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Exploitation of the non-*Saccharomyces* yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: physiological and molecular characterization

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Abstract

Nowadays, the use of non-*Saccharomyces* yeasts in combination with *Saccharomyces cerevisiae* is a state-of-the-art strategy to improve complexity and enhance the analytical composition of the wines. This application has stimulated the interest of understanding how the non-*Saccharomyces* yeasts can contribute to the quality of the wines. The study presented here explores the potential use of *Starmerella bacillaris* (synonym *Candida zemplinina*) under winemaking conditions. Physiological and genetic characterization of sixty-three isolates of *Starm. bacillaris*, previously isolated from four different varieties of grapes, were carried out. Both analyses revealed a low level of diversity between the isolates of *Starm. bacillaris*, while the fermentation trials in laboratory scale demonstrated the good enological performance of this species. The strong fructophilic character of this species and its ability to produce low quantities of ethanol and acetic acid and high amounts glycerol was confirmed. The results, presented here, demonstrated a potential application of this non-*Saccharomyces* species in mixed wine fermentations with *Saccharomyces cerevisiae*.

Keywords: non-*Saccharomyces* yeast, *Starmerella bacillaris*, *Candida zemplinina*, wine fermentation, mixed fermentation, biodiversity

1. Introduction

Non-*Saccharomyces* yeasts have a substantial role in the early stages of wine fermentations, since they can reach populations up to $10^6 – 10^8$ colony forming units (cfu)/mL depending on fermentation conditions and grape health (Heard and Fleet, 1988). With the increase of alcohol concentration, tolerant indigenous or commercial strains of *Saccharomyces cerevisiae* take over and complete the transformation of the must sugars into ethanol, CO_2 and other secondary metabolites (Fleet and Heard, 1993). The amount of biomass produced by the non-*Saccharomyces* species during the first part of fermentation is sufficient to have an impact on the wine composition and consequently their contribution during the fermentation process cannot be ignored (Fleet, 2008).

Starmerella bacillaris (synonym *Candida zemplinina*) (Duarte et al., 2102) is a non-*Saccharomyces* yeast, isolated for the first time in Napa Valley (California, USA) in 2002, under the name EJ1 (Mills et al., 2002). Interestingly this *Candida* sp. strain was able to ferment exclusively the fructose from Chardonnay must, without affecting the concentration of the glucose. One year later, Sipiczki (2003), assigned this *Candida* sp., to a novel species under the name *C. zemplinina*, due to the significant differences observed for the ribosomal RNA sequence from that of the related species *Candida stellata* (Sipiczki, 2004). *Starm. bacillaris* has since been reported to have a potentially important role in the winemaking industry, due to the extremely fructophilic character and the poor ethanol yield from sugar consumed (Magyar and Tóth, 2011). Several ecology studies have reported the presence of this species during spontaneous must fermentations in different countries (Alessandria et

al., 2013; Bokulich et al., 2013; Bokulich et al., 2014; Milanović et al., 2013; Mills et al., 2002; Rantsiou et al., 2013), suggesting the involvement of this species in the fermentation process.

Starm. bacillaris presents some very interesting characteristics from the enological point of view, such as growth at high concentrations of sugars and low temperatures (Sipiczki, 2003; Tofalo et al., 2012) and production of low levels of acetic acid, acetaldehyde and significant amounts of glycerol from consumed sugars (Magyar and Tóth, 2011). It differs from the other common non-*Saccharomyces* yeasts, since it can survive and resist until the end of the alcoholic fermentation due to its ability to tolerate high concentrations of ethanol present in the wine (Rantsiou et al., 2012). These phenotypic characteristics support the use of *Starm. bacillaris* in winemaking. It is however necessary to explore the species biodiversity and understand its behavior during the fermentation process in order to produce wines with desirable characteristics.

The goal of this study was to investigate further the potential of *Starm. bacillaris* to be employed in alcoholic fermentations. For this purpose, a collection of isolates of different origin (grape variety and geographical region of isolation) was subjected to molecular and physiological characterization, with emphasis on parameters of enological interest. Three molecular techniques, namely SAU-PCR with two different primers, SAG1 and SCA, and Rep-PCR technique, with the primer $(GTG)_5$, were applied in order to understand the genetic diversity between the isolates. Afterwards, physiological tests, which focused on growth in varying concentrations of ethanol and total SO_2 as well as production of extracellular hydrolytic enzymes, were conducted. Lastly, micro-fermentation trials were carried

out in natural grape must to evaluate the fermentation potential of *Starm. bacillaris* isolates.

2. Materials and methods

2.1. Yeast strains

In the present study we used 63 *Starm. bacillaris* isolates (Table 1), deposited in the yeast culture collection of the DISAFA (Dipartimento di Scienze Agrarie, Forestali e Alimentari, University of Torino, Italy). They were previously isolated from four different grape varieties cultivated in Italy, namely Picolit, Mondeuse, Erbaluce (Alessandria et al., 2013; Rantsiou et al., 2013; Urso et al., 2008) and Barbera (Supplementary Table 1). All the isolates were previously identified by Restriction Fragment Length Polymorphism (RFLP) analysis of the 5.8S ITS rDNA region (Granchi et al., 1999) by using the restrictions enzymes *Mbo*I and *Dra*I (Promega, Milan, Italy) as previously described by (Sipiczki, 2004) in order to distinguish *Starm. bacillaris* from *C. stellata*. Confirmation of the identification was obtained by sequencing the 26S rRNA gene, D1-D2 loop, as previously described (Kurtzman and Robnett, 1997). The isolates were stored on YPD slants (1% yeast extract, 2% bacteriological peptone, 2% glucose and 2% agar, all w/v) at 4 $^{\circ}$C.

2.2. Molecular characterization of the isolates

2.2.1. Sau-PCR analyses

One milliliter of an overnight culture was centrifuged at 14,000 rpm for 10 min. and the pellet obtained was subjected to DNA extraction by using the protocols described in Mills et al. (2002). For the molecular typing of *Starm. bacillaris* isolates,

two-hundred (200) ng of genomic DNA was digested with the restriction endonuclease *SAU3AI* (Promega, Milano, Italy) as reported by the manufacturer. Afterwards, 1 µL of the reaction was transferred into a 50 µL PCR reaction solution containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 mM of primer [either SAG1 (5' – CCGCCGCGATCAG – 3') or SCA (5' – CCGCCGCGATCCA – 3')] and 1.50 U *Taq*–polymerase (Sigma). PCR amplification was performed as described by Cocolin et al. (2004). PCR products were separated by the use of a horizontal electrophoresis, on 2% (w/v) agarose gels in 0.5 X TBE buffer solution and containing 0.5 mg/L of ethidium bromide (Sigma, Milano, Italy) at 120 V for 120 min. A molecular weight ladder (Promega) was loaded in each gel, in order to normalize the different profiles obtained. The fingerprints of the 63 *Starm. bacillaris* isolates were subject to a cluster analysis by using the computer software package BioNumerics, version 4.0 (Applied Maths, Kortrijk, Belgium). The unweight pair group with arithmetic averages (UPGMA) and the Pearson product moment correlation coefficient were used in order to calculate the similarities between the different profiles and group together genetically similar isolates of *Starm. bacillaris*.

2.2.2. Rep-PCR analysis

One hundred micrograms of genomic DNA extracted from the pure cultures of *Starm. bacillaris* were subjected to a Rep-PCR analysis, using the microsatellite oligonucleotide sequence $(GTG)_5$ as described by Lederer et al. (2013) with some modifications. Reactions were carried out in a final volume of 25 µL, containing 10 mM of Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl, 0.2 mM of each dNTP, 1 mM of primer $(GTG)_5$ (5' – GTGGTGGTGGTGGTG – 3') and 1.50 U *Taq*–polymerase

(Sigma). The PCR protocol was the as follows: initial denaturation at 95 °C for 5 minutes, 31 cycles of amplification at 94 °C for 3 seconds, 92 ºC for 30 seconds, 40 ºC for 1 minute and final extension at 65 ºC for 8 minutes. PCR products were electrophoresed, visualized and analyzed as reported above.

2.3. Physiological characterization of Starm. bacillaris isolates

2.3.1. Growth in ethanol and SO_2

The growth tests in ethanol and SO_2 were carried out in microplates as described by Arroyo Lopez et al. (2010) and Tofalo et al. (2012), with some modifications. Yeast Nitrogen Base with amino acids (YNB, 6.7 g/L, [Remel, Lenexa, KS, USA]), pH 5.5, was supplemented with 20 g/L of glucose and sterilized by filtration with a 0.2 μm membrane filter (VWR, Milan, Italy). This medium was then supplemented with different amounts of ethanol (Sigma) to reach final concentrations of 0, 8, 10, 12 and 14 % v/v. Similarly, to test growth in the presence of SO_2, different amounts of total SO_2 were added (after adjustment to pH 3.0) to reach final concentrations of 0, 25, 50 and 100 and 150 mg/L. *Starm. bacillaris* cells were prepared by inoculating one colony in 1 mL of YPD medium and after 24 h of incubation at 25 ºC, the cells were centrifuged at 9000 rpm for 10 min. The pellet was washed two times with sterile physiological solution (8 g/L NaCl) and then re-suspended in the same buffer to obtain a concentration of about 10^6 CFU/mL. The diluted cells (20 μL) were mixed with 180 μL YNB prepared as above. The microplates were incubated at 25 ºC and the optical density (OD) was measured at 630 nm using a microtiter plate reader (Savatec Instruments, Torino, Italy) every 24 hours for 2 days after an orbital shaking of 30 s, in order to re-suspend the cells in the medium before

the measurement. The cell growth was determined by the ratio (%) between the growth of the isolates in broth with and without ethanol or SO_2 at the specific incubation times. These tests were carried out in triplicate. Isolates, with a percentage of growth ratio < 10% were considered not resistant. *S. cerevisiae* strain ScBa44, a strain deposited in the collecton of the DISAFA, was used as a control.

2.3.2. H_2S production

The capacity of the isolates to produce different levels of hydrogen sulfide (H_2S) was evaluated by using the BIGGY agar medium (Oxoid). The medium was spot inoculated and incubated at 25 ºC for 48 hours. An arbitrary scale from 1 (white color = no production) to 5 (dark brown = high production) was used to evaluate the production of H_2S (Comitini et al., 2011).

2.3.3. Enzymatic activities

Esterase activity

The ability of the yeasts to hydrolyze esters was evaluated on a medium, containing 10 g/L bacteriological peptone, 5 g/L NaCl, 0.1 g/L $CaCl_2$ and 15 g/L agar. After sterilization, the medium was cooled to about 50 ºC and 5 mL of sterile Tween 80 was added. The agar plates were spot inoculated and then incubated at 30 ºC for 48 hours. Esterase activity was indicated by a visible opaque halo around the colony (Buzzini and Martini, 2002; Slifkin, 2000).

β-glucosidase activity

This activity was determined as reported by Rosi et al. (1994), by using a

medium containing 5 g/L arbutin (hydroquinone b-D-glucopyranoside, Sigma), 6.7 g/L YNB with amino acids and 20 g/L of agar. The pH of the medium was adjusted to 5.0 before the sterilization. Two milliliters of sterile ferric ammonium citrate (1%) were added to 100 mL of the medium before pouring the plates. The strains were spot inoculated onto the medium and then incubated at 25 ºC for 3 days. The presence of the β-glucosidase activity was determined by a discoloration of the medium to a brown color. Two strains of *Metschnikowia pulcherrima* W1 and W3 (Mills et al., 2002) were used as positive controls.

Glycosidase activity

Glycosidase activity was evaluated by following the method proposed by Hildebrand and Caesar (1989). Sterile petri dishes were filled with a medium containing 6.7 g/L YNB with amino acids, 1 g/L glucose, 2 g/L rutin (quercetin-3-rutinoside, Sigma) and 20 g/L agar. Isolates were spot inoculated on the surface and incubated at 25 ºC for 3 days. *M. pulcherrima* W1 and W3 (Mills et al., 2002) were used as a positive controls. A clear zone around the colony was considered as a positive reaction.

Pectinase activity

The method proposed by Charoenchai et al. (1997) was used to evaluate the ability of *Starm. bacillaris* isolates to produce extracellular pectinases. For the preparation of the medium 12.5 g/L polygalacturonic acid, 6.8 g/L potassium phosphate (pH 3.5), 6.7 g/L YNB with amino acids, 10 g/L glucose and 20 g/L agar were mixed. Isolates were spot-inoculated onto the surface and then incubated at 30 ºC for 3 days. A

clear halo around the yeast colony was a sign of the poly–galacturonate degradation.

Protease activity

Agar plates were prepared by using 3 g/L malt extract, 3 g/L yeast extract, 5 g/L bacteriological peptone, 10 g/L glucose, 5 g/L NaCl and 20 g/L agar as described by Comitini et al. (2011). Separately, an equal volume of skim milk solution (10 % w/v) was prepared by using sterile water. After the sterilization, the two media were mixed and then poured on sterile petri dishes. Before pouring the medium on the plates, pH was adjusted at pH 3.5 with the addition of 0.1 M HCl. The isolates were spot inoculated and then incubated at 25 ^{o}C for 3 days. The presence of a clear zone around the yeast colony indicated the protease activity.

2.4. Microfermentations

The enological performances of *Starm. bacillaris* were evaluated by micro-fermentations trials. Fermentations were carried out in 50 mL tubes with loose screw cap, containing 25 mL of Barbera grape must (120 g/L glucose, 124 g/L fructose, 4.39 titratable acidity as g/L of tartaric acid , pH 3.20 and absence of ethanol). Before the inoculation, the must was thermically treated at 60 °C for 50 min and the absence of viable populations was evaluated by plating 100 μL of the must after the treatment on WLN medium (Biogenetics, Milano, Italy), followed by an incubation at 28 °C for 5 days. The must was inoculated with a 24 h pre–culture grown in the same must in order to reach an initial cell concentration of 10^6 cells/mL, which was determined through a microscopical cell count. Fermentations were carried out in duplicate at 25 °C under

static conditions for 14 days. The *S. cerevisiae* strain ScBa44, was used as a control strain in these fermentation trials.

2.5. Chemical analysis

After 14 days of incubation at 25 °C, the sugars consumption (glucose and fructose) and the ethanol, glycerol and acetic acid production were directly evaluated by HPLC (Giordano et al., 2009). Acetaldehyde and total sulfur dioxide were determined by using enzymatic kits, (Megazyme International, Wicklow, Ireland, and R-Biopharm, Darmstadt, Germany, respectively) following the manufacturer's instructions.

2.6. Data analysis

DNA fragments obtained from each molecular technique used in this study were converted to a binary code matrix and the presence or absence of each band was considered as "1" and "0" respectively. Genetic structure and variability between the populations of *Starm. bacillaris* isolated from the two Italian regions (Friuli Venezia Giulia and Piedmont) was carried out through Analysis of Molecular Variance (AMOVA) and calculation of the pairwise F_{st} value (Weir and Cockerham, 1984) over all loci, by the software ARLEQUIN 3.5.1.3 (Excoffier and Lischer, 2010).

The data obtained from the results of the physiological characterization and the chemical composition of the wines was subjects to a Principal Component Analysis (PCA), in order to evaluate the intraspecific biodiversity of the 63 isolates of *Starm. bacillaris*. Statistical analyses were performed using the software package IBM SPSS Statistics (version 19.0, IBM Corp., Armonk, NY, USA).

3. Results

3.1. Molecular characterization

For the molecular characterization of the 63 *Starm. bacillaris* isolates, two different approaches were used, SAU-PCR with the primers SAG1 and SCA and Rep - PCR with the primer $(GTG)_5$. The dendrogram of similarity, which combines the results of these molecular techniques, is presented in Figure 1. All methods gave a fingerprint composed by 20 to 25 bands, based on which the dendrogram was constructed (data not shown). As it can be seen, a small degree of differentiation of the profiles was obtained when the coefficient of similarity was 70% or higher. In particular, six clusters and three single strains were distinguished. The molecular characterization, revealed a high level of similarity between isolates of *Starm. bacillaris* coming from different sources of isolation. In particular, strains of *Starm. bacillaris* isolated from grapes located in Friuli Venezia Giulia such as Picolit, were grouped together with strains isolated from grapes located in Piedmont (Barbera, Erbaluce and Mondeuse).

3.2. Population analysis

The genetic divergence between *Starm. bacillaris* isolates was carried out by AMOVA analysis and F_{st} determination, as shown in Table 2. For this analysis, the population of isolates from each Italian region (Friuli Venezia Giulia and Piedmont) was considered as a group. The contribution of variation within the two populations was always very high, whereas differences between groups constituted up to 6.84 % of variation. To further investigate associations between genetic differentiation and geographic distance, pairwise region comparison was carried out. The genetic

differentiation was very low, with F_{st} values very low up to 0.02054 that corresponds to a little genetic distance (Wright, 1978). For the analysis of variation between grape varieties, the assemblage of several populations was considered as a group and then compared with the population of every single variety (Table 2). The results of the analysis again underlined little genetic distance between *Starm. bacillaris* isolated from different grape varieties in the same Italian region.

3.3. Physiological characterization

The results of the screening for the presence of specific enzymatic activities, as well as the ability of the 63 *Starm. bacillaris* strains to produce H_2S are reported in the Supplementary Table 2. β-glucosidase activity was found in only 5% of the isolates, namely FC12, FC54 and FC55, isolated from Picolit grapes, indicating possible production and activity by these isolates also during the fermentation.-Protease activity was detected in 77% of the isolates. Seven of them, gave positive results for ester-hydrolase activity. Pectinolytic and glycosidase activity was not found in any isolate of *Starm. bacillaris*.

The ability of *Starm. bacillaris* to grow at different concentrations of ethanol (8%, 10%, 12% and 14% v/v) and total SO_2 (from 25 mg/L to 150 mg/L) at 24 and 48 hours of incubation at 25 ºC was also investigated. The growth was determined by comparing the growth with and without ethanol or total SO_2 at pH 3.0, respectively (Supplementary Table 2). Many (71%) of the isolates grew at 8% (v/v) of ethanol after 24 h, while, when the incubation time increased to 48 hours, 90% of the isolates were able to grow at all the concentrations of ethanol.-Ethanol growth was independent from the origin of the isolates.

In addition, 83% and 40% of the isolates were able to grow in the presence of 25 and 50 mg/L of SO_2 respectively, while few isolates (11%) grew at 100 and 150 mg/L of SO_2 after 24 h. With an extension of the incubation time to 48 h, the number of the isolates that were able to grow at 50 mg/L of SO_2 increased up to 54%. Interestingly, only the strain EER2C was able to grow at the highest concentrations tested, while the strain BC16 was totally inhibited by the SO_2.

The semi-quantitative determination of H_2S production demonstrated that 96% of the isolates produced a medium amount, while only 4% produced H_2S at low levels.

3.4. Microfermentation trials in grape juice

The chemical composition of the wines produced from the pure cultures of the 63 *Starm. bacillaris* isolates are reported in Table 3. Concerning residual sugars, values ranged from 1 to 140 g/L. Only the strain Cz03 (isolated from Barbera grapes) was able to consume nearly all the sugars present in the must after 14 days of alcoholic fermentation. This strain demonstrated a completely different behavior from the others studied. All isolates, except the strain BC53, were able to consume nearly all the fructose present in the must demonstrating a clear fructophilic character. In the case of strain PE3WA, isolated from Erbaluce grapes, a clear preference for fructose was observed while no glucose was consumed.

Concerning acetic acid production, quite few strains gave values greater than 0.5-0.75 g/L, with two strains showing a very low production (0.2-0.3 g/L). Fermentation purities (ratio between acetic acid and ethanol produced) were also very low (0.03 - 0.09), highlighting the good enological performance of this species. Glycerol production reached significant levels, ranging from 4.9-10.9 g/L.

Ethanol production was homogeneous: 74% of the strains produced ethanol in the range 8.0-9.5%, while 19% were able to produce more than 9.5 % (v/v) of ethanol. Interestingly, the strain Cz03 was able to produce up to 14% by volume of ethanol. Acetaldehyde was produced at medium-low quantity ranging from 1.56 to 56.02 mg/L. Finally, sulfite production was below 10 mg/L for all isolates (data not shown).

The chemical composition (residual sugar, organic acids, glycerol, ethanol and acetaldehyde) of the wines, obtained after 14 days of fermentation and the data from the growth tests at 50 mg of SO_2 after 24 hours (under oenological conditions) (Eglinton et al., 2000), presence or absence of enzymatic activities and the H_2S production were used to evaluate the physiological diversity of this species.

A Principal Component Analysis (PCA) on the physiological data and the chemical analysis of the wines was carried and the output are presented on the Figure 2. The PCA obtained explained about 75% of the total variance. The score plot of PC1 and PC2 is presented in the Figure 2. The PC1 was able to discriminate the isolates BC53 and PE3WA for the high level of residual sugars present in the wine, while the isolates Cz03 and Cz08 were differentiated from the other isolates due to the high content of ethanol produced. As shown on the PC2, one group of strains FC12, FC54 and FC55, which gave positive results for β-glucosidase activity was well differentiated

4. Discussion

In the last years, there is an increasing interest for selection of starter cultures of non-*Saccharomyces* yeasts for the winemaking industry, mainly due to their ability to enhance the analytical composition of the wines (Ciani et al., 2010; Fleet, 2008). Many

studies have proposed the potential use of *Starm. bacillaris* in wine fermentations, in combination with *S. cerevisiae* strains. Rantsiou et al. (2012) have demonstrated the possibility to use *Starm. bacillaris* in combination with *S. cerevisiae* in sweet wine fermentation to reduce the acetic acid production. In particular, the coinoculation strategy was able to decrease the acetic acid content up to 0.3 g/L of acetic acid, while sequential inoculation led to a reduction of about half of acetic acid compared the *S. cerevisiae* in pure culture. Recently, Giaramida et al. (2013) and Zara et al. (2014) have demonstrated an increase of glycerol content and a decrease of the alcoholic degree respectively, when mixed fermentations with *S. cerevisiae* were carried out in pilot scale. These applications support the use of *Starm. bacillaris,* which could be a clever choice to achieve various desired results, mainly due to its fructophilic character and the poor ethanol yield from sugar consumed (Magyar and Tóth, 2011). Given the impact of the wine yeast to the wine composition and aroma and the yeast intra-species natural biodiversity, it is important to select an appropriate *Starm. bacillaris* strain to use as a starter.

In this context, 63 isolates of *Starm. bacillaris* from grapes, musts and alcoholic fermentations of four different varieties grapes cultivated in Italy were taken into consideration. The molecular characterization revealed a high level of similarity and isolates from different grapevine cultivars grouped together. These results are in accordance, with previous findings (Pfliegler et al., 2013; Rantsiou et al., 2008; Rantsiou et al., 2012; Tofalo et al., 2012) and confirm the hypothesis of the genetic homogeneity of this species.

For the phenotypic characterization, parameters of enological interest were studied and *Starm. bacillaris* isolates were screened for the presence of enzymatic

activities and growth at different concentrations of SO_2 and ethanol. The results demonstrated β–glucosidase and protease enzymatic activity, in agreement with general observations that non-*Saccharomyces* yeasts are more probable to be in possession of extracellular hydrolytic enzymes than *S. cerevisiae* strains (Fia et al., 2005; Strauss et al., 2001). In particular, protease activity could be beneficial for the microorganisms during the fermentation progress, by liberating assimilable nutrient sources, such as amino acids and peptides. The screening for resistance at increasing concentrations of SO_2 and ethanol revealed the importance of these parameters on the potential growth of the *Starm. bacillaris* in wine. In particular, it was shown that 50 mg of total SO_2 are sufficient to inhibit the growth of *Starm. bacillaris*. On the other hand, in many cases the ethanol concentration affected the lag phase, increasing its length, in agreement with other authors (Tofalo et al., 2012). This ability of *Starm. bacillaris* to survive and grow at high concentrations of ethanol could explain the persistence of this species up to the middle-end phase of the fermentation process (Mills et. al., 2002; Rantsiou et al., 2012).

The fermentation performance of the isolates confirmed the preference of this species to consume fructose rather than glucose (Magyar et al., 2011; Mills et al., 2002; Sipiczki 2003; Soden et al., 2000) and produce relevant quantities of glycerol, low levels of acetaldehyde, acetic acid and SO_2 (Magyar and Tóth, 2011). An interesting finding of this study that deserves attention is the ethanol production by *Starm. bacillaris* isolates. Up to now *Starm. bacillaris* was considered as a low producer of ethanol (Magyar and Tóth, 2011). However, all the isolates examined in this study, demonstrated a modest to good production of ethanol reaching values as high as 14.0 % (v/v).

5. Conclusion

To our knowledge, this is the first time that a large number of *Starm. bacillaris* isolates were subjected to molecular and physiological characterization. The results obtained, support new applications of *Starm. bacillaris* that could render the organoleptic profiles of the wines more complex thanks to the enzymatic activities that this species possess. The exploitation of this yeast in combination with *S. cerevisiae* should be further investigated, in order to better understand the action of the enzymes during the fermentation process. Since all the data presented here were obtained from pasteurized natural must, the performance and consequently the dominance of the *Starm. bacillaris* in real winemaking conditions must be explored. The use of selected strains of *Starm. bacillaris* and *S. cerevisiae* in mixed wine fermentations could be further proposed since it may have a strong impact on the alcohol reduction as recently demonstrated by Giaramida et al. (2013).

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Table 1. Isolates used in this study

Geographical region	Grapevine cultivar	Number of the isolates	Isolates code
Piedmont (Italy)	Erbaluce	18	EIF1LD, EHR3B, EFR3B, EHR3C, EIF7LD, EIF5LA, EFR3A, EIF7LA, EER3C, EER2A, CBW16, ECR2D, PE3WA, EER2D, ELCFOLC, EIF5LG, PE3WE, EIF7LB
	Mondeuse	4	ECF7LC, BaF7LGg, NaF21LLE, NaF21LA
	Barbera	9	C.z 01, C.z 02, C.z 03, C.z 04, C.z 05, C.z 06, C.z 07, C.z 08, C.z 09
Friuli Venezia Giulia (Italy)	Picolit	32	C1, C2, CBE1, CBE2, CBE4, CBE6, CBE7, CBE8, CBE10, FC12, FC49, FC50, FC51, FC52, FC54, FC55, BC14, BC15, BC16, BC17, BC19, BC20, BC21, BC22, BC46, BC53, BC54, BC55, BC58, BC59, BC60, BC62

Table 2. Analysis of molecular variance (AMOVA), F_{st} values and distribution of variance components (%) among groups (AG), among populations within groups (APWG) and within populations (WP) based on the bands obtained from the fingerprinting data of the *Starm. bacillaris* populations from the two Italian regions and grape varieties. (P (random value<observed value)<0.00001).

PCR - Technique	Source of Variation	Percentage of Variation (AG)	Percentage of Variation (APWG)	Percentage of Variation (WP)	F_{st}	P (r<0)
SAU-PCR (SAG1)	Friuli Venezia Giulia and Piedmont	- 0.23	2.28	97.95	0.02054	P < 0.00001
	Variety E and other Varieties	2.41	0.54	97.05	0.02945	P < 0.00001
	Variety M and other Varieties	-1.66	2.44	99.22	0.00781	P < 0.00001
	Variety B and other Varieties	-1.15	2.56	98.59	0.01410	P < 0.00001
SAU-PCR (SCA)	Friuli Venezia Giulia and Piedmont	-1.49	1.08	100.41	-0.00406	P < 0.00001
	Variety E and other Varieties	1.22	-0.87	99.65	0.00349	P < 0.00001
	Variety M and other Varieties	-0.28	-0.04	100.32	-0.00315	P < 0.00001
	Variety B and other Varieties	0.04	0.10	100.06	-0.00060	P < 0.00001
Rep – PCR $(GTG)_5$	Friuli Venezia Giulia and Piedmont	6.84	-5.47	98.63	0.01370	P < 0.00001
	Variety E and other Varieties	2.27	-1.55	99.28	0.00720	P < 0.00001
	Variety M and other Varieties	-7.84	1.37	106.48	-0.06477	P < 0.00001
	Variety B and other Varieties	-2.46	0.85	101.65	-0.02459	P < 0.00001

E: Erbaluce, M: Mondeuse and B: Barbera.

Table 3. Chemical analysis of the wines obtained by fermentation of the pure cultures of *Starm. bacillaris* tested. Data are means ± standard deviations.

Isolates	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (% v/v)	Ethanol yield (g/g)	Sugar used for 1% ethanol production (g)	Fermentation purity[a]	Acetaldehyde (mg/L)
EIF1LD	87.32 ± 0.13	0.73 ± 0.31	9.59 ± 0.00	0.53 ± 0.05	8.88 ± 0.08	0.45 ± 0.00	17.61 ± 0.14	0.06 ± 0.01	9.03 ± 3.68
EHR3B	76.93 ± 5.80	1.26 ± 1.03	9.80 ± 0.06	0.70 ± 0.00	9.44 ± 0.25	0.45 ± 0.01	17.60 ± 0.25	0.07 ± 0.01	7.92 ± 8.40
EFR3A	80.28 ± 2.16	0.20 ± 0.28	9.97 ± 0.56	0.51 ± 0.02	9.31 ± 0.49	0.45 ± 0.02	17.62 ± 0.66	0.05 ± 0.00	24.88 ± 0.53
EIF7LA	77.39 ± 1.37	0.47 ± 0.01	9.36 ± 0.18	0.56 ± 0.04	9.35 ± 0.14	0.44 ± 0.01	17.82 ± 0.42	0.06 ± 0.00	16.67 ± 3.05
EER3C	80.13 ± 1.63	0.60 ± 0.03	9.63 ± 0.02	0.66 ± 0.03	9.28 ± 0.09	0.45 ± 0.01	17.64 ± 0.35	0.07 ± 0.01	14.91 ± 5.34
EER2A	81.01 ± 3.11	0.45 ± 0.05	9.54 ± 0.03	0.59 ± 0.03	9.26 ± 0.16	0.45 ± 0.02	17.61 ± 0.65	0.06 ± 0.00	19.64 ± 4.01
PE3WA	120.55 ± 2.08	3.51 ± 3.84	6.14 ± 0.46	0.24 ± 0.01	7.21 ± 0.49	0.45 ± 0.01	17.40 ± 0.37	0.03 ± 0.00	11.81 ± 6.68
EER2D	80.46 ± 0.79	0.23 ± 0.32	9.41 ± 0.03	0.49 ± 0.00	9.30 ± 0.10	0.45 ± 0.01	17.60 ± 0.30	0.05 ± 0.00	14.64 ± 0.76
ELCFOLC	81.20 ± 3.71	0.70 ± 0.52	9.13 ± 0.47	0.49 ± 0.03	9.30 ± 0.30	0.45 ± 0.00	17.47 ± 0.10	0.05 ± 0.00	23.98 ± 1.34
EFR3B	80.33 ± 7.37	0.79 ± 1.11	8.97 ± 0.53	0.46 ± 0.03	9.28 ± 0.50	0.45 ± 0.00	17.59 ± 0.04	0.05 ± 0.01	7.96 ± 4.68
EHR3C	76.53 ± 1.33	1.25 ± 0.43	10.03 ± 0.03	0.74 ± 0.07	9.41 ± 0.01	0.45 ± 0.00	17.71 ± 0.16	0.08 ± 0.01	31.58 ± 8.87
EIF5LA	85.90 ± 1.82	1.91 ± 0.77	8.85 ± 0.33	0.57 ± 0.01	8.76 ± 0.19	0.44 ±0.00	17.87 ± 0.09	0.07 ± 0.00	19.23 ± 6.68
EIF7LD	69.72 ± 1.59	0.14 ± 0.20	10.07 ± 0.11	0.54 ± 0.00	9.95 ± 0.10	0.45 ± 0.01	17.54 ± 0.32	0.05 ± 0.00	18.56 ± 4.96
CBW16	70.02 ± 4.67	0.47 ± 0.02	9.96 ± 0.13	0.59 ± 0.05	10.06 ± 0.20	0.46 ± 0.00	17.28 ± 0.12	0.06 ± 0.00	33.94 ± 5.57
EIF5LG	73.58 ± 2.21	1.14 ± 2.22	7.38 ± 3.11	0.66 ± 0.01	7.77 ± 2.33	0.44 ± 0.01	18.01 ± 0.24	0.09 ± 0.03	44.74 ± 8.97
ECR2D	84.83 ± 0.80	1.44 ± 0.30	9.56 ± 0.04	0.53 ± 0.01	8.94 ± 0.02	0.45 ± 0.00	17.68 ± 0.09	0.06 ± 0.00	10.81 ± 5.29
PE3WE	86.97 ± 0.18	0.72 ± 0.43	9.33 ± 0.33	0.63 ± 0.10	8.51 ± 0.32	0.43 ± 0.01	18.42 ± 0.61	0.07 ± 0.01	33.19 ± 6.97
EIF7LB	79.99 ± 0.99	0.93 ± 0.51	9.32 ± 0.45	0.58 ± 0.01	9.34 ± 0.32	0.45 ± 0.01	17.50 ± 0.44	0.06 ± 0.00	12.22 ± 6.81
BaF7LGg	85.28 ± 3.11	0.95 ± 0.63	9.36 ± 0.55	0.62 ± 0.07	9.15 ± 0.28	0.46 ± 0.00	17.29 ± 0.12	0.07 ± 0.01	34.59 ± 8.96
ECF7LC	74.32 ± 5.51	0.22 ± 0.32	9.74 ± 0.33	0.59 ± 0.00	9.61 ± 0.48	0.45 ± 0.01	17.67 ± 0.28	0.06 ± 0.00	9.41 ± 10.58
NaF21LA	78.79 ± 1.03	0.60 ± 0.20	9.49 ± 0.20	0.55 ± 0.02	9.32 ± 0.05	0.45 ± 0.00	17.71 ± 0.04	0.06 ± 0.00	24.59 ± 4.04
NaF21LLE	75.38 ± 8.27	0.28 ± 0.39	9.72 ± 0.10	0.55 ± 0.06	9.61 ± 0.24	0.45 ± 0.01	17.56 ± 0.46	0.06 ± 0.00	10.07 ± 7.33
FC55	81.75 ± 0.01	1.67 ± 0.01	9.35 ± 0.01	0.47 ± 0.00	9.03 ± 0.07	0.44 ± 0.00	17.83 ± 0.14	0.05 ± 0.00	19.56 ± 0.69
FC12	70.68 ± 16.65	0.96 ± 0.71	9.68 ± 0.36	0.58 ± 0.08	9.54 ± 0.58	0.44 ± 0.02	18.09 ± 0.72	0.06 ± 0.00	29.11 ± 6.45
BC 17	84.44 ± 1.41	5.17 ± 0.87	8.84 ± 0.01	0.49 ± 0.01	8.68 ± 0.02	0.44 ± 0.01	17.83 ± 0.22	0.06 ± 0.00	10.96 ± 4.82

C1	77.60 ± 2.53	0.88 ± 0.74	9.72 ± 0.37	0.45 ± 0.06	9.27 ± 0.32	0.44 ± 0.01	17.91 ± 0.27	0.05 ± 0.00	1.56 ± 6.43
CBE6	88.11 ± 2.57	0.24 ± 0.34	9.56 ± 0.55	0.53 ± 0.02	8.63 ± 0.20	0.44 ± 0.00	18.09 ± 0.07	0.06 ± 0.00	19.46 ± 1.61
BC53	79.77 ± 0.82	60.02 ± 0.89	4.98 ± 0.04	0.33 ± 0.01	5.11 ± 0.02	0.43 ± 0.01	18.53 ± 0.26	0.07 ± 0.00	23.58 ± 3.82
BC20	94. 47 ± 0.12	1.63 ± 0.23	8.89 ± 0.47	0.65 ± 0.02	8.28 ± 0.35	0.44 ± 0.02	17.92 ± 0.72	0.08 ± 0.01	23.72 ± 5.42
BC60	77.05 ± 12.16	0.71 ± 0.36	9.50 ± 0.01	0.59 ± 0.08	9.30 ± 0.15	0.44 ± 0.03	17.90 ± 1.05	0.06 ± 0.01	56.02 ± 5.25
CBE1	77.15 ± 2.91	0.51 ± 0.19	10.11 ± 0.08	0.48 ± 0.05	9.59 ±0.05	0.45 ± 0.01	17.39 ± 0.24	0.05 ± 0.01	29.33 ± 3.92
C2	72.42 ± 3.57	0.27 ± 0.27	9.87 ± 0.06	0.50 ± 0.01	9.87 ± 0.20	0.45 ± 0.00	17.40 ±0.04	0.05 ± 0.00	5.40 ± 8.04
FC52	81.47 ± 0.34	0.64 ± 0.06	8.79 ± 0.35	0.50 ± 0.03	9.25 ± 0.03	0.45 ± 0.00	17.54 ± 0.02	0.05 ± 0.00	32.24 ± 12.66
FC49	85.84 ± 4.14	0.77 ± 0.66	9.34 ± 0.11	0.58 ± 0.01	8.85 ± 0.11	0.44 ± 0.01	17.83 ± 0.32	0.07 ± 0.00	24.86 ± 12.05
FC51	78.51 ± 5.24	0.59 ± 0.32	8.94 ± 0.13	0.57 ± 0.08	9.38 ± 0.04	0.45 ± 0.01	17.62 ± 0.52	0.06 ± 0.01	34.02 ± 0.90
CBE10	92.05 ± 2.89	0.28 ± 0.40	9.25 ± 0.15	0.59 ± 0.00	8.60 ± 0.05	0.45 ± 0.01	17.68 ± 0.48	0.07 ± 0.00	28.13 ± 3.21
FC54	76.77 ± 0.80	0.19 ± 0.27	9.57 ± 0.19	0.61 ± 0.02	9.54 ± 0.16	0.45 ± 0.00	17.56 ± 0.19	0.06 ± 0.00	10.92 ± 5.46
CBE8	88.46 ± 1.36	0.00 ± 0.00	9.56 ± 0.01	0.59 ± 0.01	8.61 ± 0.08	0.44 ± 0.01	18.12 ± 0.33	0.07 ± 0.00	52.71 ± 0.22
BC19	75.45 ± 0.75	0.18 ± 0.25	9.57 ± 0.01	0.57 ± 0.02	9.33 ± 0.09	0.44 ± 0.01	18.09 ± 0.24	0.06 ± 0.00	26.27 ± 2.32
BC59	79.65 ± 1.98	0.88 ± 0.48	9.35 ± 0.40	0.69 ± 0.04	9.39 ± 0.25	0.45 ± 0.01	17.44 ± 0.21	0.07 ± 0.00	47.78 ± 3.47
BC14	80.28 ± 2.16	0.20 ± 0.28	9.97 ± 0.56	0.51 ± 0.02	9.31 ± 0.49	0.45 ± 0.02	17.62 ± 0.66	0.05 ± 0.00	24.88 ± 0.53
BC55	81.93 ± 8.00	0.78 ± 0.31	9.67 ± 0.19	0.69 ± 0.09	8.76 ± 0.55	0.43 ± 0.05	18.53 ± 2.10	0.08 ± 0.02	48.52 ± 9.62
BC22	83.88 ± 4.45	0.00 ± 0.00	9.80 ± 0.03	0.66 ± 0.03	9.17 ± 0.03	0.45 ± 0.01	17.51 ± 0.55	0.07 ± 0.00	12.81 ± 7.43
BC16	90.08 ± 2.72	0.55 ± 0.22	9.22 ± 0.26	0.75 ± 0.03	8.70 ± 0.15	0.45 ± 0.00	17.68 ± 0.04	0.09 ± 0.00	38.34 ± 8.46
BC15	77.09 ± 7.87	1.20 ± 0.99	9.56 ± 0.47	0.73 ± 0.11	9.56 ± 0.46	0.45 ± 0.00	17.38 ± 0.11	0.08 ± 0.01	28.57 ± 8.56
CBE2	89.58 ± 2.10	0.27 ± 0.39	9.15 ± 0.13	0.60 ± 0.11	8.67 ± 0.04	0.44 ± 0.01	17.82 ± 0.21	0.07 ± 0.01	29.64 ± 2.78
BC62	94.03 ± 1.08	1.07 ± 0.07	8.72 ± 0.01	0.52 ± 0.05	8.49 ± 0.01	0.45 ± 0.00	17.59 ± 0.10	0.06 ± 0.01	17.08 ± 6.97
CB E7	72.18 ± 2.02	0.82 ± 0.34	9.50 ± 0.03	0.57 ±0.02	9.69 ± 0.07	0.45 ± 0.00	17.68 ± 0.04	0.06 ± 0.00	18.15 ± 2.27
CBE4	73.08 ± 12.52	0.79 ± 0.59	9.93 ± 0.14	0.69 ± 0.09	9.18 ± 0.17	0.43 ± 0.02	18.56 ± 1.09	0.07 ± 0.01	30.10 ± 8.6
BC58	84.57 ± 3.62	2.29 ± 1.47	9.35 ± 0.08	0.57 ± 0.03	8.98 ± 0.06	0.45 ± 0.01	17.53 ± 0.45	0.06 ± 0.00	24.93 ± 6.08
BC21	95.21 ± 4.32	1.20 ± 1.30	8.94 ± 0.40	0.61 ± 0.02	8.20 ± 0.23	0.44 ± 0.00	18.05 ± 0.17	0.07 ± 0.00	30.03 ± 1.34
FC 50	76.19 ± 0.84	0.04 ± 0.06	9.37 ± 0.06	0.49 ± 0.03	9.50 ± 0.00	0.45 ± 0.00	17.71 ± 0.10	0.05 ± 0.00	19.72 ± 4.78
BC 46	81.74 ± 0.04	1.54 ± 0.00	9.15 ± 0.01	0.46 ± 0.00	8.86 ± 0.00	0.43 ± 0.00	18.18 ± 0.00	0.05 ± 0.00	32.87 ± 0.82
BC54	81.00 ± 5.37	1.39 ± 1.47	8.94 ± 0.41	0.53 ± 0.02	9.25 ± 0.27	0.45 ± 0.01	17.52 ± 0.22	0.06 ± 0.00	33.45 ± 4.53
C.z 01	82.49 ± 7.61	0.71 ± 0.55	9.28 ± 0.67	0.41 ± 0.02	9.24 ± 0.60	0.45 ± 0.01	17.45 ± 0.45	0.04 ± 0.00	7.36 ± 2.47
C.z 02	94.36 ± 0.44	1.54 ± 0.83	9.00 ± 0.44	0.62 ± 0.11	8.41 ± 0.22	0.45 ± 0.01	17.66 ± 0.41	0.07 ± 0.00	13.05 ± 5.77
C.z 03	1.05 ± 0.70	0.25 ± 0.06	5.91 ± 0.80	0.37 ± 0.18	14.53 ± 0.01	0.47 ± 0.00	16.73 ± 0.07	0.03 ± 0.01	51.75 ± 2.37
C.z 04	79.05 ± 10.47	0.25 ± 0.35	9.94 ± 0.37	0.56 ± 0.03	8.78 ± 0.16	0.42 ± 0.03	18.81 ± 1.49	0.06 ± 0.00	33.53 ± 10.20
C.z 05	81.80 ± 14.86	0.76 ± 0.71	9.92 ± 0.46	0.74 ± 0.27	9.23 ± 0.76	0.45 ± 0.01	17.53 ± 0.25	0.08 ± 0.02	17.86 ± 3.81

C.z 06	87.17 ± 0.67	0.44 ± 0.05	9.26 ± 0.12	0.56 ± 0.07	8.57 ± 0.36	0.43 ± 0.02	18.31 ± 0.70	0.07 ± 0.01	55.01 ± 11.11
C.z 07	80.28 ± 2.16	0.20 ± 0.28	9.97 ± 0.56	0.51 ± 0.02	9.31 ± 0.49	0.45 ± 0.02	17.62 ± 0.66	0.05 ± 0.00	24.88 ± 0.53
C.z 08	60.76 ± 5.33	0.24 ± 0.21	10.91 ± 0.25	0.78 ± 0.12	10.06 ± 0.01	0.43 ± 0.01	18.23 ±0.49	0.08 ± 0.01	30.69 ± 7.31
C.z 09	98.17 ± 23.91	2.45 ± 2.52	7.57 ± 2.33	0.41 ± 0.24	8.07 ± 1.38	0.44 ± 0.01	17.81 ± 0.23	0.05 ± 0.02	16.47 ± 4.64
ScBa44	0.64 ± 0.04	1.76 ± 0.19	7.04 ± 0.08	0.28 ± 0.04	14.57 ±0.03	0.47 ± 0.01	16.58 ± 0.05	0.02 ± 0.00	-[b]

[a]Fermentation purity: acetic acid g/L / ethanol % vol.

[b]Not determined.

Figures legends

Figure 1. Dendrogram of similarity obtained by comparison of the different fingerprinting profiles of the *Starm. bacillaris* isolates examined in this study with the two molecular techniques. The upper scale indicates the percentage of the similarity.

Figure 2. Projection of the *Starm. bacillaris* strains in the plain of the first and second principal according to the physiological characterization.

Figure 1

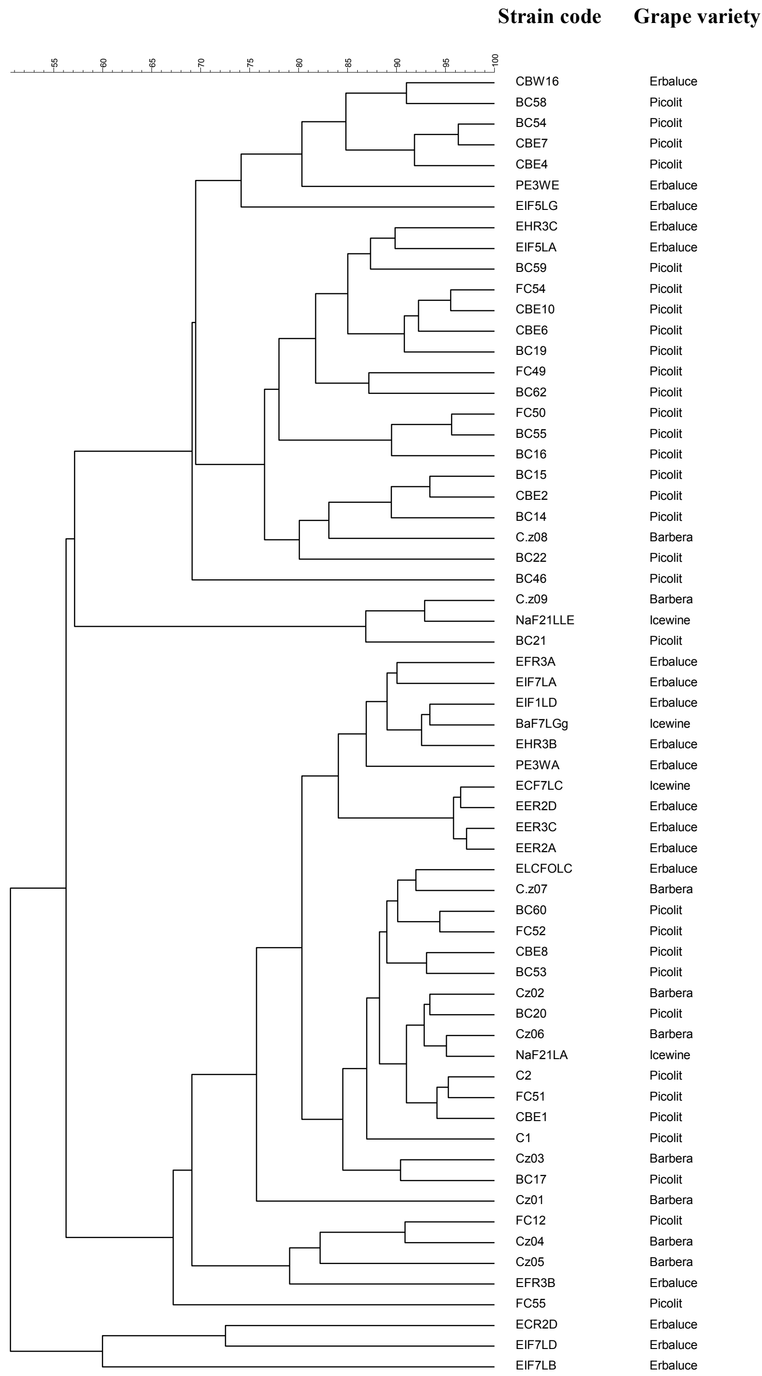
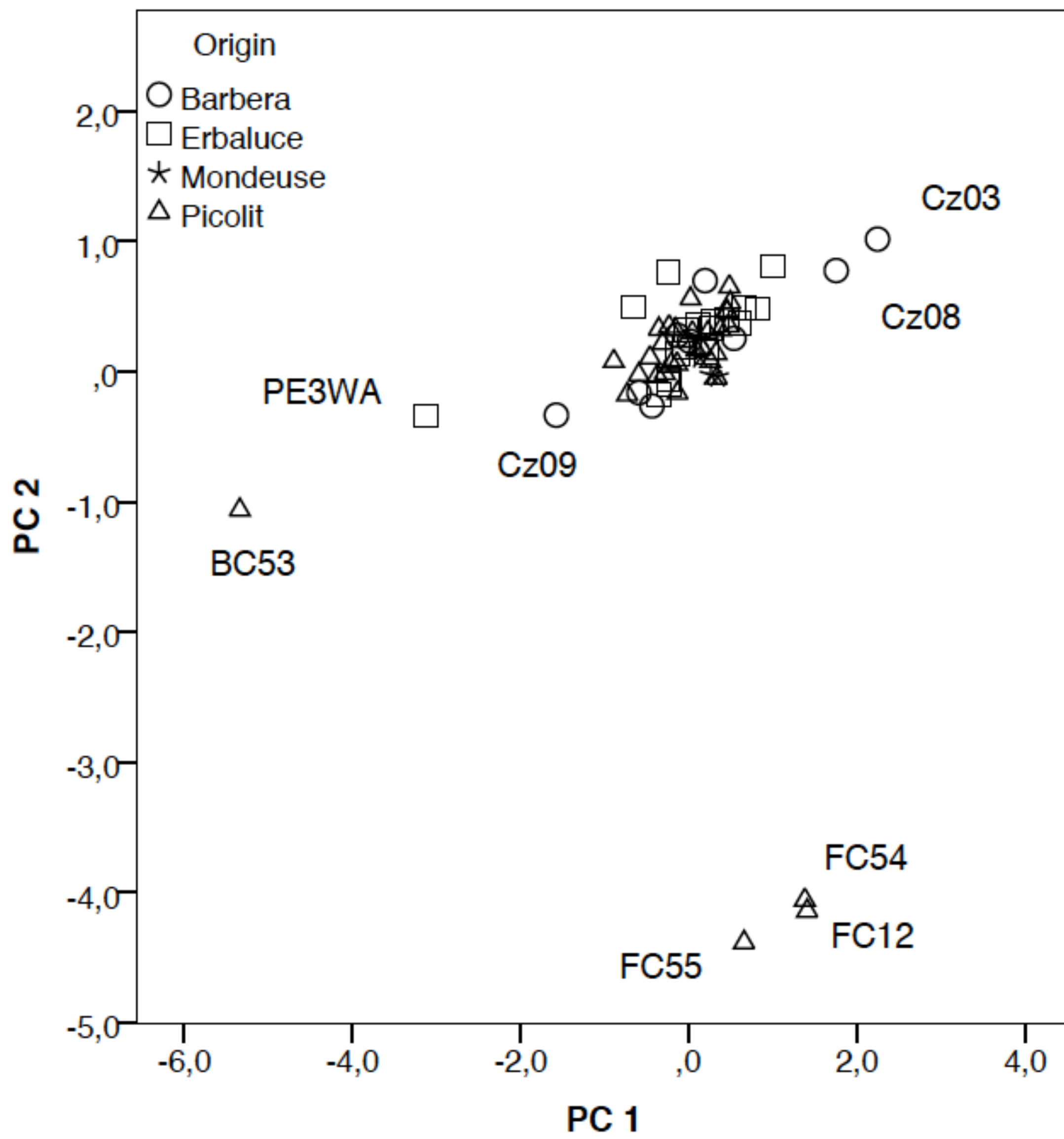


Figure 2



Supplementary table 1. List of the isolates from Barbera must fermentations and considered in this study

Strain name	Place of isolation	Source of isolation	Day of FA	Year	Vineyard	Winery code
Cz01	Italy, Asti wine area	Fermented must	1 Day	2013	Organic vineyard	Mu
Cz02	Italy, Asti wine area	Fermented must	1 Day	2013	Organic vineyard	Mu
Cz03	Italy, Asti wine area	Fermented must	1 Day	2013	Organic vineyard	Mu
Cz04	Italy, Asti wine area	Fermented must	1 Day	2013	Conventional vineyard	Isa
Cz05	Italy, Asti wine area	Fermented must	1 Day	2013	Conventional vineyard	Isa
Cz06	Italy, Asti wine area	Fermented must	1 Day	2013	Conventional vineyard	Isa
Cz07	Italy, Asti wine area	Fermented must	3 Day	2013	Conventional vineyard	Inc
Cz08	Italy, Asti wine area	Fermented must	3 Day	2013	Conventional vineyard	Inc
Cz09	Italy, Asti wine area	Fermented must	3 Day	2013	Conventional vineyard	AIV

Supplementary table 2. Results obtained from the physiological characterization of the *C. zemplinina* isolates used in this study expressed as: positive/negative for the enzymatic activities; a scale from 1 (low) to 5 (high) for H_2S production; and ratio between the growth of the isolates in broth with and without ethanol or SO_2 times 100 at the specific incubation times.

ol es	Enzymatic activities			H_2S	SO_2 growth (mg/L)								Ethanol growth (% vol.)							
	b-gluc.[a]	Prot.[b]	Est.[c]		24 hours of incubation				48 hours of incubation				24 hours of incubation				48 hours of incubation			
					25	50	100	150	25	50	100	150	8	10	12	14	8	10	12	1
F LD	-	+	-	3	100%	12%	7%	7%	100%	100%	5%	3%	53%	30%	30%	30%	100%	100%	95%	9
H 3B	-	+	+	3	100%	10%	8%	8%	100%	21%	12%	8%	24%	13%	12%	10%	76%	94%	91%	9
FR A	-	+	-	3	100%	13%	7%	8%	100%	96%	18%	14%	33%	24%	21%	21%	87%	78%	78%	6
F LA	-	+	+	3	97%	15%	8%	11%	100%	100%	7%	17%	57%	28%	21%	21%	100%	100%	100%	9
ER C	-	+	-	3	100%	26%	2%	2%	100%	88%	66%	45%	29%	10%	16%	11%	65%	99%	100%	9
ER A	-	+	+	3	99%	9%	6%	6%	100%	84%	7%	7%	33%	10%	16%	16%	84%	89%	89%	8
E3 A	-	-	-	3	87%	17%	14%	13%	100%	12%	9%	8%	20%	19%	17%	16%	60%	26%	23%	5
ER D	-	+	+	3	46%	19%	8%	8%	100%	100%	7%	7%	57%	28%	20%	8%	94%	98%	100%	8
LC OL	-	+	-	3	60%	19%	8%	7%	100%	100%	7%	7%	67%	32%	27%	18%	97%	100%	97%	6
FR B	-	+	-	3	23%	7%	9%	9%	80%	8%	2%	2%	8%	9%	18%	2%	16%	13%	10%	1
H 3C	-	+	-	3	63%	16%	4%	6%	82%	18%	1%	1%	15%	7%	9%	4%	17%	20%	19%	1
F LA	-	-	+	3	75%	28%	6%	6%	100%	100%	4%	5%	35%	25%	20%	10%	78%	66%	40%	1
F LD	-	+	-	3	81%	50%	1%	3%	40%	10%	1%	1%	1%	5%	2%	4%	2%	4%	5%	[illegible]
B 16	-	-	-	3	65%	5%	3%	3%	100%	72%	1%	1%	7%	4%	6%	9%	70%	63%	55%	5
F LG	-	-	-	3	72%	11%	3%	2%	100%	96%	5%	2%	11%	5%	7%	8%	68%	58%	48%	4
CR D	-	+	-	3	73%	8%	4%	3%	100%	80%	2%	2%	9%	5%	7%	7%	94%	94%	64%	5
E3 E	-	+	-	3	78%	7%	5%	2%	100%	73%	1%	1%	6%	3%	6%	7%	57%	56%	36%	2
F LB	-	-	-	3	83%	13%	4%	6%	100%	94%	8%	6%	18%	10%	12%	14%	70%	72%	70%	5
aF LG	-	+	-	3	27%	12%	4%	5%	69%	60%	3%	3%	14%	9%	9%	4%	49%	31%	40%	2
CF	-	+	-	3	44%	3%	4%	5%	100%	76%	3%	2%	13%	9%	9%	8%	43%	41%	39%	4

LC																				
aF L	-	+	-	3	100%	9%	4%	4%	100%	71%	2%	2%	18%	12%	13%	9%	86%	82%	79%	7
aF L E	-	+	-	3	100%	6%	3%	3%	100%	78%	1%	1%	22%	10%	9%	2%	96%	84%	84%	8
C5	+	+	-	2	96%	3%	4%	4%	96%	44%	3%	3%	64%	10%	7%	4%	23%	13%	6%	4
C1	+	+	-	2	100%	1%	1%	1%	100%	16%	0%	0%	4%	3%	2%	6%	30%	32%	36%	1
C 7	-	+	-	3	10%	10%	10%	9%	74%	4%	6%	6%	28%	16%	16%	10%	88%	76%	8%	8
l	-	+	-	3	33%	10%	10%	9%	48%	4%	7%	7%	44%	16%	16%	4%	86%	81%	78%	1
BE	-	+	-	3	100%	10%	11%	10%	100%	4%	6%	6%	58%	26%	26%	25%	95%	97%	94%	9
C5	-	+	-	3	100%	11%	12%	12%	100%	4%	6%	7%	43%	19%	17%	14%	95%	80%	77%	7
C2	-	+	-	3	100%	13%	13%	12%	100%	4%	6%	6%	35%	17%	16%	15%	89%	74%	73%	7
C6	-	+	-	3	100%	12%	11%	10%	100%	5%	6%	5%	37%	27%	22%	15%	84%	66%	61%	4
BE	-	+	-	3	100%	10%	9%	8%	100%	4%	4%	4%	26%	11%	12%	11%	83%	81%	73%	7
2	-	+	-	3	100%	8%	7%	4%	100%	100%	6%	4%	80%	38%	37%	39%	100%	100%	100%	1(
C5	-	+	-	3	100%	7%	8%	4%	100%	52%	5%	3%	53%	35%	25%	22%	100%	100%	100%	1(
C4	-	+	-	3	97%	8%	4%	5%	100%	84%	5%	5%	10%	5%	7%	12%	81%	70%	74%	9
C5	-	+	-	3	100%	7%	5%	4%	100%	100%	4%	4%	55%	25%	22%	21%	100%	100%	100%	8
BE)	-	+	-	3	100%	24%	9%	10%	100%	100%	9%	10%	65%	39%	37%	34%	100%	100%	100%	1(
C5	+	+	-	2	100%	15%	7%	7%	100%	100%	7%	8%	46%	21%	25%	18%	100%	100%	100%	1(
BE	-	+	-	3	96%	4%	3%	3%	100%	5%	4%	3%	10%	5%	6%	2%	87%	82%	72%	6
C1	-	+	-	3	100%	19%	5%	6%	100%	93%	11%	10%	100%	55%	35%	28%	100%	100%	95%	9
C5	-	+	-	3	100%	4%	5%	4%	100%	3%	2%	2%	8%	7%	7%	10%	67%	48%	39%	3
C1	-	-	-	3	50%	8%	7%	8%	100%	5%	5%	5%	30%	17%	16%	19%	91%	79%	80%	8
C5	-	+	+	3	100%	10%	9%	8%	100%	7%	6%	5%	24%	15%	13%	7%	96%	95%	74%	6
C2	-	+	-	3	86%	8%	8%	7%	95%	4%	4%	3%	26%	13%	12%	17%	71%	43%	58%	5
C1	-	-	-	3	4%	9%	9%	8%	3%	3%	3%	3%	18%	12%	11%	2%	62%	19%	40%	3
C1	-	-	-	3	91%	7%	6%	6%	100%	9%	3%	3%	16%	10%	7%	6%	70%	35%	28%	2
BE	-	-	-	3	99%	8%	8%	6%	100%	4%	4%	3%	16%	11%	9%	7%	49%	35%	25%	1
C6	-	-	-	3	100%	7%	6%	4%	100%	84%	4%	3%	63%	26%	24%	20%	100%	100%	100%	9
B 7	-	-	-	3	90%	6%	5%	4%	100%	27%	2%	2%	33%	12%	11%	6%	73%	73%	88%	5
BE	-	-	-	3	94%	4%	5%	4%	100%	2%	3%	2%	23%	11%	8%	6%	87%	80%	79%	7
C5	-	-	-	3	100%	4%	4%	3%	87%	15%	1%	1%	24%	9%	8%	3%	69%	59%	57%	5
C2	-	+	-	3	26%	4%	4%	4%	94%	61%	5%	7%	62%	50%	41%	12%	97%	96%	90%	8

C	-	+	+	3	100%	4%	4%	4%	100%	1%	1%	1%	8%	7%	7%	6%	59%	49%	43%	2
C	-	+	-	3	100%	5%	4%	3%	100%	3%	2%	2%	15%	8%	7%	4%	67%	48%	39%	2
C5	-	-	-	3	80%	8%	7%	7%	100%	3%	3%	3%	19%	11%	10%	4%	2%	46%	49%	4
Z	-	+	-	3	97%	5%	4%	3%	100%	3%	2%	2%	15%	8%	7%	4%	67%	48%	39%	2
Z	-	+	-	3	79%	3%	3%	3%	100%	39%	3%	3%	5%	6%	5%	5%	60%	57%	44%	3
Z	-	+	-	3	94%	29%	6%	5%	100%	68%	23%	16%	29%	14%	15%	6%	100%	68%	23%	1
Z	-	-	-	3	97%	27%	4%	4%	100%	61%	17%	17%	14%	11%	11%	6%	14%	11%	5%	[illegible]
Z	-	+	-	3	87%	11%	9%	9%	100%	6%	0%	0%	39%	19%	18%	19%	77%	45%	60%	3
Z	-	+	-	3	94%	8%	4%	3%	100%	20%	7%	8%	11%	6%	13%	6%	59%	49%	38%	2
Z	-	-	-	3	100%	4%	5%	4%	100%	3%	2%	2%	8%	7%	7%	6%	67%	48%	39%	3
Z	-	+	-	3	100%	24%	9%	10%	100%	100%	9%	10%	65%	48%	34%	34%	100%	100%	100%	4
Z	-	+	-	3	80%	8%	7%	7%	100%	3%	3%	3%	19%	11%	10%	4%	62%	46%	42%	4

[a] b-gluc.: b-glucosidase activity

[b] Prot.: Protease activity

[c] Est.: esterase activity