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Potential use of *Starmerella bacillaris* as fermentation starter for the production of low-alcohol beverages obtained from unripe grapes

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ABSTRACT

To obtain beverages with reduced alcohol content, the use of unripe grapes, with low sugar and high malic acid concentration, was recently explored. Due to the low sugar, ethanol and glycerol production is limited during fermentation affecting important sensory aspects such as the palate fullness of these beverages. The high acidity influences their organoleptic quality, as well.

So far, only *S. cerevisiae* starter, used in conventional fermentations, have been tested in this condition, and no selection has been performed to identify alternative yeasts suitable for unripe grape fermentation. *S. bacillaris* is known for the low ethanol tolerance, high glycerol and moderate volatile acidity production. Therefore, this non- *Saccharomyces* yeast have been investigated to improve the quality of low-alcohol beverages. Seven *S. bacillaris* strains were tested in synthetic musts with different sugar and malic acid levels, mimicking natural ripe and unripe grape musts. In all the tested conditions, *S. bacillaris* produced higher glycerol than *S. cerevisiae*. In single-strain fermentation at low sugar and high malic acid no *S. bacillaris* strains was able to transform all the sugars, although the produced ethanol was lower than that at high sugar condition. Therefore, sequential fermentations with *S. cerevisiae* were evaluated at low sugar and high malic acid. In this condition all the sugars were consumed and a significant glycerol increase was found. These results were confirmed when sequential fermentations were run in natural unripe grape must. Moreover, an increase in malic acid degradation, with respect to EC1118 single-strain fermentation, was observed.

1. Introduction

Wine is an alcoholic beverage that is the object of a strong social debate related to a responsible and moderate consumption. Among the alcoholic beverages consumed in the world, wine is in the third position. However, wine consumption worldwide has registered great reduction, determining a deficit between production and purchase (Novello and De Palma, 2013; World Health Organization, 2018). Thus, researches are seeking a way to improve the fermentative process in order to attend consumers demand (Quiróz et al., 2014; Tilloy et al., 2014). Currently, consumers are more concerned about the negative effects of alcohol on health, leading the wine industry to develop strategies for reducing alcohol content (Quiróz et al., 2014). The modern approach to the oenological researches offers possibilities for the development of grape products that are focused on the reduction of alcohol intake (Bovo et al., 2016; Chambers and Pretorius, 2010; Jolly et al., 2013; Quiróz et al., 2014).

Within the scope of controlling or reducing alcohol content without alter physicochemical and sensory characteristics of wines, many techniques have been developed such as use of membranes, distillation processes, genetically-modified strains (Cambon et al., 2006; Pickering, 2000; Tilloy et al., 2014). Since the ethanol amount is linked to sugar content (Meillon et al., 2010), strategies can take into account the reduction of sugar levels of grapes directly in the vineyard.

The reduction of the alcohol content in wine can be pursuit through the addition of selected non-conventional yeasts during fermentation

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traditionally conducted by *Saccharomyces cerevisiae* species (Bely et al., 2013; Ciani and Comitini, 2015; Englezos et al., 2017; Wang et al., 2014). These yeasts naturally present on grape surface and in oenological environment have been considered for a long time just as contaminants such as those of the following genus *Starmarella*, *Candida*, *Hanseniaspora*, *Pichia*, *Metschnikowia*, *Issatchenkia*, *Torulaspora*, *Zygosaccharomyces*, *Saccharomycodes*, *Dekkera* and *Schizosaccharomyces* (Bovo et al., 2011; Carrau et al., 2015; Contreras et al., 2014; Maturano et al., 2015; Rojas et al., 2001). Among them, *Starmerella bacillaris* (synonym *Candida zemplinina*) has been considered one of the most promising species (Englezos et al., 2017; Lemos Junior et al., 2016).

S. bacillaris presents unique characteristics. It has a strong fructophilic character, tolerates low temperatures and is able to grow at high sugar concentrations (Sipiczki, 2003). The technological properties of *S. bacillaris* strains evidenced a low ethanol yield from sugar (glucose and fructose) consumed, high glycerol and a moderate volatile acidity production (Englezos et al., 2015; Magyar and Tóth, 2011; Rantsiou et al., 2017). Recently, the whole genome sequencing of some strains of *Starmerella* genus allowed shedding light on the metabolic pathways at the basis of these technological traits (Lemos Junior et al., 2017a, 2017b; Lemos Junior et al., 2018; Matsuzawa et al., 2015). Moreover, it has been demonstrated that strains of *S. bacillaris* can biologically control *Botrytis cinerea* in grapes and *Penicillium expansum* in apples, and possess interesting technological properties that enhance cider quality (Lemos Junior et al., 2016; Nadai et al., 2018). To achieve ethanol reduction in wine, several studies proposed *Starmerella bacillaris* (synonym *Candida zemplinina*) in combination with *Saccharomyces cerevisiae*, in mixed (co-inoculated and sequential) cultures. Laboratory and pilot scale fermentations always demonstrated a limited decrease up to 1% (v/v) of ethanol and a consistent increase of glycerol (Bely et al., 2013; Englezos et al., 2016, 2017; Lemos Junior et al., 2016; Rantsiou et al., 2012, 2017; Wang et al., 2014).

An interesting approach to reduce alcohol content in wines is to use unripe grapes that are collected before the whole maturation. Unripe grapes can be obtained by cluster tinning. This agronomical practice, applied to regulate the yield levels and to help ripen the crop under poor climatic conditions or excessive crop demand (Fanzone et al., 2011), consists on the removal of about 50% of the cluster one month after bloom. Thinned clusters are usually left on the ground in the vineyard as winemaking by-products. Kontoudakis et al. (2011) and Teslić et al. (2018) evaluated the use of unripe grapes collected during cluster thinning to develop a winemaking procedure aimed at simultaneously reducing the pH and alcohol content of wine. Teslić et al. (2018) mixed unripe and ripe grape musts to decrease the sugar level and to increase acidity. *S. bacillaris* was evaluated in mixed fermentations to reduce the ethanol yield. While Kontoudakis et al. (2011) transformed unripe grape musts into a low-alcohol beverage using conventional *S. cerevisiae* strains. This product was, then, mixed with well-ripened grape juice to reach the suitable alcohol level and phenolic composition after fermentation. The main difficulties in the production of low-alcohol beverages from unripe grapes are related to the high acidity and, as sugar content is very low, a limited ethanol and glycerol production. Moreover, especially for red wines, due to the uncompleted phenolic maturity, excess of astringency and herbaceous aromas can be perceived (Kontoudakis et al., 2011). As one of the sensory effects of ethanol and glycerol, is to contribute to palate fullness (“body”) of wine (Gawel et al., 2007), in low-alcohol beverages obtained from unripe grape an evident lacking of body could be present, affecting their quality. Therefore non-*Saccharomyces* yeasts, such as *S. bacillaris*, that showed glycerol overproduction, could be very interesting to improve the sensory balance of these beverages.

Focusing on high glycerol production, in this study, seven *S. bacillaris* strains were investigated as potential yeast starters for low-alcohol beverages production starting from unripe grape must. As sugar content in unripe grape is very low, the growth at lab condition in presence of limited glucose content and different C/N ratio was evaluated. The growth ability in relation to nutrient assimilation (Van Dijken et al., 1993) was studied, considering growth kinetics parameters (lag phase duration (λ), the specific growth rate value (μ_{max}), and the maximum optical density (OD_{max})). *S. bacillaris* strains were tested in synthetic musts with different sugar levels and mimicking ripe and unripe grape musts, in single-strain fermentation and sequential fermentation with *S. cerevisiae*. Low sugar and high malic acid concentrations were 80 g/L and 12 g/L respectively, as these amounts are usually encountered in unripe grape musts obtained from cluster thinning practice (Bovo et al., 2016). High sugar and low malic acid concentrations were 200 g/L and 2 g/L, respectively. This sugar amount is considered the standard sugar concentration in grape musts (Bely et al., 1990; Lafon-Lafourcade et al., 1979) while malic acid concentration reproduces what found in juices from late-harvest grapes (Volschenk et al., 2006).

Finally, to confirm *S. bacillaris* aptitude to ferment unripe grape must, four strains were tested in sequential fermentation with *S. cerevisiae* in natural must obtained by cluster thinning practice.

2. Materials and methods

2.1. Yeast strains

The yeast strains of *S. bacillaris* used in this work, namely FRI719, FRI728, FRI729, FRI751, FRI754, FRI779 and FRI7100 were isolated from fermenting must obtained from dried grapes, as described by Lemos Junior et al. (2016). *Saccharomyces cerevisiae* EC1118 (Lallemand Italia, Castel D'Azzano, Italy) was used as control.

2.2. Growth in YPD medium

For each *S. bacillaris* strain, a stationary phase yeast preculture with approximately 10^7 to 10^8 cells/mL was obtained after 24 h incubation in YPD broth (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L) at 28 °C.

A suitable aliquot of each yeast culture (final concentration 2–4 × 10^5 cells/mL) was used to inoculate a 96-wells microplate (Greiner Bio-One, Germany) filled with 200 μL of YPD broth.

The plates were incubated at 28 °C for 25 h. Selective growth in YPD was carried out following a completely randomized design (CRD), with three repetitions for each strain analyzed. The strains were the only source of variation in the experiment.

Using a microplate reader, microbial growth was monitored by measuring the optical density at 600 nm every 30 min (Tecan, Mannedorf, Switzerland).

2.3. Growth in modified YPD medium

S. bacillaris strains were also tested in modified YPD medium, containing different concentrations of glucose and peptone. The effects of these components have been studied by means of a binary mixture design, based on the standard formulation of YPD medium, in which peptone and glucose concentrations varied while the concentration of yeast extract (precursor source of amino acids and vitamins) was kept constant. The three defined concentrations of glucose and peptone were 30, 20, and 10 g/L, according to Cruz et al. (2003) and Govindaswamy and Vane (2007).

The mixture design for the two components consisted of five assays, as shown in Table 1. All the assays were repeated at least three times.

2.4. Evaluation of strain growth kinetics in YPD

All the growth kinetics obtained in microplates test were evaluated by measuring the following parameters: lag phase duration (λ), specific growth rate value (μ_{max}), and maximum optical density D (OD_{max} 600 nm) reached by the yeast culture. All the parameters were

Table 1
Experimental design of binary mixture for glucose and peptone trials.

Assays	Coded		Concentrations (g/L)	
	Peptone	Glucose	Peptone	Glucose
1	0.333	0.333	10	10
2	1	0.333	30	10
3	0.333	1	10	30
4	1	1	30	30
5	0.666	0.666	20	20

calculated using R software (www.rstudio.com), through the grofit data library.

2.5. Fermentation trials

A loopful of a 3-day-old culture from YPD agar plate (yeast extract 10 g/L, peptone 10 g/L, glucose 20 g/L) was used to inoculate 10 mL of YPD broth in 50 mL tubes. A stationary phase culture with approximately 10^7–10^8 cells/mL, determined by OD measurements, was obtained after 24 h of incubation at 30 °C. A suitable aliquot of each yeast culture was used to inoculate 120 mL-capacity bottles, fitted with closures that enabled the carbon dioxide to escape, containing 100 mL of sterile must.

Two synthetic musts, differing in sugar and malic acid concentrations, were used: the standard synthetic must with 100 g/L of glucose, 100 g/L fructose and 2 g/L of malic acid (200 − 2), as described by Delfini and Formica (2001), and one modified synthetic must with 40 g/L glucose, 40 g/L fructose and 12 g/L malic acid (80–12). Nitrogen was supplied as casein hydrolysate (0.2 g/L), $(NH_4)_2SO_4$ (0.3 g/L), and $(NH_4)_2HPO_4$ (0.3 g/L), and pH adjusted to 3.00 with KOH. Moreover, a natural grape must, obtained from unripe Glera grapes, a white Italian variety (total reducing sugars 80 g/L, malic acid 11.2 g/L, total acidity 19 g/L, Yeast Assimilable Nitrogen concentration 182 mg/L, pH 2.8), was used.

In single-strain fermentation the inoculum concentration was 1.5–2 × 10^6 cells/mL.

In sequential fermentation the inoculum concentration was 1.5–2 × 10^7 cells/mL for *S. bacillaris* strain and 1.5–2 × 10^6 cells/mL for *S. cerevisiae* EC1118 (Bely et al., 2013). EC1118 was added 48 h after the inoculum of *S. bacillaris* in sequential fermentation in synthetic must and 72 h after in sequential fermentation in natural must.

After yeast inoculation the bottles were incubated at 20 °C for sequential fermentation in synthetic must and at 16 °C for sequential fermentation in natural must.

All experiments were performed in triplicate. CO_2 production was monitored by weighting 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.

At the end of sequential fermentations, a simple olfactory evaluation was performed by a panel of four trained judges, as described by Bovo et al. (2011a), focusing on the presence of important defects, such as volatile acidity and sulphur off-flavours. Each panelist could give a positive, neutral or negative evaluation when the presence of fermentative aromas, no defect, or negative flavour (acetic acid and/or sulphur notes), respectively, were perceived.

2.6. HPLC analysis

Concentrations of residual glucose and fructose, glycerol, acetic acid and ethanol were determined by means of HPLC analysis as described by Nadai et al. (2016). Fifty microliters of filtered (0.22 μm) samples were injected. For the separation of the components a Waters 1525 Binary HPLC Pump (Waters, Milford, MA) equipped with an Aminex HPX_87H HPLC column (Bio-Rad, Hercules, CA) was used. Waters 2414 Refractive Index Detector (Waters, Milford, MA), set at 600 nm wavelength was used for the determination of glucose, fructose, glycerol, and ethanol, while the determination of acetic acid was obtained by using a Waters 2487 Dual Absorbance Detector (Waters, Milford, MA) set to 210 nm. The concentrations, expressed as g/L, were calculated by using calibration curves of the individual compounds.

Concentration of malic acid was detected using the Hyperlab multiparametric analyzer (Steroglass, Perugia, Italy) with the L-malic acid kit (Steroglass, Perugia, Italy).

2.7. Statistical analysis

The parameters obtained by growth kinetic results with the software R Studio were submitted to simple analysis of variance (ANOVA). Regarding the growth in YPD, the results with significant differences ($p \leq 0.05$) in ANOVA were analyzed by Duncan and Fisher Test at 5% probability. With regard to the significant effect of peptone and glucose variations ($p \leq 0.05$), multiple linear, quadratic, special cubic and special quartic regression equations were used to analyze the effects of the independent variables of the process (x1, x2) on the responses (yi).

Statistical analysis of fermentations data was conducted using the software XLSTAT, vers.2016.02 (Addinsoft, Paris, France). Parametric data were submitted to simple analysis of variance (one-way ANOVA) followed by the Tukey test as “post hoc” test. Differences were considered statistically significant for p-value < 0.05.

3. Results and discussion

3.1. Effects of glucose and peptone concentrations on the cell growth

In order to evaluate strain growth at lab condition, the seven *S. bacillaris* strains were tested in YPD medium at 28 °C and OD_{600} values were measured (Supplementary material: Fig. 1 and Table 1). Results indicated a strain-dependent growth behaviours.

As shown in Table 2, the maximum specific growth rate values (μ_{max}) gathered the tested strains in three groups. A first group including FRI751, FRI754, and FRI779 strains showed the highest values, ranging from 0.189 to 0.187 h^{-1}. A second group including the strains FRI729 and FRI7100 (0.184 h^{-1}), followed by the group with lowest specific growth rate (0.181 h^{-1}) included FRI719 and FRI728 strains. The specific growth rate value indicated the change in the number of cells or biomass formed over a period of time, in relation with the assimilation of nutrients from the medium (Ciani and Picciotti, 1995; Phisalaphong et al., 2006).

Lag phase duration varied from 5.97 to 8.64 h. The strains FRI779 and FRI729 presented the lowest values (5.97 and 6.49 h respectively).

The assessment of maximum optical density was also considered. The OD_{max} expressed the highest optical density level reached by the culture, which is proportional to the cell number or biomass produced by yeasts (Ciani and Picciotti, 1995; Phisalaphong et al., 2006). The

Table 2
Evaluation of the growth kinetics parameters.

Strain	Mean ± standard deviation		
	μ_{max} (h^{-1})	λ (h)	OD_{max} (600 nm)
FRI719	0.181 ± 0.001c	7.95 ± 0.313b	1.508 ± 0.025ab
FRI728	0.181 ± 0.001c	6.88 ± 0.301 cd	1.501 ± 0.001ab
FRI729	0.184 ± 0.001b	6.49 ± 0.212de	1.459 ± 0.024c
FRI751	0.189 ± 0.001a	8.64 ± 0.214a	1.535 ± 0.022a
FRI754	0187 ± 0.001a	7.15 ± 0.311c	1.496 ± 0.012abc
FRI779	0.188 ± 0.002a	5.97 ± 0.261e	1.478 ± 0.021bc
FRI7100	0.184 ± 0.002b	7.05 ± 0.412c	1.495 ± 0.031abc

μ_{max}: maximum specific growth rate; λ: lag phase; OD_{max}: maximum optical density. Distinct letters in the same column differ by Duncan test (5% probability).

Table 3
Regression Models of the effect of glucose and peptone concentrations on the growth kinetics of *S. bacillaris*.

Strain	Parameters	Regression model or mean ± SD	R^2	P(F)
FRI719	μ_{max} (h^{-1})	$0.0168 + 0.006*P - 0.006*P^2$	0.9139	0.0003*
	OD_{max} (600 nm)	$1.264 + 0.430*G^2$	0.8338	< 0.0001*
	λ (min)	78 ± 6.36	–	0.0073*
FRI728	μ_{max} (h^{-1})	$0.016 + 0.008*G - 0.007*G^2 - 0.001*P$	0.9999	< 0.0001*
	OD_{max} (600 nm)	$0.663 + 1.679*G - 0.732*G^2 + 0.087*P$	0.9980	< 0.0001*
	λ (min)	$64.26 - 51.9*P + 58.2*P^2$	0.9987	< 0.0001*
FRI729	μ_{max} (h^{-1})	$0.016 + 0.008*P - 0.006*P^2 - 0.0006*P*G$	0.9787	< 0.0001*
	OD_{max} (600 nm)	$0.603 + 2.150*G - 1.175*G^2 + 0.054*P$	0.9999	< 0.0001*
	λ (min)	53.82 ± 18.3	–	< 0.0001*
FRI751	μ_{max} (h^{-1})	$0.019 + 0.004*G - 0.004*G^2 - 0.002*P$	0.9923	< 0.0001*
	OD_{max} (600 nm)	$0.688 + 1.964*G - 0.972*G^2$	0.9836	< 0.0001*
	λ (min)	$96 - 55.14*G + 35.16*G^2 + 16.74*P$	0.9502	< 0.0001*
FRI754	μ_{max} (h^{-1})	$0.017 + 0.007*G - 0.0006*G^2 - 0.002*P$	0.9985	< 0.0001*
	OD_{max} (600 nm)	$0.7833 + 1.4813*G - 0.6550*G^2 + 0.0780*G*P$	0.9995	< 0.0001*
	λ (min)	$47.7 + 23.88*P$	0.9839	< 0.0001*
FRI779	μ_{max} (h^{-1})	$0.019 + 0.005*G - 0.005*G^2 - 0.001*P$	0.9911	< 0.0001*
	OD_{max} (600 nm)	$0.823 + 1.350*G - 0.555*G^2$	0.9943	< 0.0001*
	λ (min)	$39.24 + 68.04*G - 55.2*G^2 + 4.2*P$	0.9961	< 0.0001*
FRI7100	μ_{max} (h^{-1})	0.017 ± 0.001	–	0.0003*
	OD_{max} (600 nm)	$1.2584 + 0.4307*G^2$	0.8880	< 0.0001*
	λ (min)	81.9 ± 12	–	< 0.0001*

Kinetic parameters: μ_{max}, λ (lag phase), and OD_{max} obtained by 600 nm absorbance. P = peptone, G = glucose, SD = standard deviation, R^2 (R-squared) = the value (0–1) of the response variable variation that is explained by a linear model. * Significant at the 5% probability.

highest value was determined for strain FRI751 (1.535), followed by FRI719 (1.508) and FRI728 (1.501). Although strain FRI751 showed longer lag phase duration, suggesting a lower capacity of adaptation in the medium, elevated values were observed for the specific growth rate and maximum optical density. Strain FRI779 exhibited the shorter time of adaptation and a very similar value for the specific growth rate, compared with strain FRI751, however the maximum optical density determined was significantly lower ($p \leq 0.05$). Despite differences, all strains grew well in YPD medium that confirmed to be suitable for *S. bacillaris* growth.

To evaluate the ability of *S. bacillaris* strains to develop at different concentrations of two basic nutrients, peptone (as nitrogen source) and glucose (as carbon source), modified YDP media were used (Table 1, Supplementary material Fig. 2). In this sense, regression models for testing if distinct concentrations of peptone and glucose could affect the cell growth parameters (λ, μ_{max}, and OD_{max}) were developed.

The regression models for each tested strain are shown in Table 3. According to the equations, μ_{max} values were highest when the concentrations of glucose and peptone were similar and at the lowest values, except for strain FRI751 and FRI719. The former showed the highest μ_{max} value when peptone and glucose were at intermediate concentrations (20 g/L of peptone and 20 g/L of glucose). For the latter the μ_{max} variation was dependent only by peptone concentration. In the case of strain FRI7100, it was not possible to fit a regression model; however the experiments clearly demonstrated the negative effect caused by peptone high concentration on FRI7100 rate of growth.

Considering the maximum optical density (OD_{max}), for strains FRI719, FRI751, FRI779 and FRI7100 the values depended only on the glucose concentration. Whereas the remaining strains showed OD_{max} values that are positively correlated with peptone, but they decrease when glucose concentration increase.

For most of the strains the lag phase (λ) was positively correlated with peptone. The lowest λ values were determined when the lowest peptone concentration was present in the medium. The growth values of strains FRI719, FRI729 and FRI7100, did not fit any regression curve, however the results demonstrated that the lag phases of FRI719 and FRI729 were not influenced by glucose concentration, showing low values when low peptone concentration was used (Supplementary material Fig. 2).

Nitrogen and carbon play important roles in the metabolism of living organisms. Thus, the cellular activity level is associated with the concentration of these compounds in the medium and their assimilation by the strains (Cruz et al., 2003). At lab conditions, *S. bacillaris* strains showed the best growth behaviour when glucose and peptone concentrations were similar, evidencing the role of C/N ratio in modulating yeast growth. This ratio is usually unfavourable in grape musts where the nitrogen source is limited (Ribéreau-Gayon et al., 2006). Regarding nitrogen availability, at tested conditions *S. bacillaris* reduced the lag phase duration when low nitrogen was present.

3.2. Fermentation performances in synthetic must at different glucose and malic acid concentrations

Fermentation performances of the 7 strains of *S. bacillaris* were evaluated in synthetic must mimicking ripe (200 g/L sugar and 2 g/L malic acid) and unripe grape juice (80 g/L sugar and 12 g/L malic acid) in single-strain fermentation. EC1118 was tested in single-strain fermentation, as a control. The CO_2 production was monitored throughout the whole fermentation processes. At high sugar - low malic acid condition (200-2), to assess strain fermentation performances, CO_2 production after 48 h of incubation was considered in order to evaluate the adaptation ability of the strains to the must conditions. CO_2 production after 336 h was considered as fermentations showed the widest range of values among the strains. As EC1118 consumed all the sugars at 624 h, at this time *S. bacillaris* fermentations were stopped (Table 4). The fermented musts were analyzed to evaluate glucose and fructose residue and the concentrations of the main fermentation products (glycerol, acetic acid and ethanol).

Fermentation performances were very similar between *S. bacillaris* strains, and none of the tested strains was able to complete the fermentation after 624 h. As expected, *S. bacillaris* strains produced limited CO_2 amounts (3.99–4.59 g/100 mL), while *S. cerevisiae* completed the fermentation producing 9.15 g/100 mL of CO_2. Between *S. bacillaris* strains, FRI779 and FRI7100 showed the highest CO_2 production at 48 h (0.32 and 0.31 g/100 mL of CO_2 respectively), and all *S. bacillaris* values were significantly lower than *S. cerevisiae* (0.89 g/100 mL). Variations in CO_2 production between strains decreased at 336 h, and at 624 h no significant differences were found. As expected, low ethanol (4.53–6.08%, v/v) production was found and acetic acid concentration (0.72–0.89 g/L) was limited, as well. The glucose residue (from 83.61 to 100.82 g/L) was higher than fructose residue (from 1.94 to 18.37 g/L), in agreement with the well-known strong fructophilic character of *S.*

Table 4
CO_2 production during fermentation. Residual glucose and fructose concentrations, and concentrations of the main fermentation products at the end of the fermentation of *S. bacillaris* strains in must with high sugar content (200-2).

Strain	CO_2/100 mL			Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (%v/v)	Ethanol/sugar yield
	48 h	336 h	624 h						
FRI719	0.03 ± 0.00c	2.76 ± 0.17bc	4.59 ± 0.14b	91.84 ± 0.53c	8.60 ± 0.33b	7.69 ± 0.04a	0.80 ± 0.01abc	5.52 ± 0.03 cd	0.44 ± 0.01
FRI728	0.03 ± 0.01c	2.24 ± 0.41c	4.10 ± 0.57b	92.17 ± 0.49c	13.65 ± 0.16 cd	7.08 ± 0.01ab	0.83 ± 0.00ab	5.13 ± 0.04de	0.43 ± 0.01
FRI729	0.09 ± 0.01c	2.42 ± 0.17c	4.22 ± 0.35b	100.82 ± 0.31a	15.72 ± 0.34d	7.73 ± 0.02a	0.89 ± 0.00a	4.68 ± 0.02ef	0.44 ± 0.01
FRI751	0.09 ± 0.01c	2.34 ± 0.23c	3.99 ± 0.46b	98.48 ± 1.93ab	18.37 ± 0.25e	6.37 ± 0.16b	0.74 ± 0.01bcd	4.53 ± 0.12f	0.43 ± 0.02
FRI754	0.08 ± 0.01c	2.70 ± 0.17bc	4.42 ± 0.22b	94.91 ± 3.03bc	11.93 ± 0.38c	7.47 ± 0.24a	0.84 ± 0.04a	5.08 ± 0.18de	0.43 ± 0.03
FRI779	0.32 ± 0.01b	3.10 ± 0.06b	4.39 ± 0.03b	90.27 ± 2.20c	3.26 ± 0.17a	7.29 ± 0.17a	0.72 ± 0.02 cd	5.87 ± 0.12bc	0.44 ± 0.01
FRI7100	0.31 ± 0.05b	3.31 ± 0.26b	4.45 ± 0.10b	83.61 ± 3.61d	4.74 ± 0.30a	7.58 ± 0.63a	0.75 ± 0.06bcd	6.08 ± 0.21b	0.43 ± 0.03
EC1118	0.89 ± 0.06a	7.42 ± 0.01a	9.15 ± 0.02a	0.68 ± 0.32e	4.50 ± 2.06a	4.35 ± 0.25c	0.65 ± 0.06d	11.12 ± 0.33a	0.45 ± 0.01

Data are expressed as the average of three replicates ± standard deviation. Different letters indicate significant differences among values (Tukey's test. $p \leq 0.05$).

bacillaris (Englezos et al., 2017; Mestre et al., 2017). With respect to the residual sugar, glycerol production (6.37 to 7.69 g/L) was high, especially if compared to *S. cerevisiae* (4.35 g/L with almost no sugar residue).

At low sugar - high malic acid condition (80-12) the same time points (48 h, 336 h, 624 h), checked at 200-2, were considered to compare *S. bacillaris* strains CO_2 production (Table 5). At this condition *S. cerevisiae* consumed all the sugars in 288 h (12 days, Table 6), but, surprisingly, none of the tested *S. bacillaris* strains was able to complete the fermentation after 624 h (26 days). This result was unexpected as at 200-2 after 624 h the average value of sugar consumed by *S. bacillaris* strains was 97 g/L, an amount that largely exceeded the concentration at 80-12 (Table 4 and Table 5). *S. bacillaris* strains showed similar fermentation behaviour, although strains FRI779 and FRI7100 confirmed the highest CO_2 production at 48 h (0.34 and 0.35 g/100 mL of CO_2 respectively).

Sugar residues highlighted the fructophilic character of *S. bacillaris* at low sugar content, as well. In fact, all fructose was consumed and glucose residues were always found (from 4.35 to 17.33 g/L) (Table 5). Moreover, ethanol/sugar yields of *S. bacillaris* strains were generally lower than *S. cerevisiae* EC1118.

The production of secondary metabolites was strongly strain dependent. Although sugar content was limited (80 g/L) the glycerol production was generally very high (from 3.54 to 4.82 g/L), with respect to EC1118 (2.55 g/L), and other *S. cerevisiae* strains found in literature. In fact, the average of glycerol concentrations of 24 *S. cerevisiae*, tested in the same condition, was reported to be 1.8 g/L (Bovo et al., 2016). No significant differences were found regarding acetic acid concentrations (from 0.46 to 0.56 g/L) that were comparable to *S. cerevisiae* tested strain (0.55 g/L).

At both 200-2 and 80-12, sugar consumption and ethanol content values were used to calculate ethanol/sugar yields (Table 4 and Table 5). In *S. cerevisiae*, comparing 200-2 with 80-12 values, an increase in ethanol/sugar yield was evident.

The increase can be explained by the different production of glycerol that is dependent on the available sugar content. In *S. bacillaris* a constant ethanol/sugar yield was observed. Due to its osmotolerant behaviour, *S. bacillars* always showed a high glycerol production that limited ethanol/sugar yield at both conditions.

3.3. Sequential fermentation performances in synthetic and natural unripe grape must

S. bacillaris strains were not able to complete the single-strain fermentation at 80-12. Therefore, at the same condition, sequential fermentations were run. *S. cerevisiae* EC1118 was added 48 h after *S. bacillaris*. Moreover EC1118 was tested in single-strain fermentation, as a control.

To assess strains performances, CO_2 production after 48 h of incubation, at the middle (190 h) and at the end of fermentation were considered (Table 6).

As expected, CO_2 production after 48 h was always lower for *S. bacillaris* than *S. cerevisiae* EC1118. Sequential fermentations were completed after 290 h (12 days) except those with FRI729 and FRI7100, that lasted 410 h (17 days). No significant differences in CO_2 produced at the end of the process, between sequential and EC1118 single-strain fermentations were observed. In general, no residual sugar was found.

Ethanol concentration in EC1118 single-strain fermentation (4.90% v/v) was not significantly different from those measured in sequential fermentations with *S. bacillaris* strains (ranging from 4.83 to 4.94% v/v), except for strain FRI7100 (4.28% v/v) that showed a very limited sugar residue.

Glycerol concentration in sequential fermentations was significantly higher than that of *S. cerevisiae*, ranging from 3.58 to 4.49 g/L, whereas EC1118 single-strain fermentation produced only 2.55 g/L of glycerol. In 80–12 sequential fermentation *S. bacillaris* allowed to reach glycerol

Table 5
CO_2 production during fermentation, residual glucose concentration, and concentrations of the main fermentation products at the end of single-strain fermentations in must with low sugar content (80-12) with *S. bacillaris* strains.

Strain	CO_2/100 mL			Residual glucose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (%v/v)	Ethanol/sugar yield
	48 h	336 h	624 h					
FRI719	0.04 ± 0.01b	2.32 ± 0.17a	3.36 ± 0.20a	10.25 ± 1.07bc	4.41 ± 0.65a	0.51 ± 0.09a	4.00 ± 0.62a	0.45 ± 0.08
FRI728	0.02 ± 0.01b	2.01 ± 0.33a	2.91 ± 0.20bc	17.33 ± 4.05a	3.54 ± 0.91a	0.47 ± 0.13a	3.33 ± 0.83a	0.42 ± 0.13
FRI729	0.13 ± 0.02b	2.21 ± 0.14a	2.98 ± 0.11bc	15.34 ± 1.51a	4.43 ± 0.50a	0.56 ± 0.07a	3.89 ± 0.45a	0.48 ± 0.07
FRI751	0.08 ± 0.04b	2.06 ± 0.09a	2.81 ± 0.07bc	13.90 ± 0.86ab	3.65 ± 0.39a	0.48 ± 0.03a	3.93 ± 0.24a	0.47 ± 0.03
FRI754	0.11 ± 0.01b	2.42 ± 0.00a	3.14 ± 0.03ab	13.00 ± 0.17abc	4.46 ± 0.07a	0.52 ± 0.04a	4.07 ± 0.12a	0.48 ± 0.01
FRI779	0.34 ± 0.06a	2.09 ± 0.04a	2.72 ± 0.05c	8.42 ± 0.40 cd	3.98 ± 0.47a	0.38 ± 0.06a	3.30 ± 0.42a	0.36 ± 0.05
FRI7100	0.35 ± 0.08a	2.27 ± 0.11a	2.96 ± 0.10bc	4.35 ± 0.16d	4.82 ± 0.21a	0.46 ± 0.02a	3.95 ± 0.15a	0.41 ± 0.02
EC1118	1.95 ± 0.10c	/	/	-	2.55 ± 0.09b	0.55 ± 0.12a	4.90 ± 0.05b	0.50 ± 0.01

Data are expressed as the average of three replicates ± standard deviation. Different letters indicate significant differences among values (Tukey's test $p \leq 0.05$). "/" fermentation already completed, "-" under detection limit.

Table 6
CO_2 production during fermentation, residual sugar concentration, and concentrations of the main fermentation products at the end of the sequential fermentation of *S. bacillaris* strains with *S. cerevisiae* EC1118 in synthetic must with low sugar content (80-12) at 20 °C.

Strain	CO_2/100 mL			Fermentation time (days)		Glycerol (g/L)	Acetic acid (g/L)	Ethanol (%v/v)
	48 h	190 h	End of fermentation		Residual sugar (g/L)			
FRI719	0.44 ± 0.01ab	2.63 ± 0.41abc	3.26 ± 0.12a	12	-	4.32 ± 0.12a	0.55 ± 0.06b	4.93 ± 0.04a
FRI728	0.62 ± 0.07a	3.05 ± 0.16ab	3.46 ± 0.11a	12	-	3.60 ± 0.12b	1.07 ± 0.03a	4.94 ± 0.01a
FRI729	0.19 ± 0.02c	2.06 ± 0.36c	3.52 ± 0.07a	17	-	4.14 ± 0.10a	0.57 ± 0.17b	4.93 ± 0.03a
FRI751	0.58 ± 0.11a	2.86 ± 0.30abc	3.55 ± 0.04a	12	-	4.26 ± 0.16a	0.87 ± 0.03a	4.83 ± 0.08a
FRI754	0.52 ± 0.10a	2.83 ± 0.47abc	3.59 ± 0.50a	12	-	4.21 ± 0.23a	0.88 ± 0.06a	4.88 ± 0.08a
FRI779	0.60 ± 0.07a	3.04 ± 0.20ab	3.47 ± 0.01a	12	-	3.87 ± 0.20ab	1.04 ± 0.12a	4.92 ± 0.08a
FRI7100	0.25 ± 0.06bc	2.14 ± 0.40bc	3.07 ± 0.34a	17	5.87 ± 0.43	3.58 ± 0.24b	0.44 ± 0.06b	4.28 ± 0.26b
EC1118	1.95 ± 0.10d	3.19 ± 0.04a	3.51 ± 0.03a	12	-	2.55 ± 0.09c	0.55 ± 0.12b	4.90 ± 0.05a

Data are expressed as the average of three replicates ± standard deviation. Different letters indicate significant differences among values (Tukey's test $p \leq 0.05$). "-" under detection limit.

concentration values very close to that obtained by EC1118 in 200-2 single-strain fermentation where high sugars were present. Bely et al. (2013) reported that during sequential-fermentation the reduction in ethanol content varied from 0.39 to 0.90% (v/v) in ripe grape must. As *S. bacillaris* showed the same ethanol/sugar yield at 200-2 and 80-12, due to the limited sugar content at 80-12 the reduction in the ethanol level was not detectable.

Acetic acid concentrations were variable, generally ranging from 0.44 to 0.88 g/L while EC1118 production was 0.55 g/L in single-strain fermentation. Only sequential fermentation with strains FRI728 and FRI779 showed acetic acid valued that exceeded 1 g/L. As *S. bacillaris* acetic acid production in single-strain fermentations was always very limited and lower than EC1118, the presence of both species seems to increase the acetic acid amount, particularly when FRI728 and FRI779 were used. Interestingly, both fermentations that showed long fermentation times (FRI729 and FRI700) exhibited low acetic acid values, comparable to EC1118. These results suggest the effect of strain-specific interaction in modulating the acetic acid concentration at this condition. Olfactory evaluation of all the fermented products obtained by sequential fermentations evidenced no defects in terms of sulphur compound and volatile acidity.

To assess the impact of *S. bacillaris* in enological conditions, sequential fermentation performances of four selected strains were evaluated in natural must obtained from unripe Glera grapes (total reducing sugars 80 g/L and malic acid 11.2 g/L). The four strains were selected on the basis of their acetic acid production excluding those that showed very high values. Fermentation performances were considered both in single-strain and sequential fermentations as well as glycerol productions. Strains FRI729 and FRI7100, showing long sequential-fermentation time and low acetic acid production, together with FRI751 and FRI754, with short sequential-fermentation time and intermediate acetic acid production were tested. Sequential fermentations were run at 16 °C. This value is the regular temperature for yeast fermentation in industrial Glera winemaking. As yeast growth is slowed down by low temperature *S. cerevisiae* EC1118 was added 72 h after *S. bacillaris* (Table 7). EC1118 was tested in single-strain fermentation, as a control.

To assess strains performances, CO_2 production after 72 h of incubation was considered. Due to presence of the same sugar amount (80 g/L) in 80-12 and Glera must, CO_2 production at 190 h and the end of the process was reported.

As expected, *S. bacillaris* CO_2 production after 72 h was always lower than EC1118, confirming results obtained in synthetic must. No significant difference in CO_2 production was observed among the *S. bacillaris* strains. All the strains completed the fermentation in about 18 days (420 h) as well as EC1118 during single-strain fermentation. At this condition no fermentation slowdown, as reported for 80-12, was observed during FRI729 and FRI7100 sequential fermentation. These results can be attributed to the composition of the natural grape must that is richer in additional nutritional elements than synthetic must. Moreover, as low temperature positively affects yeast ethanol resistance (Heard and Fleet, 1988; Gao and Fleet, 1988), fermentation at 16 °C could have differently influenced *S. bacillaris* strains ability to grow.

Ethanol concentration in EC1118 single-strain fermentation (4.62% v/v) was significantly higher than those measured in the sequential fermentations with *S. bacillaris* strains (ranging from 4.49 to 4.51% v/v), except for strain FRI751 (4.55% v/v). In this condition (natural must and low temperature), a limited ethanol reduction was observed due to the presence of *S. bacillaris* during fermentation. However, because of the low sugar concentration, a variation of only 0.1% v/v was reported.

Glycerol concentration in sequential fermentations was significantly higher than that of EC1118, ranging from 3.85 to 4.22 g/L, whereas EC1118 single-strain fermentation produced only 2.59 g/L of glycerol. Due to presence of *S. bacillaris*, an average increase of 54% in glycerol concentration was registered in sequential fermentation with respect to *S. cerevisiae* single-strain fermentation. This finding is of particular interest as one of the sensory effects of glycerol is to contribute to palate fullness (body) of wine (Gawel et al., 2007). Alcohol gives the same in-mouth impression, but when using unripe grape its concentration is

Table 7
CO_2 production during fermentation, sugar residues and concentrations of the main fermentation products at the end of the sequential fermentation of *S. bacillaris* strains with *S. cerevisiae* EC1118 in natural must with low sugar content at 16 °C.

Strain	CO_2/100 mL			Fermentation time (days)	Residual sugar (g/L)	Glycerol (g/L)	Malic acid (g/L)	Acetic acid (g/L)	Ethanol (% v/v)
	72 h	190 h	420 h						
FRI729	0.20 ± 0.02c	2.01 ± 0.38a	3.28 ± 0.10a	17.5	–	4.02 ± 0.05ab	9.59 ± 0.13b	0.68 ± 0.04a	4.49 ± 0.03b
FRI751	0.19 ± 0.02c	1.83 ± 0.41a	3.27 ± 0.08a	17.5	–	3.85 ± 0.09b	9.57 ± 0.09b	0.68 ± 0.02a	4.55 ± 0.03ab
FRI754	0.23 ± 0.02bc	1.98 ± 0.13a	3.24 ± 0.06a	17.5	–	3.89 ± 0.04b	9.57 ± 0.11b	0.69 ± 0.01a	4.49 ± 0.02b
FRI7100	0.29 ± 0.06b	2.49 ± 0.20a	3.35 ± 0.02a	17.5	–	4.22 ± 0.13a	9.30 ± 0.08c	0.68 ± 0.02a	4.51 ± 0.07b
EC1118	1.22 ± 0.04a	2.09 ± 0.03a	3.41 ± 0.01a	17.5	–	2.59 ± 0.01c	10.18 ± 0.04a	0.52 ± 0.02b	4.62 ± 0.01a

Data are expressed as the average of three replicates ± standard deviation. Different letters indicate significant differences among values (Tukey's test $p \leq 0.05$). "-" under detection limit.

very low. In this way, the lack of body could be recovered, at least partially, by glycerol increase.

Although a limited increase in acetic acid concentration was registered in sequential fermentation, the values are very similar between strains. Olfactory evaluation of the fermented products evidenced no defects in terms of volatile acidity and sulphur notes.

Malic acid content at the end of the fermentations was also evaluated.

Compared to the initial malic acid content (11.2 g/L) a decrease was observed in all the fermentations, particularly in the case of sequential fermentations (final concentration 9.30–9.59 g/L). *S. cerevisiae* species possess a poor ability to degrade malic acid and this is dependent on the must concentration (Ribéreau-Gayon et al., 2006). In this condition 9% of malic acid reduction was observed in EC1118 single-strain fermentation. In sequential fermentations an average value of 15.1% was found. Results indicated that during fermentation of unripe grape must *S. bacillaris* could give a contribution to the malic acid degradation. This activity is extremely relevant in the case of unripe grapes that have a high acidity level, mainly due to malic acid content (Dupas de Matos et al., 2017).

4. Conclusions

Among the innovative approaches to the production of low-alcohol beverages, the use of unripe grapes must obtained by cluster thinning practice has been proposed, recently. This strategy can be considered effective if it is supported by the selection of new starter yeasts that fit the peculiar composition of this must. The fermentation of low glucose and high malic acid must led to the production of an alcoholic beverage with high acidity and lacking of body due to low ethanol and glycerol level. The non- *Saccharomyces* yeast *S. bacillaris* is known for the low ethanol tolerance, high glycerol and moderate volatile acidity production. Seven *S. bacillaris* strains were screened to evaluate their growth behaviour, firstly at lab condition. In presence of different nutrient concentrations, C/N ratio has been found to modulate cell development. The best nutrient combination was found when glucose and peptone concentrations were similar and peptone present at low concentration, suggesting a limited nitrogen requirement.

At high sugar condition (200-2) during single-strain fermentation the seven *S. bacillaris* strains consumed less than half of the sugar present in the must and produced 68% more glycerol than *S. cerevisiae*. High glycerol concentration was produced by *S. bacillaris* at 80-12, as well. The average glycerol level was 64% higher than *S. cerevisiae* and comparable to that produced by *S. cerevisiae* in standard must (200-2). The low sugar level did not inhibit the growth of *S. bacillaris*, as expected from an osmophilic yeast, although all the strains were not able to complete the fermentation. Moreover no change in the ethanol/sugar yield was found between the two conditions. Fermentations at 80-12 showed an average glucose residue of 14.7%. Therefore sequential fermentations with *S. cerevisiae* EC1118 were performed at the same condition, with the aim to complete the fermentation. In this case, all the sugars were consumed and a glycerol increase from 40% to 69% was found. These results were confirmed when sequential fermentations were run in natural unripe grape must. In this condition the malic acid degradation was also evaluated. This aspect is relevant as malic acid concentration in unripe grape is usually high. Indeed, it can reach up to 25–30 g/L (Volschenk et al., 2006; Dupas de Matos et al., 2017) with most of the values in the range of 10–15 g/L (Sabir et al., 2010; Bovo et al., 2016). All *S. bacillaris* strains increased malic acid degradation with respect to EC1118 single-strain fermentation, although this was not enough to reach the suitable wine acidity. Therefore, must or wine treatment would be still required, if *S. bacillaris* is used as technological starter. Moreover, unripe grapes show inadequate phenolic balance determining excess of astringency and herbaceous aromas. Therefore, further studies will be required to overcome the lacking phenolic maturity. For example, the investigation of the yeast-derived esters in wine can be of interest. In fact esters are responsible for wine 'fruitiness' attribute, but their suitable levels can either mask 'vegetative' odours (Escudero et al., 2007).

This work demonstrated that *S. bacillaris* can be considered a valuable tool in the production of low-alcohol beverages starting from unripe grape. If used in sequential fermentation with *S. cerevisiae*, this yeast improved the product quality by means of increasing glycerol content and malic acid degradation. Indeed, these properties have a great impact on sensory aspects counterbalancing the lacking of palate fullness due to low ethanol, and partially reducing the high acidity level present in the grape must.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.05.006.

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