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The addition of wine yeast *Starmerella bacillaris* to grape skin surface influences must fermentation and glycerol production

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ABSTRACT

Starmerella bacillaris is a non-*Saccharomyces* yeast recently proposed for grape fermentation in association with *Saccharomyces cerevisiae*. Due to its high glycerol and moderate volatile acidity production this yeast can contribute to improving wine quality. Some strains have been demonstrated to exhibit antifungal activity against grey mould on grape, which is caused by *Botrytis cinerea*. The simultaneous presence of these traits in *S. bacillaris* is of great interest. Indeed, this yeast can be potentially used as a biocontrol agent in vineyards. Research on the ability of *S. bacillaris* to survive or, even to grow on the surface of grapes is a starting point in the evaluatation of its potential use in vineyards. The preliminary results of our study showed that when applied to the grape surface under laboratory condictions, inoculum sized *S. bacillaris* with antifungal activity developed and lasted for at least 6 days in high concentrations. In addition, it positively influenced the fermentation process by producing high concentrations of glycerol (average value 4.89 ± 0.47 g/L). Interestingly, a positive effect on wine quality was also observed when the inoculum size was 10 times higher or lower than the reference concentration. When sprayed on the vines in the vineyard and present on the grape skin surface after harvest, *S. bacillaris* cells can start alcoholic fermentation.

KEYWORDS

Alcoholic fermentation, biocontrol, non-Saccharomyces, sequential inoculum, wine quality

INTRODUCTION

In the past, non-*Saccharomyces* yeasts were considered to be undesirable spoilage microorganisms, because they were often isolated from stuck or sluggish fermentations, or from wines with anomalous analytical compositions or negative sensorial profiles (Jolly *et al.*, 2014; Ciani and Comitini, 2015). Throughout the past decade, they have become popular and their role has been re-considered, as many species contribute to wine fermentation and can positively affect wine quality (Zott *et al.*, 2008; Ciani and Comitini, 2015; Ivit and Kemp, 2018). Therefore, there is a growing interest in isolating and characterising non-*Saccharomyces* yeasts for the development of starter cultures that increase the diversity of flavour in wine, as well as in beer and spirits (Varela, 2016). Generally, these strains are not capable of completing alcoholic fermentation on their own. A solution to this problem is the sequential or simultaneous inoculation of non-*Saccharomyces* yeasts with *Saccharomyces cerevisiae*, thus ensuring complete fermentation (Varela and Borneman, 2017).

Starmerella bacillaris (synonym *Candida zemplinina*) is a non-*Saccharomyces* yeast commonly found in oenological environments (Bovo *et al.*, 2011; Magyar *et al.*, 2014; Varela and Borneman, 2017). During grape must fermentation, it has been tested in both sequential and mixed yeast inoculations with *S. cerevisiae* (Rantsiou *et al.*, 2012; Wang *et al.*, 2014; Englezos *et al.*, 2016; Lemos Junior *et al.*, 2016). Many studies indicate that the use of *S. bacillaris*, together with *S. cerevisiae*, influences wine stability and enhances the glycerol content of wines with moderate volatile acidity production; wine flavour and mouthfeel are improved and, due to the low ethanol yield, ethanol content is reduced (Rantsiou *et al.*, 2012; Englezos *et al.*, 2016; Lemos Junior *et al.*, 2016; Varela and Borneman, 2017; Lencioni *et al.*, 2018; Lemos Junior et al., 2020a). Recently, the whole genome comparison of two *S. bacillaris* strains shed some light on genes responsible for yeast technologically relevant properties in winemaking (Lemos Junior *et al.*, 2018). These *S. bacillaris* strains belong to a pool that have been demonstrated to exhibit antifungal activity against the grey mould caused by *Botrytis cinerea*; such activity is related to the production of volatile organic compounds such as benzyl alcohol (Lemos Junior *et al.*, 2016; Lemos Junior *et al*., 2020b), and the apple blue mould caused by *Penicillium expansum* (Nadai *et al.*, 2018). Moreover, they showed interesting technologically relevant properties that enhance wine and cider quality when they are used as unconventional starters in fermentation (Lemos Junior *et al.*, 2016, Nadai *et al.*, 2018).

Yeasts are a promising alternative to synthetic fungicides. Indeed, they have been identified by many studies as potential biological control agents, particularly due to the fact they are naturally present on surfaces of fruits and vegetables and phenotypically adapted to this niche (Piombo *et al.*, 2018; Pawlikowska *et al.*, 2019; Mukherjee *et al.*, 2020). In pre-harvest treatments, an important issue is yeast survival at a suitable concentration on the fruit surface after application (Benbow and Sugar, 1999).

As *S. bacillaris* have been proved to exhibit antifungal activity and to increase glycerol concentration in wine, it can be potentially used as a biocontrol agent by being sprayed in vineyards; after harvest, the yeast cells will start alcoholic fermentation. Research on the ability of *S. bacillaris* to survive or, possibly, to grow on the surface of grapes is a starting point for the evaluation of its potential use in vineyards and, subsequently, in the cellar. This preliminary work focused on *S. bacillaris* FRI751. This strain was previously reported to exhibit antifungal activity against *B. cinerea* and to increase glycerol content in wine when used in a sequential fermentation with *S. cerevisiae* (Lemos Junior *et al.*, 2016). The strain's ability to persist at a laboratory scale on the grape surface was tested up to 6 days after inoculum. The effects of *S. bacillaris* on the fermentation trend and products were evaluated after alcoholic fermentation of the inoculated and crushed bunches.

MATERIALS AND METHODS

1. Yeast strains

The yeast strains tested in this work were *S. bacillaris* FRI751 (Lemos Junior *et al.*, 2017), which was isolated from dried grape of the Raboso Piave variety as described by Lemos Junior *et al.* (2016), and the commercial wine strain *S. cerevisiae* EC1118 (Lallemand Inc., Montreal, Canada).

2. Yeast growth test on grape surface and after grape crushing

Healthy and undamaged grape bunches of the Incrocio Manzoni 6.0.13 variety were selected.

Grape bunches were immersed in a 0.1 % solution of sodium hypochlorite for 2 min, then the bunches were rinsed with deionised water and left to dry (Vero *et al.*, 2002; Parafati *et al.*, 2015) (Figure 1).

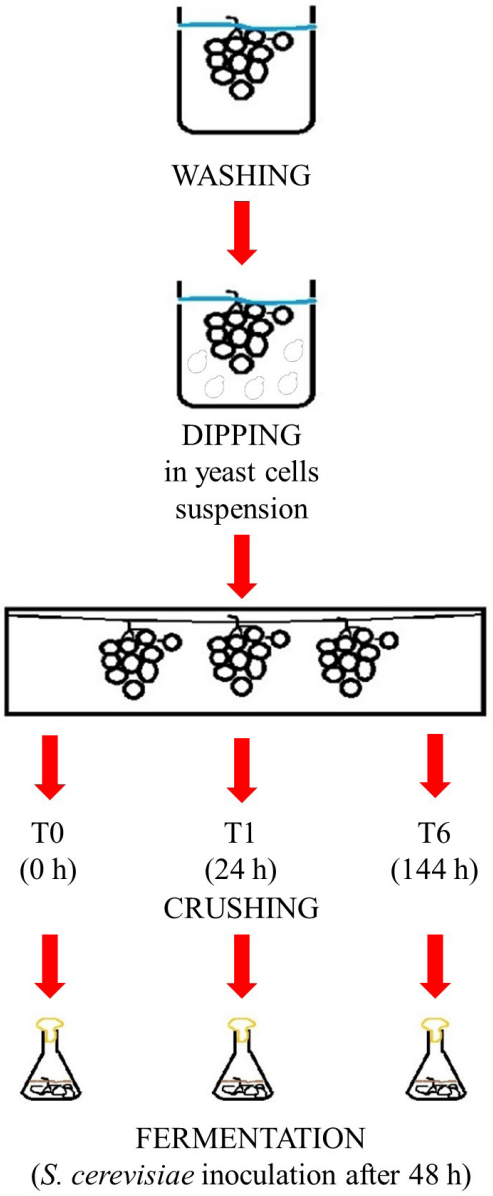

FIGURE 1. Schematic representation of the experimental procedure.

The concentration of *S. bacillaris* in a stationary phase YPD (Yeast Extract-Peptone-Dextrose, Difco, Milan, Italy) culture was determined by Thoma counter cell chamber measurements and confirmed by plate counts (CFU/mL) on a WL Nutrient Medium (Difco, Milan, Italy) after 24 h of plate incubation at 30 °C. The culture medium was removed by centrifugation and the cell pellet was resuspended in a volume of NaCl (0.9 % w/v) physiological solution, in order to achieve 1.4 x 10^8 cells/mL.

Each bunch was dipped in the yeast solution for 2 min and the volume of solution that adhered to the surface was calculated by weighing each bunch before and after dipping (Figure 1). Finally, bunches were divided into groups of five and hung from a thread without touching each other in a closed plastic box at 25 °C for 0 (T0), 24 (T1) and 144 (T6) hours after dipping. At each time point, five bunches were separately crushed in sterilised flasks and kept at 20 °C (Figure 1). Forty-eight hours after grape crushing, *S. cerevisiae* EC1118 (approximately 10^6 cells/mL) was inoculated (Figure 1). Five bunches were used as a control; they were immediately crushed after washing, and inoculated with *S. cerevisiae* EC1118. All the flasks were kept at 20 °C until the end of fermentation. The fermentations were considered completed when the sugar residue was lower than 1 g/L.

3. Microbiological analysis

Total yeast quantification was performed by plate count on a WL Nutrient Medium (Difco, Milan, Italy). Ten-fold dilutions of samples in Ringers solution (Oxoid, Milan, Italy) were spread onto WL plates. Plates were incubated at 28 °C for 5 days before plate count. The WL medium was also used to ascertain the presence of *S. bacillaris* and/or *S. cerevisiae* via the morphology and colour of the colonies: *S. bacillaris* forms flat green colonies, while *S. cerevisiae* forms umbonate creamy white colonies (Rantsiou *et al.*, 2012).

4. Fermentations trials

Pre-cultures of both strains used in this work were prepared as described by Bovo *et al.* (2016). In order to obtain final inoculum concentrations of 10^4, 10^5, 10^6, 10^7 cells/mL, suitable aliquots of *S. bacillaris* culture were used to inoculate 100 mL of Incrocio Manzoni must (204 g/L sugars, pH 3.5) in 120 mL-capacity bottles. *S. cerevisiae* EC1118 strain was used as a control in single strain fermentation (1 x 10^6 cells/mL). In the sequential fermentation, *S. cerevisiae* EC1118 (1 x 10^6 cells/mL) was added 48 h after the inoculation with *S. bacillaris*.

After yeast inoculation, the bottles were incubated at 20 °C. All experiments were performed in four replicates. CO_2 production was monitored by weighing the bottles twice a day and calculating the weight loss of each culture. The fermentations were stopped when the weight loss was lower than 0.05 g in 24 h.

5. Chemical analysis

HPLC analysis was performed to determine the concentrations of residual glucose and fructose, acetic acid, glycerol and ethanol, as described by Lemos Junior *et al.* (2019). Ten µL of sample was analysed by Waters 1525 HPLC binary pump (Waters, Milford, MA) equipped with a 300 × 7.8 mm stainless steel column packed with Aminex HPX_87H HPLC column (Bio-Rad, Hercules, CA) and a Waters 2414 Refractive Index Detector (Waters, Milford, MA). The analyses were performed isocratically at 0.6 ml/min and 65 °C with a cation-exchange column (300 by 7.8 mm [inner diameter]; Aminex HPX-87H) and a Cation H+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA), using 0.01 N H_2SO_4 as the mobile phase.

6. Statistical Analysis

The statistical data analysis was performed using XLSTAT software, vers. 2016.02 (Addinsoft, Paris, France). Data were subjected to simple analysis of variance (one-way ANOVA) followed by the Tukey's test as post-hoc analysis. Differences were considered statistically significant when the *p*-value was less than 0.05.)

RESULTS AND DISCUSSION

1. *S. bacillaris* survival on grape bunches and its growth during alcoholic fermentation

The ability of a biocontrol agent to grow and colonise a grape surface is fundamental for antifungal activity. Therefore, to investigate the persistence of *S. bacillaris* on a grape surface, a growth test on grape bunches was performed in laboratory conditions. In a previous study, the selected strain FRI751 showed strong anti-fungal activity against *Botrytis cinerea* on grape, along with good fermentation performances in must sequential fermentation with *S. cerevisiae* (Lemos Junior *et al.*, 2016).

S. bacillaris was added to the grape bunch surface by dipping bunches into a cell suspension in order to achieve a concentration of about 10^6 cells per gram of grapes. As previously reported (Lemos Junior *et al.*, 2016), this concentration significantly limited *B. cinerea* growth on grape berries. In order to verify *S. bacillaris* inoculum size for each grape bunch, the amount of cell that adhered to the grape berries surface was measured calculating the difference in the grape bunch weight before and after dipping. The measured inoculum size of each grape bunch ranged from 2.8 x 10^6 to 4.9 x 10^6 CFU/g of grape bunch. Five untreated grape bunches were kept to evaluate microbial contamination after grape washing.

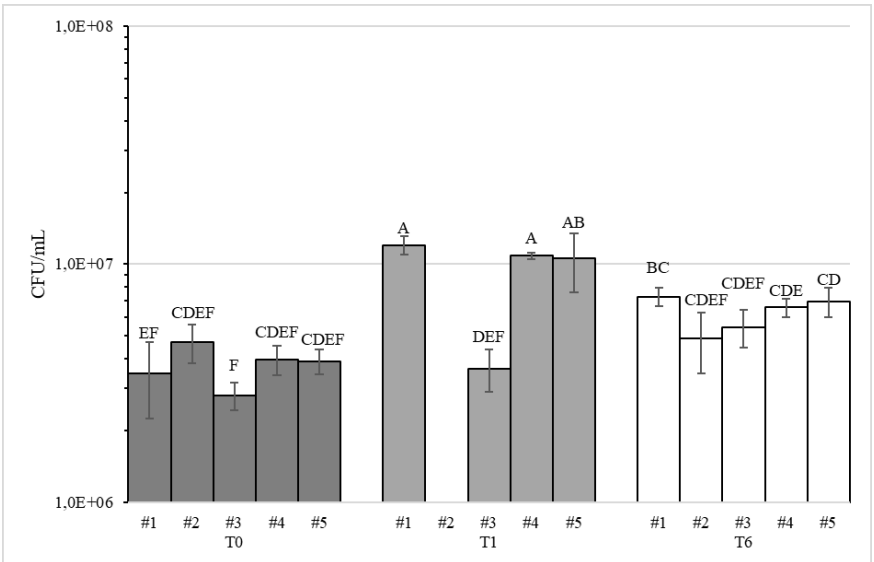

FIGURE 2. *Starmerella bacillaris* concentrations after grape bunch crushing.
Each bar represents a single grape bunch crushed 0 (T0 ■), 24 (T1 ■) or 144 (T6 □) hours after dipping. Data are expressed as the average of the replicates ± standard deviations. Different letters indicate significant differences between values ($p = 0.05$).

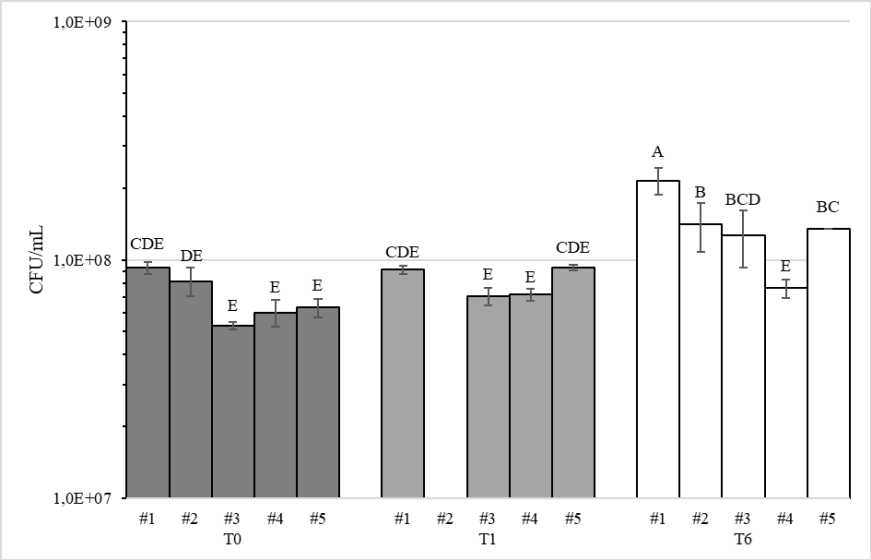

FIGURE 3. *Starmerella bacillaris* concentrations 48 hours after grape bunch crushing.
Each bar represents a single grape bunch crushed 0 (T0 ■), 24 (T1 ■) or 144 (T6 □) hours after dipping. Data are expressed as the average of the replicates ± standard deviations. Different letters indicate significant differences between values ($p = 0.05$).

The yeast population in the juice after crushing was 3.23 x 10^3 ± 1.1 CFU/g, which is about 1000 times lower than *S. bacillaris* inoculum size.

After each grape bunch incubation time (T0, T1 and T6), five bunches were separately crushed and fermentations were run. Forty-eight hours after grape crushing, the *S. cerevisiae* EC1118 strain was inoculated and the fermentation was monitored until the complete transformation of sugars. As a control, five untreated grape bunches were inoculated with *S. cerevisiae* immediately after crushing to evaluate fermentation performance in the absence of *S. bacillaris*.

S. bacillaris concentrations immediately after grape crushing are reported in Figure 2.

During the course of the test, bunch #2 of T1 was excluded due to mould contaminations. The presence of *S. bacillaris* was confirmed on all grape bunches. Three grape bunches out of four in T1 showed an increase in cells concentration, suggesting a growth of *S. bacillaris* during the 24 hours of incubation. The results indicate that *S. bacillaris* was present on the grape bunch surface up to 6 days after the inoculum, at least at concentrations comparable with the initial inoculum size. These results are promising as 10^6 CFU/mL is the concentration that had previously confirmed antifungal activity (Lemos Junior *et al.*, 2016).

S. bacillaris concentrations was determined forty-eight hours after grape crushing and before inoculating *S. cerevisiae* (Figure 3).

The concentrations ranged from 5.3 x 10^7 to 2.1 x 10^8 CFU/mL per grape bunch, although no significant differences were reported among the fermenting musts. These results evidenced a strong increase in *S. bacillaris* concentrations with respect to the inoculum size. These values are generally found when *S. bacillaris* is used as a co-starter in sequential fermentation, before adding *S. cerevisiae* (Englezos *et al.*, 2016).

Control fermentations were completed after 12 days, and *S. bacillaris* fermentations after 14 days (12 days after *S. cerevisiae* inoculation). At the end of fermentation, the yeast concentration was determined by plate count on a WL medium. Colonies with *S. cerevisae*-like morphology were dominant, while *S. bacillaris* green colonies were present only when low dilutions were plated, allowing an estimate of less than 10^5 cells/mL of grape must. In the control fermentations, *S. cerevisiae* concentration ranged from 2.1 x 10^8 to 3.5 x 10^8 CFU/mL. *S. cerevisiae* concentrations in the five fermented grape bunches crushed after 6 days from *S. bacillaris* inoculum (T6) showed no significant differences with respect to the control (Figure 4).

Conversely, in some T0 and T1 bunches yeast concentrations showed significantly lower values than the control. These results suggest an inhibitory effect of *S. bacillaris* on *S. cerevisiae* growth when the crushing is performed immediately or 24 hours after the addition of yeast to the grape surface. Since no statistical differences were found 48 hours after grape crushing among T0,

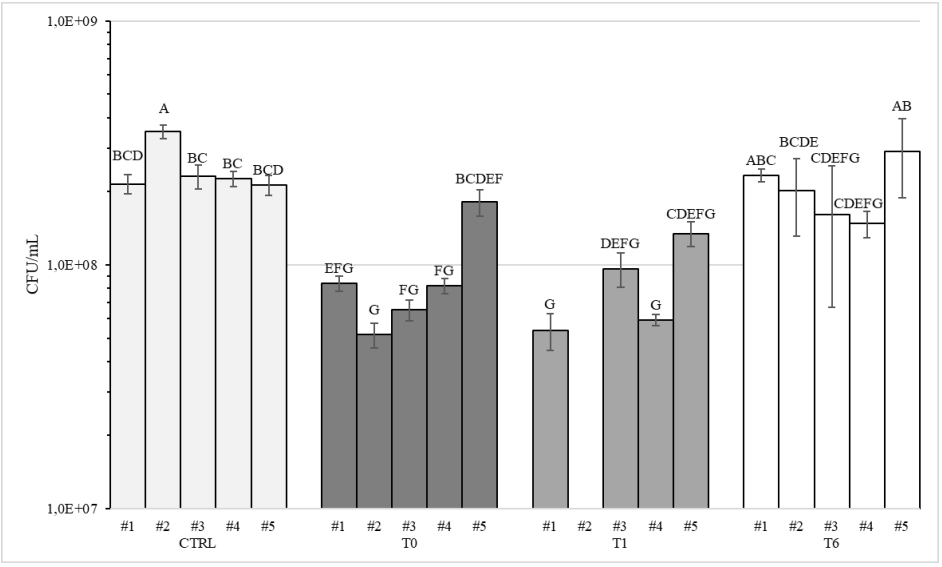

FIGURE 4. *Saccharomyces cerevisiae* concentration at the end of grape bunch fermentation.
Each bar represents a single grape bunch crushed 0 (T0 ■), 24 (T1 ■) or 144 (T6 □) hours after dipping or untreated grape bunches (Ctrl ■). Data are expressed as the average of the replicates ± standard deviations. Different letters indicate significant differences between values ($p = 0.05$).

T1, T6, *S. bacillaris* concentrations (Figure 3), the inhibitory effect could be due to *S. bacillaris* growth increase in T0 and T1 during the following days of fermentation. A previous study evidenced a reduction in the growth rate of *S. cerevisiae* in sequential fermentations with *S. bacillaris*, which is probably due to the high nutrients consumption before *S. cerevisiae* inoculation (Englezos *et al.*, 2016).

Although *S. cerevisiae* growth slowed down in the presence of *S. bacillaris*, all fermentations were completed (sugar residue < 1g/L). Due to its osmophilic properties, *S. bacillaris*produces higher glycerol concentrations than *S. cerevisiae*. This aspect is very important in wine making as high glycerol concentrations positively influence wine mouthfeel and quality (Jolly *et al.*, 2014); therefore, glycerol content was measured. *S. cerevisiae* fermentation showed the lowest glycerol production (5.7 ± 1.05 g/L), while sequential fermentations with grapes crushed at T0, T1, T6 showed 13.5 ± 1.01, 13.4 ± 1.34, 9.3 ± 0.59 g/L glycerol respectively. In all the fermentations in which *S. bacillaris* was present glycerol concentrations were significantly higher than the control.

Moreover, the T0 and T1 fermentations in which *S. cerevisiae* growth inhibition was evident showed high glycerol levels. These results indicate a cell growth higher than T6 after *S. cerevisae* inoculation.

2. Sequential fermentations in natural must at different *S. bacillaris* concentrations

The above results suggest that the concentration of *S. bacillaris* on the grape surface before crushing depends strongly on the length of the period from inoculation to grape bunch collection. This could influence the concentration of *S. bacillaris* in the grape must and its effect on alcoholic fermentation. Therefore, sequential fermentations in natural must (Incrocio Manzoni) were carried out by inoculating different concentrations of *S. bacillaris*. The lower inoculum size was 10^4 cells/mL, mimicking a vineyard treatment applied long before harvest or harsh climatic conditions that reduce the yeast presence on the grape surface. The highest inoculum size was 10^7 cells/mL, representing a vineyard treatment applied close to harvest, repeated treatments in the vineyard or good climatic conditions that promote yeast growth on the grape surface. Forty-eight hours after the fermentation start, *S. cerevisiae* was inoculated at a concentration 10^6 cells/mL. Single strain fermentation with *S. cerevisiae* was performed as a control.

The sequential fermentation rates obtained by measuring CO_2 production were very similar and all of them were slower than those for *S. cerevisiae* single strain fermentation (Figure 5).

The interaction between *S. bacillaris* and *S. cerevisiae*, which resulted in the slowing down of the overall fermentation rates, had already been

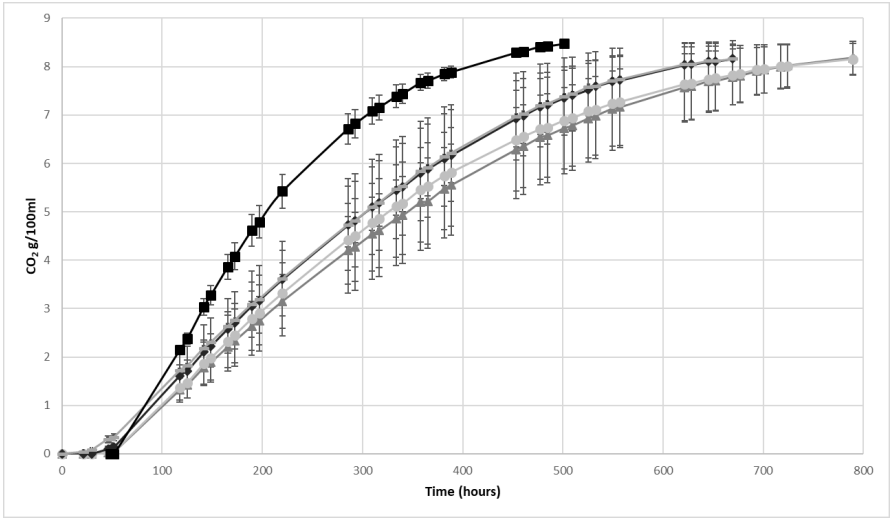

FIGURE 5. Sequential fermentations (CO_2 released/time) in natural grape must of *S. bacillaris* inoculated at different concentrations.

S. cerevisiae single-strain (10^6 cells/mL) (■), *S. bacillaris* (10^4 cells/mL) (▲), *S. bacillaris* (10^5 cells/mL) (●), *S. bacillaris* (10^6 cells/mL) (♦), *S. bacillaris* (10^7 cells/mL) (▬). *S. cerevisiae* was inoculated after 48 h at 10^6 cells/mL.

TABLE 1. Yeasts populations during fermentation, and glycerol, acetic acid and ethanol production at the end of fermentations.

Data are expressed as the average of the replicates ± standard deviations. Different letters indicate significant differences between values ($p = 0.05$). Sc = *S. cerevisiae*, Sb = *S. bacillaris*.

Starter (inoculum cell/mL)	*S. bacillaris* inoculum 10^4 CFU/mL	*S. bacillaris* at 48 h 10^7 CFU/mL	*S. cerevisiae* End of fermentation 10^6 CFU/mL	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (% v/v)
Sc (10^6)	-	-	7.2 ± 0.6 A	3.81 ± 0.15 A	0.57 ± 0.12 A	12.31 ± 0.08 A
Sb (10^4) + Sc (10^6)	1.8 ± 0.3	2.1 ± 0.2	4.0 ± 0.7 B	4.48 ± 0.12 B	0.59 ± 0.07 A	11.46 ± 0.59 A
Sb (10^5) + Sc (10^6)	15.7 ± 3.2	1.5 ± 0.1	3.9 ± 0.5 B	4.75 ± 0.02 B	0.65 ± 0.03 A	11.15 ± 0.19 A
Sb (10^6) + Sc (10^6)	130.0 ± 17.6	2.7 ± 0.7	2.8 ± 0.6 B	4.85 ± 0.22 B	0.65 ± 0.02 A	11.40 ± 0.90 A
Sb (10^7) + Sc (10^6)	1460.0 ± 340	4.6 ± 0.9	3.7 ± 0.2 B	5.59 ± 0.23 C	0.64 ± 0.02 A	11.87 ± 0.54 A

observed when the inoculum size was 10^6 cells/mL (Gobert *et al.*, 2017). Interestingly, in this study a slowdown effect also occurred when the inoculum of *S. bacillaris* was 10 and 100 times lower and 10 times higher than 10^6 cells/mL inoculum.

In all fermentations, *S. bacillaris* concentration reached 10^7 CFU/mL after 48 hours no matter the inoculum size (Table 1).

The presence of *S. bacillaris* at high concentrations confirms that it limits *S. cerevisiae* growth, as previously observed in the yeast growth test on the grape surface. Indeed, at the end of fermentation, the concentration of *S. cerevisiae* was the highest in the control in which *S. bacillaris* was not present. At the same time point in sequential fermentations, the concentration of *S. bacillaris* was less than 10^5 cells/mL (data not shown). Despite the effect on the fermentation trend, sugar

transformation into ethanol was completed in all the fermentations.

As expected, sequential fermentations resulted in higher glycerol concentrations than those in the control (Rantsiou *et al.*, 2012; Bely *et al.*, 2013; Wang *et al.*, 2014; Lemos Junior *et al.*, 2016). No significant differences were found between glycerol concentrations resulting from fermentation with *S. bacillaris* inoculum sizes of 10^4, 10^5, 10^6 cells/mL, but the production of glycerol was significantly higher (5.6 g/L) when the inoculum size was 10^7 cells/mL.

The presence of *S. bacillaris* did not influence acetic acid concentration preserving wine quality, as the detected values were lower than the acetic acid flavour threshold, 0.7 – 1.1 g/L (Lambrechts and Pretorius, 2000).

CONCLUSIONS

In conclusion, when applied to the grape surface at a concentration conducive to antifungal activity, *S. bacillaris* lasted at high concentrations for at least 6 days under laboratory conditions. These results are promising as they suggested that *S. bacillaris* is able to survive on the grape surface. However, more studies are needed to verify the dynamics of *S. bacillaris* introduced to the vineyard environment. Our results indicate that this yeast is a good candidate for being tested as a biocontrol agent close to time of harvest, when synthetic fungicide treatment must be stopped to respect the pre-harvest interval for limiting fungicide residues (Pertot *et al.*, 2016). At a concentration conducive to antifungal activity, *S. bacillaris* positively influences the fermentation process by producing high concentrations of glycerol. Interestingly, this effect on wine was also observed when the inoculum concentration was 10 times higher or lower than the reference concentration. These preliminary results lay the groundwork for further investigations involving open field trials to evaluate *S. bacillaris* as a biocontrol agent in the vineyard and its effect on wine during industrial vinification. In this condition, it would also be necessary to assess the sensory impact of *S. bacillaris* on wine quality.

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