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Cell-to-cell contact mechanism modulates *Starmerella bacillaris* death in mixed culture fermentations with *Saccharomyces cerevisiae*

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ABSTRACT

The use of mixed culture fermentations with selected *Starmerella bacillaris* and *Saccharomyces cerevisiae* strains is gaining winemaking attention, mainly due to their ability to enhance particular characteristics in the resulting wines. In this context, yeast interspecies interactions during fermentation have a fundamental role to determine the desired product characteristics, since they may modulate yeast growth and as a consequence metabolite production. In order to get an insight into these interactions, the growth and death kinetics of the abovementioned species were investigated in pure and mixed culture fermentations, using cv. Nebbiolo grape must. Trials were conducted in flasks but also in a double-compartment fermentation system in which cells of the two species were kept separate by a filter membrane. Although the two species had similar growth pattern during the first days of fermentation, *Starm. bacillaris* died earlier when tested in the flask than in the double-compartment fermentor. The early death of *Starm. bacillaris* seemed to be not caused by nutrient limitation nor by accumulation of growth inhibitory compounds (which were not measured in the present study). Rather, cell-to-cell contact mechanism, dependent on the presence of viable *S. cerevisiae* cells, appears to be responsible for the observations made. These results contribute to better understand the factors that influence *Starm. bacillaris* death during wine fermentations.

Keywords: non-*Saccharomyces; Starmerella bacillaris*; mixed cultures; cell-to-cell contact; interactions.

1. Introduction

Non-*Saccharomyces* yeasts usually dominate during the first days of alcoholic fermentation, reaching populations up to 10^8 colony forming units (cfu)/mL before dying off (Fleet, 2003). More strongly fermentative and ethanol tolerant non-*Saccharomyces* yeasts together with *Saccharomyces* spp. (predominantly *Saccharomyces cerevisiae*) then complete the transformation of sugars into ethanol (Cravero et al., 2016; Varela, 2016; Varela and Borneman, 2016). Succession of species during fermentation is largely determined by their different sensibilities to the increasing levels of inhibitory compounds like ethanol and sulfur dioxide (SO_2), temperature, depletion of nutrients, dissolved oxygen content, and killer factors (Albergaria and Arneborg, 2016; Ciani et al., 2016; Ciani and Comitini, 2015). This, in turn, will have an impact on yeast species diversity and thus on wine quality (Ciani et al., 2010; Jolly et al., 2014).

The inoculation of must with selected cultures of *S. cerevisiae* was introduced with the aim to control the fermentation process and to produce wines with desirable characteristics (Capece et al., 2010). However, there is controversy about their use due to the lack of some desirable secondary metabolites provided by spontaneous fermentations (Belda et al., 2017). In an effort to replicate the wine aroma complexity deriving from indigenous strains in spontaneous fermentations, mixed fermentations using selected non-*Saccharomyces* and *S. cerevisiae* yeasts have been proposed (Mate and Maicas, 2016). A successful mixed fermentation is achieved when the non-*Saccharomyces* is able to outcompete other yeast species and to express its peculiar metabolic characteristics, which are absent in *S. cerevisiae* (Comitini et al., 2017; Varela et al., 2016).

In this context, *Starmerella bacillaris* has gained interest in winemaking industry mainly for its unique characteristics (Englezos et al., 2017). Firstly, it has a strong

fructophilic character, is able to grow at high sugar concentrations, and tolerates low temperatures (Sipiczki, 2003). Secondly, it shows a high glycerol and low ethanol yield from sugar consumed compared to *S. cerevisiae* (Englezos et al., 2015). As a result, its use in mixed culture fermentations with *S. cerevisiae* has been proposed to improve wine quality by enhancing or reducing the production of target metabolites (Englezos et al., 2017). However, the contribution of *Starm. bacillaris* in these fermentations is variable, since strain selection, yeast interactions, physicochemical parameters (SO_2 concentration, temperature, sugar concentration, nutrients, oxygen availability and ethanol) could modulate its growth and consequently the production of target metabolites (Englezos et al., 2017, 2018).

Due to the non-sterile environment during must fermentation, different yeast species and/or strains within species are involved in several types of interactions (Ciani and Comitini, 2015). Yeast interactions can affect either positively (mutualism and commensalism) or negatively (competition and antagonism) the growth and the metabolic activity of both species (competition and mutualism) or only one species (antagonism and commensalism) (Liu et al., 2015). Moreover, yeasts can interact with each other by physical contact and by means of metabolic activity (Ciani and Comitini, 2015). Indeed, many compounds, mainly ethanol, short-chain fatty acids, killer proteins, killer-like toxins such as antimicrobial peptides (AMP_s) and SO_2 are produced by yeast cells during fermentation, and they could become selective factors for the growth of certain yeast species (Fleet, 2003). On the other hand, Miller and Bassler (2001) suggested that quorum sensing, a mechanism which allows bacteria populations to communicate, to share information about cell density and to adjust population accordingly, may play a role in the yeast-yeast interactions during fermentation. In addition, another type of interactions between yeasts has been described. Nissen and Arneborg (2003) demonstrated that the cell death of *Torulaspora delbrueckii* and *Lachancea thermotolerans* is not induced by nutrient depletion or the presence of toxic compounds, but

rather the presence of viable *S. cerevisiae* cells at high density, through a cell-to-cell contact mechanism. All these types of interaction will affect negatively or positively the metabolic activity of non-*Saccharomyces* yeasts.

Recent studies have investigated the interactions taking place between different *S. cerevisiae* strains (Pérez-Torrado et al., 2017) as well as among non-*Saccharomyces* and *S. cerevisiae* yeasts (Wang et al., 2016). Regarding *Saccharomyces* – non-*Saccharomyces* interactions, yeast species such as *L. thermotolerans, T. delbrueckii* (Branco et al., 2017; Kemsawasd et al., 2015; Nissen et al., 2003; Nissen and Arneborg, 2003), *Hanseniaspora uvarum* (Pérez-Nevado et al., 2006; Wang et al., 2015) and *Hanseniaspora guillermondii* (Branco et al., 2017; Pérez-Nevado et al., 2006) have been investigated. However, little is known about the interactions between *Starm. bacillaris* and *S. cerevisiae* strains, except for studies that focused on the impact of inoculation density, combination of strains and inoculation timing on yeast growth and metabolic activity, and as a consequence on wine composition (Englezos et al., 2017). Therefore, to increase the efficiency of mixed culture fermentations it is fundamental to understand the interactions between *Starm. bacillaris* and *S. cerevisiae* strains used.

The aim of this study was to understand the kinetics of *Starm. bacillaris* death in mixed culture fermentations with *S. cerevisiae* and to investigate the effect of physical separation of the two species during fermentation. Double-compartment fermentation was carried out to elucidate the role of cell-to-cell contact in the death of *Starm. bacillaris* occurring during mixed culture fermentations.

2. Materials and methods

2.1. Strains

The yeast species examined in this study were *S. cerevisiae* Uvaferm BC® (commercial strain from Lallemand Inc., Montreal, Canada) and *Starm. bacillaris* FC54 (autochthonous strain from the yeast culture collection of DISAFA, Department of Agricultural, Forest and Food Sciences, University of Turin, Italy). These strains were selected for their enological attributes in pure and mixed fermentations using grape must at laboratory scale (Englezos et al., 2016).

2.2. Must preparation

Vitis vinifera L. cv Nebbiolo grapes were crushed and the must obtained (consisting of grape skins, juice and seeds) was macerated at 60 ^{o}C for 1 hour to promote color extraction and to deactivate the indigenous yeast population already present in the must, as described by Boulton et al. (1996). The absence of viable yeast populations was checked by plating an aliquot of the treated must on Wallerstein laboratory nutrient (WLN) medium (Biogenetics, Milano, Italy). Nebbiolo must contained 233.5 g/L of sugars (consisting of 114.5 g/L of glucose and 120.0 g/L of fructose) and 110 mg/L of yeast assimilable nitrogen (YAN) (composed by 40 mg/L of ammonium and 70 mg/L of amino acids). Grape must was supplemented with 70 mg/L of organic nitrogen using a commercial product formulation (Fermaid O®, Lallemand Inc.) to achieve an initial YAN concentration of 180 mg/L prior to fermentation.

2.3. Inoculation procedure

For each yeast species, an aliquot of a reference stock stored at -80 °C was streaked

on YPD agar plate (per liter: 10 g Bacto yeast extract, 20 g Bacto peptone, 20 g dextrose and 15 g agar, all from Biogenetics, Milan, Italy) 96 h before fermentation. Initial cell cultures were performed by inoculating single colonies in 2 mL of sterile Nebbiolo must in 10 mL glass tubes and incubated for 24 h at 25 °C. Subsequently, these cells were used to inoculate 20 mL of fresh sterile must in 50-mL Erlenmeyer flasks at an initial cell population of 6.0 Log cells/mL, as determined by methylene blue staining and direct microscope counting using a Thoma hemocytometer chamber (BRAND GMBH + CO KG, Wertheim, Germany), and incubated another 24 h at the same temperature.

2.4. Fermentations

2.4.1. Pure and mixed culture fermentations

Three sets of fermentations were performed (Fig. 1, panel A): inoculation of each *Starm. bacillaris* and *S. cerevisiae* strain in pure culture fermentations and addition of *S. cerevisiae* after 48 h from the *Starm. bacillaris* inoculation (mixed, sequential inoculation). Each yeast species was inoculated using the abovementioned preadapted cultures to achieve an initial cell density of 6.0 Log cells/mL. Fermentations were performed at 25 °C for 14 days without agitation in 150-mL Erlenmeyer flasks containing 100 mL of sterile Nebbiolo must. Flasks were fitted with sterile air-locks containing sterile paraffin oil in order to ensure semi-anaerobic conditions. All fermentations were carried out in triplicate.

2.4.2. Mixed culture fermentations in a double-compartment fermentation system

Mixed culture fermentations were carried out in a double-compartment fermentation

system (Fig. 1, panel A). The double-compartment fermentation system consisted of two glass chambers with loose screw cap, separated by a 0.45 μm membrane filter (VWR, Milan, Italy), as previously described by Perrone et al. (2013). The growth content of each chamber was separated from the growth content of the other chamber by the abovementioned membrane, which maintained the microbiological integrity of each chamber and permitted the diffusion of YAN and main enological metabolites. The glass chambers were sterilized empty at 121°C for 15 min and sealed with filter membrane and two nylon-polyethylene discs. A polymethylmethacrylate (PMMA) system using four metal screws was designed to apply the necessary pressure in order to seal and keep the two glass chambers together (Figure S1). Each compartment was aseptically filled with 100 mL of sterile Nebbiolo must, which was then inoculated with 6.0 Log cells/mL of *Starm. bacillaris* in one compartment. *S. cerevisiae* was inoculated at the same cells amount in the other compartment 48 h after *Starm. bacillaris* inoculation. The apparatus was incubated at 25 °C without agitation. Previously, the ability of *Starm. bacillaris* and *S cerevisiae* to don't pass through the membrane was evaluated three times by inoculating the yeast cells only in one chamber. Chemical homogeneity in glucose, fructose, organic acids, ethanol, glycerol, and YAN was tested between the two compartments. However, the diffusion of other yeast secretion products like proteins through the membrane filter was not taken inconsideration in this study.

2.4.3. Addition of dead S. cerevisiae cells to pure culture fermentations of Starm. bacillaris

Cells from pure culture of *S. cerevisiae* were harvested after 48 h of fermentation and suspended in a sterile physiological solution (8 g/L NaCl). The suspension was placed at 100 °C for 10 min, and then added to 48 and 96 h-old pure cultures of *Starm. bacillaris*

presenting a cell population of 7.5 Log cells/mL (Fig. 1, panel B). The same process was carried out using heat-treated *Starm. bacillaris* cells, which were mixed in 48 and 96 h-old pure cultures of *S. cerevisiae*.

2.4.4. Addition of fresh must to mixed culture fermentations

The total amount of cells in mixed culture fermentations (section 2.4.1.) were harvested after 96 h of fermentation by a centrifugation at 3000 rpm for 5 min. The resulting cells were washed two times with the above-mentioned physiological solution. The cells were resuspended in a volume of fresh sterile Nebbiolo must equal to that (100 mL) of the discarded supernatant (Fig. 1, panel C).

2.4.5. Addition of mixed culture supernatant to pure culture fermentations

The total amount of cells in each pure culture fermentation were harvested after 48 h of fermentation by a centrifugation at 3000 rpm for 5 min, and the resulting cells were washed two times with the above-mentioned physiological solution. The supernatants from mixed culture fermentations were prepared as follows. Cells in mixed culture fermentation were harvested after 96 h of fermentation and the supernatant was collected, centrifuged at 3000 rpm for 5 min, and sterilized by filtration using a 0.45 μm membrane filter (VWR). Afterwards, the mixed culture supernatants were added to pure culture cells in a volume equal to the volume (100 mL) of supernatant that had been discarded form the pure cultures (Fig. 1, panel C).

2.5. Microbiological analysis

The viable cell population of *Starm. bacillaris* and *S. cerevisiae* during fermentation was determined by plate counting using WLN plates. Samples of 1 mL were withdrawn throughout the fermentations and appropriately diluted in sterile physiological solution (8 g/L NaCl). Plates were incubated at 28 °C for 5 days and subsequently counted. The bromocresol green present in WLN medium enables the concurrent enumeration of both species. *Starm. bacillaris* forms flat, from light to intense green colonies due to the acidogenic nature of this species (Sipiczki, 2004), whereas *S. cerevisiae* forms creamy white colonies, with different light shades of green on the top.

2.6. Chemical analysis

Sugars (glucose and fructose), organic acids (acetic and succinic acid), ethanol and glycerol concentrations were determined using an HPLC system (Agilent 1260, Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector (DAD) set to 210 nm and a refractive index detector. Analyses were performed isocratically at a flow rate of 0.7 mL/min and at 65 °C column temperature using a 300 mm x 7.8 mm i.d. cation exchange column (Aminex HPX-87H, Bio-Rad Laboratories, Hercules, CA, USA) and a Cation H^+ Microguard cartridge (Bio-Rad Laboratories). The mobile phase contained 0.0065 mol/L H_2SO_4, as described by Rolle et al. (2018). Organic and inorganic nitrogen were determined using two enzymatic assays (catalog codes: K-Large and K-PANOPA, Megazyme International, Wicklow, Ireland), according to the producer's instructions.

2.7. Statistical analyses

Significant differences between samples were established using one-way analysis of variance (ANOVA) and Tukey-b post-hoc test with the software IBM SPSS Statistics (IBM Corp., Armonk, NY, USA). Differences were considered significant when *p*-values were lower than 0.05.

3. Results and Discussion

3.1. Fermentation profiles in pure and mixed culture fermentations

The growth dynamics of pure and mixed culture fermentations are presented in Fig. 2. In all pure culture fermentations (Fig. 2a, d), both species showed similar growth dynamics during the first 2 days of fermentation reaching populations of about 7.8 – 8.1 Log CFU/mL. *Starm. bacillaris* population remained quite stable during the fermentation process (end of the monitored period: 14 days) with cell counts ranging from 7.5 to 8.0 Log CFU/mL. On the other hand, *S. cerevisiae* population remained stable and then started to decrease from day 7 onwards. After 14 days, *S. cerevisiae* cells still presented good vitality, with about 6.1 Log CFU/mL. This decline occurred only after sugar exhaustion from the fermenting medium, as previously described by Cramer et al. (2002). The chemical composition of the wines at the end of the monitored period, as well as the evolution of the main metabolites produced by yeast cells during pure culture fermentations, are presented in Table 1 and Fig. 2 (b-c, e-f), respectively. In pure culture fermentations, *Starm. bacillaris* showed a higher preference to fructose (9 days for exhaustion) than glucose with about 66.2 g/L in the medium. Ethanol and glycerol production reached relative high levels for this species (9.8 % v/v and 11.8 g/L, respectively), while acetic acid was maintained at low levels, in agreement with Rantsiou et al. (2017). On the contrary, *S. cerevisiae* consumed all the sugars present in the must in 7

days, demonstrating a higher preference to glucose than fructose since the concentration of these sugars was 55.4 and 78.5 g/L, respectively, after 2 days of fermentation. The high sugar uptake was correlated to a high ethanol (13.9 % v/v) and low glycerol (8.1 g/L) content.

In mixed culture fermentations both in the flask and in the double-compartment fermentation system (in which the cells are physically separated by a membrane), *Starm. bacillaris* and *S. cerevisiae*, both species showed different population dynamics compared to the respective pure fermentations. It is worth noting that the composition of the fermenting medium during the fermentation period was the very similar in both mixed culture fermentations. *Starm. bacillaris* and *S. cerevisiae* grew to a cell concentration of 8.0 and 7.5 Log CFU/mL, respectively, after 2 days of fermentation (Fig. 2g, j). Regardless of the physical separation of yeast cells, the initial inoculation of must with *Starm. bacillaris* FC54 had a negative impact on the growth and metabolic activity of *S. cerevisiae* Uvaferm BC®. In both fermentations, *S. cerevisiae* reached the stationary phase with about 7.5 Log CFU/mL, which is 50 % (0.5 Log CFU/mL) lower than *S. cerevisiae* in pure culture fermentation. The cells maintained the same cell viability until the end of the monitored period. Concerning their coexistence in fermentations conducted in flask, *Starm. bacillaris* FC54 dominated the fermentation process, with plate counts of 8.0 Log CFU/mL in the first 7 days and became undetectable after 9 days of fermentation. However, it should be underlined that metabolically active populations of *Starm. bacillaris* at low cell densities in mixed culture fermentations may be present in the last stages of fermentation, but they cannot be easily determined by plate counting on WLN medium when coexisting with high populations of *S. cerevisiae*. On the other hand, the dominance and survival of *Starm. bacill*aris FC54 was extended up to day 9 and the end of the monitored period, respectively in fermentations conducted in double-compartment fermentor, with cell viability above 5.5 Log CFU/mL.

The evolution of the monitored metabolites during fermentation was the same in both mixed fermentations (flask and double-compartment fermentation system) as well as in the two compartments of the double-compartment fermentation system (*Starm. bacillaris* and *S. cerevisiae* sides). In particular, the sugars and ethanol concentration at the onset of *Starm. bacillaris* death was 50 g/L and 10.2 % v/v, respectively, indicating that the death of *Starm. bacillaris* occurred long before the sugar depletion from the medium. In order to verify if the early death of *Starm. bacillaris* cells in mixed fermentations with physical contact is caused by the presence of viable *S. cerevisiae* cells at high concentrations, a new set of fermentations were carried out. For this aim, we added a high cell concentration of 7.5 Log CFU/mL of dead *Starm. bacillaris* or *S. cerevisiae* cells (metabolically and enzymatically inactive) to early (48 h) or late (96 h) exponential phase pure cultures of *S. cerevisiae* or *Starm. bacillaris*, respectively (Fig. 3). The population and metabolites production during the fermentation process were comparable to the respective control fermentations (Fig. 2a, f), indicating that the presence of dead cells at high concentrations does not induce the early death of *Starm. bacillaris* in pure culture fermentation. However, further investigations are necessary to elucidate if the addition of dead cells from mixed fermentations to pure culture of *S. cerevisiae* or *Starm. bacillaris* generate the same growth dynamics pattern, since the physical presence of another species could trigger a specific molecular or physiological response in the cells from mixed fermentation.

The results of the chemical analysis at the end of the monitored period and during the mixed cultures fermentation performed in flask and in double-compartment fermentation system are presented in Table 1 and Fig. 2 (panels h, i, k, l), respectively. As can be seen, the evolution of the metabolites was very similar between the fermentations performed with and without physical separation. In fact, in both cases the fermentation was completed in 14 days. A lower sugar uptake in the mixed compared to the pure culture fermentation with *S.*

cerevisiae was exhibited (14 days versus 7 days), which is consistent with the reduced growth of *S. cerevisiae* in these fermentations. Fructose was consumed faster compared to glucose (residual concentration of 41.5 versus 87.9 g/L) after 4 days of fermentation probably due to the dominance of *Starm. bacillaris* over *S. cerevisiae* in this period of time (Englezos et al., 2016). Fermentations in flasks and in the double-compartment fermentation system produced significantly more glycerol (14.6-14.7 g/L) than pure cultures, while the ethanol content was reduced by 0.5 % v/v with respect to the pure *S. cerevisiae* fermentation. Mixed culture fermentations were clearly differentiated from the fermentations performed using *S. cerevisiae* in pure culture because of high glycerol and low ethanol yields. Glycerol yield in mixed culture fermentations ranged from 0.0632 to 0.0639 g/g, a higher value compared to that registered for the *S. cerevisiae* in pure culture fermentation (0.0352 g/g). On the contrary, pure fermentations with *S. cerevisiae* showed the highest levels of ethanol yield. Compared to *S. cerevisiae* alone, the ethanol yields were reduced by 0.021-0.026 (g/g) in mixed culture fermentations.

3.2. The influence of fermentation associated products on the population dynamics and metabolites produced from pure and mixed cultures

To further investigate the possible factors influencing the interactions between *Starm. bacillaris* and *S. cerevisiae*, new sets of fermentations were conducted. Firstly, the cells from the mixed culture fermentation were harvested and suspended in fresh sterile must after 96 h of fermentation. Secondly, the cells from pure culture fermentations with *Starm. bacillaris* or *S. cerevisiae* were harvested and suspended in a supernatant prepared from mixed cultures after 96 h of fermentation (Fig. 1, panel C). In Fig. 4, the growth dynamics and the evolution of sugars, glycerol and ethanol during mixed and pure culture fermentations described above

are illustrated. As can be seen in Fig. 4 (a-c), the fermentation profiles of both species in mixed culture fermentation, when fresh must was added, are comparable to those obtained during fermentation with physical contact (Fig. 2, g-i). After 4 days, *Starm. bacillaris* population started to decrease and it became undetectable after 9 days, while the viability of *S. cerevisiae* achieved levels of about 6.0 Log CFU/mL at the end of the monitored period (14 days). Despite these similarities in the population dynamics, marked differences were observed in fermentation length, since sugars were consumed faster compared to control mixed fermentations (5 days (after fresh must addition) versus 14 days), and both fructose and glucose were consumed at the same speed.

Ethanol production by *S. cerevisiae* is considered to be a major factor that modulates the population size and metabolic activity of non-*Saccharomyces* species during fermentation (Fleet, 2003). Generally, the species of *Hanseniaspora*, *Pichia*, *Candida* and *Metschnikowia* found in wine fermentation are not tolerant to ethanol concentrations exceeding 5-7 % v/v, and this explains their decline and death as the fermentation progresses after mid-stage (Jolly et al., 2014). We therefore speculated that the relatively high ethanol levels in mixed culture fermentation were sufficient to induce *Starm. bacillaris* death but insufficient to induce death to *S. cerevisiae*. However, the addition of fresh must in mixed culture fermentations has reduced the amount of ethanol to values lower than 0.1 % v/v, demonstrating that the high alcohol concentration did not influence the viability loss of *Starm. bacillaris*. In fact, after 7 days of fermentation, this yeast started to lose viability even if the ethanol concentration was around 11.4 % v/v; an amount well tolerated by *Starm. bacillaris* (Englezos et al., 2015). Another possible explanation for the early death of *Starm. bacillaris* cells could be the presence of limited concentrations of different nutrients in the must (Ciani and Comitini, 2015). However, when the fermenting medium was substituted with fresh sterile must (after 96 h of fermentation), *Starm. bacillaris* still reduced its cells number to undetectable levels

earlier than in pure culture fermentation. These results show that the early death of *Starm. bacillaris* in mixed culture fermentation with *S. cerevisiae* was not due to a nutrient deficiency.

Together with ethanol and nutrients, other metabolites can modulate yeast population dynamics in mixed culture fermentations. In particular, the production of short- to medium-chain fatty acids, killer toxins, SO_2 by *S. cerevisiae* cells can negatively affect its co-existence in mixed wine fermentations with non-*Saccharomyces* yeasts (Albergaria et al., 2010; Branco et al., 2014, 2017). In order to evaluate whether the production of toxic metabolites was responsible for the death of *Starm. bacillaris* in mixed cultures, we carried out a set of fermentations in which the initial conditions resembled as closely as possible the condition in mixed cultures at the onset of the early death. For this aim, pure cultures of *Starm. bacillaris* and *S. cerevisiae* at their late exponential phase (2 days) were inoculated into supernatants, prepared from the mixed culture fermentations after 4 days of fermentation (Fig. 1, panel C). The population and metabolites evolution for these sets of fermentation are shown in Fig. 4 (panels d - i). Growth dynamics throughout fermentation were comparable to those observed for control pure culture fermentations (Fig. 2a - f). *Starm. bacillaris* produced a partially fermented wine with significant presence of residual sugars (up to 70 g/L; Fig. 4e), although fructose was totally consumed from the medium after 7 days from mixed culture supernatant addition. On the other hand, complete sugar consumption was observed in *S. cerevisiae* trial, where both glucose and fructose were consumed at the same rate (Fig. 4h). However, the chemical composition of these wines was characterised by the presence of significantly higher levels of glycerol and ethanol, compared to the respective controls due to the addition of the supernatant of mixed culture fermentations. Moreover, pure cultures of both species were able to survive at higher ethanol concentration than that present at the onset of *Starm. bacillaris* death in mixed cultures (data not shown). All these findings let us to

hypothesize that *S. cerevisiae* did not produce primary metabolites that may be considered toxic for *Starm. bacillaris* development and vice versa.

These observations, taken together with the fact that *Starm. bacillaris* survived until the end of the monitored period (9 days versus 14 days) in the double-compartment fermentor, suggest that the cell-to-cell contact, influencing the presence of viable *S. cerevisiae* cells at high density, plays a role in the decline of *Starm. bacillaris*. This is in accordance with a previous study, which demonstrated that in the early stage of fermentation, strains of *K. thermotolerans* and *T. delbrueckii* died due to the contact with viable *S. cerevisiae* cells (Nissen et al., 2003).

However, in our case we cannot exclude the production of an unknown metabolite (not measured in this study) which is not diffused efficiently between the two compartments to be responsible for the viability of both species during mixed fermentations in the double-compartment fermentor. It's well known that besides ethanol during wine fermentations yeasts can produce, other toxic compounds, namely, killer toxins, short- and medium- chain fatty acids and sulphites able to induce the death of other yeasts strains and/or species (non-*Saccharomyces* and *S. cerevisiae*) (Ciani and Comitini, 2015). In particular, Albergaria et al. (2010) reported that some 2-10 kDa peptides produced by *S. cerevisiae* could inhibit the growth of *T. delbrueckii* wine related strains. Consequently, our results do not exclude however the production by *S. cerevisiae* of any killer-like toxin (e.g. peptides, proteins and glycoproteins) or even fermentative metabolites potentially toxic for *Starm. bacillaris* strains. To get a better insight in the nature of the toxic compounds involved the early death of *Starm. bacillaris* in mixed culture fermentations with *S. cerevisiae* carried out in flasks, as well as the death mechanism underlining this phenomenon further investigation will be carried out.

4. Conclusion

The present study shows that the early death of *Starm. bacillaris* in mixed culture fermentations with *S. cerevisiae* carried out in flasks is not due to the depletion of nutrients or due to the production of toxic metabolites (not measured in the present study) by the yeasts, but rather to cell-to-cell contact mechanism. This information contributes to further understand the yeast-yeast interactions occurring during wine fermentations and allows a greater management of the production of specific metabolites to improve wine quality.

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References

Albergaria, H., Francisco, D., Gori, K., Arneborg, N., Gírio, F., 2010. *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. Appl. Microbiol. Biotechnol. 86, 965-972. http://dx.doi.10.1007/s00253-009-2409-6.

Albergaria, H., Arneborg, N., 2016. Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation processes: role of physiological fitness and microbial interactions. Appl. Microbiol. Biotechnol. 100, 2035-2036. http://dx.doi.10.1007/s00253-015-7255-0.

Belda, I., Ruiz, J., Esteban-Fernández, A., Navascués, E., Marquina, D., Santos, A., Moreno-Arribas, M., 2017. Microbial contribution to wine aroma and its intended use for wine quality improvement. Molecules. 22, 189. http://dx.doi.10.3390/molecules22020189.

Boulton, R.B., Singleton, V.L., Bisson, L.F., Kunkee, R.E., 1996. Principles and practices of wine making, first ed. Chapman & Hall, New York.

Branco, P., Francisco, D., Chambon, C., Hébraud, M., Arneborg, N., Almeida, M.G., Caldeira, J., Albergaria, H. 2014. Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. Appl. Microbiol. Biotechnol. 98, 843-853. http://dx.doi.10.1007/s00253-013-5411-y.

Branco, P., Kemsawasd, V., Santos, L., Diniz, M., Caldeira, J., Almeida, M. G., Arneborg, N., Albergaria, H., 2017. *Saccharomyces cerevisiae* accumulates GAPDH-derived peptides on its cell surface that induce death of non-*Saccharomyces* yeasts by cell-to-cell contact. FEMS Microbiol. Ecol. 93, fix055. http://dx.doi.10.1093/femsec/fix055.

Capece, A., Romaniello, R., Siesto, G., Pietrafesa, R., Massari, C., Poeta, C., Romano, P. 2010. Selection of indigenous *Saccharomyces cerevisiae* strains for Nero d'Avola wine and evaluation of selected starter implantation in pilot fermentation. Int. J. Food Microbiol. 144, 187-192. http://dx.doi.10.1016/j.ijfoodmicro.2010.09.009.

Ciani, M., Comitini, F., Mannazzu, I., Domizio, P., 2010. Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeast in winemaking. FEMS Yeast Res. 10 (2), 123-133. http://dx.doi: 10.1111/j.1567-1364.2009.00579.x.

Ciani M., Comitini F., 2015. Yeast interaction in multi-starter wine fermentation. Curr. Opin. Food Sci. 1, 1-6. http://dx.doi.org/10.1016/j.cofs.2014.07.001

Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G., Romani, P., 2016. Yeast interactions in inoculated wine fermentation. Front. Microbiol. 7, 555. http://dx.doi: 10.3389/fmicb.2016.00555

Comitini, F., Capace, A., Ciani, M., Romano, P., 2017. New insights on the use of wine yeasts. Curr. Opin. Food Sci. 13, 44-49. http://dx.doi.org/10.1016/j.cofs.2017.02.005

Cramer, A.C, Vlassides, S., Block, D.E., 2002. Kinetic model for nitrogen-limited wine fermentations. Biotechnol. Bioeng. 77, 49-60. http://dx.doi.org/10.1002/bit.10133.

Cravero, F., Englezos, V., Rantsiou, K., Torchio, F., Giacosa, S., Río Segade S., Gerbi, V., Rolle, L., Cocolin, L., 2016. Ozone treatments of post harvested wine grapes: Impact on fermentative yeasts and wine chemical properties. Food Res. Int. 87, 134-141. http://dx.doi.org/10.1016/j.foodres.2016.06.031

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., Cocolin, L., 2015. Exploitation of the non-*Saccharomyces* yeast *Starmerella bacillaris* (synonym Candida zem*plinina*) in wine fermentation: physiological and molecular characterizations. Int. J. Food Microbiol. 199, 33-40. http://dx.doi: 10.1016/j.ijfoodmicro.2015.01.009.

Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Ortiz-Julien, A., Gerbi, V., Rolle, L., Cocolin, L., 2016. *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. Applied Microbiol. Biotechnol. 100, 5515-5526. http://dx.doi: 10.1007/s00253-016-7413-z.

Englezos, V., Giacosa, S., Rantsiou, K., Rolle, L., Cocolin, L., 2017. *Starmerella bacillaris* in winemaking: opportunities and risks. Curr. Opin. Food Sci. 17, 30-35. http://dx.doi.org/10.1016/j.cofs.2017.08.007.

Englezos, V., Cravero, F., Torchio, F., Rantsiou, K., Ortiz-Julien, A., Lambri, M., Gerbi, V., Rolle, V., Cocolin, L., 2018. Oxygen availability and strain combination modulate yeast growth dynamics in mixed culture fermentations of grape must with *Starmerella bacillaris* and *Saccharomyces cerevisiae*. Food Microbiol. 69, 179-188. http://dx.doi: 10.1016/j.fm.2017.08.007.

Fleet, G.H., 2003. Yeast interactions and wine flavour. Int. J. Food Microbiol. 86 (1-2), 11-22. http://dx.doi:10.1016/S0168-1605(03)00245-9.

Jolly, N.P., Varela, C., Petronius, I.S., 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res. 14, 215-37. http://dx.doi.org/10.1111/1567-1364.12111

Kemsawasd, V., Branco, P., Almeida, M. G., Caldeira, J., Albergaria, H., Arneborg, N., 2015. Cell-to-cell contact and antimicrobial peptides play a combined role in the death of *Lachancea thermotolerans* during mixed-culture alcoholic fermentations with *Saccharomyces cerevisiae.* FEMS Microbiol. Lett. 362 (14): 1-8. http://dx.doi: 10.1093/femsle/fnv103.

Liu, Y., Rousseaux, S., Tourdot-Mareìchal, R., Sadoudi, M., Gougeon, R., Schmitt-Kopplin, P., Alexandre, H., 2015. Wine microbiome, a dynamic world of microbial interactions. Crit. Rev. Food Sci. Nutr. 57, 856-873. http://dx.doi:10.1080/10408398.2014.983591

Mate, J.J., Maicas, S., 2016. Application of non-*Saccharomyces* yeasts to wine-making process. Ferment 2, 14. http://dx.doi:10.3390/fermentation2030014.

Miller, M.B., Bassler, B.L., 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol., 55(1), 165-199. http://dx.doi.org/10.1146/annurev.micro.55.1.165.

Nissen, P., Nielsen, D., Arneborg, N., 2003. Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell–cell contact-mediated mechanism. Yeast, 20 (4), 331-341. http://dx.doi.org/10.1002/yea.965.

Nissen, P., Arneborg, N. (2003). Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. Arch. Microbiol. 180, 257-263. http://dx.doi.org/10.1007/s00203-003-0585-9.

Pérez-Nevado, F., Albergaria, H., Hogg, T., Girio, F., 2006. Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. Int. J. Food Microbiol. 108 (3), 336-345. http://dx.doi.org/10.1016/j.ijfoodmicro.2005.12.012.

Peréz-Torrado, R., Rantsiou, K., Perrone, B., Navarro- Tapia, E., Querol, A., Cocolin, L., 2017. Ecological interactions among *Saccharomyces cerevisiae* strains: insight into the dominance phenomenon. Sci. Rep. 7: 43603. http://dx.doi.10.1038/srep43603.

Perrone, B., Giacosa, S., Rolle, L., Cocolin, L., Rantsiou, K., 2013. Investigation of the dominance behavior of *Saccharomyces cerevisiae* strains during wine fermentation. Int. J. Food Microbiol. 165, 156-162. http://dx.doi.org/10.1016/j.ijfoodmicro.2013.04.023.

Rantsiou, K., Englezos, V., Torchio, F., Risse, P. A., Cravero, F., Gerbi, V., Rolle L., Cocolin, L., 2017. Modeling the fermentation behavior of *Starmerella bacillaris*. Am. J. Enol. Vitic. 68, 378-385. http://dx.doi.10.5344/ajev.2017.16108

Rolle, L., Englezos, V., Torchio, F., Cravero, F., Segade, S. R., Rantsiou, K., Giacosa, S., Gambuti, A., Gerbi, V., Cocolin, L. 2018. Ethanol reduction in red wines by technological and microbiological approaches: a comparative study. Aust. J. Grape Wine Res. 24, 62-74. http://dx.doi.org/10.1111/ajgw.12301.

Sipiczki, M., 2003. *Candida zemplinina* sp. nov., an osmotolerant and psychrotolerant yeast that ferments sweet botrytized wines. Int. J Syst. Evol. Microbiol. 53, 2079-2083. http://dx.doi.10.1099/ijs.0.02649-0.

Sipiczki, M., 2004. Species identification and comparative molecular and physiological analysis of *Candida zemplinina* and *Candida stellata*. J. Basic Microbiol. 44, 471-479. http://dx.doi.org/10.1002/jobm.200410449.

Varela, C., 2016. The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. Appl. Microbiol. Biotechnol. 100, 9861-9874. http://dx.doi.org/10.1007/s00253-016-7941-6.

Varela, C., Borneman, A.R., 2016. Yeasts found in vineyards and wineries. Yeast. 34, 111-128. http://dx.doi: 10.1002/yea.3219.

Wang, C., Mas, A., Esteve- Zarzoso, B., 2015. Interaction between *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation. Int. J. Food Microbiol. 206, 67-74. http://dx.doi. 10.1016/j.ijfoodmicro.2015.04.022.

Wang, C., Mas, A., Esteve-Zarzoso, B., 2016. The interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific. Front Microbiol, 7, 502. http://dx.doi.10.3389/fmicb.2016.00502

Table 1

Final chemical parameters of musts and wines produced by pure and mixed culture fermentations

Inoculation protocol	Residual sugars (g/L)	Succinic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Ygly (g/s) (g/g)	Yeth (eth/s) (g/g)
Grape must	233.5 ± 1.6	< 0.1	< 0.1	< 0.1	< 0.1	-	-
PCF *S. cerevisiae*	2.9 ± 0.1a	1.04 ± 0.02a	0.40 ± 0.01a	8.1 ± 0.1a	13.9 ± 0.1c	0.0352 ± 0.0003a	0.0602 ± 0.0002b
PCF *Starm. bacillaris*	66.2 ± 1.8b	0.92 ± 0.06a	0.49 ± 0.02b	11.8 ± 0.3b	9.8 ± 0.1a	0.0707 ± 0.0003c	0.0583 ± 0.0008a
MCF	3.0 ± 0.6a	0.91 ± 0.03a	0.52 ± 0.03b	14.7 ± 0.1c	13.3 ± 0.1b	0.0639 ± 0.0002b	0.0578 ± 0.0006a
CCF *S. cerevisiae* side	2.1 ± 0.1a	1.26 ± 0.03b	0.49 ± 0.02b	14.6 ± 0.1c	13.3 ± 0.2b	0.0632 ± 0.0003b	0.0576 ± 0.004a
CCF *Starm. bacillaris* side	2.8 ± 1.3a	1.33 ± 0.12b	0.54 ± 0.03b	14.6 ± 0.1c	13.4 ± 0.2b	0.0634 ± 0.0002b	0.0581 ± 0.0011a
Sign.	***	***	***	***	***	***	***

PCF: pure culture fermentation, MCF: mixed culture fermentation, CCF: compartmentalized-culture fermentations. Ygly (glycerol/sugar consumption): glycerol yield, Yeth (ethanol/sugar consumption): ethanol yield. The values are means ± standard deviations of three independent experiments. Sig: *** indicate significance at $p < 0.001$ between the wines produced.

Figure captions

Fig. 1

Schematic representation of the different sets of fermentations performed.

Fig. 2

Viable cells, sugars (fructose and glucose), ethanol and glycerol concentration during pure culture fermentations with *Starm. bacillaris* (a,b,c), *S. cerevisiae* (d,e,f) and mixed culture fermentations with *Starm. bacillaris* and *S. cerevisiae* in flask (g,h,i) and in double-compartment fermentation system (j,k,l). Data are the mean values ± standard deviations of three independent experiments.

Fig. 3

Viable cells, sugars (fructose and glucose), ethanol and glycerol concentration during pure culture fermentations with *Starm. bacillaris* (a-f), *S. cerevisiae* (g-l). In a-c and d-f, $5.0x10^7$ cells/mL dead *S. cerevisiae* cells were added after 48 and 96 h of fermentation, respectively. In g-i and j-l, $5.0x10^7$ cells/mL dead *Starm. bacillaris* cells were added after 48 and 96 h, respectively. Data are the mean values ± standard deviations of three independent experiments.

Fig. 4

Viable cells, sugars (fructose and glucose), ethanol and glycerol concentration during mixed culture fermentations with *Starm. bacillaris* and *S. cerevisiae* in flask (a,b,c) and in pure culture fermentations with *Starm. bacillaris* (d,e,f) and *S. cerevisiae* (g,h,i). In a-c, the cells were harvested and suspended in fresh sterile Nebbiolo must after 96 h of fermentation. In d-

i, the cells were harvested and suspended in supernatant prepared from mixed cultures after 96 h of fermentation. Data are the mean values ± standard deviations of three independent experiments.

Fig. 1

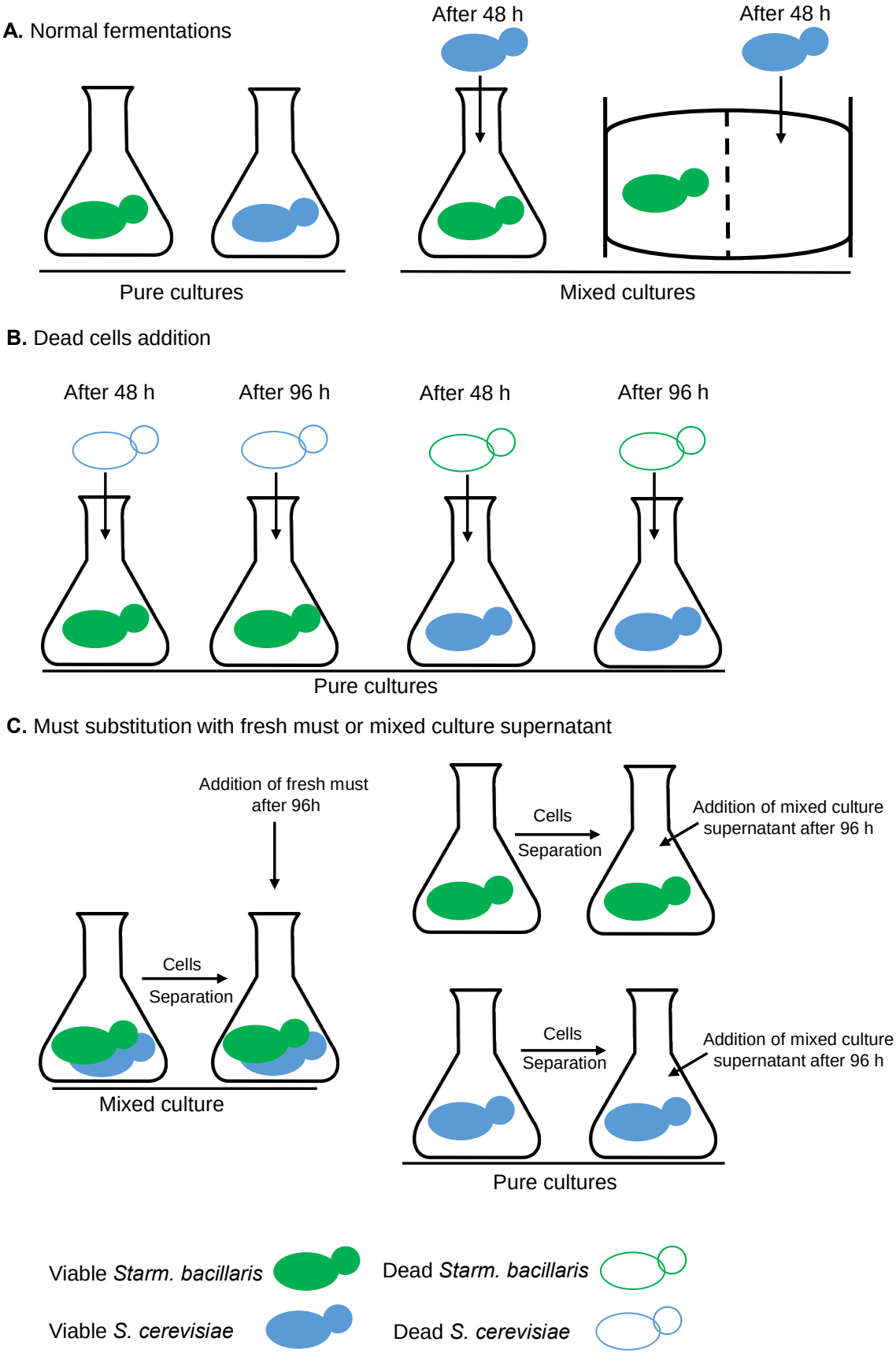


Fig. 2

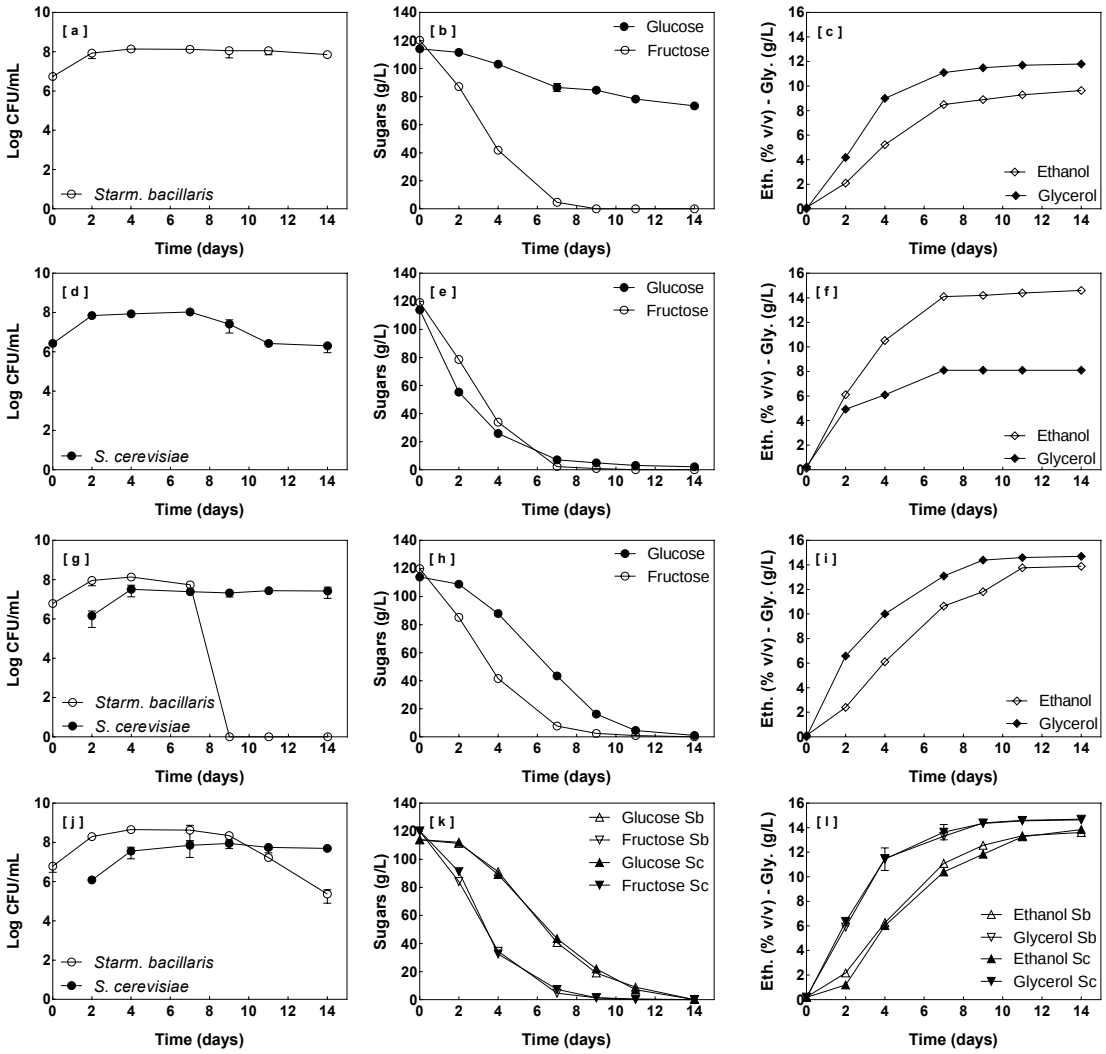


Fig. 3

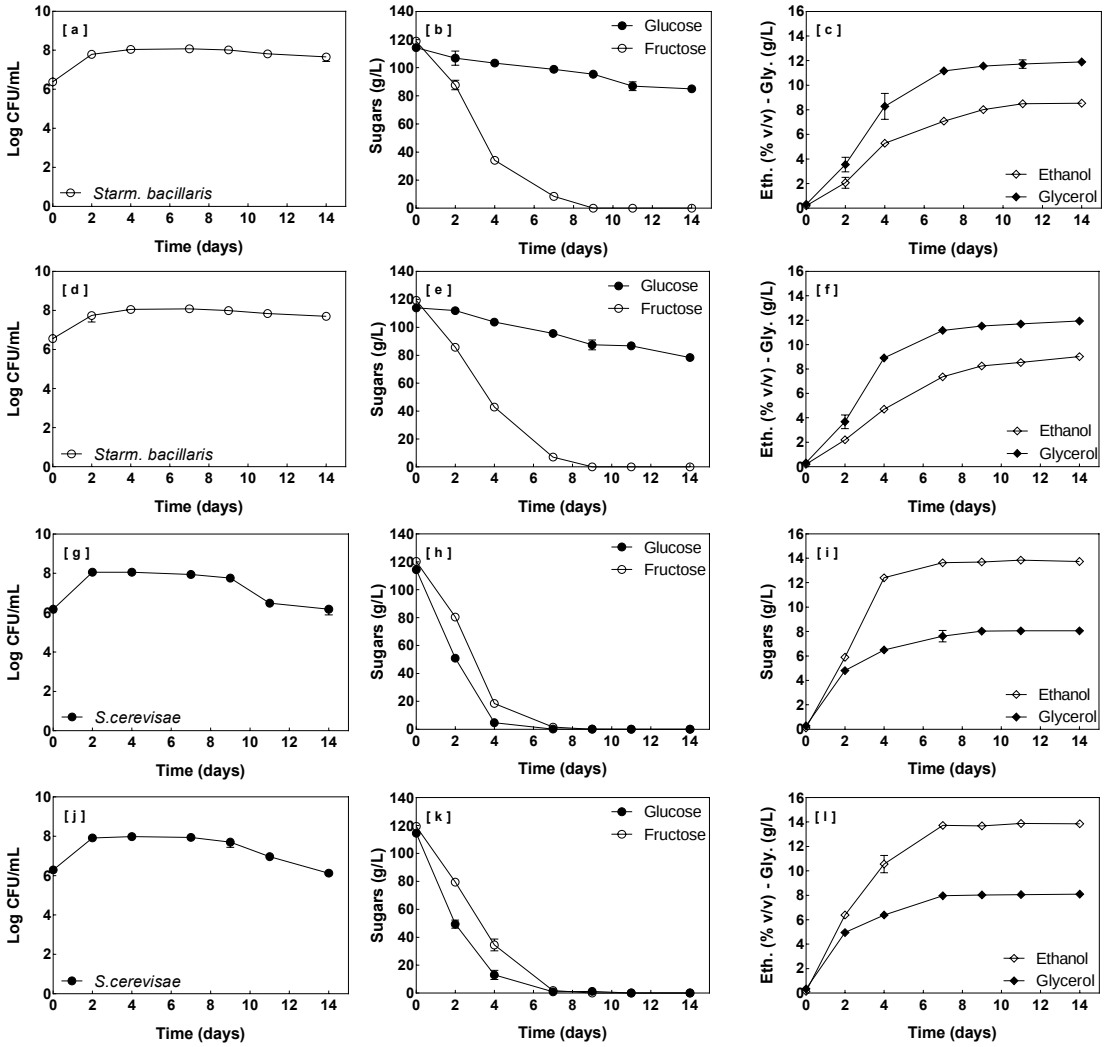
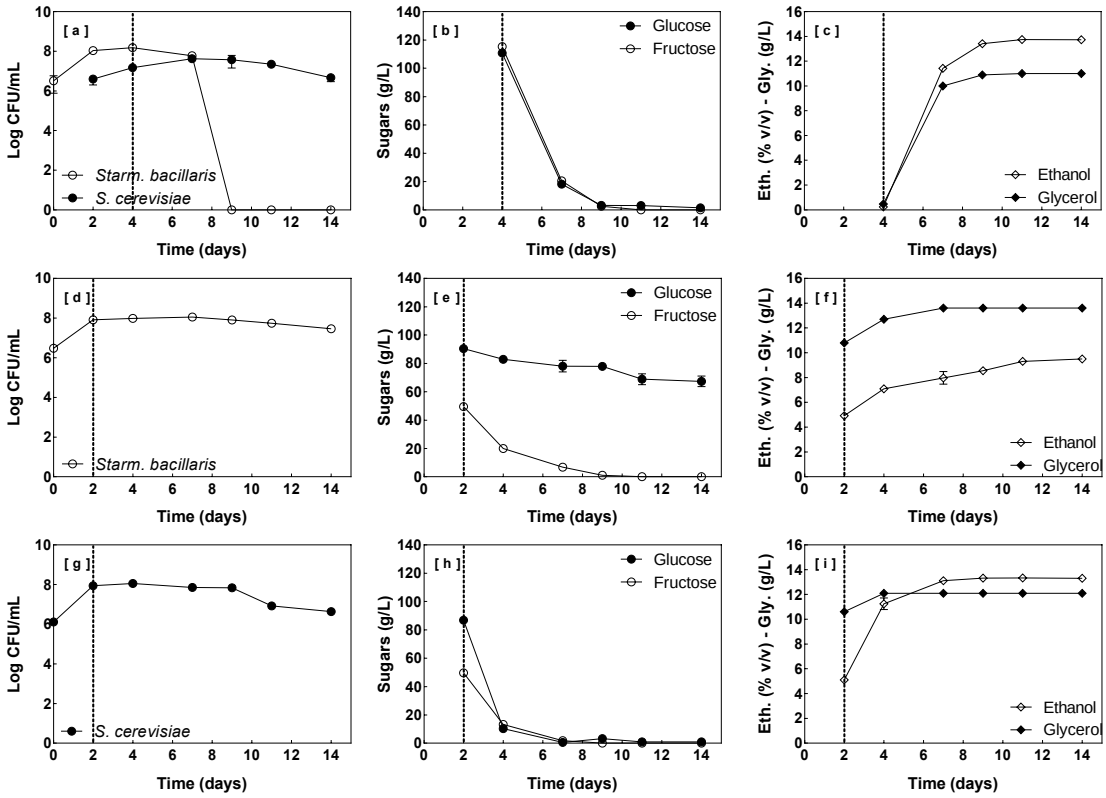


Fig. 4



Highlights

- Yeast interspecies interactions have a fundamental role to determine the desired wine characteristics
- The growth and death kinetics of *S. cerevisiae* and *Starm. bacillaris* were investigated in pure and mixed culture fermentations
- The early death of *Starm. bacillaris* is not caused by nutrient limitation or by inhibitory compounds accumulation
- Cell-to-cell contact mechanism appears to be responsible for *S. cerevisiae* dominance