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Bioprotection strategies in winemaking

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Abstract

Worldwide the interest for biological control of food spoilage microorganisms has significantly increased over the last decade. Wine makes no exception to this trend, as consumer demands for wines free of preservatives that are considered negative for human health, increase. Biological control during wine fermentation aims at producing high quality wines, while minimizing, or even eliminating, the use of chemical additives. Its success lies in the inoculation of microorganisms to prevent, inhibit or kill undesired microbes, therefore maintaining wine spoilage at the lowest level. The food industry already makes use of this practice, with dedicated commercial microbes already on the market. In winemaking, there are commercial microbes currently under investigation, particularly with the aim to reduce or replace the use of sulfur dioxide. In this review, the potential of wine yeasts and lactic acid bacteria as bioprotection agents and their mechanisms of action during wine fermentation are presented.

Keywords: Bioprotection mechanisms; Wine fermentation; Antagonism strategies; Competition strategies; Yeasts; Lactic Acid bacteria

1. Introduction

There is a growing concern about the negative effects on human health of chemical additives used in foods and drinks to improve their stability and shelf life. For this reason, several research lines are focusing on the development of alternative strategies to avoid spoilage. Bioprotection (or biopreservation or biological control) fits well in this new concept of spoilage prevention, and several microorganisms or their antimicrobial products have already been identified as bioprotection agents (Dokka et al., 2018; Mewa-Ngongang et al., 2019; Mukherjee et al., 2020; Pu et al., 2014; Singh et al., 2018;). This practice is widely used, mainly in agriculture and food industries for the protection of fruits from postharvest spoilage microorganisms, and the extension of the shelf life of food products (Ferraz et al., 2019; Keswani et al., 2019). For example, a commercial product based on *Aureobasidium pullulans* was developed to protect grapes, strawberry and tomato against *Penicillium, Botrytis* and *Monilinia,* while a product based on *Metschnikowia fructicola* is used to protect strawberry, blueberry, grape, stone fruit and pome against *Botrytis, Penicillium, Rhizopus, Aspergillus* and *Monilinia* (Zhang et al., 2020). To date, there is a patent (WO 2015/110484) related to the exploitation of a *Lactiplantibacillus plantarum* strain as bioprotective agent against undesired microorganisms in wine (Krieger-Weber et al., 2020). Different genera of bacteria (e.g. *Lactiplantibacillus, Bacillus, Pseudomonas, Streptomyces*), fungi (*Trichoderma*) and yeasts (belonging mainly to the genera*, Candida, Cryptococcus* and *Aureobasidium*) are commercially available for these purposes (Droby et al., 2016; López-Seijas et al., 2020; Mukherjee et al., 2020).

The bioprotection strategy consists of the inoculation of living microorganisms (Bio Protective Cultures, BPCs), or the addition of their metabolites (Bio Protective Metabolites, BPMs), in purified form, during the production of the food or there after. These microorganisms prevent food microbial spoilage through different bioprotection mechanisms that can be divided in passive (competition for space, nutrient and oxygen) and active antagonistic strategies (production of antimicrobial molecules) (Pandin et al., 2017). The addition of BPCs during the early stages of the

production process could also enhance the characteristics of fermented foods, such as flavour, texture and nutritional value (Gaggia et al., 2011).

In the wine industry, the most common chemical additive is sulfur dioxide (SO_2). This compound is considered an essential tool for winemakers, due to its low-cost and its antioxidant and antimicrobial properties against a wide spectrum of microorganisms (Roullier-Gall et al., 2017). Therefore, its presence during both the fermentation and the storage of the wine is desired to avoid spoilage. In spite of the advantages, high doses of SO_2 may cause consumer health problems, particularly in sensitive individuals (Guerrero and Cantos-Villar, 2015). For this reason, the World Health organization (WHO) encourages alternative methods to reduce, or even eliminate, its use in wine production. Consequently, several alternative technologies able to control microorganisms have been investigated. These include ultrasound, ultraviolet radiation, pulsed electric field, electrolyzed water, high hydrostatic pressure treatments, or the addition of lysozyme, sorbic acid, dimethyl dicarbonate and chitosan (Guerrero and Cantos-Villar, 2015). However, a definitive substitute for SO_2 has not yet been found, especially for wines undergoing long-term storage (Giacosa et al., 2019).

The application of bioprotection strategies in viticulture has increased significantly in the last years, with studies investigating its applicability against grapevine trunk diseases (Mondello and Songy, 2018), the fungal pathogen *Botrytis cinerea* causing bunch (Carbó et al., 2018; Garrido et al., 2017; Jacometti et al., 2010) and sour rot (Carbó et al., 2018) in the vineyard (Nardi et al., 2020). However, little is known about the application of bioprotection strategies during or after the completion of the fermentation process (Simonin et al., 2018). Bioprotective strategies found in wine yeasts and Lactic Acid Bacteria (LAB) are represented in Fig. 1. In this review, the current knowledge about bioprotection opportunities and mechanisms exerted by wine yeasts and LAB, during fermentation to control spoilage microorganisms, will be discussed.

2. Bioprotection mechanisms

Wine microorganisms form complex ecological interactive webs, which result in the dominance of a specific species or strains able to determine the final quality of wine. In inoculated wine fermentations, the use of a starter culture aims to establish dominance of the inoculated culture and determine wine characteristics. Conversely, the BPCs aim to prevent the growth of the spoilage microorganisms, without affecting the performances of useful microorganisms responsible for the alcoholic/malolactic fermentation (Nardi, 2020). Furthermore, BPCs may also be directly responsible for wine fermentation (Table 1). Understanding the antagonistic mechanisms that confer bioprotection activity to the inoculated microorganisms is fundamental for their successful application as BPCs. These competition strategies can be either passive or active (Bauer et al., 2018) as shown in Fig. 2. The difference is that passive competition strategies are exerted by all microorganisms, while active competition strategies are performed only by some microorganisms that, consequently, result stronger than the other and able to dominate the fermentation process.

2.1. Passive competition strategies

Passive competition strategies comprise all the mechanisms that are beneficial including strategies to take away resources from competitors (exploitative competition), or to achieve a superior positioning within the niche (Parijs and Steenackers, 2018). During wine fermentation, the factors that mainly modulate the fitness advantage of some species above others are the ability to rapidly uptake nutrients (carbohydrates, nitrogen, vitamins, sterols and microelements) and oxygen or to colonize the space (Curiel et al., 2017; Petigonnett et al., 2019; Prior et al., 2019; Rollero et al. 2018; Siedler et al., 2019; Taillandier et al. 2014).

2.1.2. Nutrients competition

In a specific environment all microorganisms compete with each other for nutrients necessary for their growth (Liu et al., 2015). Passive competitive microorganisms are able to consume the substrates faster and in a more efficient way than the competitors, producing higher levels of biomass (Boynton et al., 2019). Proteomic and transcriptomic studies have revealed that, during mixed wine fermentation, an increased resource uptake (glucose, nitrogen, oxygen, sterols, copper, iron and vitamins) occurring in several yeast strains, compared to the respective control pure culture fermentations. These yeasts belong to *Saccharomyces cerevisiae* (Alonso-del-Real et al., 2019; Barbosa et al., 2015; Curiel et al., 2017; Kosel et al., 2017; Peng et al., 2019; Rossouw et al., 2012; Shekhawat et al., 2019; Tronchoni et al., 2017;), *Torulaspora delbrueckii* (Prior et al., 2019; Su et al., 2020), even in presence of different nitrogen sources (Roca-Mesa et al., 2020), and *Lachancea thermotolerans* (Shekhawat et al. 2019). Furthermore, nutrient competition was identified as the major antagonistic strategy exhibited by *Lpb. plantarum* (Sielder et al., 2020). A metagenomic analysis revealed that during wine fermentation, this species has the largest number of phosphotransferase systems (the major mechanism used by bacteria for carbohydrates uptake) among the bacteria observed in wine (Melkonian et al., 2019). The ability of the inoculated microorganisms to subtract high levels of nutrients from the medium, could increase the microbial stability in the wines at the end of the fermentation process by inhibiting the growth of unwanted microorganisms and reduce or even avoid the use of SO_2. Since, residual nitrogen levels could favor the growth of undesirable microorganisms in unwanted steps of wine production chain and could act as precursors for the formation of biogenic ammines (Restuccia et al., 2018). However, particular attention should be paid in order to find the most appropriate strain selection that fits with the fermentation conditions, since low nitrogen concentration may lead to stuck or sluggish fermentations (Bisson et al., 1999).

Competition for nutrients can also be achieved by the secretion of extracellular molecules such as digestive enzymes, or siderophores (nutrient exclusion) (Ghoul and Mitri, 2016). In this context, antibacterial and antifungal activity of *Metschnikovia pulcherrima* and *Metschnikovia fructicola* was mainly attributed to the iron depletion caused by the insoluble maroon-red

156 pulcherrimin pigment (Lachance, 2016; Sipiczki, 2006; 2020). In particular, this antimicrobial feature
157 is the result of the reduction of the iron in the medium, due to the precipitation of iron (III) ions,
158 caused the precursor of the pulcherrimin pigment called pulcherriminic acid. As a result, low iron
159 levels in the medium could limit the growth of the microorganisms that require this compound for
160 their growth. This mechanism was found to have an inhibitory effect against the following
161 microorganisms: *Candida tropicalis*, *Candida albicans*, *Brettanomyces/Dekkera, Hanseniaspora* and
162 *Pichia* genera, *B. cinerea* and *Penicillium* (Morata et al., 2019a). While, contradictory results were
163 observed against *S. cerevisiae* (Kantor et al., 2015, Melvyda et al., 2020, Oro et al., 2019). This
164 behaviour let us to hypothesize a strain specific inhibition. Therefore, careful selection of *S. cerevisiae*
165 strains should be performed to find the most suitable partner of *M. pulcherimma* in mixed culture
166 fermentations to reduce ethanol content and enhance the aroma volatile fraction of the wines (Jolly
167 et al., 2014). However, there was not a clear correlation between the antagonistic activity and the iron
168 deprivation by pulcherrimin (Gore-Lloyd et al., 2019; Saravanakumar et al., 2008). In a recent study,
169 a genome comparison between a *M. pulcherrima* wild type and three pigmentless mutants, allowed
170 the identification of a point mutation in the *MPUL0C08850* gene, which encodes for an ortholog of
171 *S. cerevisiae SNF*2 gene. The resultant truncated form of this transcriptional regulator caused a down-
172 regulation of the transcription of *PUL* genes (pulcherrimin biosynthesis and utilisation) explaining
173 why the colourless strains showed an antimicrobial behaviour even if it was less than the wild type
174 (Gore-Lloyd et al., 2019). These authors speculated that the antagonistic activity of *M. pulcherrima*
175 was due to the combination of the effect of pulcherrimin and diverse proteins regulated by *SNF*2 such
176 as genes encoding different types of transporters or secreted proteases (Gore-Lloyd et al., 2019).
177 A similar system was also identified in many Lactobacilli, including *Lpb. plantarum*. In
178 particular, Sielder et al. (2020) demonstrated that the Lactobacilli showed an overexpression of a
179 manganese transporter (*mntH*1 gene) during milk fermentation. The depletion of this microelement
180 in the media resulted in the reduction of the growth of several yeasts (also present in wine) such as
181 *Debariomyces hansenii* and *T. delbrueckii*. These authors found that for *T. delbrueckii* the addition

of manganese restored the growth only partially and hypothesized that other mechanisms (antimicrobial compounds production) were involved in the antagonistic interactions.

2.1.3. Space competition

Another passive competition phenotype is the ability to colonize the space where competitors are established (Ghool and Mitri, 2016) or grow faster than other microorganisms. With this purpose, microorganisms are able of change their lifestyle forming multicellular structures like biofilms (Váchová and Palková, 2018). These structures may exhibit different phenotypic characteristics, compared to free-floating cells. Usually, after the adhesion of single cells to the host surface, the biofilm formation determines the modification of the cell wall properties due to the secretion of extracellular matrix and often the formation of hyphae and pseudohyphae (Freimoser et al., 2019). The biofilm formation is considered a desired characteristic in biocontrol yeasts and has been widely investigated. For example, *M. pulcherrima* and *Pichia kluyveri* are able to form biofilms on grape berries surface (Cordero-Bueso et al., 2017; Parafati et al., 2015; Pawlikowska et al., 2019). This ability confers a great competitive advantage to these yeasts to colonize both intact and damaged grapes as well as establishing themselves in grape juice and starting the fermentation process even in harsh conditions such as those found in desiccated grapes (Cordero-Bueso et al., 2017). Microorganisms with ability to form biofilms are generally characterised by a higher competitiveness against pathogens due to the higher environment persistence and higher biocontrol activity (Freimoser et al., 2019). Furthermore, it has been demonstrated that *Oenococcus oeni* is able to produce exopolysaccharides and form biofilms on cellar surfaces (Dimopoulou et al., 2015). This organization is formed by stress-tolerant cells able for efficient malolactic fermentation under winemaking conditions (Bastard et al., 2016).

2.1.4. Competition for oxygen

The presence of oxygen during the fermentation process is considered as one of the key factors that regulate the presence and population of yeast species. This molecule could positively affect ethanol tolerance and the fermentation performance of yeast cells, through its key role in the production of sterols and unsaturated fatty acids (Albergaria and Arneborg, 2016). Grape must contains relatively low dissolved oxygen levels and becomes rapidly anaerobic due to the fast consumption by the microbial consortium and the grape-derived polyphenol oxidase (Romano et al., 2019). Therefore, the concentration of oxygen may have an important role in the survival and dominance of a specific yeast during the fermentation process. Indeed, the low levels could promote the growth of species that are able to grow in anaerobic conditions, like *S. cerevisiae* (Hansen et al., 2001). While, on the other hand low oxygen levels can influence the growth and death rate of several non-*Saccharomyces* yeast species like *T. delbrueckii*, *L. thermotolerans*, *Starmerella bacillaris* and *Hanseniaspora vinae* and reduce the competitiveness against *S. cerevisiae* (Englezos et al., 2018a, Hansen et al., 2001, Yan et al., 2020). Oxygen addition is also believed to enhance the impact of non-*Saccharomyces* yeasts on the chemical and aroma composition of the wines produced by mixed culture fermentations (Englezos et al., 2018a, Hansen et al., 2001, Yan et al., 2020). While the limited addition of oxygen at the beginning of mixed fermentations with *M. pulcherrima* or *Starm. bacillaris* and *S. cerevisiae* could help to reduce the ethanol content in wines due to the higher sugar consumption of the first yeast species (Varela et al., 2017). However, a fine tuning of oxygen addition is required, since excessive oxygenation might lead to stuck fermentation due to increased antagonism between non-*Saccharomyces* and *S. cerevisiae* yeasts, as previously observed for *H. vinae* and *S. cerevisiae* (Yan et al., 2020). Interestingly, the use of selected yeasts species or strains within species could potentially protect the wine against oxidation due to the rapid consumption of oxygen, as previously demonstrated for *T. delbrueckii* (Simonin et al., 2018). The effect of oxygen on yeast interactions during mixed fermentations and as consequence on wine characteristics needs further investigation.

234 *2.2. Active competition strategies*

235

236 Generally, active competition includes the strategies exploited to confer a disadvantage to the
237 unwanted species through a direct and active interference (Ghoul and Mitri, 2016) due to the
238 production of antimicrobial compounds or through a contact dependent inhibition (Parijs and
239 Steenackers, 2018). During fermentation, yeasts release ethanol that is the most studied antimicrobial
240 compound. Its toxicity on cells is well understood (Gao and Fleet, 1988). Yeasts are also able to
241 release SO_2 as result of their metabolism (Andorrà et al., 2018). Some *S. cerevisiae* strains can
242 produce more than 100 mg/L of this compound (Rauhut, 2017), while information regarding its
243 production by non-*Saccharomyces* yeasts are scarce, probably because these yeasts are generally
244 more sensitive to SO_2 than *S. cerevisiae* (Jolly et al., 2014). However, some spoilage yeasts namely
245 *Zygosaccharomyces bailii* and *Brettanomyces* spp. are more tolerant to these conditions than *S.*
246 *cerevisiae* and therefore are still detected in wines with relatively high SO_2 levels.

247 Several studies highlighted that an array of other antimicrobial compounds are produced in
248 wine environment by yeasts (Albergaria and Arneborg, 2016; Balmaseda et al., 2018; Liu et al., 2017)
249 and bacteria (Bartle et al, 2019; Siedler et al., 2019) of oenological interest. These molecules differ
250 and possess diverse inhibition mechanisms. Antimicrobial compounds are actually subjected to an
251 extensive research with the purpose to exploit their action during wine fermentation. However, it is
252 important to consider that these compounds are often produced after the logarithmic stage and
253 therefore their competitive ability is strictly correlated with the growth performance of the inoculated
254 strain (Mazzucco et al., 2019; Singh, 2018; Villaba et al., 2016). However, the use of these
255 compounds for commercial purpose cannot be authorised without the approval by the International
256 Office of Vine and Wine (OIV) and/or the national regulators (Mehlomakulu et al., 2015). On the
257 other hand, the yeasts producing these compounds can be used if their origin is oenological. In the
258 next sections, the active antagonistic strategies used by yeasts and LAB that confer a possible
259 bioprotection in wine will be discussed. These molecules can be grouped according to the chemical

groups they possess. In particular, nitrogen antimicrobial compounds are antimicrobial peptides, killer toxins, bacteriocins and lytic enzymes as listed in the next section.

2.2.2. Antimicrobial peptides and proteins

2.2.1.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are low molecular weight oligopeptides with a varying number (from five to over a hundred) of amino acids and high sequence variability. Furthermore, the majority of AMPs present charged and hydrophobic amino acids at physiological pH, defining the amphipathic nature of these molecules (Zhang et al., 2019). AMPs are evolutionary conserved in the genome and have a broad antimicrobial spectrum of action from viruses to parasites (Bahar and Ren, 2013). Up to date, more than 5,000 AMPs have been discovered from prokaryotes (e.g., bacteria) and eukaryotes (e.g., protozoa, fungi, plants, insects, and animals) or synthesized (Zhao et al., 2013).

Several studies reported that *S. cerevisiae* is able to secrete a proteinaceous antimicrobial compound able to inhibit *O. oeni* growth and as a consequence malolactic fermentation (Comitini et al., 2005; Osborne and Edwards, 2007; Mendoza et al., 2010; Nehme et al., 2010; Rizk et al., 2016). Furthermore, the AMP "saccharomycin" produced by *S. cerevisiae* was found to act as a natural biocide (2-10kDa) against several wine-related non-*S. cerevisiae* yeasts such as *Hanseniaspora uvarum* (Pérez-Nevado et al., 2006), *Brettanomyces*/*D. bruxellensis* and LABs (Branco et al., 2014). These authors demonstrated that saccharomycin is comprises by two anionic (isoelectric point of 4.37) peptides (AMP1 and AMP2/3) able to alter intracellular pH, membrane permeability and cultivability of non-*Saccharomyces* strains. These two peptides are fragments of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH); which is considered an energy metabolism-related enzyme, able to generate NADH in glycolysis (Branco et al., 2017).

285 The production of these molecules is widespread among wine related yeasts and bacteria and
286 the use of yeasts able to release AMPs was recently proposed for the biocontrol against undesired
287 microorganisms. However, this strategy has been poorly applied to wine-related microorganisms
288 (Peña and Ganga, 2019). A recent study reported the production of an AMP by the non-
289 *Saccharomyces* strain *Candida intermedia* LAMAP1790. The application of this wine strain affected
290 the growth of several strains of *Brettanomyces bruxellensis*, without influencing *S. cerevisiae*
291 performance during fermentation (Peña and Ganga, 2019). These findings opens new prospects
292 regarding the use of these AMPs as alternatives to SO_2 in wine production industrial process
293 (Albergaria and Arneborg, 2016). Since, some spoilage microorganisms like *Pichia* spp*., Dekkera*
294 spp. and *Z. bailii* could tolerate medium-high SO_2 levels and the AMPs might have a key role in
295 controlling unwanted microorganisms without affecting the beneficial microorganisms.
296
297 *2.2.1.2 Killer toxins*
298
299 Killer toxins play an important role in the defence mechanisms of the yeast starter culture
300 against undesired microorganisms. This character consists in the production of proteins or
301 glycoproteins (molecular weight between 30 and 70 kDa) that are lethal for sensitive cells
302 taxonomically related or not. It was demonstrated that the action of these compounds is mediated by
303 the presence of two kinds of specific receptors in the sensitive microorganism cell wall (Liu et al.,
304 2015). Even if this characteristic was first observed in *S. cerevisiae*, it is well distributed in several
305 yeast genera like *Candida*, *Ogataea*~~,~~ *Pichia*, *Williopsis*, *Tetrapisispora*, *Schwanniomyces*,
306 *Debaryomyces*, *Ustilago*, *Cryptococcus*, *Metschnikowia*, *Kluyveromyces* and *Zygosaccharomyces*
307 (de Ullivarri et al., 2014; Liu et al., 2015). Killer toxins are encoded chromosomally, or their
308 production has been related with the presence of dsRNA viruses, or linear dsDNA plasmids (Belda
309 et al., 2017), however this is the case of some of the non-*Saccharomyces* species listed above. Killer
310 yeasts have been used to control pathogenic fungi in plants (Schmitt & Breinig, 2002) and to develop

311 antimycotics for the treatment of human and animal infections (Liu et al., 2015). Furthermore, they
312 were purposed as an alternative strategy of biocontrol to combat contaminating wild yeasts in food
313 industries and in particular in winemaking (Çorbacı, and Uçar, 2018; de Ullivarri et al., 2014;
314 Mazzucco, et al., 2019; Santos et al., 2011; Villaba et al., 2016;).

315 The possibility of using *S. cerevisiae* as BPCs (active antagonistic phenotype) in wine was
316 first introduced in the 1960s, when it was found that *S. cerevisiae* strains are able to release killer
317 toxins and inhibit other *S. cerevisiae* strains as well as spoilage yeast species using reduced SO_2
318 concentrations (de Ullivarri et al., 2014). These authors proposed the use of a sequential inoculation
319 in wine of two *S. cerevisiae* killer strains (Cf8 and M12) to improve the biocontrol against undesired
320 wine yeasts *Dekkera anomala* and *Z. bailii*. This new strategy showed a reduction in the growth of
321 the undesired yeasts to about 78% and 50%, respectively. Subsequently, Oro et al. (2016) found that
322 Kwkt and Pikt, two killer toxins produced by *Kluyveromyces wickerhamii* and *Wickerhamomyces*
323 *anomalus*, respectively, showed an antimicrobial activity against *B. bruxellensis*. These authors
324 demonstrated that this spoilage wine yeast, after a treatment of 0.7 mg/l of SO_2 at pH 3.2, entered in
325 a viable but non culturable state. Divergently, after treatment with Kwkt or Pikt, *B. bruxellensis* D46
326 strain completely lost its viability. Furthermore, Mehlomakulu et al. (2017) exposed this spoilage
327 yeast to the killer toxin CpKT1 produced by *Candida pyralidae* and demonstrated that the loss of
328 viability was due to the induction of cell membrane and cell wall damages. Mazzucco et al. (2019)
329 also observed an inhibition against *B. bruxellensis*. These authors, demonstrated that the killer toxin
330 SeKT produced by *Saccharomyces eubayanus* in wine, could be used for the biocontrol of four
331 common spoilage wine yeasts, namely *B. bruxellensis*, *Pichia membranifaciens*, *Pichia*
332 *guilliermondii* and *Pichia manshurica*.

333 Villaba and collegues (2016) investigated the ability of *T. delbrueckii* to release a killer toxin
334 (TdKT). In particular, the NPCC 1033 strain was tested for its killer activity against some undesired
335 wine yeasts, namely *H. uvarum*, *B. bruxellensis, P. guilliermondii* and *P. membranifaciens* (Lopes
336 and Sangorrín, 2010). It was also verified that the TdKT toxin was active in oenological conditions

337 (at different pH and temperatures values, ethanol, glucose and SO_2 concentrations simulating several
338 wine environmental conditions). It was also found that some *L. thermotolerans* strains secreted killer
339 toxins against *S. cerevisiae* (Aponte and Blaiotta 2016), while Nally et al. (2018) demonstrated that
340 two strains were able to inhibit *Aspergillus* without affecting *S. cerevisiae* in a mixed fermentation.
341 Some *P. kluyveri* strains were found able to release killer toxins in the medium inhibiting the growth
342 of some food and beverage spoilage yeast genera like *Dekkera*, *Kluyveromyces*, *Pichia*,
343 *Saccharomyces* and *Zygosaccharomyces* (with the highest activity against *D. bruxellensis*) (Labbani
344 et al., 2015; Zagorc et al., 2001). Finally, the use of killer strains in sparkling wine production was
345 proposed in order to accelerate the maturation time (Todd et al., 2000, Velázquez *et al*., 2016, 2019).
346 Since, the production of this category of wines using the traditional Methode Champenoise is
347 characterized by a secondary fermentation in a bottle of a base wine, followed by a long period of
348 aging in which the yeast cells are in contact with the fermented wine. During this period, which may
349 last several months, yeast cells undergone autolysis and release cell components into the wine with
350 positive repercussions on the aroma, mouthfeel and foaming properties of wines (Di Gianvito et al.,
351 2019). Such investigations, suggested that killer *T. delbrueckii* strains and sensitive yeasts in mixed
352 cultures could accelerate the onset of yeast autolysis, improving the organoleptic quality and foam
353 properties of sparkling wines, without affecting fermentation kinetics (Velázquez et al., 2016, 2019).
354 Another study demonstrated that the use of a combination of a killer *S. cerevisiae* strain and a sensitive
355 *Saccharomyces bayanus* induced the autolysis of the sensitive yeast in a shorter time, compared to
356 the control wine (without the addition of these yeasts), under oenological conditions (Lombardi et al.,
357 2016). In particular, in a pilot scale production, the selected strains influenced the concentrations of
358 free amino acids, total proteins and polysaccharides reaching, after 3 months of aging, the levels
359 showed by control wines after nine months of aging. These findings, raise the possibility of using a
360 biotechnological approach based on specific combinations of killer/sensitive strains or different types
361 of killer strains to reduce the time needed to release of cell components in the medium and

362 consequently the time for sparkling wine production with economic benefits for the producers
363 (Mannazzu et al., 2019).
364
365 *2.2.1.3 Bacteriocins and bacteriocin-like inhibitory substance (BLIS)*
366
367 Many wine related LAB are known for their ability to produce proteinaceous antimicrobial
368 molecules called bacteriocins, active against other bacteria that are closely related to the producing
369 organism growing in the same medium (Ndlovu et al., 2015). Several studies highlighted the ability
370 of wine LAB to produce bacteriocins, however, bacteriocin producing activity has not yet been
371 demonstrated under wine conditions (Krieger-Weber et al., 2020) and their efficacy in wine is only
372 demonstrated in combination with SO_2 (Royo-Bezares et al., 2007). In fact, the production and
373 application of bacteriocins in food is influenced by the physical and chemical conditions of food and
374 the presence of competitive microorganisms (Singh et al., 2018).
375 Knoll et al. (2008) found that both indigenous and commercial *O. oeni* strains showed an
376 antagonistic activity against several wine-related LAB strains *in vitro*. These authors identified some
377 putative bacteriocin-encoding genes, but they did not demonstrate the release of these bacteriocins in
378 wine. Recently, Lasik-Kurdyś and Sip (2019) reported the ability of one *O. oeni* strain to synthetize
379 a peptidic BLIS active against *Leuconostoc mesenteroides* and *Pediococcus pentosaceus*. In
380 particular, a synergistic effect between the presence of *O. oeni* (competition for nutrients and space)
381 the release of BLIS and other inhibition compounds (organic acids and H_2O_2) was highlighted. It was
382 further found that the removal of viable cells, as well as the neutralization of the supernatant or the
383 treatment with the catalase enzyme, significantly decreased or removed the inhibition activity against
384 some LAB indicator strains. Although several studies have indicated the use of bacteriocins in food
385 and beverages production, the application of these molecules in wine has a great potential, but it's
386 not yet approved. Interestingly, studies have proposed the use of bacteriocins to control biofilm
387 formation on stainless steel tanks and surfaces (Nel et al., 2002), suggesting that their application

388 could be a valid response to the chemical cleaning detergents with a negative impact to the
389 environment.
390
391 *2.2.1.4 Lytic enzymes*
392
393 The production of cell wall lytic enzymes by wine related yeasts and bacteria is a less known
394 form of antagonism. However, there are growing evidences that suggest the involvement of cell-wall
395 degrading enzymes in the mechanism of action of BPCs. Lytic enzymes such as *β*-glucanase
396 determine the leakage of cell contents by cleaving the β-glucan polysaccharide of fungi and bacteria,
397 such as *Penicillium expansum, Fusarium oxysporum* and *B. cinerea* (Edison et al., 2018; Wisniewski
398 et al., 1991). Cordero-Bueso et al., (2017), observed the release of lytic enzymes by *P. kluyveri* that
399 could result in the degradation of the fungal cell wall (β-1, 3-glucanase, proteolytic and pectinolytic
400 activities). Moreover, on grapes *A. pullulans* releases extracellular β-1, 3-glucanase which could play
401 a role in the biocontrol activity towards grape pathogens (Bozoudi and Tsaltas, 2018). Lastly, several
402 strains of the *M. pulcherrima* clade secrete extracellular chitinase and *β*-1, 3-glucanases able to
403 degrade the cell wall of *B. cinerea*, however their impact is strain and matrix-dependent (Sipiczki,
404 2020).
405
406 *2.2.2. Volatile Organic Compounds (VOCs)*
407
408 Among mechanisms of antagonism, VOCs are considered superior over the nonvolatile
409 compounds ones because direct contact and closeness among microorganisms are not required. In
410 fact, these small organic molecules ($<C_{20}$) can diffuse to greater distances in heterogeneous
411 environment, since are characterized by a low molecular mass (100 to 500 Da), a lipophilic moiety,
412 low solubility in the water, low boiling point and a high vapor pressure that permit a readily
413 volatilization at ambient temperatures (Zhang et al., 2021). VOCs are molecules potentially produced

414 by all living microorganisms and can be ascribed to several molecular classes, including
415 hydrocarbons, alcohols, thioalcohols, aldehydes, ketones, thioesters, cyclohexanes, heterocyclic
416 compounds, phenols, and benzene derivatives and can be produced by the primary metabolism or via
417 specialized secondary metabolic pathways.

418 It is widely known that wines produced by mixed fermentations have a complex aroma profile,
419 but the role of these volatile molecules in microorganisms' interactions is still poorly understood.
420 This is the case of the higher alcohols tryptophol, phenylethanol, and tyrosol which are produced at
421 relative high levels by *S. cerevisiae*. Recently, interactions studies highlighted their role as
422 antagonistic VOCs. In fact, through *in vitro* experiments, it was demonstrated that these molecules
423 caused the reduction of the growth of non-*Saccharomyces* like *H uvarum*, *Starm. bacillaris*, *T.*
424 *delbrueckii* and *M. pulcherrima*, even at low concentration (Gonzalez et al., 2018).

425 In another study, Farbo et al. (2018) attributed the bioprotective activity of *L. thermotolerans*
426 against toxinogenic fungi (*Aspergillu*s spp.) to the production of VOCs, particularly 2-phenylethanol.
427 These authors demonstrated that this volatile compound was able to downregulate key genes
428 implicated in Ochratoxin A biosynthesis in a species-dependent way. The implication of this higher
429 alcohol in the antagonistic role during alcoholic fermentation was also suggested by the
430 transcriptomic profile of the yeast during fermentation. In fact, it was highlighted that in a mixed
431 fermentation with *S. cerevisiae*, *L. thermotolerans* showed an up regulation of all genes involved in
432 the conversion of phenylalanine to phenylethanol (Shekhawat et al., 2019).

433 Furthermore, through *in vitro* experiments, it was demonstrated that VOCs such as isoamyl
434 acetate, isoamyl alcohol, 2-phenyl ethylacetate and 2-phenyl ethanol were responsible of the
435 bioprotective activity of *P. kluyveri* on grapes (Mewa-Ngongang et al., 2019b). These authors have
436 also observed that this yeast inhibited the growth of wine spoilage yeasts (*D. bruxellensis* and *D.*
437 *anomala*) and fruit fungi (*B. cinerea, Colletotrichum acutatum* and *Rhizopus stolonifer* (Mewa-
438 Ngongang et al., 2019a, b). A biocontrol ability on grape berries was also observed for another non-
439 *Saccharomyces* yeast, namely *Starm. bacillaris.* This species is able to survive until the last stages of

the fermentation process, contributing significantly to wine quality (Englezos et al., 2017). Its antagonistic activity was investigated against fungal infections such as *Alternaria alternata* (Prendes et al., 2018) and *B. cinerea* (Lemos Junior et al., 2016). In the latter study, through *in vivo* tests, it was found that a co-inoculation of 14 *Starm. bacillaris* strains in artificially wounded grape berries lead to a decrease of *B. cinerea* infection. A recent study attributed this bioprotection ability to the production of VOCs and in particular to benzyl alcohol (Lemos Junior et al., 2020). In line with this finding, several authors observed an increased amount of this alcohol in mixed fermentations with *S. cerevisiae* (Englezos et al., 2019a; Binati et al., 2020). Antimicrobial activity against *B. cinerea* was also observed for *M. pulcherrima* even if in a strain dependent way, due to the production of ethyl alcohol and ethyl acetate (Parafati et al., 2015; Contarino et al., 2019). The inhibitory effect of yeast-derived VOCs against postharvest food pathogens was greatly investigated and proposed as an effective biocontrol strategy to inhibit the growth of *B. cinerea, Colletotrichum acutatum, P. expansum, Penicillium digitatum* and *Penicillium italicum* (Contarino et al., 2019). VOCs could be considered as ideal antimicrobials, since contact between biocontrol agent and food pathogen is not required, to inhibit the growth of the last. Such approach could be useful on postharvest table grapes to improve shelf life and therefore reduce food loss during storage. Further studies are necessary to understand the type and the concentration of VOCs that could be considered crucial in the biocontrol mechanism.

2.2.3. Organic acids

Organic acids production by the BPCs could play an important role on their dominance over the indigenous microorganisms, increasing the microbial stability of the wine. Bagheri et al. (2018) found that in a consortium of six non-*Saccharomyces* and a *S. cerevisiae* strains, a higher concentration of *Starm. bacillaris* resulted in the disappearance of *Candida parapsilosis* and *M. pulcherrima*. This antagonistic ability was attributed to the capacity to produce high levels of organic acids, increasing

466 the total acidity and reducing pH of the wines. In wine conditions, *L. thermotolerans* bioprotective
467 ability was investigated in combination with a *Lpb. plantarum* strain (Rubio-Bretón et al., 2018). The
468 results showed the potential of this inoculation strategy as alternatives to SO_2 addition. The final
469 wines had a higher titratable acidity and esters concentration than control wines inoculated with *S.*
470 *cerevisiae*. The antimicrobial activity was attributed to the production of high lactic acid amounts by
471 both microorganisms and consequently to the decrease of the pH (Gobbi et al., 2013; Rubio-Bretón
472 et al., 2018; Vilela, 2018). This peculiar feature is of great interest in wine starter cultures, since
473 current climate change in many wine-growing regions may cause a shift in grapes composition,
474 increasing sugar content and decreasing must acidity (Mira de Orduña, 2010). In a recent study,
475 Tremonte et al., (2017) demonstrated that the highest antimicrobial activity of 106 *Lpb. plantarum*
476 strains was exerted when growing cells were present. It was also highlighted that the use of a cell free
477 supernatant reduced the bioprotective ability of all strains and in particular that the main antagonistic
478 activity was due to the production of organic acids. Interestingly, in this study a relationship between
479 the antagonistic activity and the origin of the strains was underlined, with the wine strains showing
480 the strongest growth inhibition capacity. Generally, biocontrol activity, by the decrease of pH due to
481 the organic acid production could have an impact on wine color stability, due to the enhance of SO_2
482 properties and the shift of the equilibrium of anthocyanins from the colourless to colored form
483 (Giacosa et al., 2019).
484
485 *2.2.4. Cell-to-cell contact*
486
487 Among the interaction mechanisms, different researches underlined the ability of yeasts to
488 form cellular aggregates called flocs in different environments, wine included. These flocs can be
489 formed by cells of the same strain, by different strains of the same species or by microorganisms of
490 different species (Rossouw et al., 2015). This physical association increases the metabolic exchange
491 among cells and, consequently, determines the ecological interactions like inhibition or stimulation.

492 It was demonstrated that in a wine environment this cell-to-cell contact mechanism is involved in
493 antagonistic interactions among microorganisms. This phenomenon also called co-aggregation was
494 firstly observed in *S. cerevisiae* against several wine yeasts namely *L. thermotolerans*, *T. delbrueckii*,
495 *H. uvarum* and *Starm. bacillaris* (Alonso-del-Real et al., 2019; Englezos et al., 2019b; Kemsawasd
496 et al., 2015; Nissen and Arneborg, 2003; Petigonnet et al., 2019; Pietrafesa et al., 2020; Renault et
497 al., 2013; Rossouw et al., 2018; Shekhawat et al., 2019). It was also observed between different strains
498 of *S. cerevisiae* and is involved in dominance (Pérez-Torrado et al., 2018).

499 Co-aggregation is manly studied using a double compartment bioreactor where two species
500 are separated by a membrane that permits the exchange of metabolites (Nissen and Arneborg, 2003),
501 however this system limits the nature and extent of the interactions investigated because it does not
502 permit the immediate and complete transfer of all relevant metabolites and macromolecules (Rossouw
503 et al., 2018).

504 Recently, in *S. cerevisiae* the genetic basis of this ability was mainly attributed to the
505 expression of *FLO* family genes, which encode for adhesive proteins on cell wall surface (Rossouw
506 et al., 2018). In fact, transcriptomic analyses highlighted that, during wine and sparkling wine
507 fermentation, *S. cerevisiae* is achieved in a general cell wall remodelling with the up-regulation of
508 genes involved in asexual cell aggregation (*FLO* genes) (Shekhawat et al., 2019; Di Gianvito et al.,
509 2018). Genetic analyses suggested that non-*Saccharomyces* yeasts like *H. uvarum* contain adhesion-
510 related domains in their genome (Pu et al., 2014). However, the antagonistic role in these
511 microorganisms is not yet investigated.

512

513 **3. Conclusions and future perspectives**

514

515 Success in biological control of unwanted microorganisms requires consideration of the wine
516 fermentation ecology as a balanced ecosystem, where the resident microbiota contribute to enhance
517 wine quality, while maintaining spoilage microorganisms at the lowest levels. Implementing

fermentation conditions that favour the population of microorganisms with antimicrobial activity is essential to this purpose. However, the use of chemical additives such as SO_2 may still be needed when the concentration of microbial spoilage metabolites is high, especially in wines with low or moderate ethanol levels. Despite the limitations observed in the different stages, the use of viable cells of antagonist microorganisms well adapted in specific conditions or their derived antimicrobial metabolites are of relevance in winemaking industry, since they could help to reduce or even replace chemical additives. In the same context, the selection of yeasts and bacteria of oenological interest with the double role of starter culture and BPCs represents an opportunity for winemakers to enhance wine quality and reduce the production costs, as the demand for sustainable wines will continue to grow. A better knowledge of the fermentation conditions and oenological practices which modulate microorganism's performance, will allow a greater management of specific unwanted microorganisms during the alcoholic and/or malolactic fermentation. Therefore, studies aiming to further investigate the interaction mechanisms among the starter culture and BPCs and the spoilage microorganisms will be of great value in the near future for this field of research.

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Declaration of competing interest

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Figure captions

Fig. 1 Antagonistic behaviour demonstrated in wine microorganisms. Antagonistic behaviour was investigated *in vitro* (A), on grapes (B) and in wine (C).

Fig. 2 Antagonistic strategies of wine microorganisms

1187 **Table 1**

1188 Role of starter cultures in wine and their bioprotective strategies

Yeasts	Starter ability	References	Bioprotective mechanisms		References
			Passive strategies	Active strategies	
Saccharomyces cerevisiae	- Alcoholic fermentation conduction - Positive impact on the organoleptic quality of wines	Parapouli et al., 2020	- Fast nutrient uptake - Multicellular consortia formation	- Toxic compounds production - Ethanol - SO_2 - Antimicrobial peptides (saccharomycin; killer toxins) - VOCs (higher alcohols) - Cell-to-cell contact	Alonso-del-Real et al., 2019 Tronchoni et al., 2017 Rossouw et al., 2015; 2018 Albergaria and Arneborg, 2016 Gonzàlez et al., 2018 Englezos et al., 2019b
Torulaspora delbrueckii	- Low acetic acid production - Reduction of ethanol content - Increase of glycerol content - Release of mannoproteins and polysaccharides - Production of high levels of fruity esters, thiols, and terpenes and lower amounts of higher alcohols -	Benito, 2018a	- Fast nutrient uptake	- Toxic compounds production - Ethanol - Antimicrobial peptides (Tdkt Killer toxin)	Prior et al., 2019 Su et al., 2020 Simonin et al., 2018 Villaba et al., 2016

Metschnikowia pulcherrima	- Low acetic acid production - Ethanol content reduction - Increase of glycerol content - Production of high levels of esters and higher alcohols (isobutanol and phenylethanol) - Enhances varietal and fruity aromas	Morata et al., 2019a	- Iron immobilization (pulcherrimic acid production) - high growth rate and a short lag phase - Biofilm formation	- Toxic compounds production - Ethanol - Lytic enzymes - VOCs (ethyl alcohol and ethyl acetate)	Sipiczki, 2006, Sipiczki, 2020 Gore-Lloyd et al., 2019 Kunchen et al., 2019 Cordero-Bueso et al., 2017 Pawlikowska et al., 2019 Contarino et al., 2019 Saravanakumar et al., 2009
Lachancea thermotolerans	- Increase of titratable acidity - Ethanol content reduction - Increase of glycerol content - Production of high levels of ethyl lactate, ethyl hexanoate and 2-phenylethanol - Reduction of phenylethyl acetate	Benito, 2018b Morata et al., 2019b Porter et al., 2019	- Fast nutrient uptake - Short lag phase and ability to survive until the end of fermentation - Filamentous growth/ flocculation	- Toxic compounds production - Ethanol - Lactic acid - Killer toxins - VOCs (2- phenylethanol)	Fiori et al., 2014 Farbo et al., 2018 Shekhawat et al., 2019 Rubio-Bretón et al., 2018 Vilela, 2018 Nally et al., 2018
Pichia kluyveri	- Ethanol content reduction - Increase of glycerol content - Production of thiols (hotrienol and linalool oxide) - enhance varietal aromas	Varela and Borneman, 2017	- Biofilm formation	- Toxic compounds production - Ethanol - Killer toxins - Lytic enzymes - VOCs (isoamyl acetate, isoamyl alcohol, 2-phenyl	Cordero-Bueso et al., 2017 Sipiczki, 2016 Prior et al., 2019 Labbani et al., 2015 Mewa-Ngongang et al., 2019a,b Englezos et al. (under preparation)

				ethylacetate and 2-phenylethanol)	
Starmerella bacillaris	- Ethanol content reduction - Increase of glycerol content - Production of high levels of linalool, organic acids	Englezos et al., 2017	- Relative fast amonium uptake	- Toxic compounds production - Ethanol - Killer toxins - Lytic enzymes - VOCs (benzyl alcohol)	Prendes et al., 2018 Lemos Junior et al., 2016, 2020 Englezos et al., 2018, 2019b Binati et al., 2020 Roca Mesa et al., 2020 Bagheri et al., 2018
Lactic Acid Bacteria					
Oenococcus oeni	- Decrease of titratable acidity - Diacetyl production – 'buttery' flavour - Release of secondary metabolites that impact on wine aroma and flavor		- Better adaptation to wine conditions - Biofilm formation	- Toxic compounds production - Lactic acid - BLIS	Dimopoulou et al., 2015, 2018 Bastard et al., 2016 Knoll et al., 2008 Lasik-Kurdyś and Sip, 2019
Lactiplantibacillus plantarum	- Titratable acidity decrease - Diacetyl production – 'buttery' flavour	Krieger-Weber et al., 2020 Brizuela et al., 2019	- Competitive exclusion - Nutrient competition - Biofilm formation	- Toxic compounds production - Lactic acid - Bacteriocins	Rubio-Breton et al., 2018 Melkonian et al., 2019 Sielder et al., 2020 Tremonte et al., 2017 Bartle et al., 2019

- Positive effects on the organoleptic properties of wine due to a diverse array of enzymes
- Improvement of color in red wines

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Figure captions

Fig. 1

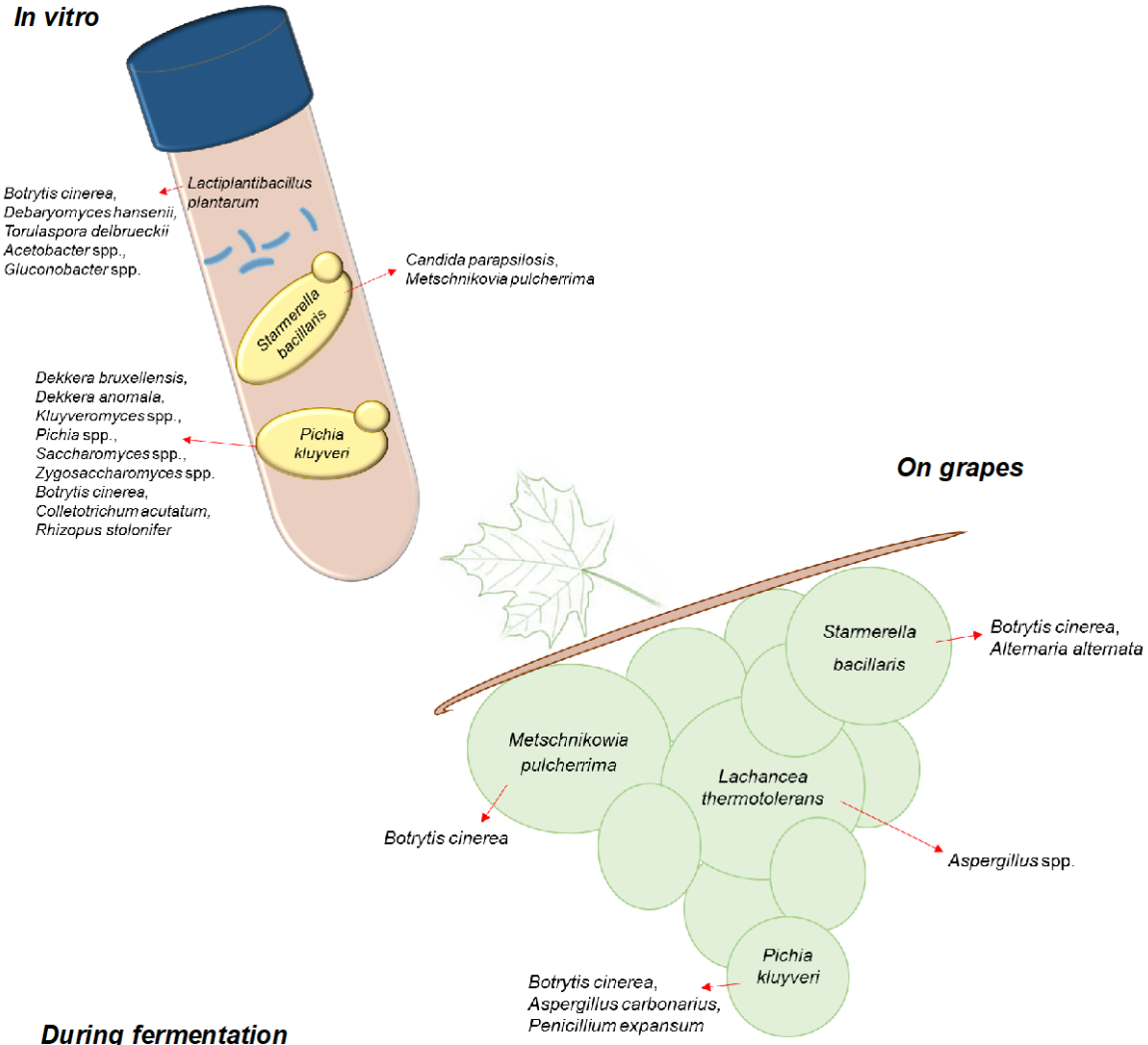

Fig. 2

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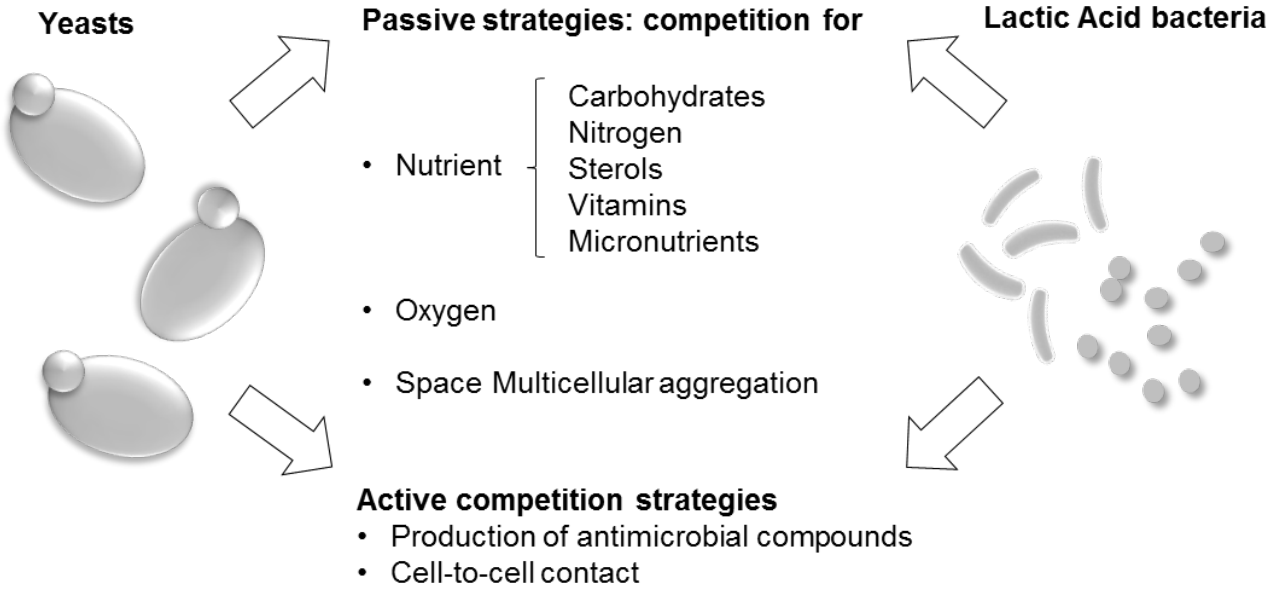

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