF can also provide nutrients for *S. cerevisiae* thanks to the passive release of amino acids and autolysis. Conversely, *S. cerevisiae* autolysis after AF may be a significant source of micronutrients for the growth of spoilage species, especially those of *Dekkera*/*Brettanomyces* (Guilloux-Benatier et al., 2001). Among the non-*Saccharomyces* yeast species, *B. bruxellensis* is better adapted than other wild yeasts to persist during AF thanks to its ethanol tolerance (Renouf et al., 2007).

Some metabolites produced by one yeast species can benefit other species. Cheraiti et al. (2005) showed that the maximum population of a mixed culture of *S. cerevisiae* and a *S. cerevisiae* x *S. uvarum* hybrid strain was much higher than the sum of the maximum populations of the two strains grown in pure cultures. They found that the mixed culture during fermentation produces large quantities of acetaldehyde that *S. cerevisiae* strain can use. *S. uvarum* produces much more acetaldehyde than *S. cerevisiae* in the resulting wine (Ciani et al., 1994; Castellari et al., 2002). The acetaldehyde produced by the *S. cerevisiae* x *S. uvarum* strain causes a shift towards lower cellular NAD(P)H levels in the *S. cerevisiae* cells. This change in redox potential is related to increases in both biomass and specific fermentation rate.



### *3.2 Yeast-bacteria Interactions*

The interactions between bacteria and yeast during AF and MLF have a direct effect on induction and completion of MLF, which is an important factor for wine quality. Various studies have addressed this interaction using different yeast/bacteria pairs, summarized in a

comprehensive earlier review (Alexandre et al., 2004). These studies reported in the review demonstrate that the type of interaction is highly dependent on the pair of strains involved. One bacterium could be inhibited and another stimulated by the same yeast strain (Nehme et al., 2008). One explanation might be that yeast strains produce different amounts of inhibitory and/or stimulatory compounds while the sensitivity of bacteria towards these compounds is strain-dependent (Hennick-Kling, 1993; Arnink & Hennick-Kling, 2005; Rosi et al., 2003; Comitini et al., 2005; Guilloux-Benatier et al., 2006; Osborne & Edwards, 2006). Here, we summarize the major elements of the earlier review of (Alexandre et al., 2004) and describe progress over the last ten years in more detail. The following types of indirect interactions will be considered with a focus on biochemical issues: antagonism, amensalism, competition and commensalism.

*3.2.1 Amensalism/Antagonism*

The ability of some wine yeasts to inhibit malolactic bacteria has been the most extensievely studied (Ribereau-Gayon & Peynaud, 1961; Lafon-Lafourcade, 1973; Wibowo et al., 1988; Osborne & Edwards, 2006). The inhibition is mediated by several bioactive yeast compounds and often involving combinatory effects:

*Ethanol*

The alcohol concentration after yeast fermentation is between 10% and 16%. All *O. oeni* strains are able to survive and proliferate at 10% v/v ethanol at pH 4.7 (Britz & Tracey, 1990). G-Alegria et al. (2004) reported that *O. oeni* and *L. plantarum* strains grow at 13% v/v ethanol at their optimal temperature (18-20°C) and Henick-Kling (1993) stated that ethanol concentrations between 10 and 14% v/v inhibit completely the growth of *O. oeni* at 25°C. Ethanol may increase

cell permeability by fluidizing membrane lipids, thereby enhancing passive proton influx and leakage of cell metabolites (da Silveira et al., 2003; Chu-Ky et al., 2005). Generally, the toxicity of ethanol increased with decreasing pH (Chu-Ky et al., 2005).

*Sulfur compounds*

At typical wine pH, $SO_2$ exists in both free forms including molecular $SO_2$, bisulfite ($HSO_3^-$) and sulfite ($SO_3^{2-}$), and as bound forms. *S. cerevisae* can produce sulfite during the sulfate reduction pathway in which sulfate is reduced to sulfite and then incorporated into sulfur-containing amino-acids (Duan et al., 2004). Sulfite efflux via the SSU1 pump is considered to be a detoxification pathway for yeast cells (Park & Bakalinsky, 2000). The sulfite released turns into bisulfite and molecular $SO_2$ in the acid wine environment. Generally there is more bisulfite at wine pH; however molecular $SO_2$ has a higher antimicrobial activity probably due to its ability to diffuse through cell membranes (Quirós et al., 2012). After entering LAB cells, molecular $SO_2$ is converted to bisulfite and sulfite thereby releasing protons and acidifying the medium (Figure 2). $SO_2$ can react with various cell components, such as ATPase and cofactor $NAD^+$ (Carreté et al., 2002), and thereby inhibit LAB growth. Its molecular mechanism of action may involve rupturing disulfide bridges in proteins (Bauer & Dicks, 2004) (Figure 2). The antimicrobial activity of molecular $SO_2$ can also affect malolactic activity (Henick-Kling, 1993; Lonvaud-Funel, 1999).

Henick-Kling & Park (1994) suggest that the $SO_2$ added to grape juice, combined with that produced by yeast, determine the success of MLF induction. In practice the amount of $SO_2$ depends on the yeast strain and the medium composition. Some strains are reported to produce

more than 100 mg/L although most currently used commercial yeast strains produce only up to 20 mg/L (Rankine & Pocock, 1969; Suzzi et al., 1985). Low pH medium enhances the inhibition since more $SO_2$ can diffuse through the membrane (Wells & Osborne, 2011).

It has been reported that the antimicrobial actions of sulfur-binding compounds are more important than previously believed (Larsen et al., 2003). Bisulfite can react with carbonyl groups, and such structures are commonly present in wine (de Azevedo et al., 2007). For example, there is substantial acetaldehyde production during exponential phase of yeast and this can quickly bind $HSO_3^-$ to form hydroxysulfonic acid (Wells & Osborne, 2011). *O. oeni* consumes acetaldehyde, thereby releasing free $SO_2$ and conseuqnelty inhibiting bacterial growth and ML activity (hypothetical pathway in Figure 2) (Osborne et al., 2000). Other $SO_2$-binding compounds, such as ketonic acids, sugars, quinones and anthocyanins, are present at only lower concentrations in wine compared to acetaldehyde and have been less well studied. In fact, sulfur compounds constitute a specific signature of the wine metabolome (Roullier-Gall et al., 2014) but their origins and roles are still unknown.

*Medium-chain fatty acids*

Medium-chain fatty acids (MCFAs) in yeast cells are precursors of long-chain membrane phospholipids and volatile esters (Saerens et al., 2010). They can be released into the extracellular environment by simple diffusion and impair both bacterial growth and malolactic activity (Alexandre et al., 2004). In LAB cells, MCFA molecules deprotonate, causing intracellular acidification and the dissipation of transmembrane gradient, thereby inhibiting ATPase, an enzyme closely associated with malolactic activity (Tourdot-Marechal et al., 1999).

The inhibition acts synergistically with low pH and with ethanol (Capucho & San Romao, 1994). It is significant that this inhibition is concentration-dependent. According to Capucho & San Romao (1994), a decanoic concentration above 12.5 mg/L and dodecanoic concentration above 2.5 mg/L cause inhibition. Below these concentrations, these compounds seem to be beneficial for bacterial growth. Additionally, the combined effect of hexanoic and decanoic acids, together with ethanol, is more inhibitory than individual MCFAs (Lonvaud-Funel et al., 1988).

*Proteins and Peptides*

Dick et al. (1992) first characterized an antibacterial factor produced by yeast as a cationic protein. Comitini et al. (2005) inferred that a MLF inhibitory compound was a protein: it was heat and protease sensitive. Nehme et al. (2010) confirmed the existence of a yeast-derived peptide fraction that was partially responsible for MLF inhibition. Recent studies have focused on active antimicrobial peptides (AMPs < 10 kDa). A $SO_2$-dependent AMP was found by Osborne & Edward (2007) and its mechanism may involve disruption of the cell membrane. Branco et al. (2014; cf. 3.1.1) using mixed cultures with TDH1-3 (GAPDH genes)-deleted *S. cerevisiae* mutants confirmed that AMP derived from GAPDH contribute to bacterial inhibition. Possible mechanisms of this inhibition include binding to bacterial DNA/RNA, thereby suppressing the DNA replication and protein synthesis (Brogden, 2005).

*Small Metabolites*

Other yeast metabolites have been found to be involved in yeast-bacteria interaction phenomena. For example, succinic acid production and malic acid consumption by yeast can modify the pH of the medium, an important determinant of bacterial growth and ML activity (Henick-Kling,

1993). 2-Phenylethanol (2-PE) can be synthesized from L-phenylalanine via the yeast *Ehrlich* pathway. The antimicrobial properties of 2-PE include inhibition of sugar and amino acid transport systems on the cell membrane (Etschmann et al., 2003) and possibly the inhibition of macromolecule synthesis by bacteria (Lucchini et al., 1993).

*Bacteria Antagonising Yeast*

It has been reported that contamination by *Lactobacillus* spp. (biomass at $4.5*10^8$ CFU/mL at 30h of AF) of yeast culture can cause a stuck AF via various mechanisms (Narendranath et al., 1997). First, the short-chain carboxylic acid produced from LAB metabolism, such as acetic acid, may acidify the yeast intracellular environment and accelerate yeast death (Bayrock & Ingledew, 2004). The existence of extracellular β-1, 3-glucanase activity implies that LAB may potentially be able to degrade yeast cell walls (Guilloux-Benatier et al., 2000). Bacteriocin-like compounds are also candidates for inhibiting yeast growth (Yurdugul, 2002; Halil et al., 2014).

*B. bruxellensis* spoilage is a serious problem for the wine industry: it confers off-odors to the wine and changes its aromatic quality. The wine after AF and before MLF is extremely apt for the growth of *B. bruxellensis* due to its microbiological instability. In practice, the use of malolactic leaven with a high *O. oeni* population density can restrict *B. bruxellensis* development, implying that this bacterium expresses antogonism towards spoilage yeast (Renouf & Murat, 2008).

*3.2.2 Competition for Nutrition*

LAB have been described as 'fastidious' with regards to their nutritional requirements due to their limited biosynthetic capabilities (Terrade & Mira, 2009). Therefore, delayed growth is possible if yeast strains have high nutrient demand during AF or a longer death phase. LAB are auxotrophic for various amino acids (e.g., glutamate, arginine and tryptophan) (Remize et al., 2006) and vitamins (e.g., biotin and pantothenic acid) (LeBlanc et al., 2011), a yeast-bacterial co-culture will have difficulty launching MLF if the yeast rapidly depletes these nutrients during AF and until the end of dead phase (Arnink & Henick-Kling, 2005). However, some studies demonstrate that the extended yeast death phase does not necessarily explain the observed inhibition of *O. oeni* (Patynowski, 2002). LAB may use up trace nutrients and survival factors (probably protein in nature) in continuous fermentation, resulting in acceleration of death and sluggish fermentation (Bayrock & Ingledew, 2004). The biochemical basis of competition between yeast and LAB is still not fully understood.

*3.2.3 Commensalism*

*Nitrogen compounds*

Stimulation of malolactic bacteria by yeast has been studied in less detail. In practice, the antagonistic effects of yeast on malolactic bacteria usually decrease when wine is left in contact with lees after AF. The bacteria probably benefit from the release of nutrients, especially nitrogen compounds, during yeast autolysis. Among the nitrogenous fractions of yeast autolysate, the smallest (<1kDa) is the most effective for stimulating bacterial growth (Feuillat et al., 1977). This fraction contains important amino acids, such as arginine, isoleucine, glutamic acid and tryptophan (Guilloux-Benatier & Chasagne, 2003). Bigger fractions containing

macromolecules, such as cell wall polysaccharides and proteins, may shorten the lag phase and stimulate the growth of *O. oeni* (Guilloux-Benatier et al., 1995). Yeast macromolecules in the medium can induce aminopeptidase activity in *O. oeni* (Guilloux-Benatier et al., 1993). The protease activity of the strain X2L has been studied under starved conditions (Faria & Manca, 2000). These bacterial proteases are responsible for the hydrolysis of yeast proteins into essential amino acids and peptides, and thereby enrich the medium in nitrogenous nutrients.

Studies on the yeast side have focused on the cell wall glycoproteins, such as mannoproteins, produced during AF and autolysis (Fleet, 1991). These proteins can adsorb toxic MCFAs (Guilloux-Benatier & Feuillat, 1991) and phenolic compounds from the grape must (Vasserot et al., 1997), some of which have an inhibitory effect on LAB growth (Reguant et al., 2000). *O. oeni* possesses α-glucosidase, β-glucosidase, N-acetyl β-glucosamidase and peptidase activity and can thus release sugars and amino acids from these macromolecules (Cavin, 1988). The proteolytic activity expresses by yeast also has a direct effect on the nitrogen composition of the medium (Guilloux-Benatier et al., 2006).

*Smaller Metabolites*

Activities of various glycosidases produced by *O. oeni* suggest that LAB may be able to release free sugars as carbohydrate source from yeast-derived polysaccharides and glycoconjugated compounds (Grimaldi et al., 2005). Other yeast metabolites, such as vitamins, nucleotides and long chain fatty acids, may have stimulatory effects on malolactic bacteria growth and activities. However, this issue has not been extensively studied.

Yeast-bacteria interaction is a complex field of study. Various factors, such as pH and ethanol, act in synergy with others. Many yeast compounds involved in LAB stimulation/inhibition are still unidentified or uncharacterized. The future studies, thanks to new tools or methodologies, will reveal how and even whether these factors can be exploited for wine-marking, by chosing/engineering of strains, or adapting medium composition and fermentation conditions, to ensure successful MLF.



*3.3 Bacteria-bacteria interactions*

MLF generally occurs naturally after AF, usually due to *O. oeni*. However, members of other LAB genera, notably *Pediococcus*, *Lactobacillus* and *Leuconostoc*, are also present in must and wine and may have positive or deleterious effects on wine quality (Osborne & Edwards, 2006). Despite the importance of these bacteria, very little is known about how they interact.

Bacteria are auxotrophs for certain amino acids and secreted proteolytic activity to the extracellular medium to generate the amino acids necessary to sustain their growth (Remize et al., 2006; Ritt et al., 2008). It is thus likely that the amino acids released by extracellular protease from one strain promotes the growth of others. Unfortunately, this type of interaction has never been studied. The amino acids released by extracellular proteases are also precursors for Biogenic Amines (BA) production, affecting the hygienic and sensorial quality of the wine (Spano et al., 2010). Aredes-Fernandez et al. (2010) report that co-culturing of *O. oeni* and *L. hilgardii* strains diminished the growth yield of *O. oeni* but this decrease was not due to inhibitory substances or low pH. The competitive interaction between the two microorganisms

appears to involve the consumption of arginine, a stimulant for the growth of *O. oeni* (Aredez-Fernandez et al., 2010).

An example of mutualism between *Pediococcus* and *Oenococcus* has also been reported: a mutualistic growth response due to the proteolytic system of *O. oeni* was observed (Fernandez & Nadra, 2006). An analysis of BA production indicated that *L. hilgardii* produced more histamine in mixed cultures with *O. oeni* than in pure culture (Aredez-Fernandez et al., 2010).

Wine LAB in presence of oxygen produce $H_2O_2$, which oxidizes thiol groups. A consequence of this reaction is the denaturation of various enzymes (Byczkowski & Gessner, 1988). $H_2O_2$ also leads to membrane lipid peroxidation and could serve as the precursor for the formation of superoxide and hydroxyl radicals that damage DNA (Byczkowski & Gessner, 1988). Hydrogen peroxide production by *L. hilgardii* has been shown to restrict *O. oeni* growth (Rodriguez & Manca de Nadra, 1995).

Other compounds that have received great attention are bacteriocins. Bacteriocins produced by LAB are involved in antagonistic reactions between bacteria. Some LAB of oenological origin, such as *L. plantarum* and *P. pentosaceus*, produce bacteriocins (Rojo-Bezares et al., 2007; Knoll et al., 2008). Most bacteriocins act by forming pores and destabilizing the cell membrane. Exogenous added bacteriocins affect LAB in wine (Lonvaud & Joyeux, 1993; Rojo-Bezares et al., 2007; Diez et al., 2012). Pediocin PD-1 can successfully remove *O. oeni* biofilms from stainless steel surfaces in contact with Chardonnay must (Bauer et al., 2003). Diez et al. (2012) reported for the first time that a non-enological bacterium produces a well-known bacteriocin (pediocin PA-1) under enological conditions or in the presence of ethanol and grape juice. However, production of bacteriocin in wine by enological LAB has never been demonstrated.

Consequently, it is still unknown if this family of compounds plays a role in the interactions between bacteria in wine.

Although it is not a bacteria-bacteria interaction, interactions between bacteria and phage are pertinent. This is the sole example of parasitism known for bacteria in wine. Phages have been found in the wine-related species *Lactobacillus* (*L. casei*, *L. fermentum*, *L. plantarum*), *Leuconostoc* (*Leuconostoc mesenteroides*) and *Oenococcus* (*O. oeni*) (Neve & Josephsen, 2004). They can cause stuck MLF (Poblet-Icart et al., 1998). A high prevalence of lysogeny in the *O. oeni* species and the existence of four distinct groups of temperate bacteriophages was reported (Jaomanjaka et al., 2013). These recent findings illustrating the diversity of phages infecting *O. oeni* suggest that it would be valuable to reassess their impact on winemaking.

*3.4 Signaling based interactions and cell-cell contact*

Quorum sensing (QS) is a term used to describe cell-to-cell communication. This sensing mechanism is based on the production, secretion, and detection of small signalling molecules, whose concentration correlates with the abundance of secreting microorganisms in the medium (Choudhary & Schmidt-Dannert, 2010). Perception of the signal leads to various responses, such as the secretion of virulence factors, initiation of biofilm formation, sporulation, competence, mating, root nodulation, bioluminescence and production of secondary metabolites. Several classes of signaling molecules of microbial origin have now been identified, including *N*-acyl homoserine lactones (AHLs), furanosyl borate diester, and autoinducing peptides which are the best studied such molecules in bacteria (Cataldi et al., 2013). For yeast, bicarbonate, acetaldehyde, ammonia, farnesol, tryptophol and phenylethanol have been identified as QS molecules (Ivey et al., 2013). There is no evidence for a role in wine of tyrosol, tryptophol, or 2-

phenylethanol as QS molecules during AF by *S. cerevisiae*. However, during AF, QS molecules are secreted during the shift from exponential to stationary phase, which is the moment when starvation mechanisms initiate (Zupan et al., 2013). It has been suggested that these QS molecules could be involved in yeast-yeast interactions and responsible for early growth arrest of non-*Saccharomyces* yeasts in co-culture with *S. cerevisiae* (Nissen et al., 2003). The same authors propose that the early growth arrests of *K. thermotolerans* and *Torulaspora delbrueckii* in co-culture with *S. cerevisiae* are not due to a QS effect, but rather, that the yeasts possess a cell-cell contact mechanism regulating their growth in mixed cultures. However, such cell-cell contact is not the sole mechanism responsible for the observed effect. Indeed, in another study, Nissen et al. (2004) reported that glucose uptake and oxygen availability regulated *Torulaspora delbrueckii* and *S. cerevisiae* interactions. Evidence of a cell contact mechanism regulating *Torulaspora delbrueckii* cell density in co-culture with *S. cerevisiae* has been reported: Renault et al. (2013) observed a much higher viability of *Torulaspora delbrueckii* when physically separated from *S. cerevisiae* (co-cultures of the two yeasts in double fermenters) than in standard mixed co-culture. Acetaldehyde has been identified as playing a role in cell-cell communication: it affects biomass, by-product formation, and fermentation kinetics (cf. 3.1.2).

A major cell-cell contact mechanism is flocculation, defined as cells adhering in clumps that are rapidly separated from the medium by sedimentation. Efficient yeast flocculation after AF can lead to compacted sediments and facilitate the clarification process (Govender et al., 2011). Interestingly, strains which do not flocculate alone can co-flocculate when mixed together (Nishihara & Imamura, 2000). Sosa et al. (2008) showed that flocculent *K. apiculata* interacts with a non-flocculent strain of *S. cerevisiae* in mixed fermentations, inducing co-flocculation of

both strains. *S. cerevisiae*, *Dekkera* spp. and *K. apiculata* have been found to co-flocculate with several bacteria (Peng et al., 2001). All types of co-flocculation seem to be mediated by a lectin-carbohydrate binding system (Nishihara & Imamura, 2000; Peng et al., 2001; Sosa et al., 2008). There has been no study of bacteria cells in wine conditions regarding the existence of either cell-cell contact or QS mechanisms, so it is not known whether either phenomenon operates in fermentation conditions. Double fermentors are useful tools for investigating the cell-cell contact mechanisms and QS for both yeast and bacteria. Another approach likely to be informative is the use of microfluidic devices that allow the study of interactions at the level of the cell.

*3.5Horizontal gene transfer*

The potential of microbes to exchange genetic information through horizontal gene transfer (HGT) is a major factor in their genetic adaptation and evolution. Generally, successful HGT events between microbes are those leading to increased fitness for the receiving microorganism. The transfer of genes between bacteria is well documented, although research studies have focused on horizontal (or lateral) gene transfer between pathogens, particularly the spread of multi-drug resistance (Ochman et al., 2000). Diverse bacteria and yeast species are in close contact on grapes, and during AF and MLF, and this might promote HGT. The *S. cerevisiae* EC1118 genome sequence contains three gene clusters resulting from horizontal transfers (Novo et al., 2009). Genes in these clusters encode key functions linked to the winemaking process, such as carbon and nitrogen metabolism, cellular transport and the stress response. These observations strongly suggest that HGT is one of the mechanisms by which wine yeast strains

adapted to their high-sugar, low-nitrogen environment. The donor of some of the genes is *Zygosaccharomyces bailii*, a major wine spoilage microorganism, consistent with the idea that the coexistence of microbes in wine facilitates genetic exchange. Sequencing the genome of the commercial wine yeast strain EC1118 revealed a gene encoding a protein very similar to that encoded by the *S. pastorianus*-specific fructose symporter gene FSY1. This gene encodes a high-affinity fructose/H+ symporter (Galeote et al., 2010). The presence of a high-affinity fructose symporter in *S. cerevisiae*, not previously suspected, might confer an adaptive advantage during the fermentation of grape must (Galeote et al., 2011).

There is also evidence of HGT between wine bacteria. Indeed, some *L. plantarum* strains such as WCFS1 and ATCC 14917 do not carry the *tyrDC* and *tyrP* genes involved in BA production, however, recently, Bonnin-Jusserand et al., (2012) demonstrated that other *L. plantarum* such as IR BL0076 can produce the BA tyramine thanks to the presence of *tyrDC* and *tyrP* genes in its genome. It seems that this ability to produce tyramine was acquired by HGT. Indeed, the phylogenetic tree based on the sequence divergence of TyrDC and TyrP reveals that *L. plantarum* TyrDC and TyrP are closely related to those of *L. brevis* proteins and that these two species form a clearly separated cluster. From a physiological point of view, BA production may help LAB to survive in acidic conditions by the production of metabolic energy. Evidence of HGT is also available for *O. oeni*: genes possibly acquired from *L. plantarum* are associated with fitness and are stress responsive in wine (Bon et al., 2009).

## 4. Influence of microbial interactions on sensorial properties of wine

The nature of the interactions in wine is determinant for the sensorial and hygiene properties of the wine (cf. 3.1.2). Depending on the type of interactions, different species will have their growth stimulated, or alternatively inhibited. Different yeast species have different aromatic properties, so the nature of the species present, those microbes that successfully outcompete the other microorganisms, condition the final quality of the wine. Various microbes are present on grapes, in the must and during AF and MLF. Non-*Saccharomyces* yeast species are not considered as good candidates for high quality wine when present in pure culture, they may be of biotechnological value in mixed culture (Ciani et al., 2010; Sadoudi et al., 2012). Many studies involving controlled co-cultures have demonstrated the impact of interactions between yeast species on the wine composition, as reviewed by Ciani et al. (2010) and Jolly et al. (2014). To summarize, the presence of non-*Saccharomyces* yeast together with *S. cerevisiae* can result in a lower alcohol concentration, and increased concentrations of terpenoids, esters, higher alcohols, glycerol, acetaldehyde, acetic acid and succinic acid. The presence of specific enzymes in non-*Saccharomyces* yeast, such as glycosidase not encoded by *S. cerevisiae*, has consequences for flavor compounds (Rosi et al., 1994; Fernandez-Gonzalez et al., 2003). This enzyme releases volatile compounds from non-volatile precursors (Jolly et al., 2014). Other non-*Saccharomyces* extracellular enzymatic activities, such as proteolytic and pectinolytic polygalacturonase enzymes, contribute to the differences observed between results with pure cultures of *S. cerevisiae* and mixed culture with non-*Saccharomyces*. The literature on the organoleptic effects of such co-cultures (co-fermentation) is very rich, however, links between these organoleptic features and yeast-yeast interactions have not been reported.

At the end of the AF, the abundance of each aroma compound depends on several factors: the properties and biomass of each yeast species present, the survival time of each yeast species, the fermentation rate and of course the mechanisms of interaction between yeast species. Sadoudi et al. (2012) have shown recently that when aroma compound concentrations are normalized to total biomass, the biomass effect can be distinguished from interaction effects. The authors then demonstrate the existence of a synergistic effect (positive interaction) between *M. pulcherrima* and *S. cerevisiae* leading to the concentrations of aromatic compounds being higher than the sum of those for the same aromatic compounds in each mono-culture, independent of biomass. *Torulaspora delbrueckii*/*S. cerevisiae* co-culture is a model of passive interaction: the aromatic profile generally corresponds to the mono-culture profiles. The lower concentration of aromatic compounds in *Candida zemplinina*/*S. cerevisiae* co-culture than *Candida zemplinina* mono-culture suggests a negative interaction between these two yeasts. Some interaction mechanisms are known, such as competition for nutrients and oxygen, however, the molecular mechanisms underlying the higher production of aroma compound or lower production of acetic acid independently from the biomass have not been discovered.

The consequences of yeast co-culture for the aroma profile has been extensively studied, the influence of LAB and especially *O. oeni* on yeast has received less attention. For successful MLF, various strategies can be used. MLF could be completed by indigenous LAB either during AF or after AF. Another possibility is to inoculate must (co-inoculation with yeast) or wine (sequential inoculation after completion of AF) with LAB, generally *O. oeni*. Simultaneous inoculation can be an effective alternative to overcome potential inhibition of LAB by various factors as described above. The sensorial profile of the wine will differ depending on the choice

of strategy. However, contradictory results have been reported. Some studies indicate that yeast-bacteria co-inoculation can lead to stuck or sluggish fermentation due to antagonistic interactions, resulting in wines with high acetic acid concentrations and production of off-odors (Henick-Kling & Park, 1994; Edwards et al., 1999). On the other hand, several reports describe improvement of wine quality due to co-inoculation of yeast and bacteria (Mendoza et al., 2011). Izquierdo et al. (2012) report that total acidity and lactic acid content were higher in wines following co-inoculation than sequential inoculation for two different grape varieties (Tempranillo and Merlot). The co-inoculated wines contained less furfuryl alcohol and tyramine and more ethyl lactate than wines obtained by sequential inoculation. Differences between co-inoculation and sequential inoculation have also been confirmed from a sensorial point of view (Izquierdo et al., 2012); this study also revealed that concentrations of some BAs like cadaverine and tyramine were lower in wines produced by co-inoculation. The origin of these differences is not known and needs to be investigated.



## 5. Future perspectives

Genomics, transcriptomics, proteomics, metabolomics and other omics techniques provide static or dynamic representations how a single cell reacts in a microbial community and how microbial species interact with each other, and with the environment. These techniques have been used for investigations in waste water ecology (Werner et al., 2011), plant-soil ecology (Charles, 2010), the food industry (Mounier et al., 2008) and health-related host-microbiome ecology (Faith et al., 2011), where they have provided a clearer understanding and better prediction of the interaction mechanisms.

*5.1 Omics approaches*

A central goal of studies of these systems is to understand the population dynamics of different species. In the past 20 years, technologies for profiling microorganisms have developed, largely due to the availability of relatively inexpensive and efficient sequencing techniques; these technique have provided insight into microbial community composition and their temporal changes in response to environmental perturbation. The classical approach begins with isolation of a single species from a community, followed by culture and DNA/RNA extraction. The DNA/RNA is used for both individual biomass determination (Diguta et al., 2010) and functional studies to discover genes related to interactions with other species (Araújo et al., 2001; Shelburne et al., 2010). However this approach is time-intensive for understanding community composition and interaction-related genes. More importantly, only small fraction of microorganisms are successfully isolated and cultured (Hugenholtz, 2002). Consequently, currently strategies are shifting towards community analysis based on the total DNA/RNA extracted, hopefully from all microorganisms. A common isolation-free technique involves sequencing the 16S rRNA gene (18S rRNA for eukaryotes), because it contains conserved primer-binding sites and signature sequences for different bacterial species (Schmidt et al., 1991). This technique captures a rapid image of the composition of a microbial population at particular stage (Junicke et al., 2014). More recently, genome-wide sequencing approaches, notably whole-metagenome shotgun (WMS) sequencing and RNA-Seq (Whole Transcriptome Shotgun Sequencing), in which the whole genome of microorganism is explored instead of single rRNA gene, have added information about gene functions and expression levels. These metagenomic approaches could provide insight into the roles of different microbes within communities (Streit & Schmitz, 2004)

and predict the metabolic potential of communities (Larsen et al., 2011). Examples of applications include analyses of gut microbiome interactions with respect to the host (Qin et al., 2010; Rosenthal et al., 2011), plant-microbe interactions (Charles, 2010) and bacteria-fungi interactions in mixed-culture fermentations (Sieuwerts et al., 2008). The popularity and effectiveness of these techniques has increased substantially with the development of next generation sequencing and related bioinformatics tools. To detect microbial interactions through meta-omics profiles, several similarity metrics have been developed to identify combinations of microorganisms that reveal co-presence or mutual exclusion patterns according to samples from different locations or time points. Such bioinformatics tools include correlation networks (Friedman et al., 2012; Chaffronet al., 2010; Eiler et al., 2012) and multivariate statistics (Rudi et al., 2007; Raes et al., 2011). This type of approach could also be used to assess differentially abundant pathways within the community (Segeta et al., 2013). The main impediments to bioinformatics in this field are the compositionality bias after abundance normalization and the sparsity of data matrix (Aitchison, 2003).

Proteomics and metabolomics approaches have been developed to enhance gene function annotations, and improve the catalogs of inter-microbial small molecule and peptide signaling mechanisms. Protein biomarkers identified by proteomics approaches provide a clearer and more reliable picture of metabolic function of a microbial species than was previously possible (Wilmes & Bond, 2006). High throughput mass spectrometry has been used in an interesting meta-proteomics approach to study community proteomics in a natural acid mine drainage (AMD) microbial biofilm (Ram et al., 2005). Once the community protein is sequenced, it can be aligned to corresponding genomic sequences, thereby linking metabolic functions to individual

microbial species (Rastogi et al., 2011). By looking at the functions of proteins, the various roles of community members can be elucidated. The study also predicts the function of unknown proteins based on their localization in the cell, their abundance and protein-protein interactions. In more complex systems, like the human gut, the human microbiome project (HMP) has discovered previously unknown proteins and thereby microbial pathways, highlighting novel interactions within gut microbiome (Turnbaugh et al., 2007).

The metabolome, the complete set of metabolites produced by a microbe, presumably reflects its metabolic pathways and thereby provides an accurate snapshot of its physiological state (Garcia et al., 2008; Mashego et al., 2007). Untargeted meta-metabolomics reveals synergistic relationships, exchanges of metabolites and cell-to-cell signaling between species within a community (Raes et al., 2008; Jansson et al., 2009). Thanks to unprecedented ultra-high precision of mass measurements, meta-metabolomics combined with microbiome analysis further allows the identification of yet unknown metabolite markers through networks-based approaches (Walker et al., 2014).

*5.2 Post-omics modeling*

Thanks to advanced high-throughput technologies, a large number of omics projects arise. It is now possible to consider combining data from all the diverse omics approaches and thereby to interpret all the pathways of individual microbial species and even of entire microbial ecosystems (Witting & Schmitt-Kopplin, 2014). One possibility is to develop an interaction model composed of strains that have sequenced genomes in which products exchanged between strains are inferred biochemically and genetically (Stolyar, 2007). The idea generates genome-

scale metabolic models (GEM) for each species which allows working directly with metabolic networks instead of pathways (Marcotte, 2001). The reconstruction of GEM requires not only network-wide omics data, such as annotated whole genomes, but also detailed information about microorganisms and biochemical reactions (Feist et al., 2008; Borodina & Nielsen, 2005).

Once the reconstructed network is converted into a mathematical representation, it should allow the use of computational tools to study the properties of the network. Constraint-based analysis, such as flux balance analysis (FBA), is preferred for studying microbial interactions in this type of model due to its ability to predict a solution space for metabolic flux at steady-state of metabolite concentration using solely stoichiometric constraints. The advantage of this approach to investigations over pathway kinetic analysis is the ability to maintain prediction accuracy even in a complex network (Price et al., 2003; Feist & Palsson, 2008). Stolyar (2007) presented the first multispecies stoichiometric model to study the syntrophic growth of two microorganisms: *Desulfovibrio vulgaris* and *Methanococcus maripaludis*. The concept is to create a system of three compartments: the central metabolism of each organism is described by one compartment, and the third describes metabolite transfer in culture medium. The solution space was optimized by maximization of total biomass, with a priority on the dominant species *Desulfovibrio vulgaris*. The model confirms the fact that hydrogen transfer was essential for syntrophic growth. Zhuang (2011) extended dynamic FBA (Mahadevan et al., 2002) to dynamic multi-species metabolic modeling (DMMM). This method, unlike Stolyar's, could also be applied to non-interdependent relations, such as competition, because a separate FBA model is used for each microbial species in community and the solution space is optimized for maximum growth of each species. DMMM is able to predict the population dynamics and changes of extracellular

metabolite concentrations (Figure 3). Zomorrodi & Maranas (2012) further developed a multilevel optimization framework called OptCom. The inner problems, such as the biomass maximization of one species, are linked to the community-level /outer-stage problems through both flow constraints in the shared metabolite pool and community objective realization, such as maximization of total biomass in cases of mutualism. The framework integrates both species- and community-level fitness criteria and measures trade-offs between selfish and altruistic driving forces in a microbial ecosystem (Figure 3). The framework has been applied and adapted for a yeast co-culture model (Hanley & Henson, 2013) where it successfully predicts the inoculum concentration and aeration level that improves batch ethanol productivity. The model further suggests molecular engineering of the xylose transport system would allow similar improvements.

*5.3 Future wine omics*

The focus of studies on microbial interactions is shifting from compositional to functional, from targeted to untargeted, from static to dynamic and from descriptive to predictive, thanks to the exploitation of diverse omics data (Kau et al., 2011). The study of interactions between wine microbes is a major beneficiary of these developments (Cocolin et al., 2000; Mendes et al., 2013; Rossouw et al., 2014). Although GEM models of *S. cerevisae* are one of earliest reconstructed models (Förster et al., 2003), high-quality GEM models for other wine microorganisms are lacking (Mills et al., 2005). Although wine composition has a huge variability, further development in this field might lead to partial dynamic wine microbial modeling. It is expected that such models would help to predict the population dynamics and biochemical activities of

microbes and give informations regarding the aromatic profile of wine over the whole winemaking process; this would allows a better control of yeast and bacteria mixed-starter culture processes. Synthetic communities obtained by genetic engineering of one member or by removal/addition of one species in the mixed-starter culture could be used to improve wine sensory properties (Dunham, 2007).



**6. Conclusion**

This review presents the state of the art in research on microbial interactions in wines and highlights the existing gaps in our understanding of the mechanisms underlying interactions between microbes.

As stated in introduction, a better control of natural fermentation or fermentation by multi-starters requires a better understanding of the interaction mechanisms. There are still many questions to answer. It is clearly established that when two yeasts co-ferment, the aromatic compound profile is affected, but we still do not know why. We do not know why apart from ethanol and some other known compounds, non-*Saccharomyces* yeast dies early during co-culture with *S. cerevisiae*, and very little is known about the existence or effects of cell-cell contact or QS between yeast or bacteria in wine. A multidisciplinary approach is needed to find the answers to these and other questions. Here, we suggest various strategies that we believe should help unravel some of mechanisms that govern interactions among microbe in wine.

We are convinced that research in the field of wine microbiome would have tremendous consequences for monitoring wine fermentations. Interaction studies in wine would also

constitute a model that could benefit other fields like dairy, brewing, and bakery. In particular, we believe that the economic spinoff would be very substantial.



 

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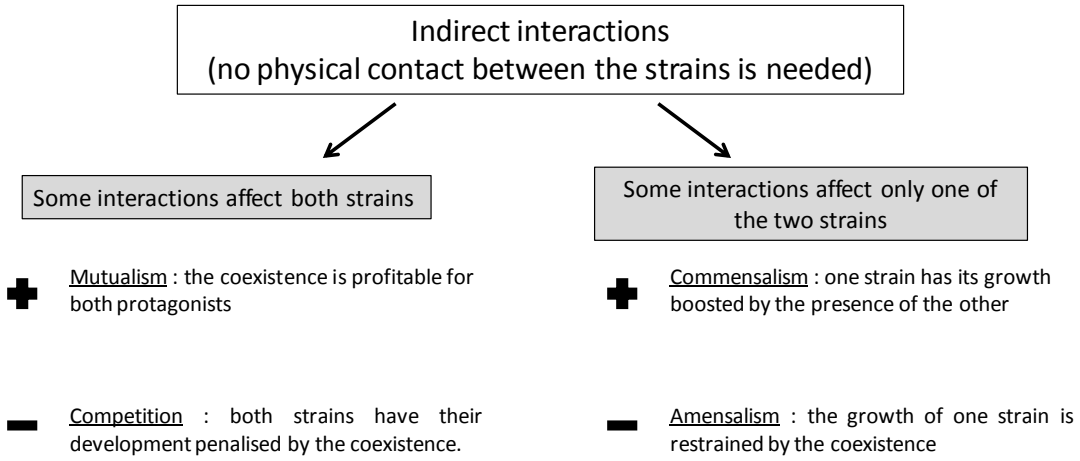


Figure 1: Schematic representation summarizing indirect interactions during the wine-making process

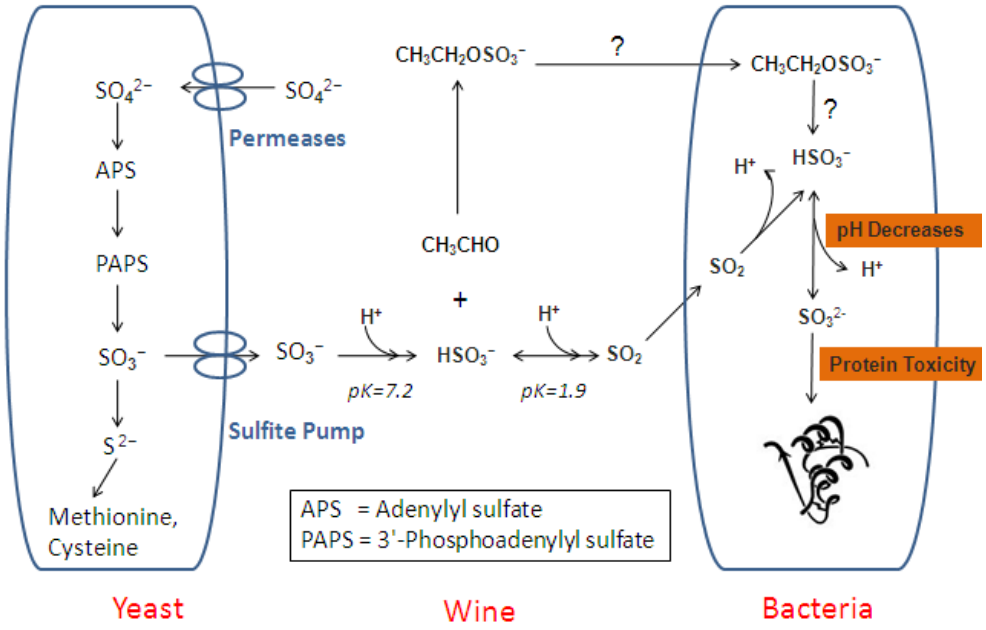


Figure 2: Effect on bacteria of $SO_2$ produced by yeast.

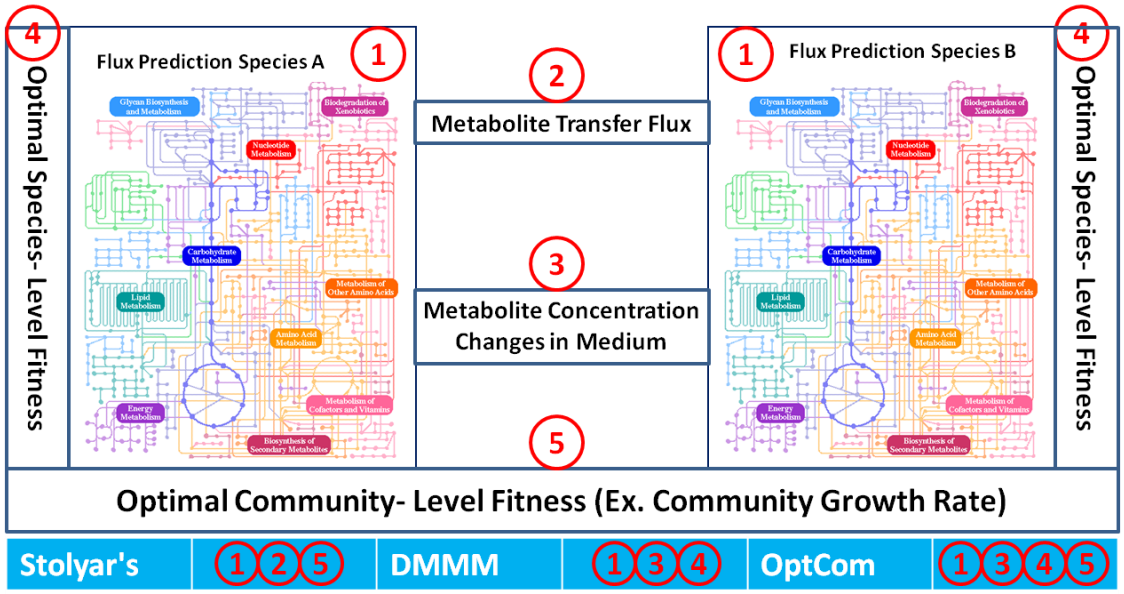


Figure 3: Goals of different multispecies FBA models.