Microbial interactions in winemaking: Ecological aspects and effect on wine quality

Microbial interactions in winemaking: ecological aspects and effect on wine quality

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ABSTRACT

Background: Wine microbiota is a dense and diverse ecosystem that is directly involved in the production and synthesis of many metabolites of oenological interest thereby directly affecting wine composition. The biodiversity and successional evolution of yeast and lactic acid bacteria (LAB) species and strains within species during alcoholic (AF) and malolactic fermentation (MLF) is greatly influenced by the complexity of the wine environment. Consequently, the successful prediction of wine characteristics is limited.

Scope and approach: The use of starter cultures has allowed better control of the fermentation process and the production of wines with desired characteristics. Mixed culture fermentations with selected non-*Saccharomyces* and *Saccharomyces* yeasts has regained attention in recent years due to their potential to modulate a wide range of metabolites of oenological interest. In this context, interactions among yeast species and LAB throughout the AF and MLF are known to influence the main enological parameters and aromatic profile of the wines. Studies have been conducted to uncover the nature of these interactions, with the aim to better control the AF and MLF.

Key findings and conclusions: This review provides an overview of microorganism interactions during the different steps of the winemaking process. This gives wine producers the ability to control and fine-tune microorganism population dynamics and therefore the fermentation process and finally wine quality.

Keywords: Wine yeasts; Lactic acid bacteria; Interactions; Selection; Fermentation

1. Introduction

Grapes and fermenting must for wine production represent a complex ecological niche that determines the presence and activity of specific yeast and bacteria species (Ciani et al., 2016). Despite the frequent dominance of *Saccharomyces cerevisiae*, it is generally accepted that a wide variety of non-*Saccharomyces* yeasts and lactic acid bacteria (LAB) are also present during spontaneous and inoculated wine fermentations. The non-*Saccharomyces* yeast and LAB also contribute significantly to the transformation of grape sugars into ethanol, carbon dioxide and other secondary metabolites essential to the flavour profile of wine (Dzialo, Park, Steensels, Lievens, & Verstrepen, 2017).

Currently, the use of inoculated mixed-cultures, based on the incorporation of multiple *S. cerevisiae* strains or specifically the addition of non-*Saccharomyces* yeasts and/or LAB (either in co-inoculation and sequential inoculation strategy), has been proposed as a solution to achieve the benefits of spontaneous fermentation while reducing the risks of spoilage and/or stuck fermentation (Fig. 1) (Padilla, Gil, & Manzanares, 2016). The benefits include improved wine complexity by increasing the diversity of chemical compounds present. Generally, wines with increased complexity are more preferred by consumers, and in mixed-culture fermentations yeasts produce aromas and flavours in way that cannot be reached with a single pure starter culture of *S. cerevisiae* (Jolly et al., 2014). Despite these positive factors, the fermentation conditions in which yeasts are subjected to need to be carefully controlled to achieve the desired results (Albergaria et al., 2016).

Successful mixed-culture fermentations can be achieved by increasing the contribution of the non-*Saccharomyces* yeasts by enhancing their metabolic activity and survival time (Morrison-Whittle, Lee, Fedrizzi, & Goddard, 2020). However, several scientific publications have reported contrasting results, even when the same species were studied (Albertin et al., 2017; Benito, 2019). Until recently, scientists generally believed that non-*Saccharomyces* yeasts "die off" and disappear during the early stages of AF, due to their low capacity to resist the changes in fermenting must composition (increasing ethanol levels, nutrient depletion). However, detailed studies have shown that the survival time and the reason for the disappearance of non-*Saccharomyces* yeast and bacteria includes several types of antagonistic interactions among the microorganisms (Liu et al., 2017).

These interactions can be passive (nutrients, oxygen and space competition) or active (antimicrobial compounds, volatile organic compounds, organic acids, cell-to-cell contact) (Di Gianvito et al., 2022). More recently it was demonstrated that some wine-related strains such

101 as *S. cerevisiae* (Legras et al., 2018), *Lachancea thermotolerans* (Hranilovic et al., 2018) and
102 *Torulaspora delbrueckii* (Albertin et al., 2014a), were able to survive until the end of wine
103 fermentation because they underwent a domestication event that made them highly adapted to
104 this man-made environment. Furthermore, it was demonstrated that in a wine environment
105 positive interactions also took place through the formation of mixed-species biofilms,
106 aggregation and/or cross-feeding (the product of one strain's metabolism may be utilised in the
107 nutrition of another). These interactive phenotypes were observed between *S. cerevisiae* and
108 *Lactobacillus* sp. (Xu et al., 2021) and between yeasts such as *Hanseniaspora vineae* (Bagheri
109 et al., 2017), *Saccharomyces uvarum* (Cheraiti et al., 2005), *Metschnikowia pulcherrima*
110 (Seguinot et al., 2020) or *Torulaspora delbrueckii* (Renault et al., 2016).
111 Scientific publications reporting on the impact of different non-*Saccharomyces* yeasts
112 with selected *S. cerevisiae* strains in mixed culture fermentations has increased significantly in
113 the last years. In both co-inoculation and sequential inoculation approaches it has been shown
114 that there are numerous chemical and physical interactions that influence compatibility and the
115 success of fermentation. The *S. cerevisiae*/non-*Saccharomyces* fermentation process presents
116 a new environment in which malolactic fermentation (MLF) needs to take place. Although the
117 effects of population dynamics during non-*Saccharomyces/S. cerevisiae* and LAB//*S.*
118 *cerevisiae* mixed-culture fermentations have received extensive attention, little is known about
119 the ability of LAB to perform MLF during or at the end of the fermentation of the wines
120 produced by mixed-yeast cultures. This review summarizes the current knowledge on
121 microbial interactions during wine making, with a focus on yeast-yeast and yeast-bacteria
122 interactions during alcoholic and MLF. The impact of mixed culture fermentations on LAB
123 involved in MLF and the most important factors that modulate these interactions, as well as
124 their impact on wine production, are also considered.
125
126 **2. Impact of non-*Saccharomyces* species on wine quality**
127
128 The impact that non-*Saccharomyces* yeasts have on wine quality largely depends on
129 the initial population (microbial numbers and species diversity) in the fermenting juice, albeit
130 from a natural population or inoculated strain (commercial or other) (Table 1). Must
131 characteristics such as osmotic pressure (sugar level), ratio of glucose to fructose, yeast
132 assimilable nitrogen (YAN), presence of sulfur dioxide (SO_2), temperature, degree of
133 clarification (for white musts) and presence/absence of inoculated *S. cerevisiae* all affects the
134 activity of the initial non-*Saccharomyces* population (Padilla et al., 2016). The degree of non-

135 *Saccharomyces* activity in turn determines the concentrations of metabolites formed. The
136 impact of more robust and ethanol tolerant non-*Saccharomyces* species can be expected to be
137 greater than more sensitive ones. However, as large strain diversity exists within species (Liu
138 et al., 2017) conclusions on the contribution after investigating a single strain, cannot necessary
139 be extrapolated to the entire species.

140 Wine flavour (aroma and taste) is made up of primary flavours derived from
141 compounds in the grapes themselves, secondary flavours due to yeast and LAB metabolites
142 and yeast mediated aromas from non-volatile precursors (Dzialo et al., 2017; Sumby et al.,
143 2019). Depending on concentration, these compounds can contribute either positively or
144 negatively to wine flavour. The range of flavour compounds produced or mediated by non-
145 *Saccharomyces* yeasts includes esters, higher alcohols, glycerol, terpenoids, acetic acid,
146 succinic acid, volatile fatty acids, carbonyl and sulfur compounds (Dzialo et al., 2017).

147 More than 160 esters have been identified with a positive effect on wine quality,
148 especially in wines produced from neutral grape varieties (Dzialo et al., 2017). Non-
149 *Saccharomyces* yeasts form varying levels of esters. Yeasts known to produce higher levels of
150 esters include *Hansenula anomala* (*Pichia anomala*), *Hanseniaspora uvarum* (*Kloeckera*
151 *apiculata*) and *Metschnikowia pulcherrima* (*Candida pulcherrima*) being regarded as higher
152 producers (Jolly et al., 2014). Higher alcohols produced from amino acid catabolism through
153 the Erlich pathway are generally not desired in wine, since high levels are strongly correlated
154 with unpleasant sensory attributes. However, low levels of higher alcohols can impart fruity
155 characters to wine and contribute to the wine's overall complexity (multiple identifiable
156 sensory elements). Although there is a large strain variability, non-*Saccharomyces* yeasts often
157 form lower levels of higher alcohols than *S. cerevisiae* (Jolly et al., 2014).

158 After ethanol, glycerol is the next major metabolite produced by yeast during wine
159 fermentation. Glycerol is important for regulating redox potential in the yeast cell but can also
160 contribute to mouth-feel, sweetness and complexity in wines (Dzialo et al., 2017). Extrinsic
161 factors such as grape variety and wine style determines the extent to which increased glycerol
162 levels impact on the wines' quality. Spontaneously fermented and non-*Saccharomyces*
163 inoculated wines often have higher glycerol levels than *S. cerevisiae* inoculated wines,
164 indicating a contribution by non-*Saccharomyces* yeasts (Jolly et al., 2014). Several non-
165 *Saccharomyces* yeasts, such as *Lachancea thermotolerans* and *Starmerella bacillaris* (also
166 known in older literature as *Candida zemplinina* or *Candida stellata*), consistently produce
167 high glycerol concentrations (up to 14 g/L) during wine fermentation (Table 1; Fig. 2).
168 However, increased glycerol production is linked to increased acetic acid (volatile acidity)

169 production (Dzialo et al., 2017). Volatile acidity is generally not desired in wine. However,
170 decreased volatile acidity and acetic acid concentration can be obtained when using some non-
171 *Saccharomyces* yeast in mixed fermentations with *S. cerevisiae* (Table 2). Volatile acidity is
172 especially a problem during production of wines from botrytized and/or high-sugar musts using
173 *S. cerevisiae* (Benito, 2019). A non-*Saccharomyces* yeast solution has been proposed whereby
174 *Torulaspora delbrueckii* and *Starm. bacillaris* could be used, in combination with *S. cerevisiae*,
175 to obtain wines with decreased levels of volatile acidity (Table 1).

176 Some non-*Saccharomyces* yeasts are linked to increased total acidity, a useful
177 characteristic where natural acidity in wine is lacking due to variances in temperatures during
178 grape ripening (Vilela 2019). *L. thermotolerans* is well known for its ability to produce lactic
179 acid that can be beneficial to wines produced in geographical regions affected by global
180 warming where grapes are characterized by low natural acidity (Binati et al. 2020; Balicki et
181 al. 2016). Increases in acidity due to the metabolism of *T. delbrueckii* is a result of the
182 production of succinic acid (Benito, 2019). However, as succinic acid is a harsher acid than
183 lactic acid and has a 'salt-bitter-acid' taste, excessive levels could be detrimental to wine
184 quality.

185 Wine aroma can also be affected when glycosylated flavourless precursors, present in
186 grapes, are hydrolysed by β-glucosidase enzymes to form free flavour-active volatiles (Dzialo
187 et al., 2017). These enzymes are not encoded by the *S. cerevisiae* genome (Maicas & Mateo,
188 2016). However, non-*Saccharomyces* yeasts belonging to the genera *Debaryomyces,*
189 *Hansenula*, *Candida, Pichia, Starmerella* and *Hanseniaspora* variably possess β-glucosidase
190 activity (Maicas & Mateo, 2016) so can play a role in the expression of wine aroma.

191 Wines from some grape varieties are more amenable to improvement by the
192 contribution of non-*Saccharomyces* yeasts than other varieties. For Chardonnay, co-
193 fermentation with *Debaryomyces pseudopolymorphus* and *S. cerevisiae* led to increased
194 concentrations of terpenols (citronellol, nerol and geraniol) in wine (Mateo & Maicas, 2016),
195 although the effect on wine aroma was not investigated. Similarly, co-fermentation with
196 *Debaryomyces vanriji* and *S. cerevisiae* produced Muscat wines with increased concentrations
197 of several terpenols (Mateo & Maicas, 2016), that could make a positive contribution to the
198 Muscat wine aroma. It was also shown that mixed cultures of *Starm. bacillaris* and *S. cerevisiae*
199 or *T. delbrueckii* and *S. cerevisiae* produced Sauvignon Blanc wines with high concentrations
200 of terpenols compared to reference wines fermented with only *S. cerevisiae* (Jolly et al., 2014).
201 Varieties such as Sauvignon Blanc and Chenin Blanc depend on volatile thiols to contribute to
202 the varietal character of the wine. It has been shown that non-*Saccharomyces* yeasts such as

203 *Starm. bacillaris* and *Pichia kluyveri* can produce significant amounts of the volatile thiols 3-
204 sulfanyl hexanol (3SH) and 3-sulfanyl hexyl acetate (3SHA), respectively, in Sauvignon Blanc
205 wines (Anfang, Brajkovich, & Goddard, 2009). Similarly, *T. delbrueckii*, *M. pulcherrima* and
206 *L. thermotolerans* have also been described as being able to produce significant amounts of
207 3SH during Sauvignon Blanc fermentation (Fig. 2, Table 1).

208 Ethanol, although the main product of alcoholic fermentation, is a cause for concern for
209 modern consumers, who now demand wines containing low to moderate alcohol levels. The
210 use of non-*Saccharomyces* yeasts in fermentation can lead to lower ethanol yields due to lower
211 sugar-ethanol transformation efficiencies when compared to *S. cerevisiae.* A possible counter
212 effect is a high residual sugar concentration. Another natural approach to decease wine ethanol
213 levels is to take advantage of the respiratory metabolism found in some non-*Saccharomyces*
214 species (Gonzalez, Quirós, & Morales, 2013). It has been shown that using an aeration regime,
215 alcohol content could be lowered by 1.5, 2.0 and 3.8% by *T. delbrueckii, Zygosaccharomyces*
216 *bailii* and *M. pulcherrima,* respectively (Contreras et al., 2015). The trials were done in a
217 chemically defined medium, so the effect of aeration on wine aroma was not established. With
218 more intensive aeration, the use of *Williopsis saturnus* in a laboratory-scale protocol could
219 produce a 3.0 % (v/v) ethanol wine from a 15% (w/v) total sugar grape juice that was judged
220 to have an interesting, but acceptable estery and fruity sensory profile (Jolly et al., 2014).

221 Non-*Saccharomyces* yeasts have also been reported to affect the mouth-feel properties
222 (texture or body) and colour of wine (Table 1; Fig. 1) by increasing polysaccharides
223 concentrations (Domizio et al., 2011;) and affecting phenolic composition, respectively
224 (Escribano-Viano et al., 2019). Polysaccharides can affect wine taste and mouth-feel positively
225 by increasing the perception of wine viscosity and fullness on the palate. Specifically strains
226 of *Hanseniaspora osmophila*, *Pichia fermentans*, *Saccharomycodes ludwigii*, Z. *bailii* and/or
227 *Zygosaccharomyces florentinus* as mixed cultures with *S. cerevisiae* were found to produce
228 wines with increased polysaccharides concentrations (Domizio et al., 2011).

229 Wine astringency, bitterness and colour is determined by phenolic content. Yeast cell
230 walls can adsorb anthocyanins during fermentation. These anthocyanisn can then interact with
231 mannoproteins and arabinogalactans in the wine. The degree of adsorption is generally
232 dependent on the yeast species and strain Non-*Saccharomyces* yeasts therefore affect the
233 composition of polyphenols (Escribano-Viano et al., 2019).

234 Sequential fermentation of grape juice enriched with anthocyanins using *Pichia*
235 *guilliermondii* and *S. cerevisiae* lead to increased formation of vinylphenolic
236 pyranoanthocyanins molecules, which showed greater wine colour stability (Benito, Morata,

237 Palomero, Gonzalez, & Suarez-Lepe, 2011). *T. delbrueckii* has also been shown to improve
238 colour (anthocyanins) and mouthfeel (flavanols) of red wine, but this was dependent on grape
239 variety and as already mentioned, the specific yeast strain (Escribano Viana et al., 2019).
240 Some non-*Saccharomyces* can also play a non-fermentative role in the wine production
241 process by producing extracellular proteolytic and pectolytic (polygalacturonase) enzymes.
242 These enzymes could potentially play a role in reducing wine protein levels with the
243 accompanying increase in wine protein stability (Belda et al., 2016). Therefore, lower doses of
244 extraneous enzymes would be needed bringing about cost savings to the producer. Non-
245 *Saccharomyces* yeast can also deplete essential nutrients in the fermenting must adversely
246 affecting the ability for *S. cerevisiae* to complete a sequential fermentation. However, contrary
247 to this is the death and lysis of weaker non-*Saccharomyces* yeast cells during the earlier phases
248 of fermentation that can in turn be a source of nutrients, especially nitrogen, for *S. cerevisiae*
249 (Prior, Bauer, & Divol, 2019).
250 Non-*Saccharomyces* yeast metabolites acting against spoilage organisms e.g.
251 *Brettanomyces bruxellensis,* is another area receiving attention (Mewa Ngongang et al., 2019;
252 Di Gianvito et al., 2022). This has potential for application during wine production, maturation
253 and storage to preserve the wine quality. The success of the use of non-*Saccharomyces* yeast
254 in research wines has led to the commercialisation of a number of species. The phenotypic
255 traits of the commercial yeast available in the market are shown in Fig. 2.
256
257 **3. Mixed yeast alcoholic fermentations and their effect on LAB and malolactic**
258 **fermentation**
259
260 MLF is a secondary fermentation process that plays an important role in the production
261 of many red and full-bodied white wines. During this secondary fermentation LAB are
262 responsible for the enzymatic decarboxylation of L-malic to L-lactic acid thereby providing
263 deacidification, with a concomitant increase in pH (Sumby, Bartle, Grbin, & Jiranek, 2019).
264 Other benefits are the enhancement of microbial stability through removal of nutrients from
265 the medium, and contributing to the flavour profile of the wine (Sumby et al., 2019). The LAB
266 responsible for MLF, include the genera *Oenococcus*, *Lactobacillus sensu lato*,
267 *Lactiplantibacillus*, *Pediococcus* and *Leuconostoc*. Over recent years, various reviews have
268 been published giving increasing amounts of information on bacterial metabolism during MLF
269 and the coexistence and compatibility of the LAB with yeast starter cultures (Krieger-Weber,
270 Heras, & Suarez, 2020; Sumby et al., 2019). The phenotypic traits of the commercial LAB

271 available in the marker are presented in Fig. 2. *Oenococcus oeni* is best adapted to the harsh
272 conditions found during fermentation, which includes high ethanol, low pH and the presence
273 of SO_2. Concomitantly, the majority of the commercial LAB starter cultures belong to this
274 species. In recent years, *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) has
275 also been considered to be a promising LAB to be used as a malolactic starter culture (Krieger-
276 Weber et al., 2020). This is mainly due to its ability to conduct MLF and produce a wide range
277 of extracellular enzymes like glucosidases, b-glucosidases, esterases, phenolic acid
278 decarboxylase (PAD) and citrate lyases able to enhance the sensorial properties of the wines to
279 higher levels than that achieved by *O. oeni* strains. The glucosidase activity of *L. plantarum*
280 strains are greatly affected by the environmental factors such as pH, ethanol and temperature
281 of the medium, while it was found to be strain-dependent (Kriger-Weber et al., 2020). Previous
282 studies on *L. plantarum* isolated from grape and wine samples demonstrated that 60% of the
283 overall isolates possess genes encoding for esterases.
284 The selection of LAB species and strains within species, as well as the inoculation
285 protocol (co-inoculation or sequential inoculation), is crucial to ensure a fast and successful
286 MLF. This is due the interactions between LAB and yeasts having a direct effect on LAB
287 growth and malolactic activity (Bartle, Sumby, Sundstrom, & Jiranek, 2019). Table 2 reports
288 a summary of the main outcomes of these interactions on wine composition. Wine is considered
289 a selective medium for LAB, especially when they are inoculated at the end of the alcoholic
290 fermentation, due mainly to the presence of high levels of inhibitory compounds such as
291 ethanol, SO_2 and organic acids (Sumby et al., 2019). Conducting MLF by controlled co-
292 inoculation of yeasts together with LAB starter cultures has gained attention in recent years,
293 due to the potential reduction of the duration of MLF. The selection of compatible yeast and
294 LAB strains is fundamental in order to ensure a successful AF and MLF (Liu et al., 2017), as
295 yeast species have been found to have either stimulatory, inhibitory or neutral effect on LAB
296 and vice versa. These interactions are mainly associated with the ability of the yeast to consume
297 or release nitrogen compounds and/or to produce metabolites that affect LAB metabolism (Liu
298 et al., 2017). Most of the studies evaluated the interactions between S. *cerevisiae* and LAB,
299 mainly *O. oeni*. Using different *S. cerevisiae* strains and two LAB species (*O. oeni* and *L.*
300 *plantarum*), Englezos et al. (2019a) and Lucio, Pardo, Krieger-Weber, Heras, & Ferrer (2016)
301 concluded that co-inoculation of *S. cerevisiae* with the above-mentioned microorganisms
302 clearly affect lactic acid and titratable acidity in a LAB species-dependent manner. More
303 specifically, wines that underwent MLF with *L. plantarum* completed MLF faster and
304 contained higher levels of lactic acid compared to the respective wines inoculated with *O. oeni*.

305 However, the amount of lactic acid formed, was also dependant on the *S. cerevisiae* strain used
306 to conduct AF.
307 The use of mixed starter cultures with non-*Saccharomyces* and *S. cerevisiae* can result
308 in wines with chemical compositions that differ in ways that cannot be attained by *S. cerevisiae*
309 in pure culture fermentations (Table 1). This concept is not new. However, the focus of interest
310 has now moved to the specific phenotypic characteristics of the non-*Saccharomyces* yeasts
311 aligned to consumption of nitrogen compounds and production of metabolites that positively
312 or negatively affects the LAB starter culture (Gobert, Tourdot-Maréchal, Sparrow, Morge, &
313 Alexandre, 2019). To date only a few studies have investigated how non-*Saccharomyces*
314 species (*Starm. bacillaris*, *H. uvarum*, *M. pulcherrima*, *L. thermotolerans,* and *T. delbrueckii*)
315 affect the growth and malolactic activity of LAB in MLF performed by *O. oeni* (Capozzi,
316 Berbegal, Tufariello, Grieco, & Spano, 2019; Du Plessis et al., 2017) and *L. plantarum* (Du
317 Plessis et al., 2019; Russo et al., 2020).
318 In general, it was found that co-inoculation with LAB does not affect yeasts behaviour
319 during alcoholic fermentation (Russo et al., 2020). In contrast, non-*Saccharomyces* yeast
320 influence LAB development and consequently, the MLF in terms of both technological i.e.
321 fermentation time and compositional aspects i.e. primary and secondary metabolite production,
322 in a species and strain dependent manner (du Plessis et al., 2017; Russo et al., 2020). In
323 particular, it was observed that in pure culture fermentation with *Starmerella stellata*
324 (previously *Candida stellata*) the MLF took longer to complete due to the yeast inhibiting the
325 bacteria and reducing their cell numbers (du Plessis et al., 2017). Divergently, other non-
326 *Saccharomyces* yeasts (*L. thermotolerans*, *M. pulcherrima* and *Starm. bacillaris*) had a
327 beneficial effect on MLF duration in pure and mixed fermentations with *S. cerevisiae*, leading
328 to wines with improved quality parameters, such as improved body (du Plessis 2019; Russo et
329 al., 2020). A particular case is represented by *H. uvarum.* The fermentation of grape must with
330 this non-*Saccharomyces* yeast in pure culture led to a slight inhibitory effect on MLF, possibly
331 due to depletion of essential nutrients for the LAB, or the production of toxic metabolites
332 against the LAB. In mixed fermentation with *S. cerevisiae*, *H. uvarum* had a positive effect on
333 the growth of inoculated and naturally occurring LAB in comparison to *S. cerevisiae* only (du
334 Plessis et al., 2019). This further illustrates how different yeast/bacteria interactions varyingly
335 affect fermentation processes.
336
337 **4. Microbial interactions during alcoholic and malolactic fermentations**
338

339 As in several natural environments, wine microorganisms often form complex
340 ecological ecosystems that result in the dominance of a specific species or a strain within a
341 species, which then determines the final quality of wine (Knight, Klaere, Fedrizzi, & Goddard,
342 2015). These interactions are presented in Fig. 3 and may mediate one-way, two-way, and
343 multi-way communications, which in turn could be intra-species, inter-species or inter-
344 kingdom interactions (Arneborg, Appels, & Howell, 2019). Starting from the surface of the
345 grapes in the vineyard, these interactions continue during both the primary AF and the
346 secondary MLF leading to the hegemonic role of *S. cerevisiae* and *O. oeni*, respectively
347 (Knight, Karon, & Goddard, 2020; Liu et al., 2018). Yeasts and LAB interactions are strongly
348 influenced by several factors that will be discussed below and in Fig. 1.
349
350 *4.1 Environmental conditions*
351
352 One of the most important factors that should be considered in the study of interactions
353 among different wine microorganisms is the role of environmental conditions. Furthermore, it
354 is also important to remember that the wine ecosystem is continually changing due to the
355 utilisation of compounds e.g. sugar and the production of alcohol, organic compounds, fatty
356 acids, peptides and antimicrobial compounds by the microorganism involved (Branco, Viana,
357 Albergaria, & Arneborg, 2015). As a result, compatibility between yeasts and LAB is affected
358 by chemical and physical parameters that are strain and cultivar specific (Bartle et al., 2019).
359 Several studies have investigated the effect of grape variety and vineyard management
360 practices (organic, bio-dynamic or conventional) on the composition, number and biodiversity
361 of indigenous yeasts and bacteria on grape berries (Martins et al., 2012). Although these studies
362 all showed an effect on the diversity of yeasts and bacteria, the results cannot be generalized
363 and are often contradictory.
364 Another important factor that can influence population dynamics is must composition.
365 It was demonstrated that even small changes in must composition results in a critical affect on
366 the growth and metabolism of wine yeasts and LAB, and thus affects the formation of aroma
367 compounds. For example, Brou Taillandier, Beaufort, & Brandam, (2018) showed that a
368 modification of nutrient concentration completely reversed the domination of *S. cerevisiae* in
369 a mixed fermentation with *T. delbrueckii* and *S. cerevisiae*. In particular, they found that an
370 increase in lipids affected growth and fermentation performance that was dependant on the
371 nature of the lipid mixture, the yeast genus and the medium composition. Fatty acid content,
372 as well as an increase in SO_2 addition, as part of winemaking, and a decrease in pH also

373 influence LAB ethanol tolerance (Bartle et al., 2019). Additionally, pH directly affects the
374 growth and fermentation rate of yeasts and LAB, and the constitution of fermentation products
375 (Bartle et al., 2019; Ciani et al., 2016). Consequently, this parameter is a determinant factor
376 when choosing *O. oeni* or *L. plantarum* to conduct MLF. *O. oeni* is well adapted to low pH
377 fermentations (pH below 3.5), while *L. plantarum* shows the best performances at higher pH
378 values (pH above 3.5) (Krieger-Weber et al., 2020).
379 A decisive variable of microrganisms interactions is the nitrogen content of the must.
380 Nitrogen depletion can lead to slow or sluggish alcoholic fermentations. Therefore, the addition
381 of exogenous nitrogen sources is a common practice in wineries. Grape musts contain a wide
382 range of YAN (yeast assimilable nitrogen) sources, including, not only amino acids and
383 ammonium, but also urea and small peptides (Gobert et al., 2019). The YAN content is
384 dependent on many factors including rootstock, irrigation, grape variety, climate, vine growing
385 conditions and grape processing. During fermentation, a diverse pattern of nitrogen
386 consumption has been observed for different yeasts species and strains (Englezos et al., 2018b;
387 Su et al., 2019). Such diverse behaviour is related to both the nature of the nitrogen source
388 (amino acids or ammonium) (Englezos et al., 2018b; Kemsawasd, Viana, Ardö, & Arneborg,
389 2015), as well as the type of amino acids required (Englezos et al., 2018b; Medina, Boido,
390 Dellacassa, & Carrau, 2012; Su et al., 2020). Su et al. (2020) found that proline, generally
391 considered an unassimilable nitrogen source for *S. cerevisiae* under anaerobic conditions, was
392 consumed by non–*Saccharomyces* yeasts. Furthermore, in mixed fermentations with sequential
393 inoculums, the non-*Saccharomyces* yeast species release significant amounts of nitrogen (and
394 probably other nutrients) supporting the growth and fermentation of *S. cerevisiae* (Englezos et
395 al., 2018b; Su et al., 2019) and LAB (Bartle et al., 2019).
396
397 *4.2 Winemaking practices*
398
399 Wine production involves numerous practices that affect the dynamics of microbial
400 populations during fermentation. The most important are: harvesting (hand-picked or machine
401 harvested grapes), manner of transportation to the winery, pre-fermentation operations such as
402 method of crushing and/or juice extraction (pressing), juice clarification and SO_2 addition, and
403 yeast/LAB inoculation. In winemaking, pre-fermentation operations comprise the time
404 between grape harvest until the start of AF. This phase can last from a few hours to several
405 days and leads to substantial changes in the indigenous biota (Albertin et al., 2014b). Albertin
406 et al. (2014b) showed that pre-fermentation operations had a great impact on species with high

407 initial population in a Chardonnay grape must, such as *Hanseniaspora* spp. and *Starm.*
408 *bacillaris*. In contrast, these two yeasts were less affected by cold settling of white grape juice
409 than *H. anomala*, *Issatchenkia terricola* and *S. cerevisiae* (Grangeteau et al., 2017).
410 Maceration may also affect the grape must microbiota. In general, the dominance of
411 yeasts and LAB starter cultures is easier to achieve in white musts than in red. This is probably
412 due to contact with grape skins in red wine maceration that increase the quantity of yeasts
413 naturally present that are able to compete with the starter culture. In fact, some authors found
414 that the duration of inoculated MLF in sterile-filtered red wine samples was reduced, in
415 comparison to the non-sterile must, due to reduction or complete elimination of competing
416 microorganisms (Cinquanta, De Stefano, Formato, Niro, & Panfili, 2018). Furthermore,
417 Guzzon Malacarne, Larcher, Franciosi, & Toffanin, (2020) found that carbonic maceration
418 (delayed crushing for some days while grapes are anaerobically stored in fermentation vats),
419 used in some wine regions like Beaujolais and the Rhone Valley in France or Rioja in Spain,
420 had a strong impact on the evolution of the microbiota during fermentation. In that study,
421 carbonic maceration, and consequently the unavailability of oxygen, affected the biodiversity
422 and the development of the microbial groups usually found during fermentation. It was
423 especially *Saccharomyces* spp. that were characterized by a slow development. Other
424 researchers studied the effect of grape juice saturation with CO_2 and highlighted that growth
425 of *H. uvarum* and *Starm. bacillaris* was strongly inhibited, while *Metschnikowia* spp., *P.*
426 *kluyveri* and *T. delbrueckii* species were promoted (Chasseriaud, Coulon, Marullo, Albertin, &
427 Bely, 2018).
428 Oxygen concentration is one of the main forces driving microbial growth during
429 fermentation (Guzzon et al., 2020) and consequently, yeast and bacteria interactions. During
430 fermentation the decrease in levels of oxygen are dependent on the shape and size of the vats,
431 as well CO_2 released. However, oxygen can be supplied to fermenting must to facilitate yeast
432 biomass accumulation and to promote colour extraction in red wines (Gonzalez et al., 2013).
433 Several authors demonstrated that during wine fermentation, changes in the initial aeration
434 regime had a strong impact on the growth of non-*Saccharomyces* yeasts in mixed culture
435 fermentations. In particular, *M. pulcherrima* (Morales, Rojas, Quirós, & Gonzalez, 2015),
436 *Starm. bacillaris* (Englezos et al., 2019), *Hanseniaspora vinae*, *T. delbrueckii*, *L.*
437 *thermotolerans* (Yan, Zhang, Joseph, & Waterhouse, 2020) and *Saccharomyces kudriavzevii*
438 (Arroyo-López, Pérez-Través, Querol, & Barrio, 2011) were able to survive and coexist for
439 longer period with *S. cerevisiae* when oxygen was added to the fermentation medium.
440 Recently, oxygen addition to fermenters, under a controlled flowrate, was applied to promote

441 the respiratory consumption of sugars by non-*Saccharomyces* yeasts in order to reduce alcohol
442 content in the wines (Gonzalez et al., 2013; Alonso-del-Real, Contreras-Ruiz, Castiglioni,
443 Barrio, & Querol, 2017a). Judicious addition of oxygen could help increase the overall impact
444 of non-*Saccharomyces* yeasts on wine quality, accelerate transformation of phenols to reduce
445 astringency and avoid the excessive production of unpleasant metabolites, such as acetic acid.
446 Another oenological practice that can influence interactions between microorganisms
447 is the fermentation temperature, due to its effect on microbial performance. This evidence was
448 widely reported for yeasts in pure and mixed fermentations (Arroyo-López, Orlić, Querol, &
449 Barrio, 2009). During wine fermentation, temperatures naturally increase mainly due to *S.*
450 *cerevisiae* fermentative activity. Although the fermentation temperature is usually controlled
451 in modern wineries, any increase represents an inhibition factor for temperature sensitive
452 species (Liu et al., 2017). However, at lower temperatures e.g. 10°C and 15°C, ethanol
453 tolerance of non-*Saccharomyces* yeasts is higher enabling a stronger contribution in low-
454 temperature fermentations (Jolly et al., 2014). This phenomenon is also evident within the
455 *Saccharomyces* genus. Alonso- del- Real, Lairón-Peris, Barrio, & Querol (2017b) evaluated
456 the performance of *S. cerevisiae* and *Saccharomyces* non-*cerevisiae* strains in mixed culture
457 fermentations at different temperatures. These authors revealed that cryotolerant
458 *Saccharomyces* non-*cerevisiae* particularly *S. uvarum*, has a notable effect on *S. cerevisiae*
459 dominance at low and intermediate temperatures (8, 12 and 20°C). This clarifies why *S. uvarum*
460 can replace *S. cerevisiae* during wine fermentations in European regions with oceanic and
461 continental climates (Alonso- del- Real et al., 2017b), where *S. uvarum* can be found naturally
462 on grapes.
463 The use of SO_2 as an antioxidant and antimicrobial agent is known since Roman times
464 where it was used to prevent food and beverage spoilage. In winemaking, SO_2 is often added
465 at the end of the fermentation process or before bottling to act as a preservative agent, however,
466 it is mostly used before the start of the fermentation. At this stage, it promotes the establishment
467 of *S. cerevisiae* as the dominant yeast because generally non-*Saccharomyces* yeasts (*Candida*,
468 *Cryptococcus*, *Hanseniaspora* and *Metschnikowia*), LAB and acetic acid bacteria are more
469 sensitive to SO_2 (Albertin et al., 2014b). In this context, Cinquanta et al. (2018) found that SO_2
470 has a major effect against LAB at low pH where there is a high percentage of SO_2 in the
471 molecular form. Additionally, during wine fermentation yeasts can release SO_2 due to their
472 metabolism. Generally, *S. cerevisiae* strains can produce more than 100 mg/L SO_2. Information
473 regarding non-*Saccharomyces* yeasts is lacking.

474 Given the importance of SO_2 and the synergic effect of pH together with ethanol on the
475 survival of specific microorganisms, knowledge of the tolerance of this metabolite by the
476 microorganisms present during the fermentation process is necessary. This can lead to the
477 desired reduction of added SO_2 levels in wine (to satisfy consumers) while avoiding the
478 inhibition of the microorganisms necessary during the winemaking process.

479 The development of large-scale fermentations, as often required in commercial
480 wineries, highlighted the unpredictability and complexity of spontaneous fermentations due to
481 the interactions among microorganisms. Therefore, to maintain repeatable results, the use of
482 selected cultures tailored to complete AF and MLF has become the norm in commercial
483 wineries. However, the dominance of a specific starter culture depends on factors such as the
484 species/strain used, the yeast/yeast or yeast/bacteria combination chosen, the inoculum size and
485 ratio, and the rehydration conditions. The species that inhabit the must ecosystem have
486 different responses to wine fermentation parameters and the behaviour of the non-
487 *Saccharomyces* yeast is influenced by *S. cerevisiae* and vice versa (Bagheri, Bauer, & Setati,
488 2017). Generally, in the presence of *S. cerevisiae*, populations of *Wickerhamomyces anomalus*,
489 *M. pulcherrima*, *Pichia terricola*, and *Candida parapsilosis* decrease in the early stages of the
490 fermentation, while *L. thermotolerans, T. delbrueckii* and *Starm. bacillaris* survive until late
491 stages of fermentation. The presence of non-*Saccharomyces* yeasts in the initial stages of the
492 alcoholic fermentation could limit the growth of *S. cerevisiae* yeasts by utilizing large
493 quantities of nitrogen and oxygen from the must (Liu et al., 2017). However, in contrast,
494 growth of *H. vineae* is promoted by the presence *S. cerevisiae* suggesting a positive interaction
495 between these two yeasts (Bagheri et al., 2017).

496 The diversity of yeasts involved in the AF affects the growth of LAB and their capacity
497 to conduct MLF (Du Plessis et al., 2017, 2019; Capozzi et al., 2019). Du Plessis et al. (2017)
498 found that *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* possessed a larger inhibitory effect
499 on the levels of the naturally occurring LAB than *Starm. bacillaris* and *H. uvarum.* The reduced
500 MLF duration in mixed fermentations using *Starm. bacillaris* co-inoculated with *S. cerevisiae*
501 was probably due to the chemical composition of the medium. Firstly, *Starm. bacillaris* was
502 found to produce less ethanol compared to sugar consumed, implying that *O. oeni* had more
503 favourable environmental conditions for growth and consumption of malic acid. Secondly,
504 *Starm. bacillaris* consumed less nitrogen compounds, compared to *S. cerevisiae,* further
505 benefiting the growth of the LAB. Results from *L. thermotolerans* trials were conflicting
506 thereby highlighting that interactions are also strain-specific and not only species-specific
507 (Bagheri et al., 2017; Du Plessis et al., 2017). The MLF inoculation strategy is also important

508 and Capozzi et al. (2019) found that some *O. oeni* strains showed better malolactic activity
509 when co-inoculated with the selected yeasts at 0% (v/v) ethanol or added up to 4% (v/v) of
510 ethanol.

511 The size and ratio of the yeast inoculum is a key parameter for a successful pure and
512 mixed (multistarter) fermentation (Comitini et al., 2011). In a multistarter fermentation,
513 inoculum ratios of 10:1, 100:1 and 10,000:1 (non-*Saccharomyces*:*S. cerevisiae*) caused a
514 reduced or delayed growth of *S. cerevisiae*. In contrast, an inoculum ratio of 1:1 between non-
515 *Saccharomyces* yeasts (*C. zemplinina*, *L. thermotolerans*, *M. pulcherrima* and *T. delbrueckii*)
516 and *S. cerevisiae* did not affect the performance of the second yeast (Comitini et al., 2011;
517 Medina et al., 2012). However, inhibition was not observed between *S. kudriavzevii* and *S.*
518 *cerevisiae* in low temperature fermentation (Alonso-del-Real et al., 2017a) demonstrating the
519 synergistic effect of temperature and inoculum sizes.

520 Co- or sequential inoculation of *S. cerevisiae* has a great impact on the performance of the non-
521 *Saccharomyces* yeasts. In general, when simultaneously inoculated, *S. cerevisiae* shows a
522 highly antagonistic behaviour and reduces the other population in comparison to sequential
523 inoculations (Table 1). The chemical composition of the wine produced from simultaneously
524 inoculated fermentations is very similar to the respective pure fermented wine with *S.*
525 *cerevisiae* only. On the contrary, in sequential fermentations the initial growth of the non-
526 *Saccharomyces* yeasts enables further modulation of metabolites of oenological interest due
527 the ability of this group of species to achieve higher population levels and be present for a
528 longer time, in comparison to the respective co-inoculated fermentations.

529

530 *4.3 Interaction mechanisms*

531

532 In the wine ecosystem, microorganisms interact at different levels. Firstly, they are
533 driven by the need to consume nutrients. Secondly, their existence necessarily leads to physical
534 contact with each other as well as the production of metabolites that can affect other
535 populations, either as a source of nutrients, or by producing inhibitory factors. In the following
536 two sections the mechanisms responsible for the above-mentioned interactions will be further
537 discussed in relation to the various steps of the winemaking process.

538

539 *4.3.1 Interactions concerning substrate*

540

541 During wine fermentation, all microorganisms must consume nutrients from the same
542 source, so competition between different populations takes place. Starting from the grape
543 crushing, yeasts consume oxygen, sugars, nitrogen, vitamins and lipids (Brou et al., 2018)
544 thereby determining the inhibition level for other species. In general, spontaneous and co-
545 inoculated wine fermentations end with the dominance of the glucophilic *S. cerevisiae,* due to
546 its extensively reprogrammed gene expression during the first phases of the fermentation. This
547 change results in an enhanced nutrient uptake and an up-regulation of genes involved in amino
548 acids, vitamins and lipids uptake. This behaviour was observed in competition against bacteria,
549 non-*Saccharomyces* and *Saccharomyces* non-*cerevisiae* yeasts (Bartle et al., 2019). Yeasts
550 such as *Starm. bacillaris* and *H. uvarum* can probably survive during fermentation due to their
551 fructophilic nature (ability to consume fructose preferentially to glucose as a carbon source)
552 enabling them to compete against *S. cerevisiae* (Fig. 1). Divergently, when a sequential
553 inoculation is followed, *S. cerevisiae* performs poorly in comparison to a pure fermentation
554 equivalent. This is independent from the non-*Saccharomyces* used (Medina et al., 2012; Lleixà,
555 Manzano, Mas, & Portillo, 2016). A sluggish or stuck fermentation has been attributed to
556 nutrient unavailability (Gobert et al., 2019). This was observed for S. *cerevisiae* in mixed
557 fermentations with *Hanseniaspora* spp., *M. pulcherrima* and *T. delbrueckii*, and was probably
558 due to nitrogen and vitamin depletion (Medina et al., 2012). In a mixed fermentation between
559 *L. thermotolerans* and *S. cerevisiae*, Petigonnet et al. (2019) showed that the non-
560 *Saccharomyces* yeast consumed most of the oxygen and approximately 68% of the *β*-sitosterol,
561 14% of the stigmasterol and all the campesterol content present in the must in only 24 h of
562 fermentation. Consequently, *S. cerevisiae* growth was slow, as ergosterol and unsaturated fatty
563 acids biosynthesis were inhibited due to the oxygen unavailability (enzymes for their formation
564 are oxygen-dependent) and because phytosterols needed to replace ergosterol in the membrane
565 had been consumed (Petigonnet et al., 2019).
566 Competition for nutrients have differing outcomes dependant on the yeast species
567 involved. Yeasts with complex nutrient requirements show an increased antagonistic behaviour
568 with LAB (Bartle et al., 2019). Some *S. cerevisiae* strains and non-*Saccharomyces* yeasts (*T.*
569 *delbrueckii*, *Starm. bacillaris*, *M. pulcherrima*, *I. orientalis* and *Schizosaccharomyces* spp.)
570 are able to consume L-malic acid that then becomes unavailable for LAB during MLF
571 (Balmaseda, Bordons, Reguant, & Bautista-Gallego, 2018). Nutrient depletion has an essential
572 role in promoting wine shelf life. During fermentation LAB consume L-malic acid and other
573 nutrients. This impoverishes the wine and prevents the development of contaminant
574 microorganisms (Balmaseda et al., 2018).

575 During wine fermentation, nutrients may also lead to mutualism (positive interactions
576 between species). Some yeasts are able to produce or release amino acids and vitamins that can
577 stimulate LAB growth (Ivey, Massel, & Phister, 2013). This cross feeding was observed
578 between *S. cerevisiae* and *L. plantarum* in grape juice (Ponomarova et al., 2017). It was
579 highlighted that the yeast released amino acids and other metabolites able to stimulate the
580 growth of the LAB strain. Furthermore, it was demonstrated that this metabolic dependency of
581 *L. plantarum* was unidirectional and was conserved among diverse yeast isolates. Another
582 nutrient source is a consequence of yeast autolysis when weaker yeast cells die off during
583 fermentation. This phenomenon is characterized by the release of extra nitrogen sources that
584 can be used by LAB as nutrient source during MLF, and by *S. cerevisiae* when it is added in
585 the wine towards the end of fermentation (Lleixà et al., 2016).
586
587 *4.3.2 Chemical-physical interactions*
588
589 Throughout the last decade, many studies demonstrated that the reduction in the
590 numbers of non-*Saccharomyces* yeasts during early to late stages of mixed culture wine
591 fermentations involves physical cell-to-cell contact. In non-*Saccharomyces/S. cerevisiae*
592 mixed culture fermentations conducted in double compartment fermentors (in which a
593 membrane separates the cells of the two species), the disappearance of non-*Saccharomyces*
594 yeasts was not associated with nutrient limitation or the presence of inhibitory compounds. It
595 was concluded that the reduction was induced by direct physical contact through
596 receptor/ligand like interactions. This phenomenon was observed when *S. cerevisiae*
597 populations reached high cell densities in fermentation with *Starm. bacillaris* (Englezos et al.,
598 2019b), *L. thermotolerans* (Petitgonnet et al., 2019), *T. delbrueckii* (Branco et al., 2017a),
599 *Hanseniaspora* spp. (Rossouw et al., 2015), *K. marxianus* (Lopez, Beaufort, Brandam, &
600 Taillandier, 2014), *H. uvarum*, *M. fructicola*, *P. kudriavzevii* or *Cr. flavescens* (Borded et al.,
601 2020; Rossouw et al., 2015). Other authors observed that a contact-dependent mechanism also
602 occurs in intra-species competition, highlighting that physical contact is a prerequisite for
603 dominance (Pérez-Torrado et al., 2017). It was also shown by Kemsawasd et al. (2015) that the
604 association between cell-to-cell contact and other inhibitory factors (antimicrobial peptides
605 [AMPs]) was responsible for *L. thermotolerans* death during mixed-culture fermentation with
606 *S. cerevisiae*.
607 Antimicrobial compounds (AMCs) such as fatty acids, peptides, proteins, SO_2 and
608 other molecules are produced by yeasts (Liu et al., 2017). Additionally, LAB in wine are able

609 to excrete carboxylic acids, proteases, glucanases and bacteriocins (Balmaseda et al., 2018;
610 Bartle et al, 2019). The aforementioned can all have an effect on the yeast and bacterial
611 population in the wine. The use of antimicrobial compounds is an attractive topic for many
612 researchers, due to consumer demands for safer alternatives to SO_2. However, sometimes it is
613 difficult to understand which molecules are responsible for the inhibition. Simonin et al. (2018)
614 found that the inoculation of *T. delbrueckii* at the start of AF induced a decrease in must
615 biodiversity, spoilage microorganisms included. However, they could not explain the cause of
616 this observation. In addition, Mewa-Ngongang et al. (2019) demonstrated that *C. pyralidae* and
617 *P. kluyveri* showed growth inhibition activity against spoilage yeasts and fungi namely *D.*
618 *bruxellensis*, *D. anomala*, *Z. bailii*, *Botrytis cinerea*, *C. acutatum* and *Rhizopus stolonifera in*
619 *vitro* and on fruits (grapes and apples). These authors found that both direct contact and
620 extracellular volatile organic compounds (VOCs) were two of the mechanisms of inhibition.
621 VOCs include alcohols, organic acids and esters previously described with antimicrobial
622 properties. However, it was not clear which compound, or combinations were responsible for
623 the growth inhibition activity.

624 Antimicrobial compounds, and specifically AMPs have been proposed for use in the
625 biocontrol of undesired microorganisms during winemaking. Peptides are generally used as
626 host defence molecules, but some microorganisms are able to produce AMPs with the purpose
627 of ensuring survival (Mahlapuu, Håkansson, Ringstad, & Björn, 2016). However, this
628 biocontrol strategy has not been thoroughly investigated against wine-related spoilage
629 microorganisms (Di Gianvito et al., 2022).

630 *S. cerevisiae* is able to release an AMP called "Saccharomycin" (Kemsawasd et al.,
631 2015; Branco et al. 2017a). This is a natural biocide (2–10 kDa) active against several wine-
632 related non-*Saccharomyces* yeasts and LAB (Branco et al., 2014, Kemsawasd et al. 2015;
633 Branco et al., 2017a, 2019). Branco et al. (2017a) demonstrated that "Saccharomycin" is a
634 fragment of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an
635 energy metabolism-related enzyme. These authors revealed that during wine fermentation this
636 peptide is involved in the death of non-*Saccharomyces* yeasts by cell-to-cell contact, because
637 GAPDH-derived AMPs accumulate on the *S. cerevisiae* cell surface at the end of the growth
638 phase (24 - 48 h). With reference to non-*Saccharomyces* yeasts, a recent study reported the
639 release of an AMP by the *C. intermedia* strain LAMAP1790. This peptide affected the growth
640 of several strains of the spoilage yeast, *B. bruxellensis*, without influencing the *S. cerevisiae*
641 performance during fermentation (Peña & Ganga, 2019).

642 The production of yeast killer toxins, a characteristic first observed in *S. cerevisiae*, is
643 well distributed in several yeast genera such as *Candida*, *Hansenula*, *Pichia*, *Williopsis*,
644 *Tetrapisispora*, *Schwanniomyces*, *Debaryomyces*, *Ustilago*, *Cryptococcus*, *Metschnikowia*,
645 *Williopsis*, *Kluyveromyces* and *Zygosaccharomyces* (Liu et al., 2017). Yeast killer toxins are
646 effective under wine conditions and for this reason the production of killer toxins are often a
647 sought after characteristic for wine yeast starter culture selections. Killer toxins are able to
648 inhibit *S. cerevisiae* as well as spoilage yeast species in the presence of reduced SO_2
649 concentrations (Di Gianvito et al., 2022). Oro, Ciani, Bizzaro, & Comitini (2016) found that
650 Kwkt and Pikt, two killer toxins produced by *K. wickerhamii* and *W. anomalus*, respectively,
651 had an antimicrobial activity against *B. bruxellensis*. Furthermore, Mehlomakulu, Prior, Setati,
652 & Divol (2017) exposed this spoilage yeast to the killer toxin CpKT1 produced by *C. pyralidae*
653 and revealed that the loss of viability was due to damages to the cell membrane and cell wall.
654 Mazzucco, Ganga, & Sangorrín (2019), also observed an inhibition against *B. bruxellensis*.
655 These authors studied the killer toxin SeKT, produced by *Saccharomyces eubayanus,* in wine,
656 demonstrating that this protein could be used for the biocontrol of four common spoilage wine
657 yeasts (*B. bruxellensis*, *Pichia membranifaciens*, *P. guilliermondii* and *Pichia manshurica*).
658 However, further studies are necessary to understand the efficacy against undesired
659 microorganisms under real winemaking conditions. A factor missing in some investigations is
660 the determination whether populations die off or if they enter in a viable but not culturable
661 (VBNC) state. In this context, Branco et al. (2015) demonstrated that interactions through
662 excreted compounds determined the VBNC status of *Hanseniaspora guilliermondii* during
663 fermentation.
664
665 **5. Conclusion and future perspectives**
666
667 Currently, mixed fermentations with selected non-*Saccharomyces* and *S. cerevisiae*
668 starter cultures are considered a state of art strategy to modulate the production of target
669 metabolites. Investigations on how these species interact with each other and/or with LAB are
670 developing fast, highlighting the potential future directions in this research area. However,
671 more comprehensive data is needed to further uncover the nature of these interactions. This
672 will permit the management of starter cultures in specific inoculation protocols and
673 winemaking conditions in order to increase their metabolic activity and survival time
674 (Morrison-Whittle, Lee, Fedrizzi, & Goddard, 2020). This will ensure their dominance and
675 enhance their contribution to the final wine.

676 A relatively unexplored field that requires more detailed investigation is the impact of
677 mixed culture fermentations on the LAB responsible for MLF. Recent studies demonstrated
678 that certain non-*Saccharomyces* yeast caused a strong inhibition or stimulated the growth and
679 malolactic activity of *O. oeni* and *L. plantarum*, but more clarification is required before a
680 practical application can be devised.

681 In recent years, there has been a rapid increase in omics-methodology based studies,
682 with the aim to extract more information from the wine microbiome. The integration of
683 multiple-omics approaches has revealed molecular based information and enhanced the
684 existing knowledge regarding microbial diversity during the various steps of wine production.
685 This knowledge will help to further understand the complex interactions between
686 microorganisms, the substrate and physical fermentations conditions. However, the overall
687 potential of combining the different omics approaches remains underexploited and there are
688 significant challenges to be addressed before any of these techniques become a routine
689 procedure (Siren et al., 2019). Among the different challenges is the difficulty to extract RNA
690 from grape must and wine, due to the increased levels of inhibitors, such as polyphenols and
691 polysaccharides. Furthermore, since most of the studies are performed in a synthetic grape must
692 medium, the optimization of omics-analysis in samples of natural origin need attention. This
693 could help reveal the effect of specific stress conditions during the fermentation process and
694 identify metabolic pathways that lead to the formation of metabolites responsible for wine
695 quality. Such approaches could help to predict the population dynamics and biochemical
696 activities of yeasts and bacteria and allow better control of their growth during the fermentation
697 process. This will have a positive impact on wine quality, according to the needs of wine
698 producers.

699 Continued advances in the knowledge of microbial interactions could provide many
700 opportunities for innovation and adaptation to a changing market, as recently proposed by Di
701 Gianvito et al. (2022). This will enable the development of new products based on the ability
702 of the starter cultures to control the growth of spoilage microorganisms. More research is
703 required to identify the mechanisms of action exerted by wine yeasts and LAB during the
704 different steps of wine production. Mainstream consumer's demand for diverse wine styles and
705 their increasing concern of the effects of chemical preservatives (such as SO_2) on human health
706 present new challenges for innovation in wine industries. Legislation regarding permitted
707 additives to wine and the continuous search for wines without, or reduced levels of SO_2, are
708 likely to have a cascading effect on microbial community dynamics during wine production.
709 Consequently, a comprehensive understanding of microbial interactions during wine

fermentation will be a key factor for the future elaboration of quality wines. This will assist in addressing the challenges and opportunities (Table 3) that lie ahead in winemaking industries.

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1209 **Figure Captions**

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1211 **Fig. 1** Wine mycobiota and their evolution during the various steps of wine production

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1213 **Fig. 2** Phenotypic traits of the commercial yeasts and lactic acid bacteria available for wine
1214 production (except *Starmerella bacillaris*)

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1216 **Fig. 3** Factors affecting microrganisms interactions in wine

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1237 **Table 1.** Summary of recent studies evaluating the influence of mixed fermentations with different non-*Saccharomyces* yeasts and *Saccharomyces*
1238 *cerevisiae* on wine composition
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Species	Inoculation protocol	Trial conditions	Grape *cultivar*	Impact on chemical composition or sensory attributes	Reference
Lachancea thermotolerans	Sequential	- Fermentation temperature: 20-23°C - pH: 3.23 - Total SO_2: 60 mg/L - Inoculation size: *L. thermotolerans*: 1 × 10^7 cells/mL, *S. cerevisiae* 1 × 10^6 cells/mL	Cabernet Sauvignon	- Increase in lactic acid Increase in terpenes (linalool and geraniol)	Zhang et al., 2021
	Sequential	- Fermentation temperature: 22 °C - pH: not reported - No SO_2 addition - Inoculation size: 1 × 10^6 CFU/mL	Pinot Grigio	- Reduction in ethanol content - L-lactic acid production ranged from 0.53 to 4.42 g/L -- Decrease in fatty acids	Binati et al., 2020
	Co-inoculation and sequential	- Fermentation temperature: 18 °C - pH: 3.37 - Total SO2: not reported - Inoculation size: 5 × 10^6 cells/mL	Emir	- Increase in total acidity Sequential fermentations: - Increase in n-propanol, acetaldehyde and decrease in esters and higher alcohols (except n-propanol) Co-inoculation: - Increase in isoamyl acetate	Balikci et al., 2016
	Sequential	- Fermentation temperature: 25° C - pH: 3.8 - Total SO_2: 60 mg/L - Inoculation size: 1 × 10^6 CFU/mL Malolactic fermentation: YES	Shiraz	- Increase in 2-methyl propanoic acid and some of its esters - Increase in esters (isoamyl lactate, acetic acid, butyl ester, butanoic acid, pentylester, 3-nonenoic acid, ethyl ester, propanoic acid, and 2-hydroxy-, ethyl ester)	Whitener et al., 2017
	Sequential	- Fermentation temperature: 20-23 °C - pH: 3.23 - Total SO_2: 60 mg/L - Inoculation size: *T. delbrueckii*: 1 × 10^7 cells/mL, *S. cerevisiae* 1 × 10^6 cells/mL	Cabernet Sauvignon	- Decrease in acetic acid - Increase in 2-phenylethyl alcohol and esters	Zhang et al., 2021
	Co-inoculation and sequential	- Fermentation temperature: 18 °C - pH: 3.16 - Total SO_2: 60 mg/L - Inoculation size: *T. delbrueckii*: 1 × 10^7 cells/mL, *S. cerevisiae* 1 × 10^6 cells/mL	Cabernet Sauvignon	- Decrease in acetic acid - Reduction in ethanol content from 0.05% to 0.82% (v/v) - Decrease in succinic acid - Decrease in fatty acids - Increase in Phenylethyl alcohol - Decrease in volatile phenols	Zhang et al., 2018a
	Sequential	- Fermentation temperature: 20 °C	Verdejo	- Ethanol reduction content (0.52% (v/v))	Belda et al., 2017

		- pH: 3.42 - No SO_2 addition - Inoculation size: 1×10^6 cells/mL		- Increase in glycerol - Increase in pyruvic acid (from 27 to 52 mg/L) - Decrease in acetic acid - Decrease in higher alcohols - Increase in 2- phenylethyl acetate and 2-phenyl-ethanol - Volatile thiols release (3-sulfanylhexan-1-ol and methyl-4-sulfanylpentan-2-one)	
Torulaspora delbrueckii	Co-inoculation and sequential	- Fermentation temperature: 20 °C - pH: 3.42 - Total SO_2: 40 mg/kg - Inoculation size: 1×10^6 cells/mL	Tempranillo	- Decrease in total acidity - Increase in pH (higher malic acid consumption) - Decrease in ethanol content - Increase in glycerol - Mannoproteins release - Decrease in higher alcohols	Belda et al., 2015
	Co-inoculation and sequential	- Fermentation temperature: 24 °C - pH: 3.15 - Total SO_2: 60 mg/L -Inoculation size: *T. delbrueckii*: 2×10^7 cells/mL, *S. cerevisiae* 1×10^6 cells/mL	Sauvignon Blanc	- Volatile thiols release (3-Sulfanylhexan-1-ol and its acetate, 3-sulfanylhexyl acetate increase)	Renault et al., 2016
	Co-inoculation	- Fermentation temperature: 22 °C - pH: 3.5 -Total SO_2: 10 mg/L - Inoculum ratio: 1:1, 3:1 T.d/S.c, 1:3 T.d/S.c (5×10^6 cells/mL)	Moscato Branco	- Increase in 2-Phenylethanol and ethyl esters (Ratio 3:1 NS/S) - Increase in acetaldehyde - Release of Linalyl acetate (Ratio 3:1 NS/S and 1:1 NS/S)	Marcon et al., 2018
	Sequential	- Fermentation temperature: 16°C - pH: 3.0 - No SO_2 addition - Inoculation size: 10^6-10^7 cells/mL	Pinot blank	- Decrease in ethanol content - Increase in2-phenylethanol, diethyl succinate, phenylethyl acetate and 3-methylbutanoic acid	Ženišová et al., 2021
	Co-inoculation and sequential	- Fermentation temperature: 16° C - pH: 3.35 - High sugar content (400 g/L) - SO_2: 80 mg/L - Inoculation size: 5×10^6 cells/mL	Vidal	- Ethanol reduction - Decrease in fatty acid ethyl esters	Zhang et al., 2018b
	Sequential	- Fermentation temperature: 20 °C - pH: 3.31 - No SO_2 addition - Inoculation size: 1×10^6 CFU/mL	Verdejo	- Decrease in ethanol content (0.6% v/v) - Decrease in acetaldehyde - Decrease in higher alcohols (increase on for phenylethanol) - Varietal thiols release (4-methyl ulfanylpentan-2-one)	Ruiz et al., 2018

	Sequential	- Fermentation temperature: 22 °C - pH: not reported - No SO_2 addition - Inoculation size: 1×10^6 CFU/mL	Pinot Grigio	- Increase in the esters and higher alcohols - Decrease in volatile phenols	Binati et al., 2020
Metschnikowia pulcherrima	Sequential	- Fermentation temperature: 22.5 °C - pH: not reported - No SO_2 addition - Inoculation size: 5×10^6 cells/mL	Chardonnay / Semillon blend	- Decrease in acetic and ethanol - Increase in ethanol and fumarate - Increase in acetate esters (mainly ethyl acetate, isoamyl acetate and phenylethyl acetate) - Increase in higher alcohols and monoterpenoids	Hranilovic et al., 2020
	Sequential	- Fermentation temperature: 22.5 °C - pH: 3.5 -No SO_2 addition -Inoculation size: 5×10^6 cells/mL	Merlot	- Reduction in ethanol content from 1.0 to 1.1% (v/v) - Increase in total acidity - Decrease in pH - Increase in higher alcohols (1-Propanol, 2-Phenylethanol)	Aplin et al., 2021
	Sequential	- Fermentation temperature: 16°C - pH: 3.0 - No SO_2 addition - Inoculation size: 10^6-10^7 CFU/mL	Pinot blank	- Increase in 2-phenylethanol and diethyl succinate - Decrease in acetaldehyde	Ženišová et al., 2021
	Sequential	- Fermentation temperature: 20° C - pH: 3.90 (Shiraz), 3.88 (Cabernet Sauvignon) - Total SO_2: 50 mg/L - Inoculation size: 5×10^6 cells/mL - Malolactic fermentation: YES	Shiraz, Cabernet Sauvignon	Shiraz: - Increase in total esters carbon disulphide Cabernet Sauvignon: - Decrease in volatile acids and dimethyl sulphide - Increase in total esters	Varela et al., 2021
	Sequential	- Fermentation temperature: 25° C - pH: 3.8 - SO_2: 60 mg/L - Inoculation size: 1×10^6 cells/mL - Malolactic fermentation: YES	Shiraz	- Increase in esters (butyl octanoate, isobutyl acetate, pentanoic acid, 4-methyl-, ethyl ester, hexanoic acid, 2-methylpropyl ester, 6-octen-1-ol, 3,7-dimethyl-, acetate, acetic acid, methyl ester, and cis-3-hexen-1-ol)	Whitener et al., 2017
	Sequential	- Fermentation temperature: 20 °C - pH: 2.9 - No SO_2 addition - Inoculation size: 10^6 cells/mL	Riesling	- Malic acid degradation - Reduction in ethanol content - Increase in glycerol - Increase in ethyl hexanoate and ethyl octanoate	Dutraive et al., 2019
	Sequential	-Fermentation temperature: 25° C -pH: 3.8 -SO_2: 60 mg/L -Inoculation size: 1×10^6 cells /mL Malolactic fermentation: YES	Shiraz	- Increase in acetic acid -Increase in acetaldehyde, ester and butyl octanoate	Whitener et al., 2017

	Sequential	- Fermentation temperature: 20 °C - pH: 2.9 - No SO_2 addition - Inoculation size: 10^6 cells/mL	Riesling	- Reduction in ethanol content - Increase in glycerol content - Increase in total esters (ethyl acetate, isoamyl acetate and 2-methyl butyl acetate, 2-Phenyl-ethyl acetate) - Increase in higher alcohols (2-phenylethanol, 3-methyl-butanol and 2-methylbutanol) - Increase in valeric acid	Dutraive et al., 2019
	Simultaneous	- Fermentation temperature: 24 °C - pH: 3.5 - No SO_2 addition - Inoculation size: 2 x 10^6 cells/mL	Pinot noir	- Increase in esters (ethyl acetate, isobutyl acetate, isoamyl acetate, hexyl acetate, benzyl acetate, 2-phenylethyl acetate, ethyl hexanoate and ethyl octanoate) - Increase in higher alcohols (isobutyl alcohol, isoamyl alcohol, benzyl alcohol) - Increase in volatile fatty acids (isobutyric acid, hexanoic acid)	Hu et al., 2022
Pichia kluyveri	Sequential	- Fermentation temperature: 22 °C - pH: not reported - No SO_2 addition - Inoculation size: 1.0 × 10^6 CFU/mL	Pinot Grigio	- Reduction in ethanol content - Increase in glycerol (average value 5.83 g/L) - Decrease in acetaldehyde - Decrease in fatty acids - Increase in production of nerol, benzyl alcohol and (E)-3-hexen-1-ol	Binati et al., 2020
	Sequential	- Fermentation temperature: 25° C - pH: 3.8 - SO_2: 60 mg/L - Inoculation size: 1 × 10^6 cells/mL Malolactic fermentation: YES	Shiraz	- Terpens release (linalool and geraniol amount increase) - Increase in δ -valerolactone and pentolactone as well as 2-hexenoic acid and 2-hexanoic acid, ethyl ester	Whitener et al., 2017
	Co-inoculation and sequential	- Fermentation temperature: 25° C - pH: 3.44 - Total SO_2: 30 mg/L - Inoculation size: 10^6- 10^7 cells/mL	Kotsifali and Mandilar (3:1)	- Reduction in ethanol content - Increase in glycerol - Increase in ethyl esters (ethyl octanoate, 2-Phenylethyl acetate, Hexyl acetate) - Decrease in higher alcohols	Nisiotou et al., 2018
Starmerella bacillaris	Sequential	- Fermentation temperature: 20° C - pH: Chardonnay 3.99, Muscat 3.81, Riesling 3.82, Sauvignon blanc 3.56 - No SO_2: addition -Inoculation size: 5 x 10^6- 10^7 cells/mL	Chardonnay, Muscat, Riesling and Sauvignon blanc	- Reduction in ethanol content - Increase in glycerol - Decrease in acetic acid - Increase in higher alcohols and esters in Sauvignon blanc (ethyl octanoate and ethyl decanoate) - Increase 2-Phenylethanol in Riesling and Sauvignon blanc	Englezos et al., 2018

				- Decrease in esters in Chardonnay and Muscat Increase - Decrease in terpenes - Increase in 3-mercapto-1-hexanol (3MH)	
	Co-inoculation	- Fermentation temperature: 25° C - pH: 3.52 - No SO_2 addition -Inoculation size: *Starm. bacillaris* 1 x 10^6 cells/mL, *S. cerevisiae*: 1 x 10^4 cells/mL	Negroamaro	- Increase in glycerol - Increase in terpenes	Truffariello et al., 2020
	Sequential	-Fermentation temperature: 28° C -pH: 3.4 -No SO_2 addition -Inoculation size: *Starm. bacillaris* 1 x 10^6 cells/mL, *S. cerevisiae*: 1 x 10^4 CFU/mL	Sangiovese	- Reduction in ethanol content - Increase in glycerol content - Decrease in anthocyanins and flavan-3-ols - Increase in Vitisin A and Vitisin B	Mangani et al., 2020

1259 **Table 2.** Summary of recent studies evaluating the influence of lactic acid bacteria on wine composition

Species	Inoculation protocol	Trial conditions	Must/wine	Quality advantages	References
Oenococcus oeni	After AF	- Fermentation temperature: 21° C - pH: 3.73 - No SO_2 addition - Inoculation size: 5 x 10^7 CFU/mL - Malic acid: 2 g/L - Ethanol: 14.5% (v/v) - Malic acid: 2.00 g/L	Pinot noir	- Increase in colour intensity and redness - Increase in procyanidin - Increase in esters (ethyl hexanoate, ethyl octanoate and ethyl cinnamate) - Increase in octanoic and n-decanoic fatty acids - Increase in 4-ethyl phenol - Increase in vanillin	Brizuela et al., 2021
	After AF	- Fermentation temperature: 21° C - pH: 3.52 - No SO_2 addition - Inoculation size: 3 × 10^7 CFU/mL - Ethanol: 13.3 % (v/v) - L-malic acid: 3.17 g/L	Tempranillo	- Increase in acetate and ethyl esters (isoamyl acetate, phenylethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate) - Increase in ethyl succinate and ethyl lactate - Increase in terpenes (linalool, a-Terpineol, citronellol and nerolidol)	Diez-Ozaeta et al., 2021
	After AF	- Fermentation temperature: 23° C - pH: 3.46 - No SO_2 addition - Inoculation size: 10^8 CFU/mL - Ethanol: 12.5 % (v/v) - L-malic acid: 2.51 g/L	Cabernet Gernischt	- Decrease in phenolic compounds - Production of caffeic acid and 4-hydroxycinnamic acid - Production of ethyl lactate and isoamyl lactate - Accumulation of 3,4-dimethylbenzaldehyde - Accumulation of linalool and α-terpineol accumulation	Wang et al., 2020
	Co-inoculation	- Fermentation temperature: 22° C - pH: 3.7 - No SO_2 addition - Inoculation size: 1 x 10^6 CFU/mL - Ethanol: 15.0 % (v/v) - L-malic acid: 1.91 g/L	Tinto Fino (Tempranillo)	- Prevention of the increase of histamine values during wine aging	Pérez-Magariño et al., 2021
	After 24h from the beginning of AF	- Fermentation temperature: 23° C - pH: 3.32 - SO_2 addition: 30 mg/L - Inoculation size: 1 x 10^6 CFU/mL - Malic acid: 1.85 g/L	Barbera	- Decrease of yellow/blue coordinate (b*) and increase of red/green coordinate (a*)	Englezos et al., 2019a
Lactiplantibacillus plantarum	Co-inoculation	- Fermentation temperature: 25° C - pH: 3.52 - No SO_2 addition - Inoculation size: 10^6 CFU/mL - Malic acid: 2.17 g/L	Negroamaro	- Increase in higher alcohols (1-Hexanol, phenylethanol, benzyl alcohol) - Production of ethyl lactate and diethyl succinate	Truffariello et al., 2020

		- Ethanol: 12.1 % (v/v)			
	After AF	- Fermentation temperature: 21° C - pH: 3.73 - No SO_2 addition - Inoculation size: 5 x 10^7 CFU/mL - Malic acid: 2 g/L - Ethanol: 14.5 % (v/v)	Pinot noir	- Increase in neutral polysaccharides - Increase in procyanidin - Increase in esters (diethyl succinate and ethyl cinnamate) - Increase in β -citronellol - Increase in 2-phenylethyl alcohol - Increase in vanillin	Brizuela et al., 2021
	After AF	- Fermentation temperature: 23° C - pH: 3.46 - No SO_2 addition - Inoculation size: 1 x 10^8 CFU/mL - Malic acid:3.5 g/L - Ethanol: 12.5% (v/v)	Cabernet Gernischt	wine color stabilization: - Increase in pyranoanthocyanins increase - Decrease in total anthocyanins - Vitisin B release - b* and H* values decrease	Wang et al., 2018
	After AF	- Fermentation temperature: 23° C - pH: 3.46 - No SO_2 addition - Inoculation size: 10^8 CFU/mL - Ethanol: 12.5 % (v/v) - L-malic acid: 2.51 g/L	Cabernet Gernischt	- Decrease in phenolic compounds - Decrease in biogenic amines reduction (strain dependent) - Release of 2-hydroxyisovaleric acid ethyl ester - Increase in esters (isoamyl hexanoate) - Production of ethyl lactate and isoamyl lactate - Accumulation of ((E)-3-hexen-1-ol, 2-nonanol and 2,3-butanediol) - Release of 4-ethylphenol	Wang et al., 2020

1275 **Table 3.** Factors affecting microrganisms interactions in wine

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Challenges[a]	**Opportunities**[a]
Further investigate the interaction mechanisms among wine microorganisms	Control the fermentation process and greater management of specific microorganisms
Integrate the knowledge of microbial dynamics and their impact on wine	Modulate specific metabolites concentration
Explore the potential of omics-based technologies in wine production	Omics could help to better predict the behavior of microorganisms during fermentation
Produce wines with less SO_2 by using bioprotective microorganisms	Fulfil consumer demands for wines free of chemical additives which are considered negative for health
Accessing low SO_2 addition to microbial interactions	

1277 [a] Ciani et al., 2016, Di Gianvito et al., 2022, Liu et al., 2017, Siren et al., 2019.

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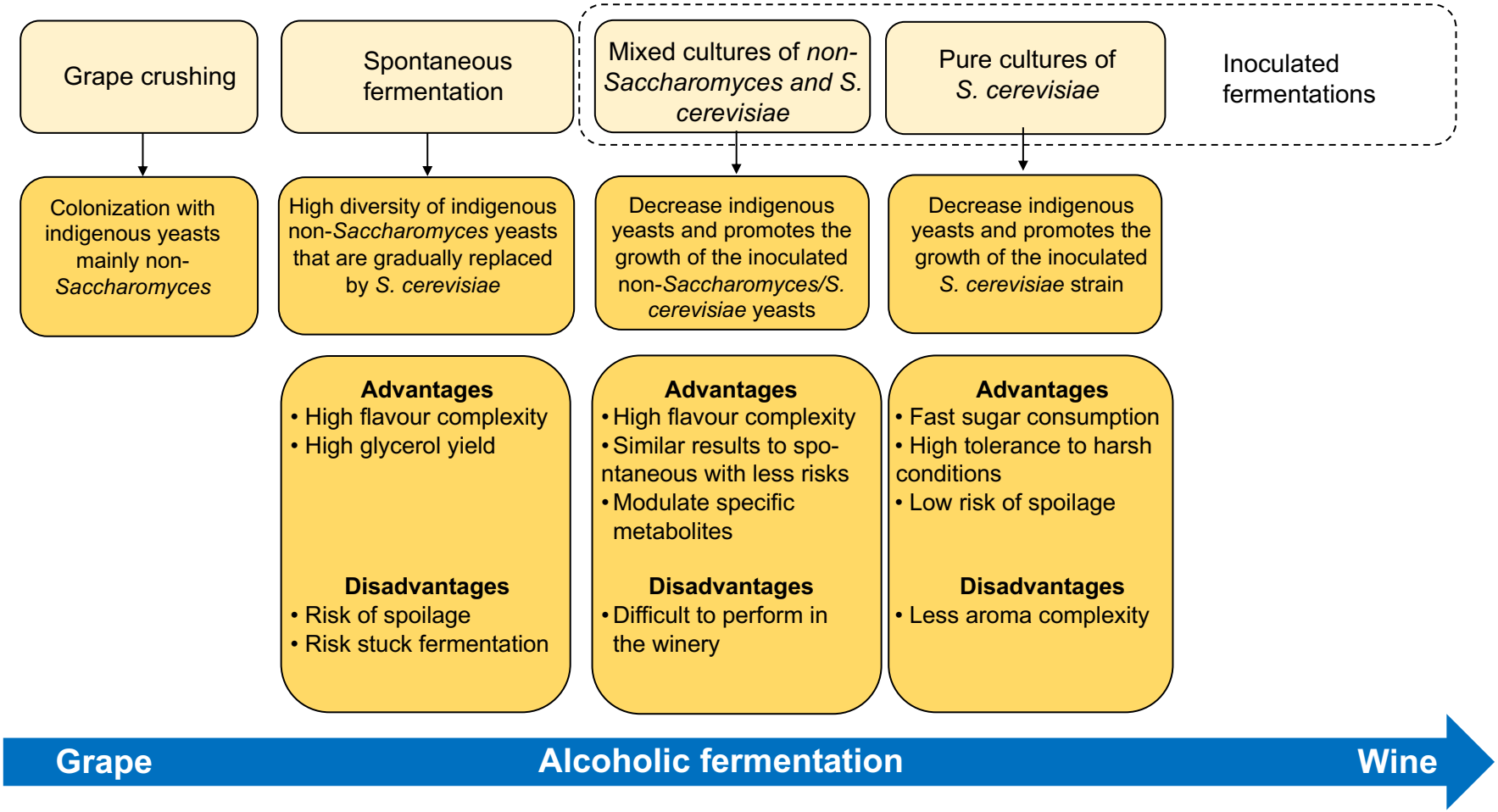
1290 **Figures**

1291 **Fig. 1**



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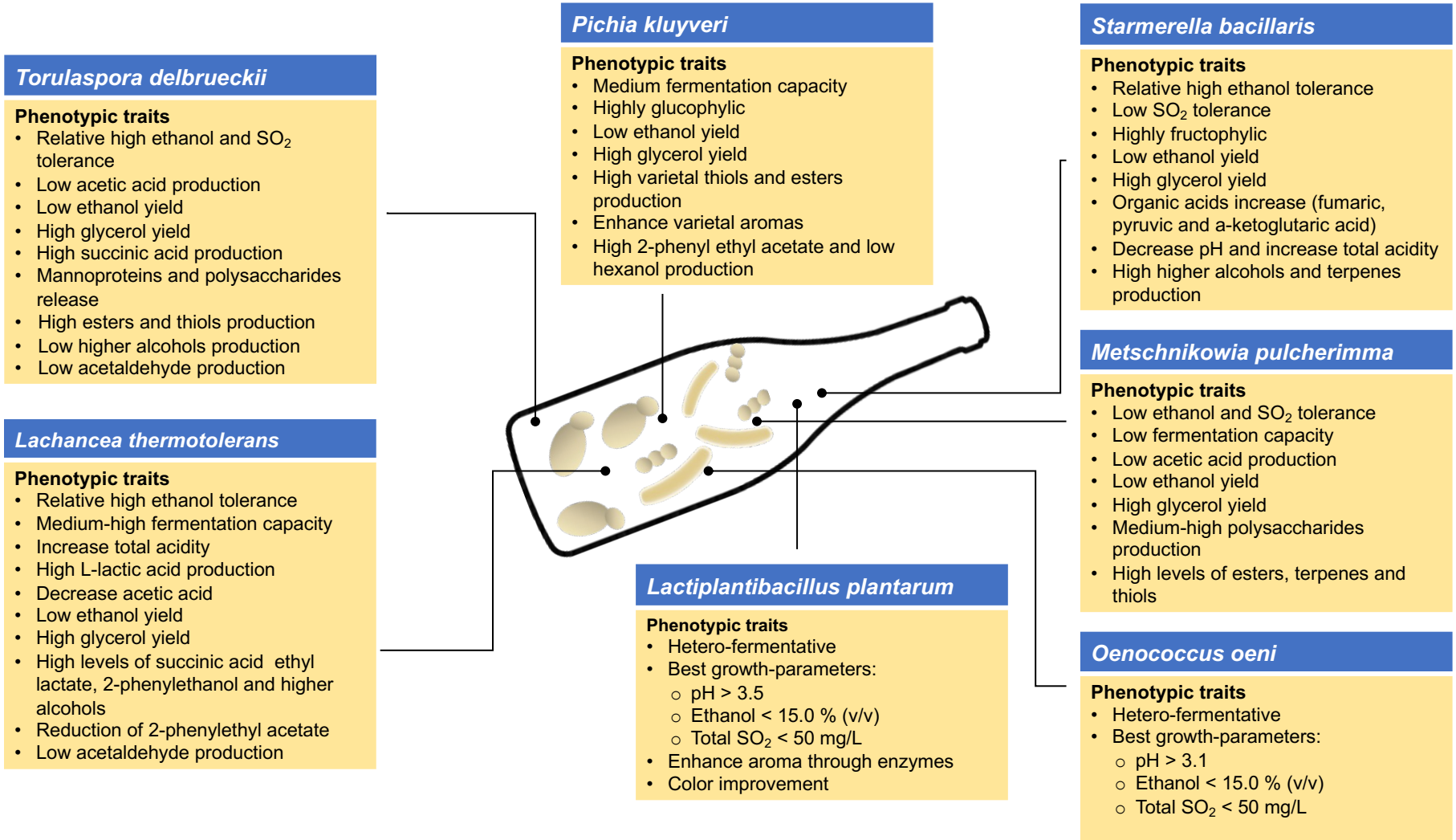
1293 **Fig. 2**



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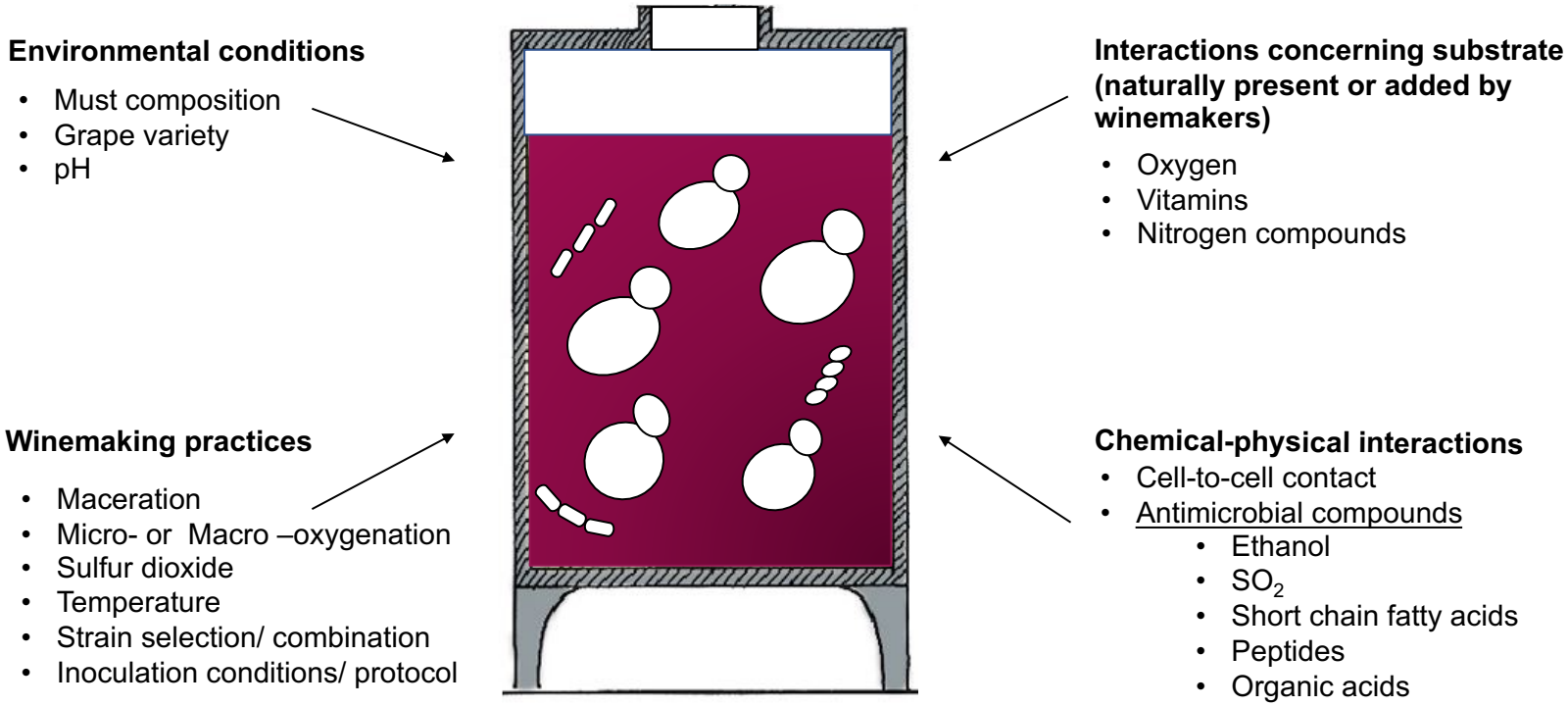
1296 **Fig. 3**



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