Università degli Studi di Torino

Scuola di Dottorato in
Scienze della Natura e Tecnologie Innovative

Dottorato in
Scienze Agrarie, Forestali ed Agroalimentari

Ciclo: X X X

Exploitation of the non-*Saccharomyces* yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation

Vasileios Englezos

Docente guida:
Prof. Luca Simone Cocolin

Coordinatore del ciclo:
Prof. Aldo Ferrero

Anni:
2015, 2016, 2017

Abstract

Negli ultimi anni c'è un crescente interesse globale per l'utilizzo di lieviti selezionati non-*Saccharomyces* nel settore enologico, principalmente per la loro capacita di migliorare la complessità e le caratteristiche specifiche dei vini. Queste applicazioni hanno stimolato l'interesse a comprendere il contributo dei singoli lieviti non-*Saccharomyces* nel vino. *Starmerella bacillaris* (sinonimo *Candida zemplinina*) è spesso presente nelle fermentazioni vinarie spontanee in diversi paesi. La potenzialità dell'utilizzo di questo lievito in fermentazioni miste è stata ampiamente approfondita negli ultimi anni, soprattutto per il suo carattere fruttosofilo e la produzione di metaboliti di interesse enologico. In questo contesto, le condizioni ambientali, il protocollo di inoculo e la selezione dei ceppi hanno un ruolo fondamentale per ottenere vini con caratteristiche desiderabili, poiché essi potrebbero impattare la crescita dei lieviti e di conseguenza la produzione di metaboliti. Ulteriori conoscenze sulla biodiversità intraspecifica di questa specie e il suo comportamento in diverse condizioni ambientali è essenziale per produrre vini caratteristiche desiderabili.

Parole chiave: non-*Saccharomyces*; *Starmerella bacillaris*; fermentazioni vinarie; produzione di metaboliti

Abstract

In recent years there is an increasing global interest for the use of selected non-*Saccharomyces* yeasts in winemaking industry, mainly due to their ability to improve the complexity and specific traits of the wines. These applications stimulated the interest for understanding the contribution of individual non-*Saccharomyces* yeasts in the wine. *Starmerella bacillaris* (synonym *Candida zemplinina*) is frequently found in spontaneous wine fermentations in different countries. The potential use of this yeast species in mixed wine fermentations have been extensively investigated in the last years, mainly due to its fructophilic character and the production of metabolites of oenological interest. In this context, environmental conditions and inoculation protocol and strain selection have a fundamental role to obtain a wine with desirable characteristics, since they can modulate yeast growth and as a consequence metabolite production. Further knowledge on the species intraspecific biodiversity as well as their behavior in different environmental conditions is essential in order to produce wines with desirable characteristics.

Keywords: non-*Saccharomyces*; *Starmerella bacillaris*; wine fermentation; metabolites production

Contents

Introduction

Alcoholic fermentation is the transformation of grape sugars, mainly fructose and glucose, into ethanol, carbon dioxide and other secondary metabolites. This process usually is carried out by successional evolution of yeast species or single strains of them, present on grapes and winemaking equipment, however it is more complex than what it seems (Bokulich et al., 2015, Ciani et al., 2010). Since, a list of physical, chemical and physicochemical reactions are occurring to turn grape juice into wine (Fleet, 2008). Besides ethanol, several metabolites are transformed or synthesized by yeasts, including a list of metabolites such as glycerol, higher alcohols and esters (Moreno-Arribas and Polo, 2009).

A large diversity of yeast species are involved in winemaking. Generally, spontaneous fermentations start by the simultaneous growth of various non-*Saccharomyces* species, which are generally characterized by low fermentative power (Fleet, 2008). The growth of many of them is generally limited to the first days of fermentation, after which they die off. At this time, more strongly fermentative and more ethanol tolerant non-*Saccharomyces* (mainly *Hanseniaspora* - anaform *Kloeckera*, *Metschnikowia*, *Torulaspora*, *Candida* and *Kluyveromyces*) together with *Saccharomyces* spp. (predominantly *S. cerevisiae*) take over the fermentation (Cravero et al., 2016, Varela and Borneman, 2016, Varela, 2016). This successional evolution of strains and species during fermentation, is largely determined by their different sensibilities to the increasing levels of ethanol, temperature, dissolved oxygen content, and killer factors (Ciani and Comitini, 2015, Ciani et al., 2016, Albergaria and Arneborg, 2016). This, in turn, will have an impact on yeast biodiversity and thus on wine quality, possibly affected by pleasant or unpleasant attributes (Ciani et al., 2010, Jolly et al., 2014). The adoption of fermentation practices, which limit the production of undesirable metabolites by favoring the growth of desirable yeasts, is fundamental in order to enhance wine quality.

Spontaneous and inoculated fermentations

Spontaneous fermentations are the result of the local yeast populations of the winery or vineyard where the grapes are grown (Varela and Borneman, 2016, Bokulich et al., 2016, Bokulich et al., 2013). This fermentation practice allows the wines to express the complexity of the vineyard biology, and allows the wine consumers to experience the nuances between different vineyards and vintages (Álvarez-Pérez et al., 2012). The extra degree of complexity is derived from the array of by-products produced from the different native non-*Saccharomyces* and *S. cerevisiae* yeasts (Jolly et al., 2014, Pretorius, 2016). Despite these advantages, agronomical practices evolved during these years, and also climate variations increased the average mean temperature in many wine regions (Mira de Orduña, 2010). The consequence is an increase of sugars content in the must, with the result that some producers who wanted to avoid inoculation now they have to do that to avoid stuck fermentations. Not to mention that some wild yeasts can play a negative role in the character of the product, through the production of undesirable metabolites, such as acetaldehyde, hydrogen sulphide and volatile acidity. All these facts indicate that spontaneous fermentations lacks reproducibility (Ciani et al., 2010, Jolly et al., 2014).

To this regard, many winemakers inoculate the musts with commercial *S. cerevisiae* strains, which ensure a rapid increase of their cell number, by improving the fermentation rate and producing more predictable wines with established criteria. However, there is some controversy about the use of commercial starters, due to the lack of some desirable metabolites provided by spontaneous fermentations (Belda et al., 2017). In an effort to replicate the aroma complexity, derived from indigenous strains in spontaneous fermentations, mixed fermentations using selected non-*Saccharomyces* and *S. cerevisiae* were proposed (Mate and Maicas, 2016, Tofalo et al., 2016, Varela et al., 2016). It is generally acknowledged that wines produced with or without inoculated yeasts are the outcome of mixed fermentations, which involves contributions from many indigenous species and strains. The presence of higher percentage of non-*Saccharomyces* yeasts appears to be one of the reasons for the higher complexity found in spontaneously fermented wines (Ciani et al., 2010, Jolly et al., 2014). With understanding, yeast ecology of wine fermentations, winemakers are seeking to increase

specific metabolites of wines and predictability by controlled fermentations with different species.

The role of non-*Saccharomyces* yeasts in the winemaking industry

Non-*Saccharomyces* yeasts usually predominate during the first days of alcoholic fermentation by reaching populations of 10^7 colony forming units (cfu)/mL or more, before dying (Fleet, 2008). By this time, they utilized a not negligible amount of sugars and nutrients, and generated moderate amounts of beneficial and detrimental metabolites, which have an impact in the wine character. Among the latter productions, the production of high levels of acetic acid, ethyl acetate, acetoin, acetaldehyde and volatile phenols, generally prevents their use as starters. However, the contribution of each species in both spontaneous and inoculated fermentations shows distinct differences, since strain selection, yeast interactions, physicochemical parameters (SO_2, temperature, sugar concentration, nutrients, oxygen availability and ethanol) could modulate yeast growth and consequently the production of target metabolites (Ciani and Comitini, 2015, Ciani et al., 2016, Kemsawasd et al., 2015, Wang et al., 2016). Indeed, over the last two decades several studies have been revaluating the role of these yeasts to the analytical composition and sensorial characteristics of wines, and they demonstrate their ability to improve quality (Tofalo et al. 2016, Whitener et al., 2016, 2017). This is mainly, due to the ability of non-*Saccharomyces* yeasts to produce and secrete enzymes (such as esterases, β-glycosidases, proteases), which enhance the release of aroma metabolites with positive notes (Englezos et al., 2015, Maicas and Mateo, 2015). Moreover, they could promote a high production of glycerol, mannoproteins, organic acids that contribute to total acidity, volatile esters with pleasant notes and decrease the production of acetic acid and ethanol, thus possibly increasing wine quality (Domizio et al., 2014, Suzzi et al. 2012, Padilla at al., 2016, Gobbi et al. 2013, Benito et al., 2105). However, few non-*Saccharomyces* strains are capable of fermenting high sugar levels in pure culture fermentation. Therefore, two inoculation strategies have proposed to ensure complete fermentation: co-inoculation with *S. cerevisiae* strains and sequential inoculation in which *S. cerevisiae* is inoculated successively (Ciani et al., 2010, Jolly et al., 2014). A successful inoculation is

considered the fermentation, which enables the non-*Saccharomyces* to demonstrate its peculiar metabolic characteristics, which are absent in *S. cerevisiae* (Varela, 2016, Belda et al., 2017). Besides, non-*Saccharomyces* yeasts, *Starmerella bacillaris* (synonym *Candida zemplinina*) can tolerate relative concentrations of ethanol, making them more suitable for mixed fermentations with selected *S. cerevisiae* strains (Ciani et al., 2010, Jolly et al., 2014, Englezos et al., 2015).

Contribution of *Starmerella bacillaris* to wine quality

In 2011, many strains of *Candida stellata* were reclassified to *Starmerella bacillaris* (synonym *Candida zemplinina,* Kurtzman et al., 2011). Since its identification, *Starm. bacillaris* has been found to be one of the most abundant species during the various stages of spontaneous fermentations in relative high population of 10^4-10^6 cfu/mL all over the world, and gained interest in winemaking industry mainly for its peculiar characteristics (Englezos et al., 2015, Masneuf-Pomarede et al., 2015, Mestre et al., 2017, Englezos et al., 2016a. *Starm. bacillaris* is an acidogenic, psychrotolerant, osmotolerant yeast and therefore adapted to sweet wine fermentations (Pfliegler et al., 2014, Rantsiou et al., 2012). Most of the strains of this species are tolerant to high ethanol levels and can survive and persist up to the middle-end phase of the fermentation process (Englezos et al., 2015, Rantsiou et al., 2012, Englezos et al., 2016b. Its strongly fructophilic character, is associated with a lower ethanol yield production from the sugar consumed as compared with *S. cerevisiae* strains (Mestre et al., 2017, Englezos et al., 2016a). Thus reduction in ethanol yield can be partially explained by the production of secondary metabolites alternative to ethanol. Among them, it has been well described that many of the studied strains exhibit a high glycerol yield (Suzzi et al., 2012, Zara et al., 2014). Such modification in sugar ethanol yield is particularly interesting due to the increasing attention of wine consumers for low ethanol wines, and the increased levels of sugars in the grapes due to the aforementioned factors (Mira de Orduña, 2010, Good et al., 2016). Additionally, it has been found that *Starm. bacillaris* could increase the total acidity in the wines, mainly due to the relative high production of pyruvic acid (Magyar et al., 2014). Some publications reported that *Starm. bacillaris* could produce less acetic acid than *S.*

cerevisiae and in sequential fermentations with *S. cerevisiae* can also reduce the acetic acid produced by the latter (Rantsiou et al., 2012). However, the production of this metabolite is strain-dependent and may vary with the fermentation environment such as the temperature, nitrogen composition, inoculation density and inoculation protocol applied.

Many studies have proposed the potential use of *Starm. bacillaris* in wine fermentations, in combination with *S. cerevisiae* strains, which involvement during the fermentation process is essential in order to convert all the sugars in ethanol (Fleet and Heard, 1993). 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 to *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). However further exploitation of this non-*Saccharomyces* species either in pure and mixed (co-inoculated and/or sequential) cultures is essential in order to produce with desirable characteristics.

References

Albergaria, H., Arneborg, N. (2016). Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation processes: role of physiological fitness and microbial interactions. *Applied Microbiology and Biotechnology* 100, 2035-2046.

Álvarez-Pérez, J.M., Campo, E., San-Juan, F., Coque, J.J.R., Ferreira, V., Hernández-Orte, P. (2012). Sensory and chemical characterisation of the aroma of Prieto Picudo rosé wines: The differential role of autochthonous yeast strains on aroma profiles. *Food chemistry* 133, 284-29.

Belda, I., Ruiz, J., Esteban-Fernández, A., Navascués, E., Marquina, D., Santos, A., Moreno-Arribas, M.V. (2017). Microbial contribution to wine aroma and its intended use for wine quality improvement. *Molecules* 22, 189.

Benito, S., Hofmann, T., Laier, M., Lochbühler, B., Schüttler, A., Ebert, K., Fritsch, S., Röcker, J., Rauhut, D. (2015). Effect on quality and composition of Riesling wines fermented by sequential inoculation with non-*Saccharomyces* and *Saccharomyces cerevisiae*. *European Food Research Technology* 241, 707-717.

Bokulich, N.A., Thorngate, J.H., Richardson, P.M., Mills, D.A. (2013). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proceedings of the National Academy of Science USA* 111, 139-148.

Bokulich, N., Swadener, M., Sakamoto, K., Mills, D.A., Bisson, L.F. (2015). Sulfur dioxide treatment alters wine microbial diversity and fermentation progression in a dose dependent fashion. *American Journal of Enoligy and Viticolture* 66, 73-79.

Bokulich, N.A., Collins, T.S., Masarweh, C., Allen, G., Heymann, H., Ebeler, S.E., Mills, D.A. (2016). Associations among wine grape microbiome, metabolome, and fermentation behavior suggest microbial contribution to regional wine characteristics. *mBio* 7, e00631-16.

Ciani, M., Comitini, F., Mannazzu, I., Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Research* 20, 123-133.

Ciani, M., Comitini, F. (2015). Yeast interactions in multi-starter wine fermentation. *Current Opinion in Food Science and Technology* 1, 1-6.

Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G., Romano, P. (2016). Yeast interactions in inoculated wine fermentation. *Frontiers in Microbiol*ogy 7, 555.

Cravero, F., Englezos, V., Torchio, F., Giacosa, S., Segade, S.R., Gerbi, V., Rantsiou, K., Rolle, L., Cocolin, L. (2016). Post-harvest control of wine-grape mycobiota using electrolyzed water. *Innovative Food Science and Emerging Technol*ogies 35:21-28.

Domizio, P., Liuc. Y., Bisson, L.F., Barile, D. (2014). Use of non-*Saccharomyces* wine yeasts as novel sources of mannoproteins in wine. *Food Microbiology* 43, 5-15.

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., Cocolin, L. (2015). Exploitation of the non-*Saccharomyces* yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: physiological and molecular characterizations. *International Journal of Food Microbiology* 199, 33-40.

Englezos, V., Torchio, F., Cravero, F., Marengo, F., Giacosa, S., Gerbi, V., Rantsiou, K., Rolle, L., Cocolin, L. (2016a). Aroma profile and composition of Barbera wines obtained by mixed fermentations of *Starmerella bacillaris* (synonym *Candida zemplinina*) and *Saccharomyces cerevisiae*. *LWT-Food Science and Technology* 73, 567-575.

Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Ortiz-Julien, A., Gerbi, V., Rolle, L., Cocolin, L. (2016b). *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. *Applied Microbiology and Biotechnology* 100, 5515-5526.

Fleet, G.H. and Heard, G.M. (1993). Yeasts – growth during fermentation. In: 'Wine microbiology and biotechnology'. Ed. G.H. Fleet (Harwood Academic Publishers, Chur, Switzerland) pp. 27-54.

Fleet, G.H. (2008). Wine yeasts for the future. *FEMS Yeast Research* 8, 979-995.

Giaramida, P., Ponticello, G., Di Maio, S., Squadrito, M., Genna, G., Barone, E., Scacco, A., Corona, O., Amore, G., di Stefano, R., Oliva, D. (2013). *Candida zemplinina* for production of wines with less alcohol and more glycerol. *South African Journal of Enology and Viticulture* 34, 204-211.

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M. (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: a strategy to enhance acidity and improve the overall quality of wine. *Food Microbiology* 33, 271–281.

Goold, H.D., Kroukamp, H., Williams, T.C., Paulsen, I.T., Varela, C., Pretorius, I.S. (2016). Yeast's balancing act between ethanol and glycerol production in low-alcohol wines. *Microbial Biotechnology*, in press.

Jolly, N.P., Varela, C., Pretorius, I.S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Research* 14, 215-237.

Kemsawasd, V., Viana, T., Ardö, Y., Arneborg, N. (2015). Influence of nitrogen sources on growth and fermentation performance of different wine yeast species during alcoholic fermentation. *Applied Microbiology and Biotechnology* 99, 10191-10207.

Kurtzman, C.P., Fell, W.F., Boekhout, T. (2011). The Yeasts, a Taxonomic Study. Elsevier Science Publishers.

Magyar, I. and Tóth, T. (2011). Comparative evaluation of some oenological properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. *Food Microbiology* 28, 94-100.

Magyar, I., Nyitrai-Sárdy, D., Leskó, A., Pomázi, A., Kállay, M. (2014). Anaerobic organic acid metabolism of *Candida zemplinina* in comparison with *Saccharomyces* wine yeasts. *International Journal of Food Microbiology* 178, 1-6.

Maicas, S. and Mateo, J.J.M. (2015). Enzyme contribution of non-*Saccharomyces* yeasts to wine production. *Universal Journal of Microbiology Research* 3, 17-25.

Mate, J.J. and Maicas, S. (2016). Application of non-*Saccharomyces* yeasts to wine-making process. *Fermentation* 2, 14.

Mestre, M.V., Maturano, Y.P., Combina, M., Mercado, L.A., Toro, M.E., Vazquez. F. (2017). Selection of non-*Saccharomyces* yeasts to be used in grape musts with high alcoholic potential: a strategy to obtain wines with reduced ethanol content. *FEMS Yeast Research*, fox010. doi: 10.1093/femsyr/fox010, in press.

Masneuf-Pomarede, I., Juquin, E., Miot-Sertier, C., Renault, P., Laizet, Y., Salin, F., Alexandre, H., Capozzi, V., Cocolin, L., Colonna-Ceccaldi, B., Englezos, V., Girard, P., Gonzalez, B., Lucas, P., Mas, A., Nisiotou, A., Sipiczki, M., Spano, G., Tassou, C., Bely, M., Albertin, W. (2015). The yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) shows high genetic diversity in winemaking environments. *FEMS Yeast Research* 15, fov045. doi: 10.1093/femsyr/fov045.

Mira de Orduña, R.L. (2010). Climate change associated effects on grape and wine quality and production. *Food Research International* 43, 1844-1855.

Moreno-Arribas, M.V., Polo, M.C. (2009). Wine chemistry and biochemistry. Springer-Verlag;

Padilla, B., Gil, J.V., Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Frontiers in Microbiology* 7, 411.

Pfliegler, W.P., Horvath, E., Kallai, Z., Sipiczki, M. (2014). Diversity of *Candida zemplinina* isolates inferred from RAPD, micro/minisatellite and physiological analysis. *Microbiology Research* 169, 402-410.

Pretorius, I.S. (2016). Conducting wine symphonics with the aid of yeast genomics. *Beverages* 2, 36.

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., Suzzi, G., Rolle, L., Cocolin, L. (2012). *Candida zemplinina* can reduce acetic acid production by *Saccharomyces cerevisiae* in sweet wine fermentations. *Applied and Environmental Microbiology* 78, 1987-1994.

Suzzi, G., Schirone, M., Sergi, M., Marianella, R.M., Fasoli, G., Aguzzi, I., Tofalo, R. (2012). Multistarter from organic viticulture for red wine Montepulciano d' Abruzzo production. *Frontiers in Microbiology* 3, 135.

Tofalo, R., Patrignani, F., Lanciotti, R., Perpetuini, G., Schirone, M., Di Gianvito, P., Pizzoni, D., Arfelli, G., Suzzi, G. (2016). Aroma profile of Montepulciano d' Abruzzo wine fermented by single and co-culture starters of autochthonous *saccharomyces* and non-*Saccharomyces* yeasts. *Frontiers in Microbiol*ogy 7, 610.

Varela. C., Borneman, A.R. (2016a). Yeasts found in vineyards and wineries. *Yeast*, doi: 10.1002/yea.3219.

Varela, C. (2016b). The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. *Applied Microbiology and* Biotechnology 100, 9861-9874.

Varela, C., Sengler, F., Solomon, M., Curtin, C. (2016c). Volatile flavour profile of reduced alcohol wines fermented with the non-conventional yeast species *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Food Chemistry* 209, 57-64.

Wang, C., Mas, A., Esteve, B. (2016). The interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific. *Frontiers in Microbiology* 7, 502.

Whitener, M.E.B., Stanstrup, J., Panzeri, V., Carlin, S., Divol, B., Du Toit, M., Vrhovsek, U. (2016). Untangling the wine metabolome by combining untargeted SPME–GCxGC-TOF-MS and sensory analysis to profile Sauvignon blanc co-fermented with seven different yeasts. *Metabolomics* 12, 1-25.

Whitener, M.E.B., Stanstrup, J., Carlin, S., Divol, B., Du Toit, M., m Vrhovskek, U. (2017). Effect of non-*Saccharomyces* yeasts on the volatile chemical profile of Shiraz wines. *Australian Journal of Grape and Wine Research*, in press.

Zara, G., Mannazzu, I., Del Caro, A., Budroni, M., Pinna, M.B., Murru, M., Farris, G.A., Zara, S. (2014). Wine quality improvement through the combined utilisation of yeast hulls and *Candida zemplinina*/*Saccharomyces cerevisiae* mixed starter cultures. *Australian Journal of Grape and Wine Research* 20, 199-207.

Aims of the PhD thesis

The PhD activity was aimed to investigate further the potential application of *Starm. bacillaris* in the winemaking industry. For this purpose, the objectives of the activity were:

- Evaluate the molecular and physiological diversity of *Starm. bacillaris* strains.
- Improve the knowledge of how strain selection and inoculation delay of *S. cerevisiae* in mixed fermentations with *Starm. bacillaris*, can affect the overall fermentation performance and wine composition.
- Develop a new inoculation protocol able to modulate glycerol production and ethanol reduction.
- Evaluate the impact of mixed fermentations with *Starm. bacillaris* and *S. cerevisiae* on the final composition of Barbera wines, in terms of aroma and flavor.

List of publications

The following publications constitute the basis for this thesis. Each paper is published in a ISI-indexed journal.

I. Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., Cocolin, L. Exploitation of the non-Saccharomyces yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: physiological and molecular characterizations. *International Journal of Food Microbiology* 199, 33-40.

II. Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Ortiz-Julien, A., Gerbi, V., Rolle, L., Cocolin, L. (2016). *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. *Applied Microbiology and Biotechnology* 12, 5515-5526.

III. Englezos, V., Torchio, F., Cravero, F., Marengo, F., Giacosa S., Gerbi, V., Rantsiou, K., Rolle, L., Cocolin, L. (2016). Aroma profile and composition of Barbera wines obtained by mixed fermentations of *Starmerella bacillaris* (synonym *Candida zemplinina*) and *Saccharomyces cerevisiae*. *LWT - Food Science and Technology* 73, 567-575.

Paper I

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

Vasileios Englezos, Kalliopi Rantsiou, Fabrizio Torchio, Luca Rolle, Vincenzo Gerbi and Luca Cocolin*

University of Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Agricultural Microbiology and Food Technology Sector, Largo Paolo Braccini, 2 - 10095 Grugliasco, Torino, Italy.

*Corresponding author

Published on International Journal of Food Microbiology

doi.org/10.1016/j.ijfoodmicro.2015.01.009

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 ethanol, acetic acid and high quantities of 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

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.

Materials and methods

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 °C

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

Molecular characterization of the isolates

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*.

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.

Physiological characterization of *Starm. bacillaris* isolates

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]), 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 uL) were mixed with 180 uL 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 by the ratio (%) 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 was used as positive control.

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).

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 ~~above~~ the medium and then incubated at 25 ºC for 3 days. The presence of the -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.

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 fermentations trials.

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.

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).

Results

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).

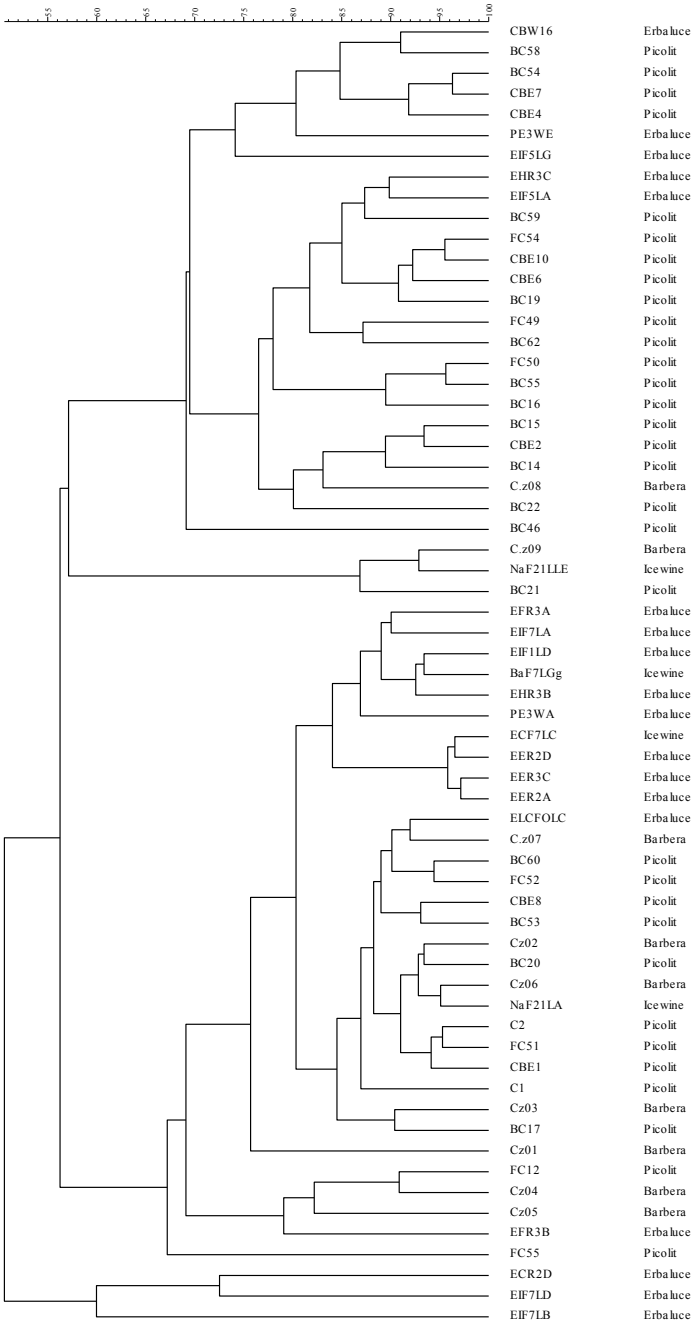

Fig. 1. Dendrogram of similarity obtained by comparison of the different fingerprinting profiles of the *Starm. bacillaris* isolates examined in this study with the two molecular techniques. The upper scale

indicates the percentage of the similarity.

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.

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	-7.84	1.37	106.48	-0.06477	P < 0.00001

	Varieties Variety B and other Varieties	-2.46	0.85	101.65	-0.02459	P < 0.00001

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

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 H2S are reported in the Supplementary Table 2. B-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.

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

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~~ing~~ 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.

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[b]	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

A Principal Component Analysis (PCA) on the physiological data and the chemical analysis of the wines was carried and the outputs are presented on the Figure 2. The PCA obtained from the first four variables 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.

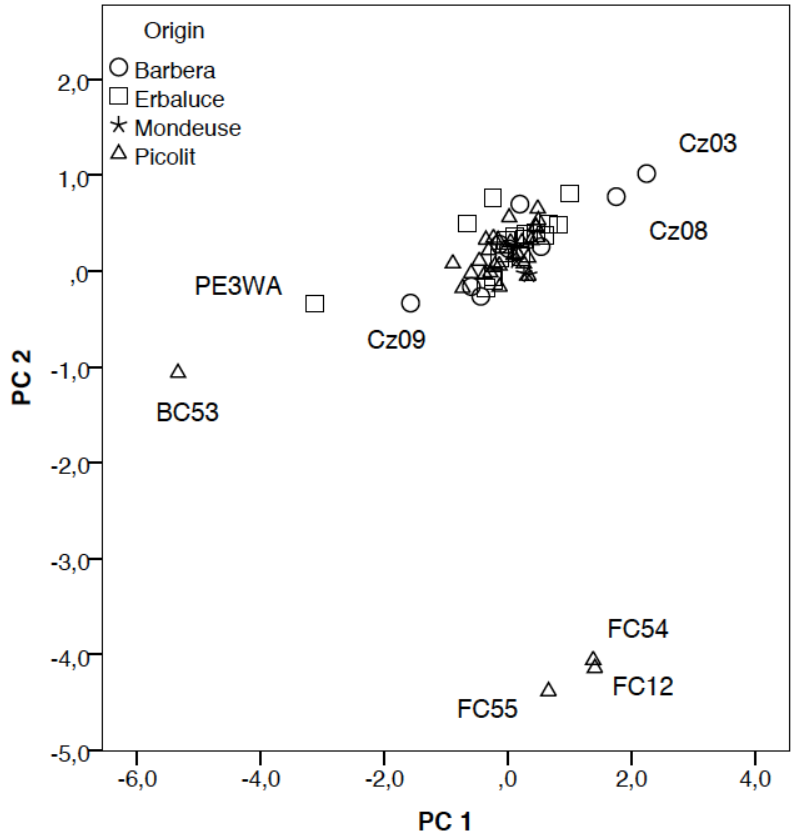

Fig. 2. Projection of the *Starm. bacillaris* strains in the plain of the first and second principal according to the physiological characterization.

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).

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).

Acknowledgements

The authors would like to thank the Professor George Banilas (Department of Oenology and Beverage Technology, Technological Educational Institute of Athens, Greece) for his helpful advices with the AMOVA analysis. The conducted research received funding from the EU (FP7/2007-2013), under grant agreement no. 315065-WILDWINE (www.wildwine.eu). The information in this document only reflects the authors' views and the Community is not liable for any use that may be made of the information contained herein.

References

Alessandria, V., Giacosa, S., Campolongo, S., Rolle, L., Rantsiou, K., Cocolin, L. (2013). Yeast population diversity on grapes during on-vine withering and their dynamics in natural and inoculated fermentations in the production of icewines. *Food Research International* 54, 139-147.

Arroyo-Lopez, F.N., Salvadó, Z., Tronchoni, J., Guillamón, J.M., Barrio, E., Querol, A. (2010). Susceptibility and resistance to ethanol on *Saccharomyces* strains isolated from wild and fermentative environments. *Yeast* 27, 1005-1015.

Bokulich, N.A., Ohta M., Richardson, P.M., Mills, D.A., (2013). Monitoring Seasonal Changes in Winery-Resident Microbiota. *PLoS ONE* 8, e66437.

Bokulich, N.A., Thorngate, J.H., Richardson, P.M., Mills, D.A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proceedings of the National Academy of Science USA* 111, 139-148.

Buzzini, P. and Martini, A. (2002). Extracellular enzymatic activity profiles in yeast and yeast-like strains isolated from tropical environments. *Journal of Applied Microbiology* 93, 1020-1025.

Charoenchai, C., Fleet, G.H., Henschke, P.A., Todd, B.E.N. (1997). Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. *Australian Journal of Grape*

and Wine Research 3, 2-8.

Ciani, M., Comitini, F., Mannazzu, I., Domizio P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Research* 10, 123-133.

Cocolin, L., Pepe, V., Comitini, F., Comi G., Ciani, M. (2004). Enological and genetic traits of *Saccharomyces cerevisiae* isolated from former and modern wineries. *FEMS Yeast Research* 5, 237–245.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, L., Ciani, M. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiology* 28, 873-882.

Duarte, F.L., Pimentel, N.H., Teixeira, A., Fonseca, Á. (2012). *Saccharomyces bacillaris* is not a synonym of *C. stellata*: reinstatement as *Starmerella bacillaris* comb. nov. *Antonie van Leeuwenhoek* 102, 653–658.

Eglinton, J.M., McWilliam, S.J., Fogarty, M.W., Francis, I., Kwiatkowski, M.J., HØJ, P.B., Henschke, P.A., (2000). The effect of *Saccharomyces bayanus*-mediated fermentation on the chemical composition and aroma profile of Chardonnay wine. *Australian Journal of Grape and Wine Research* 6, 190-196.

Excoffier, L. and Lischer, H.E.L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Research* 10, 564-567.

Fia, G., Giovanni, G., Rosi, I. (2005). Study of *β*-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzyme activity. *Journal of Applied Microbiology* 99, 509-517.

Fleet, G.H. and Heard, G.M. (1993). Yeasts – growth during fermentation. In: 'Wine microbiology and biotechnology'. Ed. G.H. Fleet (Harwood Academic Publishers, Chur, Switzerland) pp. 27-54.

Fleet, G.H. (2008). Wine yeasts for the future. *FEMS Yeast Research* 8, 979–995.

Giaramida, P., Ponticello, G., Di Maio, S., Squadrito, M., Genna, G., Barone, E., Scacco, A., Corona, O., Amore, G., di Stefano, R., Oliva, D. (2013). *Candida zemplinina* for production of wines with less alcohol and more glycerol. *South African Journal of Enology and Viticulture* 34, 204–211.

Giordano, M., Rolle, L., Zeppa, G., Gerbi, V. (2009). Chemical and volatile composition of three Italian sweet white passito wines. *Journal International des Sciences de la Vigne et du Vin* 43, 159-170.

Granchi, L., Bosco, M., Messini, A., Vincenzini, M. (1999). Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. *Journal of Applied Microbiology* 87, 949-956.

Heard, G.M. and Fleet, G.H. (1988). The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. *Journal of Applied Bacteriology* 65, 23-28.

Hildebrand, D.C. and Caesar, A. (1989). The widespread occurrence of rutin glycosidase in fluorescent phytopatogenic Pseudomonas. *Letters in Applied Microbiology* 8, 117-119.

Kurtzman, C.P. and Robnett, C.J., 1997. Identification of clinically important ascomycetous yeast based on nucleotide divergence in the 5' and of the large-subunit (26S) ribosomal DNA gene. *Journal of Clinical Microbiolgy* 35, 1216-1223.

Lederer, M.A., Nielsen, D.S., Toldam-Andersen, T.B., Herrmann, J.V. Arneborg, N. (2013). Yeast species associated with different wine grape varieties in Denmark. *Acta Agriculturae Scandinavica, Section B – Soil and Plant Science* 63, 89-96.

Magyar, I. and Tóth, T. (2011). Comparative evaluation of some oenological properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. *Food Microbiology* 28, 94-100.

Milanović, V., Comitini, F., Ciani, M. (2013). Grape berry yeast communities: Influence of fungicide treatment. *International Journal of Food Microbiology* 161, 240-246.

Mills, D.A, Johannsen E.A., Cocolin L. (2002). Yeast diversity and persistence in botrytis affected wine fermentations. *Applied and Environmental Microbiology* 68, 4884-4893.

Pfliegler, W.P., Horváth, E., Kállai, Z., Sipiczki, M. (2013). Diversity of *Candida zemplinina* isolates inferred from RAPD, micro/minisatellite and physiological analysis. *Microbiological Research* 169, 402-410.

Rantsiou, K., Mills, K.B., Cocolin, L. (2008). Lieviti fruttosifili ad interesse enologico: diversità molecolare di *Candida zemplinina*. *Quaderni di Scienze Viticole ed Enologiche, University of Turin* 30, 99-109.

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., Suzzi, G., Rolle, L., Cocolin, L. (2012). *Candida zemplinina* can reduce acetic acid production by *Saccharomyces cerevisiae* in sweet wine fermentations. *Applied and Environmental Microbiology* 78, 1987-1994.

Rantsiou, K., Campolongo, S., Alessandria, V., Rolle, L., Torchio, F., Cocolin, L. (2013). Yeast populations associated with grapes during withering and their fate during alcoholic fermentation of high-sugar must. *Australian Journal of Grape and Wine Research* 19, 40-46.

Rosi, I., Vinella, M., Domizio, P. (1994). Characterization of betaglucanase activity in yeasts of oenological origin. *Journal of Applied Microbiology* 77, 519-527.

Sipiczki, M. (2003). *Candida zemplinina* sp. nov., an osmotolerant and psychrotolerant yeast that ferments sweet botrytized wines. *International Journal of Systematic and Evolutionary Microbiology* 53, 2079-2083.

Sipiczki, M., 2004. Species identification and comparative molecular and physiological analysis of *Candida zemplinina* and *Candida stellata*. J. Basic Microbiol. 44, 471-479.

Slifkin, M. (2000). Tween 80 opacity test responses of various *Candida* species. *Journal of Clinical Microbiology* 38, 4626-4628.

Soden, A., Francis, I.L., Oakey, H., Henschke, P.A. (2000). Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. *Australian Journal of Grape and Wine Research* 6, 21-30.

Strauss, M.L.A., Jolly, N.P., Lambrechts, M.G., van Resemburg, P. (2001). Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. *Journal of Applied*

Microbiology 91, 182-190.

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., Suzzi, G., 2012. Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiology* 29, 18-26.

Urso, R., Rantsiou, K., Dolci, P., Rolle, L., Comi, G., Cocolin, L., 2008. Yeast biodiversity and dynamics during sweet wine production as determined by molecular methods. *FEMS Yeast Research* 8, 1053-1062.

Weir, B. and Cockerham, C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution* 38, 1358-1370.

Wright, S. (1978). Evolution and the genetics of populations, variability within and among natural populations. *University of Chicago Press*, Chicago.

Zara, G., Mannazzu, I., Del Caro, A., Budroni, M., Pinna, M.B., Murru, M., Farris, G.A., Zara, S. (2014). Wine quality improvement through the combined utilisation of yeast hulls and *Candida zemplinina*/*Saccharomyces cerevisiae* mixed starter cultures. *Australian Journal of Grape and Wine Research* 20, 199-207.

Starmerella bacillaris and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine

Vasileios Englezos[1], Kalliopi Rantsiou[1], Francesco Cravero[1], Fabrizio Torchio[2], Anne Ortiz-Julien[3], Vincenzo Gerbi[1], Luca Rolle[1], Luca Cocolin[1*].

[1]University of Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Agricultural Microbiology and Food Technology Sector, Largo Paolo Braccini 2, 10095 Grugliasco, Torino, Italy.

[2]Istituto di Enologia e Ingegneria Agro-Alimentare, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy.

[3]Lallemand SAS, Blagnac, France.

[*]Corresponding author

Published on Applied Microbiology and Biotechnology

doi:10.1007/s00253-016-7413-z

Abstract

Decreasing the ethanol content in wine is a current challenge, mainly due to the global climate change and to the consumer preference for wines from grapes with increased maturity. In this study, a central composite design and response surface methodology approach was used to investigate the potential application of *Starmerella bacillaris* (synonym *Candida zemplinina*) in combination with *Saccharomyces cerevisiae*, in mixed (co-inoculated and sequential) cultures, to understand better the mechanism of co-habitation and achieve the objective of reducing the ethanol in wines. Laboratory scale fermentations demonstrated a decrease up to 0.7 % (v/v) of ethanol and an increase of about 4.2 g/L of glycerol when *S. cerevisiae* was inoculated with a delay of 48 h with respect to the inoculation of *Starm. bacillaris.* Pilot scale fermentations, carried out in winemaking conditions, confirmed the laboratory results. This study demonstrates that the combination of strains and inoculation protocol, could help to reduce the ethanol content in wines.

Keywords: Non-*Saccharomyces* yeast; *Starmerella bacillaris*; Central Composite Design; Response Surface Methodology; Ethanol content reduction.

Introduction

In the last 20 years, there has been an increasing global attention for ethanol content in wines, influenced mainly by the media and the government programs, due to the marketing, social and health associated reasons (Saliba et al. 2013). Wine consumption, in light to moderate amounts (1-2 glass of wine per day), has been well demonstrated to be beneficial for the human health (German and Walzem 2000; Yoo et al. 2010). In opposition, high levels of ethanol consumption and irregular drinking has been shown to be casually correlated with more than sixty different medical conditions (Room et al. 2005). The production of well-structured and full bodied red wines nowadays, is more difficult than previously thought, especially in warm climate wine regions (Jones et al. 2005). Usually, winemakers in order to achieve the optimum phenolic maturation and tannin concentration, necessary for the quality of these wines, postpone the harvest

time, which results in a high, to excessive, sugar concentration in the over ripe grapes (Mira de Orduňa 2010). As a consequence, the excessive sugar content could be translated to wines with elevated levels of ethanol, by increasing the perception of bitterness, hotness and decreasing the perception of some wine aromas and flavour attributes (Goldner et al., 2009).

Facing the climate change, human health and the constant growing demand for full bodied red wines, it is important to anticipate further increase. In this way, several technological approaches have been proposed, to reduce ethanol content in wine (Pickering 2000), ranging mainly from pre-fermentation (selection of grapevine clones and vineyard management) to post-fermentation approaches (spinning cone column, reverse osmosis etc.), which however, could increase the production costs and affect negatively wine quality (Pickering 2000). One the other hand, in recent years intervening on the yeast ecology during must fermentation is gaining more attention and this is carried out mainly by decreasing the sugar-ethanol yield transformation through the selection of wine yeasts (Contreras et al. 2015; Gobbi et al. 2014; Quirós et al. 2014). For non-*Saccharomyces* yeasts, the quantity of sugar used to produce 1 % (v/v) of ethanol is higher (17.0 – 40.0 g/L) (Englezos et al. 2015; Magyar and Tóth 2011) due to their ability to utilize the carbon to produce biomass and by-products. As a consequence, through their metabolism ethanol concentration does not increase (Contreras et al. 2014; Contreras et al. 2015ab; Gobbi et al. 2015; Gonzalez et al. 2013; Morales et al. 2015; Quiros et al. 2014).

Among, the non-*Saccharomyces* species of oenological interest, *Starmerella bacillaris* (synonym *Candida zemplinina*) (Duarte et al. 2012) is considered as one of the most promising species to achieve the objective described above. *S. bacillaris* is supposed to be one of the best candidates, due to its ability to produce less ethanol from sugar consumed, tolerate high concentrations of ethanol present in the wine and produce low levels of biogenic amines (Englezos et al. 2015; Magyar and Tóth 2011; Rantsiou et al. 2012; Suzzi et al. 2012; Tristezza et al. 2013). These phenotypic characteristics support the potential use of this wine yeast, in combination with *S. cerevisiae* either in co-inoculated or sequential inoculated fermentations to reduce the potential ethanol content in wine (Giaramida et al. 2013; Gonzalez et al. 2013; Masneuf-Pomarede et al. 2015). However, strain selection and establishment of inoculation protocols are

essential in order to moderate yeast growth and produce wines with the aspects described above.

In this context, the aim of this study was to understand the appropriate time of *S. cerevisiae* addition after *S. bacillaris* inoculation in order to achieve a high level of ethanol reduction. A central composite design (CCD) and response surface methodology approach (RSM) was used for this final goal, in order to optimize and find the appropriate inoculation protocol.

Materials and methods

Yeast strains

Two *S. bacillaris* (FC54 and C.z 03) and one *S. cerevisiae* (ScBa49) isolate were obtained from the Yeast Culture Collection of the DISAFA (Dipartimento di Scienze Agrarie, Forestali e Alimentari University of Torino, Italy). *S. bacillaris* MUT 5705 came from the Mycotheca Universitatis Taurinensis - MUT (DBIOS - University of Torino, Italy), while a commercial *S. cerevisiae* wine yeast Uvaferm BC® (Lallemand SA, Montreal, Canada) was used as a reference strain (Table 1). *S. bacillaris* strains were selected for their physiological and enological performance (Englezos et al. 2015) and routinely cultivated on YPD slants (1% yeast extract, 2% bacteriological peptone, 2% glucose and 2% agar, all w/v) or stored at -80 °C in YPD broth supplemented with 20% glycerol (Sigma, Milano, Italy).

Table 1 Strains used in this study

Strain	Species	Geographical region of isolation	Collection
FC54	*S. bacillaris*	Friuli Venezia Giulia (ITALY)	DISAFA
MUT 5705	*S. bacillaris*	Friuli Venezia Giulia (ITALY)	MUT[a]
C.z 03	*S. bacillaris*	Piedmont (ITALY)	DISAFA
ScBa49	*S. cerevisiae*	Piedmont (ITALY)	DISAFA
Uvaferm BC®	*S. cerevisiae*	France	LALLEMAND

[a]MUT= Mycotheca Universitatis Taurinensis (DBIOS - University of Torino, Italy)

Wine fermentations

Laboratory scale fermentations

Grape must of Barbera cultivar (*Vitis vinifera* L.) without the grape skin was obtained from the experimental winery of the University of Torino. Grape must contained 233.2 g/L sugars (116.4 g/L glucose, 116.8 g/L fructose), titratable acidity 8.20 g/L (expressed as tartaric acid), pH 3.20 and absence of ethanol. The initial Yeast Available Nitrogen (YAN) was 197 mg/L composed by the sum of 116 mg/L of AUG (ammonium + urea + L-arginine) and 81 mg/L of PAN (primary amino nitrogen). The must was pasteurized in a water bath at 60˚C for 1 hour and the sterility was checked by plating 100 µL of must on WL Nutrient Agar medium (Biogenetics, Milano, Italy) and incubated them at 28 ^{o}C for 5 days. Under sterile conditions, 25 mL of the pasteurized must was distributed onto 50 mL tubes with loose screw cap for all the fermentations performed in this work.

Pure fermentations

The oenological performance of the three *S. bacillaris* and two *S. cerevisiae* strains was evaluated by micro-vinification trials in pure culture fermentations. The inoculum of the five yeast strains was prepared by pre-adaptation of the strains in the same must as described above for 48 hours at 25 °C. Afterwards, the yeast cells were stained with methylene blue dye and immediately the viable cell population was counted by using a Thoma hemocytometer chamber (BRAND GMBH + CO KG, Wertheim, Germany). Before inoculation, appropriate amounts of inoculum were calculated and subsequently used to inoculate the musts at an initial cell population of 1.0 x 10^6 cells/mL. All the fermentations were carried out in duplicate under static conditions at 25 °C for 21 days.

Central Composite Design

Two factorial CCD were used to understand the appropriate experimental plan to model the delay of *S. cerevisiae* inoculation and the sampling time for the chemical analyses

during the fermentation period, as previously described by Torchio et al. (2011). A matrix was generated with two factors, delay of *S. cerevisiae* inoculation (hours) and time of chemical analyses (days of fermentation) at five levels (-α, -1, 0, +1, +α), where α was equal to 1.41 factorial units. The corresponding values were calculated in the decoded matrix based on the limit of the design -α and +α. In this study, it was decided that 0 (co-inoculation) and 48h (sequential inoculation) delay would be the extreme values of the *S. cerevisiae* addition and 0 and 21 days for the time of chemical analyses (Table S1 in the Supplementary Material).

Table S1 Matrix used for the CCD analysis

Experiments	Inoculation delay (hours)	Chemical analyses (days)	Inoculation delay (hours)	Chemical analyses (days)
1	-1	-1	7	3.0
2	1	-1	41	3.0
3	-1	1	7	18.0
4	1	1	41	18.0
5	-1.41	0	0	10.5
6	1.41	0	48	10.5
7	0	-1.41	24	0.0
8	0	1.41	24	21.0
9-13	0	0	24	10.5

After running Response Surface Methodology (RSM), a second-order polynomial regression equation was fitted to the subsequent equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$$

Where, Y is the predicted response of the dependent variable, X_1 (inoculation delay) and X_2 (sampling time) are the independent variables that correspond to the response of Y, b_0 is the value in the central point conditions, b_1 and b_2 represent the linear regression coefficient associated with each variable, b_{11} and b_{22} are the quadratic regression coefficient of each independent value, while b_{12} is the regression coefficient of the interaction effect between the two variables. The second-order polynomial equations used to generate the surface curves are presented in Supplementary Material (Table S2 in the Supplementary Material).

Table S2 Second-order polynomial regression equations used to generate the surface curves for the three couples of *S. bacillaris* and *S. cerevisiae*.

Chemical compounds	Couples	b_0	b_1	b_2	b_{11}	b_{22}	b_{12}
Glycerol	FC54 and Uvaferm BC®	-4.941	0.2513	1.849	-5.217E-03	-6.280E-02	3.900E-03
	MUT 5705 and Uvaferm BC®	-4.767	0.2443	1.781	-5.167E-03	-6.036E-02	5.390E-03
	C.z03 and Uvaferm BC®	-4.941	0.2513	1.849	-5.217E-03	-6.281E-02	3.929E-03
Ethanol	FC54 and Uvaferm BC®	3.238	-0.1163	1.563	7.870E-04	-4.892E-02	1.111E-03
	MUT 5705 and Uvaferm BC®	2.398	-0.0506	1.701	-4.563E-04	-5.708E-02	2.460E-03
	C.z03 and Uvaferm BC®	2.256	-0.0301	1.721	-7.940E-04	-5.922E-02	2.939E-03

Mixed fermentations

Mixed fermentations were carried out by inoculating the three *S. bacillaris* and two *S. cerevisiae* strains in combination, according to the X_1 of CCD pattern. Five inoculation strategies were carried out: inoculation of the two species simultaneously (co-inoculation) and addition of the *S. cerevisiae* at 7, 24, 41 and 48 hours after *S. bacillaris* inoculation (sequential inoculation). In each case, the musts were inoculated with 48 hours pre-adapted cultures grown in the same must, with the same cell relative density of 1:1 as described above for the pure cultures. Fermentations were carried out in duplicate under static conditions at 25 °C for 21 days.

Pilot-scale fermentations

Fermentations were performed in 2-hL stainless-steel fermenters with Barbera grape, at the experimental winery of the University of Torino. The must composition was as follows: 250.4 g/L of sugars (126.1 g/L glucose, 124.3 g/L fructose), titratable acidity 10.21 g/L (expressed as tartaric acid), pH 3.09, total sulphur dioxide 20 mg/L. The initial YAN was 145 mg/L composed by the sum of 55 mg/L of AUG and 90 mg/L of PAN. The best performing couple and inoculation strategy according to the laboratory fermentations were selected for these trials: a pure culture fermentation of *S. cerevisiae* Uvaferm BC® was used as control and a sequential mixed culture which *S. cerevisiae* Uvaferm BC® was inoculated with 48 hours delay after *S. bacillaris* MUT 5705 inoculation. Both strains were inoculated with an initial cell population of 1.0 x 10^6 cells/mL as described above for the laboratory scale trials. Fermentations were

performed in duplicate at 25 ± 2 °C. Must was pumped up twice a day and racking was carried out when residual sugars were less than 2 g/L. Malolactic fermentation was carried at 20 °C in stainless steel tanks, by inoculating the commercial *Oenococcus oeni* Lalvin VP41® strain (Lallemand SA, Montreal, Canada), according the manufacturer's instructions. At the end of the malolactic fermentation, wines were clarified, supplemented with 50 mg/L of total SO_2 and then bottled and subjected to chemical analysis.

Microbiological and molecular analysis

Samples were collected in duplicate at 0, 1, 2, 4, 7, 14 and 21 days from the beginning of fermentation, serially diluted in Ringer's solution (Oxoid, Milan, Italy) and plated on WL Nutrient Agar medium (Biogenetics). Plates were incubated at 28 °C for 5 days and the two types of colonies were differentiated visually as described previously (Rantsiou et al. 2012) and subsequently counted. The enumeration of non-*Saccharomyces* yeasts in the pilot-scale fermentations was carried out using lysine agar medium (Oxoid, Milan, Italy). Concerning pilot-scale trials, 5 putative colonies of *S. bacillaris* and *S. cerevisiae* from each sampling point (30 for each ferment), were isolated and then subjected to molecular characterization by Rep and interdelta-PCR, as suggested by Englezos et al. (2015) and Charpentier et al. (2002) respectively, in order to understand strain dynamics over the fermentation process.

Chemical analysis

Ethanol, glycerol, acetic acid production, as well as the glucose and fructose consumption were determined by HPLC using an Agilent 1260 Infinity HPLC apparatus (Milford, MA, USA) as described by Rolle et al. (2012). The concentration of total YAN was determined by using two enzymatic kits (Megazyme International, Wicklow, Ireland) following the kit manufacturer instructions. In particular, total YAN concentration was calculated by the sum of ammonium, urea and L-arginine (AUG) and the concentration of the primary amino nitrogen (PAN).

Statistical analyses

The data obtained from the different inoculation strategies, were subjected to one-way analysis of variance (ANOVA) by using the statistical software package IBM SPSS Statistics (version 21.0. IBM Corp., Armonk. NY, USA). The ANOVA analysis was coupled by the Duncan test ($p < 0.05$), in order to evaluate the significant differences between the data obtained.

The RSM was performed with the statistical software STATISTICA™, program version 10.0 (StatSoft Inc. Tulsa, USA) to evaluate the results obtained by the CCD pattern applied. The regression models were performed only with R^2 values greater than 0.8 indicating that the variability could be explained by the second-order model equations.

Results

Laboratory scale fermentations

Growth dynamics

The growth dynamics of the *S. bacillaris* and *S. cerevisiae* strains when inoculated in pure cultures in Barbera must are presented in Fig. 1. The five strains grew similarly and reached a cell population of about 10^8 colony forming units (CFU)/mL on the second day of fermentation. On the seventh day, viable cell population started to decrease and no *S. bacillaris* population was observed at the last sampling point (<10 CFU/mL on WLN medium plates), while populations of the *S. cerevisiae* strains (ScBa49 and Uvaferm BC®) ranged from 10^6 to 10^7 CFU/mL, respectively.

In Fig. 2 the growth dynamics of the mixed fermentations with *S. cerevisiae* ScBa49 are illustrated. In the co-inoculated and sequential fermentation (7 hours delay), all *S. bacillaris* and *S. cerevisiae* couples showed comparable growth dynamics, reaching a population of 10^7 to 10^8 CFU/mL in 2 days. At this point, *S. cerevisiae* ScBa49 maintained this population through the fermentation, while *S. bacillaris*

populations started to decrease. When the other three inoculation strategies (24, 41 and 48 hours delay) were carried out, all *S. bacillaris* strains used in this study competed with *S. cerevisiae* ScBa49 during the first 7 days of fermentation. After this day, *S. bacillaris* started to decrease and the population became undetectable (<10 CFU/mL on WLN medium plates) after 14 (24 hours delay) and 21 days (41 and 48 hours delay), while the viability of the *S. cerevisiae* cells remained stable at 10^6 to 10^7 CFU/mL throughout the whole fermentation process.

The population dynamics for the mixed fermentations with *S. cerevisiae* Uvaferm BC® are shown in Fig. 3. Population dynamics within the first four days of fermentation, for the co-inoculated and sequential inoculated trials (7 hours delay), were comparable to those observed for *S. cerevisiae* ScBa49. Afterwards, the population of *S. cerevisiae* Uvaferm BC® started to decrease, with counts that ranged from 10^5 to 10^6 CFU/mL at the end of the monitored period (21 days), in contrast with the *S. cerevisiae* ScBa49 population that remained stable during all the monitored period. Interestingly, sequential inoculations with the highest delay (24, 41 and 48 hours), showed similar population dynamics as for the couples previously tested.

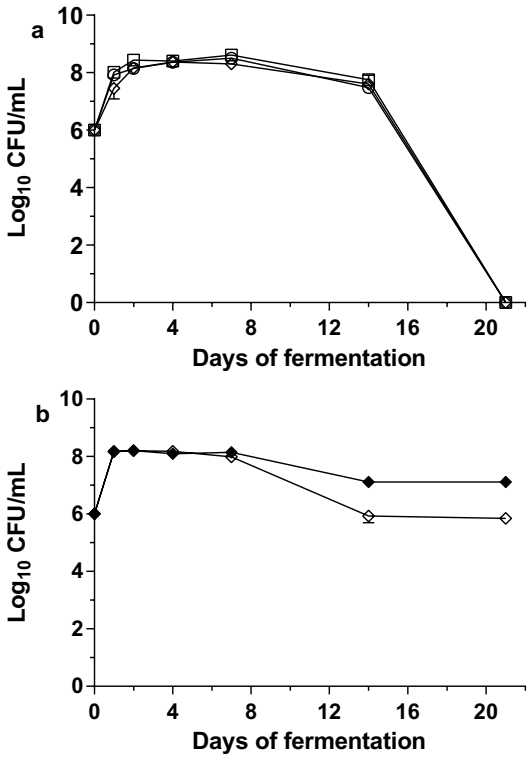

Fig. 1 Growth dynamics of *S. bacillaris* (A) and *S. cerevisiae* (B) strains in pure cultures. *S. bacillaris* strains: FC54 (*white circle*), MUT 5705 (*white diamond*), C.z 03 (*white square*) and *S. cerevisiae* strains: ScBa49 (*black diamond*) and Uvaferm BC® (*white diamond*). Counts are the mean CFU/mL values ± standard deviations of two independent experiments.

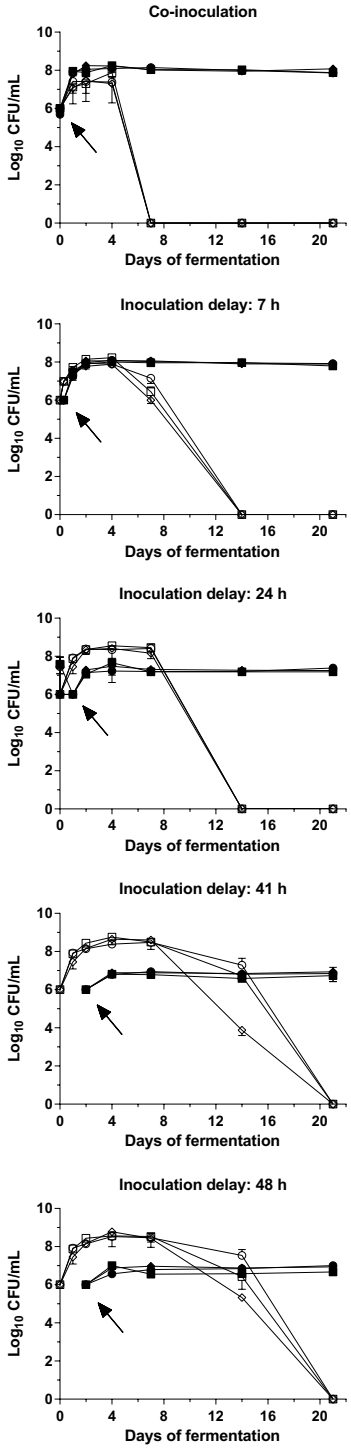

Fig. 2 Growth dynamics of mixed fermentations performed with the three *S. bacillaris* strains and the *S. cerevisiae* strain ScBa49. *S. bacillaris*/*S. cerevisiae* couples: FC54 (*white circle*), ScBa49 (*black circle*), MUT 5705 (*white diamond*) and ScBa49 (*black diamond*), C.z 03 (*white square*) and ScBa49 (*black square*). Counts are the mean CFU/mL values ± standard deviations of two independent experiments. The arrow indicates the *S. cerevisiae* inoculation.

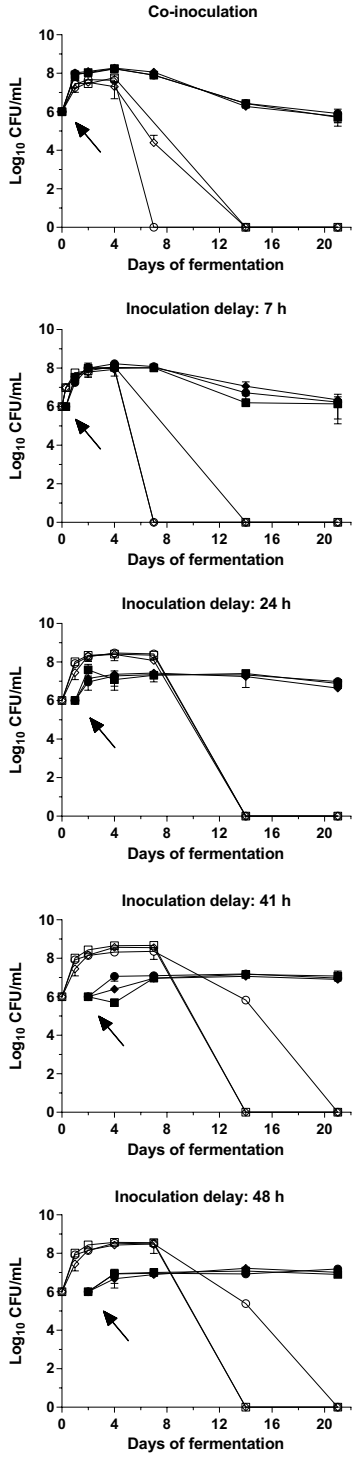

Fig. 3 Growth dynamics of mixed fermentations performed with the three *S. bacillaris* strains and the *S. cerevisiae* strain Uvaferm BC®. *S. bacillaris*/*S. cerevisiae* couples: FC54 (*white circle*) and Uvaferm BC® (*black circle*), MUT 5705 (*white diamond*) and Uvaferm BC® (*black diamond*), C.z 03 (*white square*) and Uvaferm BC® (*black square*). Counts are the mean CFU/mL values ± standard deviations of two independent experiments. The arrow indicates the *S. cerevisiae* inoculation.

Chemical composition of the wines

The mean concentration of sugars, glycerol, organic acids and ethanol in the must and wines obtained from the pure cultures of *S. bacillaris* and *S. cerevisiae*, after 21 days of fermentation, are presented in Table 2. *S. bacillaris* in pure cultures produced partially fermented wines with significant presence of residual sugars (up to 32.6 g/L glucose), while the fructose was totally consumed (<1.0 g/L). Complete fermentation of the sugars was observed only for *S. cerevisiae* strains in pure cultures. The chemical composition of these wines was characterized from the presence of lower levels of glycerol (7.8 – 8.3 g/L) and higher levels of ethanol (13.8 – 14.0) % (v/v) compared to *S. bacillaris* pure cultures. Compared to wines produced with *S. cerevisiae* in pure culture, wines fermented with *S. bacillaris* presented significantly higher glycerol yields and lower potential ethanol concentrations.

The chemical composition of the wines produced from mixed fermentations carried out with *S. cerevisiae* strains ScBa49 and Uvaferm BC® are presented in Tables 3 and 4, respectively. In mixed fermentations with ScBa49, the five different inoculation protocols resulted in a different consumption of sugars. As it can be seen, inoculation delay up to 7 hours, always allowed consumption of all sugars (< 2.9 g/L) from the must at day 21, regardless of the *S. bacillaris* strain used. On the contrary, inoculation delays of 24, 41 and 48 hours, always performed poorly, leaving significant higher quantities of sugars (14.1 – 27.6 g/L), mainly glucose. A different behaviour was observed for the Uvaferm BC® commercial strain, since all couples fermented all the sugars from the must (< 3.9 g/L) after 21 days from the beginning of fermentation, independently of the inoculation delay applied.

The glycerol production was also influenced by time of *S. cerevisiae* addition and the sampling time (Table 4). Compared to wines produced by *S. cerevisiae* Uvaferm BC® in pure culture, wines produced by mixed yeast species contained more glycerol. In particular, with a delay of 48 hours the glycerol content of the wines increased up to 4.2 g/L (Table 4). The modeling of glycerol production with RSM reflects that its increase is correlated with the increase of the inoculation delay of *S. cerevisiae*. The production of this metabolite was linearly increased when *S. cerevisiae* yeast strain Uvaferm BC® was inoculated in the first 24 hours (R^2=0.985) after *S. bacillaris*

addition. Conversely, minor differences were found by increasing the sequential inoculation between 24 and 48 hours. Acetic acid production, was also influenced by the inoculation strategy, however all the couples tested in this study maintained values at levels lower than 0.50 g/L.

Regarding ethanol production, in sequentially inoculated fermentations, only the couple *S. bacillaris* MUT 5705 and *S. cerevisiae* Uvaferm BC® produced wine with 0.7 % (v/v) less ethanol compared to *S. cerevisiae* Uvaferm BC® in pure culture (Tab. 4). In this case, the must was initially inoculated with *S. bacillaris* followed by *S. cerevisiae* after 24, 41 and 48 hours. Additionally, an interesting observation was the increasing quantity of sugars (g/L) consumed by these couples to produce 1% (v/v) of ethanol, with the increase of inoculation delay. This was particularly evident when the inoculation delay moved to 48 hours, highlighting the contribution of *S. bacillaris* to the ethanol reduction. For all the couples tested, the shape of the surface curves (Fig. 4, right panel) also confirmed this trend, indicating a significant linear decrease of the ethanol content when *S. cerevisiae* was inoculated with a delay of minimum 4 hours. However, this decrease was improved with the inoculation delay, with a maximum value at 48 hours (maximum monitored). Taking in consideration these findings and the results from growth dynamics, we hypothesized that the most suitable protocol able to reduce the ethanol at industrial scale could be the sequential inoculation with 48 hours delay. This is also in line with previous studies, in which indigenous *S. cerevisiae* started to grow after 2 days from *S. bacillaris* inoculation (Giaramida et al. 2013). Extended delays were not tested because considered not applicable in real wine-making settings.

Table 2 Concentration of sugars, glycerol, organic acids and ethanol in the must and wines obtained from pure fermentations of *S. bacillaris* and *S. cerevisiae* strains

Treatment	Residual sugars (g/L)	Glucose (g/L)	Fructose (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Fermentation efficiency[1]	Potential ethanol[2] (% v/v)	Glycerol yield (g/L)[3]
Must	233.2 ± 0.1	116.4 ± 0.1	116.8 ± 0.1	< 0.1	< 0.1	< 0.1	/	/	/
S. bacillaris									
FC54	21.7 ± 10.4^{b}	21.5 ± 10.6	0.2 ± 0.2	0.40 ± 0.02	11.8 ± 0.8	12.1 ± 0.7	18.1 ± 0.7^{c}	13.4 ± 0.0^{a}	0.06 ± 0.00^{b}
MUT 5705	29.7 ± 9.9^{b}	29.7 ± 10.0	0.1 ± 0.1	0.41 ± 0.02	12.9 ± 0.3	12.0 ± 0.6	17.0 ± 0.1^{ab}	13.7 ± 0.0^{b}	0.07 ± 0.01^{b}
C.z 03	32.6 ± 11.2^{b}	32.4 ± 11.4	0.2 ± 0.2	0.37 ± 0.03	12.6 ± 0.4	11.7 ± 0.7	17.1 ± 0.1^{b}	13.7 ± 0.0^{b}	0.07 ± 0.01^{b}
S. cerevisiae									
ScBa49	1.0 ± 0.2^{a}	0.3 ± 0.1	0.7 ± 0.0	0.26 ± 0.04	7.8 ± 0.1	13.8 ± 0.0	16.8 ± 0.0^{ab}	13.9 ± 0.0^{c}	0.03 ± 0.00^{a}
Uvaferm BC®	0.5 ± 0.1^{a}	0.1 ± 0.1	0.4 ± 0.0	0.14 ± 0.01	8.3 ± 0.3	14.0 ± 0.2	16.6 ± 0.2^{a}	14.1 ± 0.2^{d}	0.04 ± 0.01^{a}
Sig[4]	***	/	/	/	/	/	***	***	***

[1]Fermentation efficiency (sugars used to produce 1.0 % of ethanol (v/v)): initial and residual sugar concentrations were used to calculate the fermentation efficiency, [2]Potential ethanol (% v/v) = ethanol produced + ((residual glucose + residual fructose) * 0.06), [3]Glycerol yield = glycerol produced / (initial sugar concentration – final sugar concentration). All data are expressed as average value ± standard deviation (n = 2). Different superscript letters within the same column indicate significant differences among the strains according to the Duncan test ($p < 0.05$). Sig: *** indicate significance at $p < 0.001$.

Table 3 Concentration of sugars, glycerol, organic acids and ethanol in the wines obtained from mixed fermentations using the *S. cerevisiae* strain ScBa49

Strains and inoculation strategy	Residual sugars (g/L)	Glucose (g/L)	Fructose (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Fermentation efficiency[1]
Pure fermentation ScBa49	$1.0 \pm 0.2^{aA\alpha}$	$0.3 \pm 0.1^{aA\alpha}$	0.7 ± 0.1	$0.26 \pm 0.05^{aA\alpha}$	$7.8 \pm 0.1^{aA\alpha}$	$13.8 \pm 0.1^{bC\gamma}$	16.6 ± 0.3^{A}
Couple: FC54 and ScBa49							
Co-inoculation	2.0 ± 0.1^{a}	0.2 ± 0.1^{a}	1.7 ± 0.1	0.34 ± 0.02^{b}	8.2 ± 0.3^{a}	13.8 ± 0.1^{b}	16.6 ± 0.1
Inoculation delay: 7 h.	2.4 ± 1.4^{a}	0.4 ± 0.1^{a}	2.0 ± 1.3	0.39 ± 0.01^{b}	9.6 ± 0.3^{b}	13.7 ± 0.2^{b}	16.7 ± 0.2
Inoculation delay: 24 h.	23.1 ± 0.1^{b}	22.6 ± 0.9^{b}	0.4 ± 0.1	0.51 ± 0.01^{c}	11.8 ± 0.2^{c}	12.4 ± 0.2^{a}	16.8 ± 0.3
Inoculation delay: 41 h.	26.3 ± 7.9^{b}	26.2 ± 7.9^{b}	0.4 ± 0.1	0.49 ± 0.01^{c}	12.0 ± 0.5^{c}	12.3 ± 0.5^{a}	17.1 ± 0.1
Inoculation delay: 48 h.	23.2 ± 8.5^{b}	23.2 ± 8.5^{b}	0.4 ± 0.1	0.49 ± 0.01^{c}	12.0 ± 0.3^{c}	12.1 ± 0.5^{a}	17.1 ± 0.5
Sig[2]	**	**	NS	***	***	**	NS
Couple: MUT 5705 and ScBa49							
Co-inoculation	1.8 ± 0.2^{A}	0.3 ± 0.1^{A}	1.5 ± 0.3	0.32 ± 0.06^{AB}	8.5 ± 0.2^{B}	14.0 ± 0.1^{D}	16.4 ± 0.1^{A}
Inoculation delay: 7 h.	2.9 ± 0.1^{A}	0.3 ± 0.1^{A}	2.6 ± 0.1	0.40 ± 0.02^{B}	9.5 ± 0.1^{C}	13.8 ± 0.1^{C}	16.7 ± 0.3^{AB}
Inoculation delay: 24 h.	14.1 ± 2.7^{B}	13.5 ± 2.9^{B}	0.5 ± 0.2	0.50 ± 0.01^{C}	11.7 ± 0.3^{D}	12.8 ± 0.1^{B}	17.3 ± 0.1^{BC}
Inoculation delay: 41 h.	24.5 ± 0.3^{C}	24.1 ± 0.3^{C}	0.4 ± 0.1	0.51 ± 0.01^{C}	12.2 ± 0.1^{E}	12.4 ± 0.1^{A}	17.3 ± 0.4^{BC}
Inoculation delay: 48 h.	27.6 ± 0.7^{D}	27.2 ± 0.1^{D}	0.6 ± 0.3	0.51 ± 0.05^{C}	12.8 ± 0.1^{F}	12.3 ± 0.1^{A}	17.5 ± 0.2^{C}
Sig[2]	***	***	NS	**	***	***	*
Couple: C.z 03 and ScBa49							
Co-inoculation	$0.9 \pm 0.1^{\alpha}$	$0.3 \pm 0.1^{\alpha}$	0.7 ± 0.1	$0.34 \pm 0.02^{\beta}$	$8.5 \pm 0.1^{\beta}$	$13.9 \pm 0.2^{\gamma}$	16.8 ± 0.4
Inoculation delay: 7 h.	$1.3 \pm 0.1^{\alpha}$	$0.3 \pm 0.1^{\alpha}$	1.0 ± 0.1	$0.40 \pm 0.02^{\beta}$	$9.7 \pm 0.3^{\gamma}$	$13.8 \pm 0.1^{\gamma}$	16.7 ± 0.1
Inoculation delay: 24 h.	$20.0 \pm 1.4^{\beta}$	$19.6 \pm 1.4^{\beta}$	0.4 ± 0.1	$0.47 \pm 0.01^{\gamma}$	$12.5 \pm 0.2^{\delta}$	$12.6 \pm 0.1^{\beta}$	16.9 ± 0.3
Inoculation delay: 41 h.	$23.6 \pm 1.2^{\gamma}$	$23.6 \pm 1.2^{\gamma}$	0.4 ± 0.1	$0.49 \pm 0.01^{\gamma}$	$12.6 \pm 0.2^{\delta}$	$12.1 \pm 0.1^{\alpha}$	17.2 ± 0.1
Inoculation delay: 48 h.	$25.8 \pm 0.1^{\delta}$	$25.8 \pm 0.1^{\delta}$	0.4 ± 0.1	$0.47 \pm 0.02^{\gamma}$	$12.5 \pm 0.4^{\delta}$	$12.3 \pm 0.1^{\alpha}$	17.2 ± 0.1
Sig[2]	***	***	NS	***	***	***	NS

[1]Fermentation efficiency (sugars used to produce 1.0 % of ethanol (v/v)): initial and residual sugar concentrations were used to calculate fermentation efficiency. All data are expressed as average value ± standard deviation (n = 2). Different superscript Latin, UPPER Latin and Greek letters within the same column indicate significant differences among the couples FC54 and ScBa49, MUT 5705 and ScBa49 and C.z 03 and ScBa49 according to the Duncan test ($p < 0.05$) respectively. Sig: *, **, *** and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant respectively.

Table 4 Mean concentration of sugars, glycerol, organic acids and ethanol in the wines obtained from mixed fermentations with the *S. cerevisiae* strain Uvaferm BC®

Strains and inoculation strategy	Residual sugars (g/L)	Glucose (g/L)	Fructose (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Fermentation efficiency[1]
Pure fermentation Uvaferm BC®	$0.5 \pm 0.1^{A\alpha}$	$0.1 \pm 0.1^{A\alpha}$	0.4 ± 0.1	$0.14 \pm 0.01^{aA\alpha}$	$8.3 \pm 0.3^{aA\alpha}$	14.0 ± 0.2^{B}	16.6 ± 0.2^{A}
Couple: FC54 and Uvaferm BC®							
Co-inoculation	0.8 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.19 ± 0.05^{a}	8.8 ± 0.1^{ab}	14.0 ± 0.1	16.6 ± 0.1
Inoculation delay: 7 h.	0.6 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.19 ± 0.05^{a}	9.5 ± 0.1^{b}	14.0 ± 0.2	16.7 ± 0.2
Inoculation delay: 24 h.	1.3 ± 0.6	1.0 ± 0.5	0.3 ± 0.1	0.37 ± 0.01^{b}	12.5 ± 0.2^{c}	13.8 ± 0.3	16.8 ± 0.3
Inoculation delay: 41 h.	3.9 ± 2.5	3.6 ± 2.4	0.3 ± 0.1	0.46 ± 0.09^{b}	12.5 ± 0.3^{c}	13.4 ± 0.2	17.1 ± 0.1
Inoculation delay: 48 h.	3.1 ± 1.9	2.8 ± 1.9	0.3 ± 0.1	0.41 ± 0.05^{b}	12.6 ± 0.6^{c}	13.5 ± 0.3	17.0 ± 0.5
Sig[2]	NS	NS	NS	**	***	NS	NS
Couple: MUT 5705 and Uvaferm BC®							
Co-inoculation	0.7 ± 0.1^{AB}	0.2 ± 0.1^{A}	0.5 ± 0.1	0.19 ± 0.05^{A}	9.1 ± 0.1^{B}	14.1 ± 0.1^{B}	16.4 ± 0.1^{A}
Inoculation delay: 7 h.	0.6 ± 0.1^{A}	0.2 ± 0.1^{A}	0.3 ± 0.1	0.19 ± 0.05^{A}	9.9 ± 0.1^{C}	13.9 ± 0.2^{B}	16.7 ± 0.3^{AB}
Inoculation delay: 24 h.	0.8 ± 0.2^{AB}	0.5 ± 0.2^{AB}	0.3 ± 0.1	0.34 ± 0.01^{B}	12.5 ± 0.2^{D}	13.4 ± 0.1^{A}	17.3 ± 0.1^{BC}
Inoculation delay: 41 h.	1.7 ± 0.4^{C}	1.3 ± 0.4^{C}	0.4 ± 0.1	0.42 ± 0.03^{B}	12.9 ± 0.1^{D}	13.4 ± 0.3^{A}	17.3 ± 0.4^{BC}
Inoculation delay: 48 h.	1.1 ± 0.1^{B}	0.9 ± 0.0^{B}	0.3 ± 0.1	0.42 ± 0.04^{B}	12.5 ± 0.2^{D}	13.3 ± 0.1^{A}	17.5 ± 0.2^{C}
Sig[2]	**	**	NS	***	***	*	*
Couple: C.z 03 and Uvaferm BC®							
Co-inoculation	$0.7 \pm 0.1^{\beta}$	$0.2 \pm 0.1^{\alpha}$	0.5 ± 0.1	$0.17 \pm 0.05^{\alpha}$	$9.1 \pm 0.4^{\beta}$	13.9 ± 0.4	16.8 ± 0.4
Inoculation delay: 7 h.	$0.5 \pm 0.1^{\alpha}$	$0.2 \pm 0.1^{\alpha}$	0.4 ± 0.1	$0.19 \pm 0.04^{\alpha}$	$9.5 \pm 0.1^{\beta}$	13.9 ± 0.1	16.7 ± 0.1
Inoculation delay: 24 h.	$1.1 \pm 0.1^{\gamma}$	$0.7 \pm 0.1^{\beta}$	0.4 ± 0.1	$0.34 \pm 0.03^{\beta}$	$12.8 \pm 0.1^{\delta}$	13.7 ± 0.2	16.9 ± 0.3
Inoculation delay: 41 h.	$1.9 \pm 0.1^{\delta}$	$1.6 \pm 0.1^{\gamma}$	0.3 ± 0.1	$0.41 \pm 0.03^{\beta}$	$12.2 \pm 0.3^{\gamma\delta}$	13.5 ± 0.1	17.2 ± 0.1
Inoculation delay: 48 h.	$2.5 \pm 0.1^{\varepsilon}$	$2.2 \pm 0.1^{\delta}$	0.3 ± 0.1	$0.40 \pm 0.01^{\beta}$	$12.0 \pm 0.2^{\gamma}$	13.4 ± 0.1	17.2 ± 0.1
Sig[2]	***	***	NS	***	***	NS	NS

[1]Fermentation efficiency: (sugars used to produce 1.0 % of ethanol(v/v)): initial and residual sugar concentrations were used to calculate fermentation efficiency. All data are expressed as average value ± standard deviation (n = 2). Different superscript Latin, UPPER Latin and Greek letters within the same column indicate significant differences among the couples FC54 and Uvaferm BC®, MUT 5705 and Uvaferm BC® and C.z 03 and Uvaferm BC® according to the Duncan test ($p < 0.05$) respectively. Sig: *, **, *** and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant respectively.

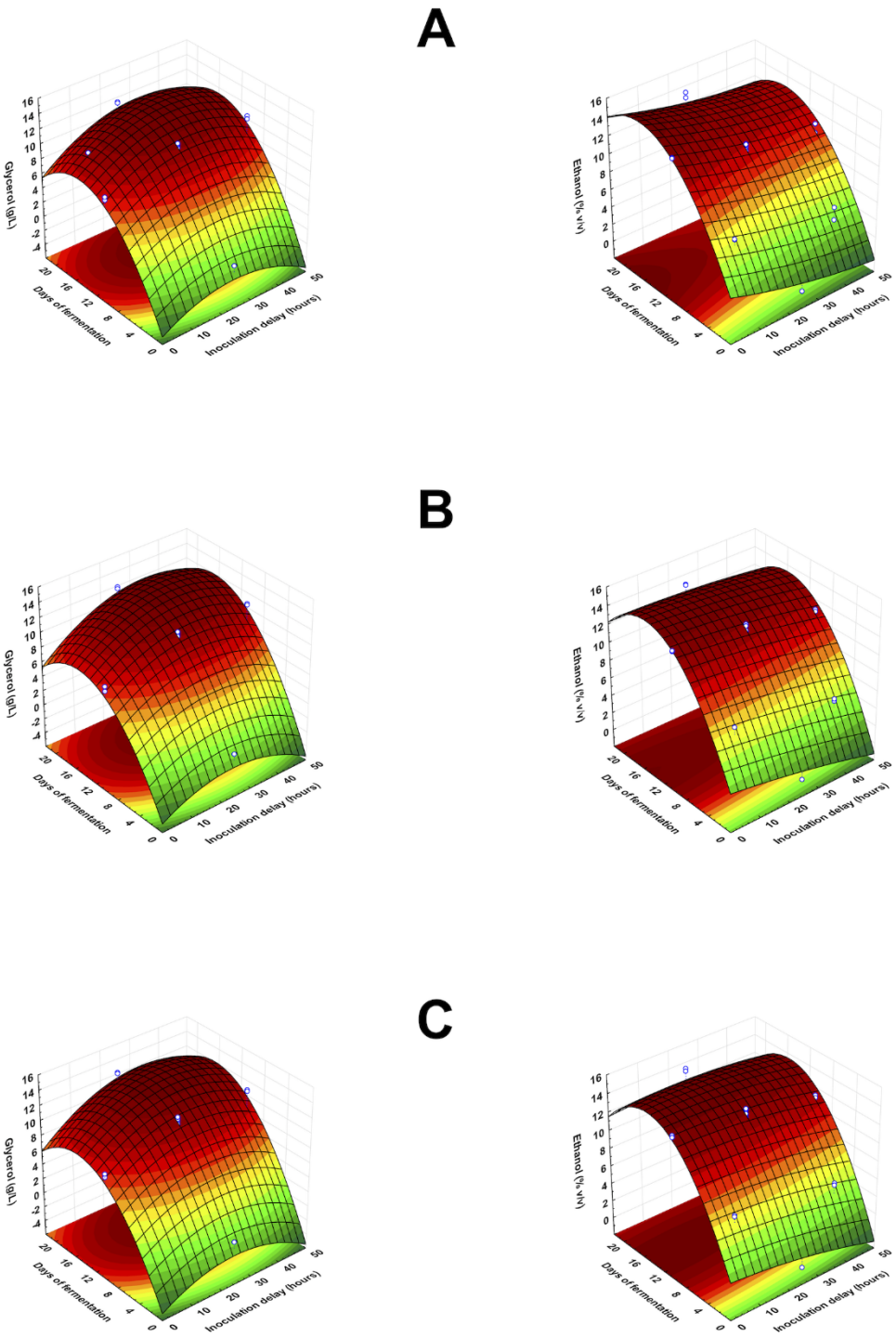

Fig. 4 Response surface curves fitted to experimental data points corresponding to the glycerol (g/L) (left panel) and ethanol (% v/v) (right panel) production as a function of *S. cerevisiae* inoculation and time of the chemical analyses. *S. bacillaris*/*S. cerevisiae* couples: FC54 and Uvaferm BC® (panel A), MUT 5705 and Uvaferm BC® (panel B), C.z 03 and Uvaferm BC® (panel C).

Pilot-scale fermentations

In order to validate the results obtained at laboratory scale, the best performing couple (MUT 5705 and Uvaferm BC®) and the inoculation strategy of 48 h delay were selected to ferment Barbera must at pilot-scale fermentations at 25 °C. Pure fermentation with Uvaferm BC® was used as control. Both inoculation rates and procedures were as close as possible to laboratory scale fermentations, in order to obtain a better reproducibility. Molecular typing of *S. bacillaris* and *S. cerevisiae* isolates by Rep and interdelta-PCR amplification respectively, revealed that these fermentations were guided by the inoculated strains (data not shown). The cell population of Uvaferm BC® in pure culture reached a concentration of about 10^8 CFU/mL on the second day, which was maintained to these levels during the whole fermentation period (Fig. S1 in the Supplemental Material, panel a). Indigenous non-*Saccharomyces* yeasts were detected at concentration of 10^5 - 10^6 CFU/mL during the first two days, after which they rapidly decreased to undetectable levels (< 10 colonies on lysine medium) on the fourth day. In addition, wild *S. bacillaris* strains, which were determined to be different from the inoculated MUT 5706 by Rep-PCR profiling, were found in the must at concentrations up to 10^6 CFU/mL during the first four days, after this point a remarkable decrease of cell population was observed. When *S. cerevisiae* Uvaferm BC® was inoculated with 48 hours delay after *S. bacillaris* MUT 5705 inoculation (Fig. S1 in the Supplemental Material, panel b), Uvaferm BC® cell population was affected by MUT 5705 and it was not able to reach counts of 10^8 CFU/mL. This allowed MUT 5705 to maintain relative high cell population (about 10^6 – 10^7 CFU/mL) until the seventh day of fermentation. The same pattern was seen for the non-*Saccharomyces* during the first four days, afterwards rapidly decreased to undetectable levels.

The chemical composition of the wines produced from the pilot-scale fermentation is given in Table 5. Sequential fermentations consumed sugars slower than Uvaferm BC® in pure culture (10 days vs 7 days). The wine produced from sequential inoculation contained significantly more glycerol (13.4 g/L vs 12.0 g/L) than Uvaferm BC®, while the ethanol content was reduced by 0.5 % (v/v). On the contrary, acetic acid production after malolactic fermentation for the sequential inoculation was reduced compared to that observed for Uvaferm BC® (0.34 g/L vs 0.47 g/L). A significant increase of 0.5 in total acidity was seen for the sequentially inoculated wine, with a parallel decrease of pH.

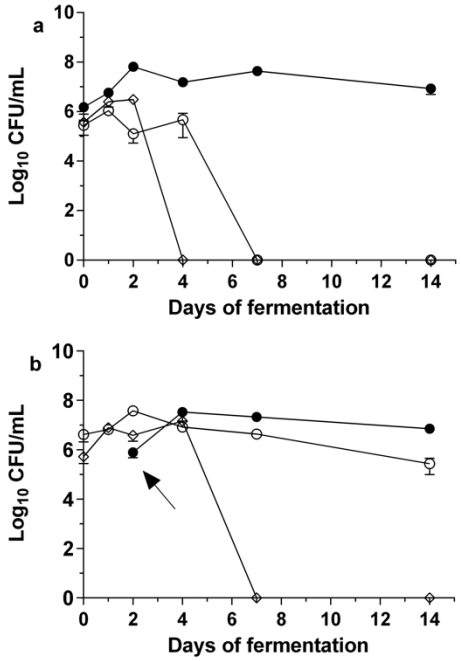

Fig. S1 Growth dynamics of yeasts during pilot scale fermentations (a) Control culture fermentation, (b) Sequential fermentation. *S. bacillaris* (white circle), *S. cerevisiae* (*black circle*), and indigenous non-*Saccharomyces* yeasts (white diamond). Counts are the mean CFU/mL values ± standard deviations of two independent experiments. The arrow indicates the *S. cerevisiae* inoculation.

Table 5 Mean concentration of sugars, glycerol, organic acids and ethanol in the wines produced from pure (Uvaferm BC®, control) and sequential (MUT 5705 and Uvaferm BC®) culture fermentations at pilot scale (2hL).

Parameter	Must	Control	Sequential	Sign[1]
Residual sugars (g/L)	250.4 ± 2.5	< 2.0	< 2.0	NS
Acetic acid (g/L)	< 0.1	0.47 ± 0.07	0.34 ± 0.04	**
Glycerol (g/L)	< 0.1	12.0 ± 0.4	13.4 ± 0.1	***
Ethanol (% v/v)	< 0.1	15.4 ± 0.0	14.9 ± 0.1	***
pH	3.09 ± 0.01	3.38 ± 0.00	3.35 ± 0.00	***
Titratable acidity (g/L)	10.21 ± 0.14	6.71 ± 0.04	7.18 ± 0.08	***

All data are expressed as average value ± standard deviation (n = 2)

[1]Sig: **, *** and NS indicate significance at $p < 0.01$, $p < 0.001$ and not significant respectively, between control and sequential fermented wines.

Discussion

One possible approach to reduce the ethanol content of wines is by fine-tuning yeast ecology during must fermentation. The selection and use of non-*Saccharomyces* wine yeasts can potentially lead to a reduction of the overall sugar-ethanol yield during alcoholic fermentation. In the study presented here, the possibility of using *S. bacillaris* in combination with *S. cerevisiae* was investigated in order to regulate the ethanol production in a must with a high initial sugar concentration (233.2 g/L). In order to find the appropriate time of *S. cerevisiae* addition after *S. bacillaris* inoculation, a CCD approach was selected to model the chemical composition of the wine produced with particular attention on the ethanol and glycerol concentration. With RSM, several combinations of *S. bacillaris*/*S. cerevisiae* at different inoculation times were tested simultaneously with a small number of experiments able to generate large amounts of information, according to the CCD experiment plan, which permits to uncover interactions between variables (Bezerra et al. 2008).

In pure fermentations, *S. bacillaris* strains produced wines with residual sugars, composed exclusively by glucose, confirming the preference of this species for fructose (Englezos et al. 2015; Magyar and Tóth 2011). The impact of this non-*Saccharomyces* yeast on the chemical composition of the wine was evident with a higher production of glycerol, in agreement with previous studies (Englezos et al. 2015; Magyar and Tóth 2011; Magyar et al. 2014), higher amounts of sugars used to produce 1% of alcohol and slightly higher

production of acetic acid compared to the *S. cerevisiae* strains, in accordance with previous studies (Sadoudi et al. 2012; Soden et al. 2000).

When mixed fermentation trials were performed, the co-inoculation of the two species did not show a significant reduction of ethanol content and the chemical composition of these wines was very similar to the control wines produced by the *S. cerevisiae* strains in pure cultures. This behavior is confirming the high competitive ability of *S. cerevisiae* over non-*Saccharomyces* yeast cells, probably due to the depletion of nutrients present in the must, cell-to-cell contact-mediated mechanisms or due to the production of toxic metabolites (Andorrà et al. 2010; Nissen et al. 2003; Pérez-Nevado et al. 2006) and underlines the need to understand better the mechanism of this co-habitation.

In this context, the early growth of *S. bacillaris* in the sequential inoculations with the highest delays (24, 41 and 48 hours) limited the subsequent growth of the two *S. cerevisiae* strains. One possible explanation for this behaviour, is that *S. bacillaris* decreased the nutrient concentration by subtracting large quantities of organic nitrogen from the must (data not shown) (Andorrà et al. 2010; Medina et al. 2012). Indeed, since only the Uvaferm BC® commercial strain totally consumed the sugars in these fermentations, it can be hypothesized that this strain has probably lower demands in nutrients (e.g. nitrogen) compared to ScBa49 wild strain. These results suggest that nutrient concentration and strain selection have a fundamental role on the fermentation rate of the mixed fermentations with *S. bacillaris* and *S. cerevisiae*.

Sequential fermentations performed with the strain Uvaferm BC®, changed positively the chemical composition of the wines produced, especially in terms of glycerol. Glycerol production was influenced by the time of *S. cerevisiae* addition and the sampling time (Table 4). The higher concentration of glycerol is in agreement with previous studies (Giaramida et al. 2013; Suzzi et al. 2012).

Interestingly, the inoculation delay changed dramatically the sugar to ethanol conversion rate of alcoholic fermentation. More specifically, in the sequentially inoculated fermentations, yeasts consumed more sugars to produce 1.0 % (v/v) of ethanol, compared to *S. cerevisiae* Uvaferm BC® in pure culture highlighting the impact of *S. bacillaris* for ethanol reduction (Bely et al. 2013; Englezos et al. 2015; Giaramida et al. 2013). However, the results revealed that only the couple MUT 5705 and Uvaferm BC® sequentially inoculated with a minimum of 24 hours delay was able to consume up to 17.5 g/L of sugars to produce 1.0% of ethanol, while the official European Economic Community (EEC) ethanol conversion factor is 16.83 g/L (Ribéreau Gayon et al. 2006). The coefficient of determination

(R^2) was 0.88 indicating a good correlation between the inoculation delay and fermentation efficiency (g/L of sugar used for 1 % v/v ethanol production).

These results let us to hypothesize that *S. bacillaris* diverts carbon derived from glycolytic pathway away from ethanol production to the synthesis of biomass and production of by-products, in order to maintain intracellular NADH/NAD+ redox balance and ensure continuous operation of metabolic process. These products include glycerol, monocarboxylic acids (acetic acid and pyruvic acid), dicarboxylic (succinic acid and α-ketoglutaric acid) tricarboxylic acids (citric acid and isocitric acid) and aroma volatile compounds (van Dijken and Scheffers 1986). The overproduction of titratable acidity observed in the pilot scale sequential fermentation could result from the swift of carbon flux towards organic acid production, since *S. bacillaris* is considered high producer of α-ketoglutaric acid and pyruvic acid (Magyar et al. 2014; Mangani et al. 2011).

In order to confirm laboratory scale fermentations, the best performing couple (MUT 5705 and Uvaferm BC®) and inoculation strategy (48 hours delay) were used to ferment Barbera must in pilot scale fermentations. The presence and dominance of the inoculated yeast strains was confirmed using Rep-PCR and interdelta PCR amplification for the *S. bacillaris* and *S. cerevisiae*, in order to exclude contributions of indigenous strains. Pilot-scale results confirmed the findings observed in laboratory settings, with the only exception of acetic acid production, which was registered to be higher in pure culture fermentation. The effect of MUT 5705 on wine composition was apparent. As in the laboratory scale fermentation, production of glycerol was higher in the sequential trial than in pure culture fermentation, while ethanol production showed a significant reduction. The pH was also lower and the titratable acidity higher, for wine produced from sequential than this produced from pure culture fermentation.

In summary, this study presents a fermentation protocol tested under both laboratory and pilot scale conditions to reduce ethanol levels in wines. This protocol is based on the inoculation of the grape must with *S. bacillaris* MUT 5705 and *S. cerevisiae* Uvaferm BC® after 48 hour from the beginning of the fermentation. Furthermore, the exploitation of this inoculation protocol could be further investigated using other varieties of grape musts, in order to understand the impact of the co-habitation of these species to wine composition in terms of aroma and flavor. In the future, the decrease of ethanol as described here, could help winemakers to decide the appropriate time to harvest their grapes, without the risk of excessive sugar content, which can be converted in high levels of ethanol in wine.

Acknowledgments

This work was funded by the (FP7/2007-2013) under grant agreement no. 315065 – WILDWINE (www.wildwine.eu). The information in this document reflects only the author's views and the Community is not liable for any use that may be made of the information contained herein.

References

Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J.M., Esteve-Zarzoso, B. (2010). Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *European Food Research Technology* 231, 215-224.

Bely, M., Renault, P., Da Silva, T., Masneuf-Pomerade, I., Albertin, W., Moine, V., Coulon, J., Sicard, D., De Vienne, D., Marullo, P. (2013). Non-conventional yeasts and alcohol level reduction. In: Teissedre PL (eds) Alcohol level reduction in wine. *Vigne et Vin Publications Internationales*, France, pp 33-37

Bezerra, M.A, Santelli, R.E., Oliveira, E.P., Villar, L.S., Escaleria, L.A. (2008) Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta* 76, 965-977.

Charpentier, C., Colin, A., Alais, A., Legras, J.L. (2009). French Jura flor yeasts: genotype and technological diversity. *Antoine van Leeuwenhoek* 95, 263-273.

Contreras, A., Hidalgo, C., Henschke, P.A., Chambers, P.J., Curtin, C., Varela, C. (2014). Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Applied and Environmental Microbiology* 80, 1670-1678.

Contreras, A., Curtin, C., Varela, C. (2015a). Yeast population dynamics reveal a potential "collaboration" between *Metschnikowia pulcherrima* and *Saccharomyces uvarum* for the production of reduced alcohol wines during Shiraz fermentation. *Applied Microbiology and Biotechnology* 99, 1885–1895.

Contreras, A., Hidalgo, C., Schmidt, S., Henschke, P.A., Curtin, C., Varela, C. (2015b). The application of non-*Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content. *International Journal of Food Microbiology* 205, 7-15.

Duarte, F.L., Pimentel, N.H., Teixeira, A., Fonseca, Á. (2012). *Saccharomyces bacillaris* is not a synonym of *C. stellata*: reinstatement as *Starmerella bacillaris* comb. nov. *Antonie van Leeuwenhoek* 102, 653–658.

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., Cocolin, L. (2015). Exploitation of the non-*Saccharomyces* yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: Physiological and molecular characterizations. International Journal of Food Microbiology 199:33-40.

German, J.B. and Walzem, R.L. (2000). The health benefits of the wine. *Annual Review of Nutrition* 20, 561-593.

Giaramida, P., Ponticello, G., Di Maio, S., Squadrito, M., Genna, G., Barone, E., Scacco, A., Corona, O., Amore, G., Di Stefano, R., Oliva, D. (2013). *Candida zemplinina* for production of wines with less alcohol and more glycerol. *South African Journal of Enology and Viticulture* 34, 204-211.

Gobbi, M., De Vero, L., Solieri, L., Comitini, F., Oro, L., Giudici, L., Ciani, M. (2014). Fermentative aptitude of non-*Saccharomyces* wine yeast for reduction in the ethanol content in wine. *European Food Research Technology* 239, 41-48.

Goldner, M.C., Zamora, M.C., Di Leo Lira, P., Gianninoto, H., Bandoni, A. (2009) Effect of ethanol level in the perception of aroma attributes and the detection of volatile compounds in red wine. *Journal of Sensory Studies* 24, 243-257.

Gonzalez, R., Quirós, M., Morales, P. (2013). Yeast respiration of sugars by non-Saccharomyces yeast species: A promising and barely explored approach to lowering alcohol content of wines. *Trends in Food Science and Technology* 29, 55-61.

Jones, G.V., White, M.A., Cooper, O.R., Storchmann, K. (2005). Climate change and global wine quality. *Climate Change* 73, 319-343.

Magyar, I. and Tóth, T. (2011). Comparative evaluation of some oenological properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. *Food Microbiology* 28, 94-100.

Magyar, I., Nyitrai-Sárdy, D., Leskó, A., Pomázi, A., Kállay, M. (2014). Anaerobic organic acid metabolism of *Candida zemplinina* in comparison with *Saccharomyces* wine yeasts. *International Journal of Food Microbiology* 178, 1-6.

Mangani, S., Buscioni, G., Collina, L., Bocci, E., Vincenzini, M. (2011). Effects of microbial populations on anthocyanin profile of Sangiovese wines produced in Tuscany, Italy. *American Journal of Enology and Viticulture* 62, 487-494.

Masneuf-Pomarede, I., Juquin, E., Miot-Sertier, C., Renault, P., Laizet, Y., Salin, F., Alexandre, H., Capozzi, V., Cocolin, L., Colonna-Ceccaldi, B., Englezos, V., Girard, P., Gonzalez, B., Lucas, P., Mas, A., Nisiotou, A., Sipiczki, M., Spano, G., Tassou, C., Bely, M., Albertin, W. (2015). The yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) shows high genetic diversity in winemaking environments. *FEMS Yeast Research* 15, fov045.

Medina, K., Boido, E., Dellacassa, E., Carrau, F. (2012). Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *International Journal of Food Microbiology* 157, 245-250.

Mira de Orduña R, (2010). Climate change associated effects on grape and wine quality and production. *Food Research International* 43, 1844-1855.

Morales, P., Rojas, V., Quirós, M., Gonzalez, R. (2015). The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture. *Applied Microbiology and Biotechnology* 99, 3993-4003.

Nissen, P., Nielsen, D., Arneborg, N. (2003). Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell-cell contact-mediated mechanism. *Yeast* 20, 331-341.

Pérez-Nevado, F., Albergaria, H., Hogg, T., Girio, F. (2006). Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentation with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology* 108, 336-345.

Pickering, G.J. (2000). Low-and reduced-alcohol wine: a review. *Journal of Wine Research* 11, 129-144.

Quirós, M., Rojas, V., Gonzalez, R., Morales, P. (2014). Selection of non-*Saccharomyces* yeast strains for

reducing alcohol levels in wine by sugar respiration. *International Journal of Food Microbiology* 181, 85-91.

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., Suzzi, G., Rolle, L., Cocolin L. (2012). *Candida zemplinina* can reduce acetic acid production by *Saccharomyces cerevisiae* in sweet wine fermentations. *Applied and Environmental Microbiology* 78, 1987-1994.

Ribéreau Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A. (2006). The microbiology of wine and vinifications. Handbook of enology, vol. 1, 2nd edn. Wiley, Chichester, England

Rolle, L., Giordano, M., Giacosa, S., Vincenzi, S., Río Segade, S., Torchio, F., Perrone, B., Gerbi, V. (2012) CIEL*a*b* parameters of white dehydrated grapes as quality markers according to chemical composition, volatile profile and mechanical properties. Analytica Chimica Acta 732, 105-112.

Room, R., Babor, T., Rehm, J. (2005). Alcohol and public health. *The Lancet* 365:519-30.

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J., Ballester, J., Vichi, S., Guérin-Schneider, R., Caixach, J., Alexandre, H. (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiology* 32, 243-253.

Saliba, A.J., Ovington, L.A., Moren, C.C. (2013). Consumer demand for low-alcohol wine in an Australian sample. *International Journal of Wine Research* 5, 1-8.

Soden, A., Francis, I.L., Oakey, H., Henschke, P.A. (2000). Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. Wine Australian Journal of Grape and Wine Research 6, 21-30.

Suzzi, G., Schirone, M., Sergi, M., Marianella, R.M., Fasoli, G., Aguzzi, I., Tofalo, R. (2012). Multistarter from organic viticulture for red wine Montepulciano d'Abruzzo production. Frontiers in Microbiology 3, 1-10.

Torchio, F., Rio Segade, S., Gerbi, V., Cagnasso, E., Rolle, L. (2011). Changes in chromatic characteristics and phenolic composition during winemaking and shelf-life of two types of red sweet sparkling wines. *Food Research International* 44, 729-738.

Tristezza, M., Vetrano, C., Bleve, G., Spano, G., Capozzi, V., Logrieco, A., Mita, G., Grieco, F. (2013). Biodiversity and safety aspects of yeast strains characterized from vineyards and spontaneous fermentations in the Apulia Region, Italy. *Food Microbiology* 36, 335-342.

Van Dijken, J.P., Scheffers, W.A. (1986). Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiology Letters* 32, 199-224.

Yoo, Y.J., Saliba, A.J., Prenzler, P.D. (2010). Should red wine be considered a functional food? *Comprehensive Reviews in Food Science and Technology* 5, 530-551.

Paper III

Aroma profile and composition of Barbera wines obtained by mixed fermentations of *Starmerella bacillaris* (*synonym Candida zemplinina*) and *Saccharomyces cerevisiae*

Vasileios Englezos[1§], Fabrizio Torchio[2§], Francesco Cravero[1], Fabio Marengo[1], Simone Giacosa[1], Vincenzo Gerbi[1], Kalliopi Rantsiou[1], Luca Rolle[1] and Luca Cocolin[1*]

[1]University of Torino, Department of Agricultural, Forest and Food Science, University of Turin, Agricultural Microbiology and Food Technology Sector, Largo Paolo Braccini 2, 10095 Grugliasco, Torino, Italy.

[2]Istituto di Enologia e Ingegneria Agro-Alimentare, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy.

[*]Corresponding author

§The authors contributed equally to this paper.

Published on LWT - Food Science and Technology

doi.org/10.1016/j.lwt.2016.06.063

Abstract

In recent years there is an increasing global interest for the use of selected non-*Saccharomyces* yeasts by the winemaking industry, mainly due to their positive contribution to the wine complexity. In this study, *Starmerella bacillaris* (synonym *Candida zemplinina*) and *Saccharomyces cerevisiae* were evaluated in mixed (co-inoculated and sequentially) inoculated fermentations with the aim of improving the aroma profile of Barbera wine. The different inoculation protocols and combination of strains tested, influenced the interactions and the fermentation behaviour of the two yeast species. The wines produced with mixed cultures contained higher amounts of glycerol and pleasant esters compared to the wine fermented with *S. cerevisiae* alone. The use of mixed culture fermentations with selected yeast strains and appropriate inoculation strategies could be considered as a tool to enhance the aroma profile of wines produced from non-floral grape varieties like Barbera.

Keywords: Non-*Saccharomyces*; *Starmerella bacillaris*; Wine fermentation; Mixed fermentation; Aroma

Introduction

The Barbera grape is Piedmont's most widely planted red variety of *Vitis vinifera* L. Barbera vineyards are located mainly in two big areas, which produce the most outstanding wines of this grape variety, the region near the town of Alba (Barbera d'Alba) and Asti (Barbera d'Asti). Even if the Nebbiolo-based wines (Barolo and Barbaresco) are considered as the most renowned red wines of this region, Barbera is the quintessential "wine of the people". It is meant to be enjoyed young during the meals. The sensory quality of young fresh wines, produced from non-aromatic grape varieties, like Barbera, depends greatly on numerous chemical constituents, mainly extracted during the pre-fermentation and fermentation process (Delfini et al., 2001).

Among other parameters, the volatile aroma compounds need special attention since it has a substantial influence on the wine quality and its acceptance by the wine consumers (Bruwer, Saliba, & Miller, 2011; Swiegers, Bartowksy, Henschke, & Pretorius, 2005). Aroma is considered as one of the main parameters that is affected by

innumerable variations during wine production, ranging from viticulture to winemaking. Particularly the nature and amount of the volatile compounds can be influenced by environmental factors, cultivar and vineyard management, fermentation conditions and lastly by the microbial community consisting of non-*Saccharomyces* and *S. cerevisiae* yeast species which take over the fermentation (Fleet, 2003; González-Barreiro, Rial-Ortero, Cancho-Grande, & Simal-Gándara, 2015; Lambrechts & Pretorius, 2000; Swiegers, Francis, Herderich, & Pretorius, 2006).

Wine yeasts found on grapes and consequently in the grape juice, have a strong impact on the wine quality and composition, since are responsible for the production of hundreds of secondary products, which contribute collectively, or individually, to the wine character and composition (Fleet 2003; Lambrechts & Pretorius, 2000; Romano, Fiore, Paraggio, Caruso, & Capece, 2003).

Wine production is based on spontaneous or inoculated fermentation and in both cases, the dominance of *S. cerevisiae,* either indigenous or inoculated, is desired in order to ensure a complete consumption of sugars. However, the presence of non-*Saccharomyces* yeast has been documented (Fleet, 2008), at significant levels (up to 10^7 -10^8 CFU/mL), during fermentation progress and for longer periods than previously thought (Bokulich, Swadener, Sakamoto, Mills, & Bisson, 2015; Cocolin, & Mills 2003). Few years ago, it was believed that the presence of non-*Saccharomyces* yeasts, could make the wine defective due to the production of metabolites of unpleasant origin (Romano, Suzzi, Comi, & Zironi, 1993). Nowadays, this trend is changing and the inoculation of mixed cultures of selected non-*Saccharomyces* yeasts in combination with highly fermentative *S. cerevisiae* strains able to ensure the complete consumption of sugars, is gaining attention and considered as an up-to-date inoculation strategy to enhance wine complexity and avoid unwanted compounds to be produced (Ciani & Comitini, 2015; Fleet, 2008; Jolly, Varela, & Pretorius, 2013) In this context, over the last years there has been an increasing interest regarding non-*Saccharomyces* yeasts and in order to improve the chemical composition and sensory aspect of the wines (Andorrà, Berradre, Mas, Esteve-Zarzoso, & Guillamón, 2012; Gobbi et al., 2013; Sadoudi et al., 2012; Soden, Francis, Oakey, & Henschke, 2000).

The increasing interest of winemakers in improving the complexity of young fresh wines produced from non-aromatic grape varieties requires further effort into

understanding the metabolic profiles of specific non-*Saccharomyces* yeast species. To gain an insight into the contribution of these species to wine aroma, the aim of this work was to evaluate the use of controlled multi-starter fermentation cultures of *Starmerella bacillaris* and *Saccharomyces cerevisiae* to enhance the analytical composition of Barbera wine. Two inoculation protocols were investigated: i) inoculation of both species at the beginning of the fermentation process (co-inoculation), and ii) inoculation of *S. cerevisiae* two days after *Starm. bacillaris* inoculation (sequential inoculation). Control wines were also produced by fermenting the same must with each of the *S. cerevisiae* and *Starm. bacillaris* strains in pure culture. Metabolic profiles of wines produced were compared, in order to highlight the effect of the inoculation strategy and strain selection on the final product.

Materials and Methods

Yeast strains

Four *Starm. bacillaris* (FC54, BC60, EFR3B and C.z 02) and two *S. cerevisiae* (ScBa49 and ScBa50) strains from the yeast culture collection of the Department of Agricultural, Forest and Food Science (DISAFA, University of Turin, Italy) were used in this study. *Starm. bacillaris* strains were isolated from grape and musts of different varieties and were selected for their oenological attributes in laboratory scale fermentations (Englezos et al., 2015).

Must preparation

Barbera grapes were harvested, destemmed and crushed. The must with grape skins was heated to 60 °C for 1h to promote color extraction in a process called *thermovinification* (Boulton, Singleton, Bisson, & Kunkee, 1996) and to deactivate indigenous yeast populations already present in the must. The grape juice was then separated using a stainless steel sieve, cooled down and frozen at -20 ^{o}C until use. The efficiency of the pasteurization was checked by plating 100 μL of the treated must on WLN medium (Biogenetics, Milan, Italy) and then incubated at 28 °C for 5 days. The

unfermented must had the following composition: pH 3.20; titratable acidity 5.39 (expressed as g/L of tartaric acid); sugar concentration 244.4 g/L.

Inoculation procedure

For each strain, an aliquot of a stock in YPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose with 20 % glycerol), stored at – 80 ^{o}C was streaked onto a WLN medium, 48 h before the preparation of inoculum. Afterwards, one fresh single colony was selected to inoculate 10 mL of sterile must. After 24 h of incubation at 25 °C, 30 mL of sterile must were added to the activated inoculum and then incubated for another 24 h at the same temperature. Finally, the preadapted inoculum was added in 360 mL of fresh sterile must.

Microfermentation trials

Three sets of fermentations were performed: inoculation of each *Starm. bacillaris* and S. *cerevisiae* strains in pure culture fermentations, simultaneous inoculation of both yeast species (co-inoculation) and inoculation of *S. cerevisiae* after 48 hours from the *Starm. bacillaris* inoculation (sequential inoculation). Mixed fermentations were carried out, using 8 different combinations of *Starm. bacillaris* and *S. cerevisiae*, according to the experimental plan reported in Table 1. Fermentations were carried in 500 mL sterile glass bottles, containing 400 mL of sterile must per bottle. Pure and mixed culture fermentations were inoculated with the abovementioned preadapted cultures, to achieve an initial cell population of about 1 x 10^6 cells/mL which was determined through plate counts on WLN medium. The bottles were equipped with sterile glass air locks containing sterile paraffin oil, to allow the carbon dioxide evolved during the fermentation process to escape from the fermenting juice. Fermentations were performed twice, under static conditions at 25 ± 1 °C (semi-anaerobic conditions). Fermentations were stopped when the weight loss remained stable for two days. Wines from both pure and mixed fermentations were then refrigerated for two days at 4 °C to remove solid parts. Afterwards, a solution of

potassium metabisulfite was added to the wines, to achieve a total sulfur dioxide concentration of 50 mg/L, which were stored at -4 °C until analysis.

Table 1 Experimental plan used in this study

Pure fermentations[1]	Mixed fermentations[1,2]	
	Co-inoculation	Sequential inoculations
Strains	Couples	Couples
S. cerevisiae	FC54 and ScBa49	FC54 and ScBa49
ScBa49	FC54 and ScBa50	FC54 and ScBa50
ScBa50	EFR3B and ScBa49	EFR3B and ScBa49
Starm. bacillaris	EFR3B and ScBa50	EFR3B and ScBa50
FC54	C.z 02 and ScBa49	C.z 02 and ScBa49
EFR3B	C.z 02 and ScBa50	C.z 02 and ScBa50
C.z 02	BC60 and ScBa49	BC60 and ScBa49
BC60	BC60 and ScBa50	BC60 and ScBa50

[1]Inoculum size: 1.0 x 10^6 cells/mL, [2]Inoculum ratio: 1:1

Microbiological Analyses

From each bottle, 1 mL samples were collected in duplicate at 0, 1, 2, 4, 7, 14 and 21 days from the beginning of fermentation to evaluate the viable cell populations. One hundred microliter aliquots of serial dilutions were plated on WLN medium, which allows the visual differentiation of the two yeast species. Plates were incubated at 28 °C and the two types colonies were visually differentiated as described previously by Rantsiou et al. (2012) and subsequently counted.

Chemical analyses

Determination of standard chemical parameters

The production of glycerol, alcohol and acetic acid, as well as glucose and fructose consumption, were directly quantified by HPLC using an Agilent 1260 Infinity HPLC system (Milford, MA, USA), equipped with a UV detector set to 210 nm and a refractive index detector, as described in Rolle et al. (2012). Fermentation purity was calculated as the amount of acetic acid produced in relationship to ethanol produced (acetic acid (g/L) / ethanol (% v/v)) (Ciani, & Macarelli, 1998).

Volatile compounds determination

Volatile aroma compounds from wines produced using pure and mixed cultures of yeasts, were directly analysed by Head Space Solid Phase Micro-Extraction (HS-SPME), coupled by Gas Chromatography-Mass Spectroscopy (GC-MS) as previously reported (Rolle, Torchio, Giacosa, & Río Segade, 2015; Whitener et al., 2015), with some modifications. Five mL of each wine sample were placed into 20 mL glass vials with a headspace screw cap containing, 5 mL of water, 2 g of NaCl and 1-heptanol solution (200 µL of 15.5 mg/L solution in 10% v/v ethanol) as an internal standard (IS). The sealed vials were carefully shaken to dissolve the NaCl before the analysis. Silicon septa (Supelco, Bellefonte, PA, USA) were used with 18 mm diameter screw caps to seal the glass vials. The fiber used for the extraction of the volatile compounds was the 50/30 µm DVB/CAR/PDMS fiber (Supelco) and the procedure was performed with Gerstel MPS2 XL auto sampler (Gerstel, Baltimore, MD, USA). The sample vial was placed at 40 °C for 10 min, then the SPME were exposed to the headspace of the capped vial for 20 min at 40 °C. Afterwards the fiber was inserted into the injection port of the GC apparatus for the thermal desorption. The thermal adsorption of the analytes from the fibre was carried out, in splitless mode at 250 °C for 5 min.

The analyses were performed using an Agilent 7890C gas chromatograph (GC) (Little Falls, DE, USA) equipped with an Agilent 5795 mass selective detector (MS) and a DB-WAX capillary column (30 m x 0.25 mm inner diameter, 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA, USA). Helium was used as carrier gas, with a flow-rate of 1 mL/min. The injection port temperature was 250 °C, the ion source temperature was 150 °C and interface was 280 °C. The GC oven program used was as follows: 40 °C for 5 min, and an increase to 200 °C (at a rate of 2 °C/min) for 10 min followed by an increase of 5 °C/min to 220 °C. The detection was carried out by electron impact mass spectroscopy in total ion current (TIC) mode, using ionization energy of 70 eV. The analyses were performed in a scan range between m/z 33-330. Identification of the volatile compounds was carried out using mass spectra and retention indices, reported in the literature and in the database (http://webbook.nist.gov/chemistry/) and pure standards when available (2,3-butanediol isomers mixture, 2-ethyl hexanol, 2-methyl-1-propanol, 1-octanol, 2-phenylethanol, diethyl succinate, ethyl acetate, ethyl decanoate, ethyl dodecanoate, ethyl heptanoate, ethyl hexanoate, ethyl nonanoate, ethyl octanoate, ethyl phenylacetate, hexanal,

hexanoic acid, hexyl acetate, linalool, methyl decanoate, methyl salicylate, octanoic acid and β-damascenone were supplied by Sigma (Milan, Italy)).

For semi-quantification purposes, the relative peak area of each identified compound was measured and then compared with the relative peak area of the added internal standard.

Statistical analysis

The quantities of the metabolites produced were subjected to one-way ANOVA to uncover statistical differences between the wines produced from the different inoculation protocols. The significant differences among the data obtained were established through the use of Tukey-b test, at $p < 0.05$. Principal Component Analysis (PCA) based on the concentration of the volatile compounds formed from each inoculation strategy and couple of strains tested, was also carried out in order to enlighten the relationship between samples and variables. Statistical analyses were performed with the statistical software package IBM SPSS Statistics (version 21.0, IBM Corp., Armonk, NY, USA).

Results and discussion

Yeast growth during fermentation

The growth dynamics of the pure culture fermentations conducted with *Starm. bacillaris* and *S. cerevisiae* are summarized in Fig.1. As can be seen, both species grew equally reaching a cell population of around 10^8 CFU/mL in two days. The cell population remained stable for seven days and then started to decline, with plate counts ranging from 10^6 to 10^7 CFU/mL for *S. cerevisiae* strains, probably due to the nutrient depletion (Cramer, Vlassides, & Block, 2002) or/and the presence of significant levels of alcohol present (Alexandre & Charpentier, 1998), while *Starm. bacillaris* population became undetectable after 21 days.

In Fig. 2, the growth dynamics of the mixed culture fermentations are illustrated. Remarkably, all couples showed comparable growth dynamics. When both yeasts were

co-inoculated (Fig. 2, panel A) the two population dynamics were similar in all cases (for all couples of strains tested). They achieved the stationary phase (almost 5 x 10^7 CFU/mL) in two days. A remarkable decrease of *Starm. bacillaris* population was registered on day 4, while *S. cerevisiae* population remained stable throughout the whole period. The early death of *Starm. bacillaris* cells appeared to be the result of the antagonistic effect of *S. cerevisiae* strains upon non-*Saccharomyces* yeasts, as also reported by Andorrà et al. (2010) in mixed fermentations with *Starm. bacillaris*, *H. uvarum* and *S. cerevisiae*.

A completely different picture emerged when *S. cerevisiae* was sequentially inoculated (Fig. 2, panel B). Compared to fermentations which *S. cerevisiae* strains pure inoculated, *Starm. bacillaris* caused a small reduction in rate of growth and the maximum population (remaining below 10^8 CFU/mL), achieved by both *S. cerevisiae* strains. There appeared to be an inhibitory effect of *Starm. bacillaris*, probably due to the high consumption of nutrients prior to *S. cerevisiae* inoculation, in agreement with the findings of Englezos et al. (2016). The cell viability of both yeasts, remained relative high during the fermentation and started to decrease from day 14 onwards. The capability of the *Starm. bacillaris* to dominate *S. cerevisiae* strains and persist up to the middle-end phase of the fermentation process was previously observed in laboratory scale fermentations (Cocolin, & Mills 2003; Rantsiou et al., 2012).

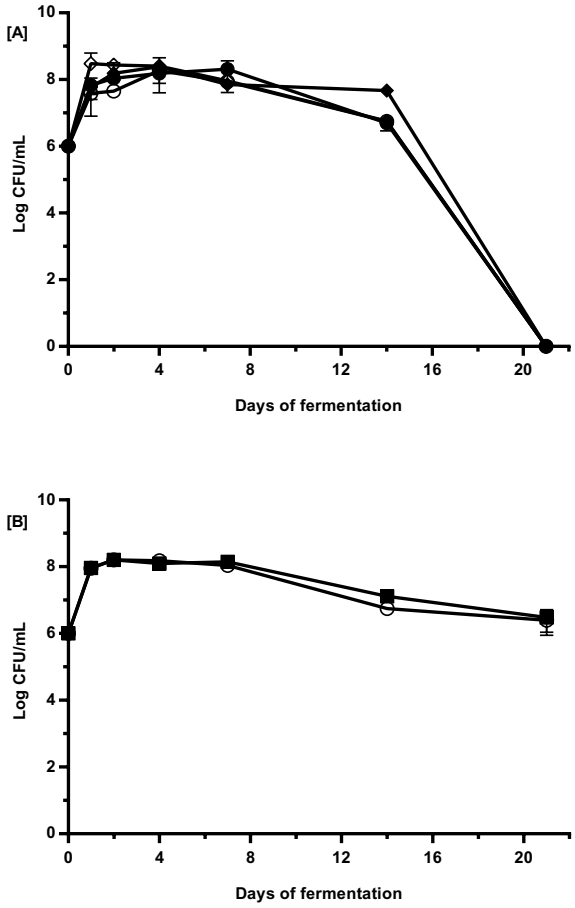

Fig. 1. Growth dynamics of *Starm. bacillaris* (A) and *S. cerevisiae* (B) strains in pure cultures. *Starm. bacillaris* strains: FC54 [*black circle*], EFR3B [*white circle*], C.z 02 [*black diamond*] and BC60 [*white diamond*]*, S. cerevisiae* strains: ScBa49 [*black square*] and ScBa50 [*white square*]. Counts are the mean CFU/mL values ± standard deviations. Data are representative of two independent experiments.

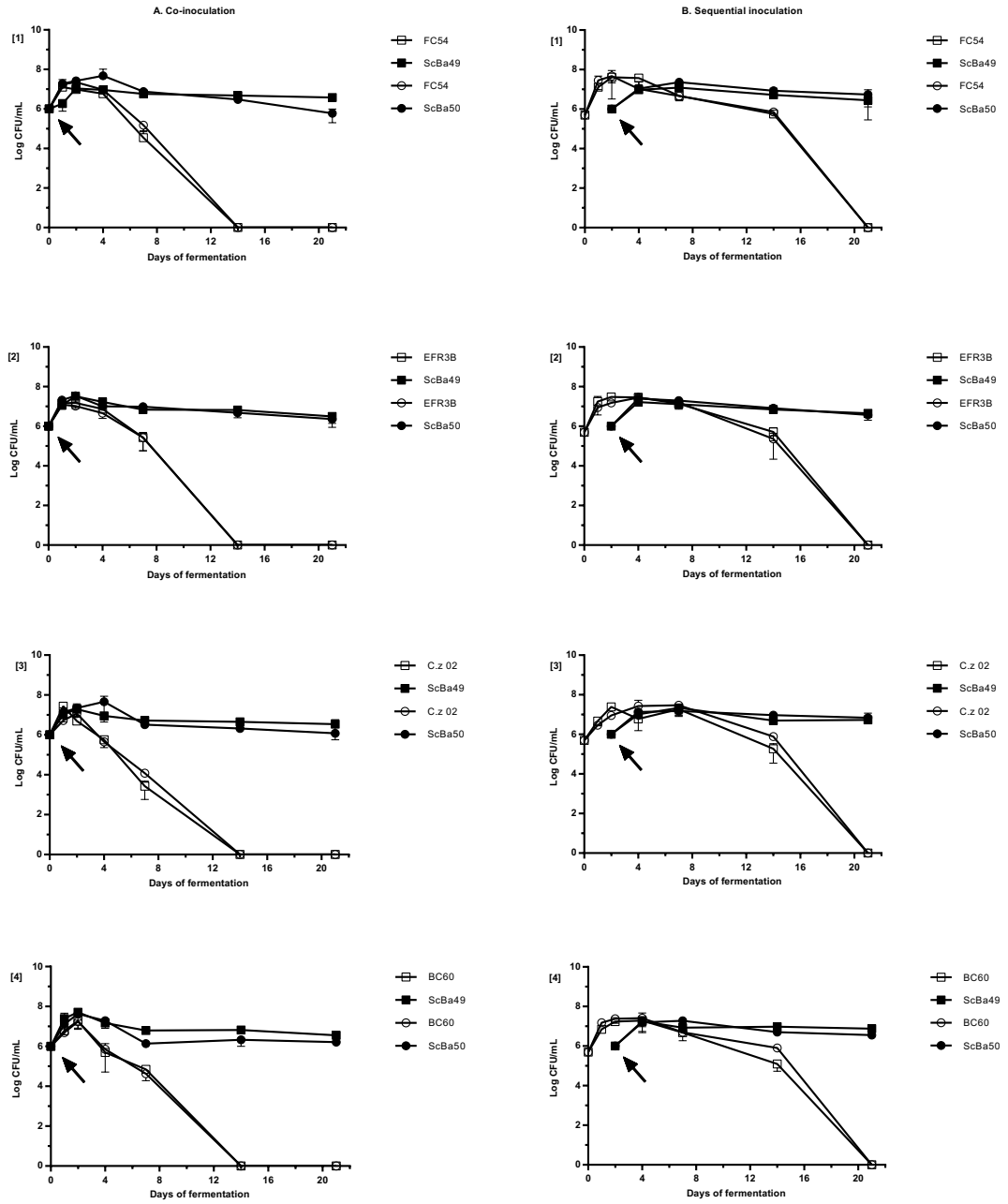

Fig. 2. Growth dynamics of the *Starm. bacillaris* (FC54 [1], EFR3B [2], C.z 02 [3] and BC60 [4]) co-inoculated (panel A) or sequentially (panel B) inoculated with *S. cerevisiae* (ScBa49 and ScBa50) strains. The arrows indicate *S. cerevisiae* inoculation. Counts are the mean CFU/mL values ± standard deviations. Data are representative of two independent experiments. Mixed fermentations: A. *Starm. bacillari*s (*white square*) with *S. cerevisiae* strain ScBa49 (*black square*) and B. *Starm. bacillaris* (*white circle*) with *S. cerevisiae* strain ScBa50 (*black circle*).

Aroma composition of the wines

Table 2 Semi-quantitative concentration of the volatile compounds (ratio among volatile compounds and internal standards) produced from the different inoculation protocols.

Compounds	***Kovats index***	*S. cerevisiae*	*Starm. bacillaris*	Co-inoculation	Sequential inoculation	Sig[1]	Odour description[2]
Alcohols							
(R,R)-2,3-Butanediol	1552	0.615 ± 0.010^{b}	0.525 ± 0.052^{ab}	0.433 ± 0.068^{ab}	0.472 ± 0.050^{ab}	**	Butter, creamy
(R,S-meso)-2,3-Butanediol	1587	0.164 ± 0.035^{b}	0.148 ± 0.016^{ab}	0.122 ± 0.016^{a}	0.134 ± 0.013^{ab}	*	Butter, creamy
2-Ethyl-hexanol	1501	0.005 ± 0.002^{a}	0.016 ± 0.002^{c}	0.013 ± 0.002^{b}	0.013 ± 0.002^{bc}	***	Sweet, floral , citrus
Hexanol	1367	0.145 ± 0.006^{a}	0.195 ± 0.003^{b}	0.193 ± 0.011^{b}	0.205 ± 0.006^{b}	***	Resin, flower, green
Isoamyl alcohol	1231	5.712 ± 0.153^{ab}	3.908 ± 0.416^{a}	6.466 ± 1.467^{b}	7.935 ± 2.033^{b}	**	Fusel, fruity, banana
2-Methyl-1-propanol	1113	0.002 ± 0.001^{a}	0.298 ± 0.021^{b}	0.380 ± 0.087^{b}	0.515 ± 0.176^{c}	***	Ethereal
1-Octanol	1568	0.019 ± 0.001	0.021 ± 0.003	0.018 ± 0.006	0.023 ± 0.002	NS	Floral, citrus, rose
2-Phenylethanol	1885	7.08 ± 0.688^{ab}	4.468 ± 0.599^{a}	7.460 ± 2.787^{ab}	10.116 ± 3.483^{b}	*	Floral, rose, sweet
1-Propanol	1052	0.003 ± 0.001^{a}	0.013 ± 0.005^{b}	0.013 ± 0.006^{b}	0.011 ± 0.003^{b}	**	Alcohol, pungent
Σ Alcohols		13.745 ± 0.971^{ab}	9.596 ± 0.965^{a}	15.097 ± 4.367^{ab}	19.422 ± 5.691^{b}	**	
Esters							
Diethyl succinate	1684	0.113 ± 0.010^{b}	0.053 ± 0.018^{a}	0.068 ± 0.029^{a}	0.089 ± 0.024^{ab}	**	Fruity
Ethyl acetate	nd	5.654 ± 0.021^{a}	6.132 ± 0.340^{a}	6.83 ± 1.229^{ab}	8.072 ± 0.968^{b}	**	Vanish, nail polish, fruity
Ethyl butanoate	1040	0.110 ± 0.008^{a}	0.111 ± 0.013^{a}	0.171 ± 0.034^{b}	0.203 ± 0.035^{b}	***	Sweet, fruity
Ethyl decanoate	1648	18.462 ± 3.763	18.065 ± 5.151	21.989 ± 9.634	20.830 ± 4.338	NS	Waxy, fruity, apple, grape
Ethyl 9-decenoate	1697	0.119 ± 0.011	0.082 ± 0.047	0.108 ± 0.055	0.129 ± 0.035	NS	Fruity
Ethyl dodecanoate	1834	3.244 ± 0.527^{a}	4.602 ± 0.942^{ab}	6.426 ± 3.597^{ab}	7.682 ± 0.928^{b}	*	Sweet, waxy

Ethyl heptanoate	1344	0.003 ± 0.001[a]	0.008 ± 0.003[a]	0.010 ± 0.002	0.011 ± 0.003[b]	**	Fruity, cognac
Ethyl hexadecanoate	2122	0.354 ± 0.047[b]	0.325 ± 0.016[b]	0.203 ± 0.085[a]	0.427 ± 0.092[b]	***	Waxy
Ethyl hexanoate	1249	1.685 ± 0.172[a]	2.709 ± 0.511[ab]	3.521 ± 0.783[bc]	4.027 ± 0.865[c]	***	Apple peel, fruit
Ethyl nonanoate	1543	0.016 ± 0.003	0.014 ± 0.006	0.016 ± 0.005	0.016 ± 0.003	NS	Fruity, rose, waxy
Ethyl octanoate	1445	16.577 ± 2.700	17.434 ± 5.562	22.392 ± 6.055	21.939 ± 5.577	NS	Fruity, fatty
Hexyl acetate	1286	0.209 ± 0.019[a]	0.293 ± 0.079[ab]	0.425 ± 0.148[b]	0.451 ± 0.153[b]	*	Fruit, herb
Isobutyl decanoate	1758	0.002 ± 0.002[a]	0.005 ± 0.002[ab]	0.011 ± 0.006[b]	0.011 ± 0.005[b]	*	Cognac, brandy, apricot,
Methyl decanoate	1599	0.047 ± 0.010	0.043 ± 0.013	0.047 ± 0.016	0.045 ± 0.011	NS	Winey, fruity, floral
3-Methyl-1-butyl acetate	1130	2.705 ± 0.604[ab]	1.662 ± 0.493[a]	3.159 ± 0.902[ab]	3.76 ± 1.287[ab]	*	Fruity, banana
2-Methylbuthyl octanoate	1664	0.040 ± 0.008[a]	0.039 ± 0.008[a]	0.103 ± 0.066[b]	0.097 ± 0.029[b]	*	Fruity
3-Methylbutyl pentadecanoate	1846	0.072 ± 0.011[a]	0.098 ± 0.008[ab]	0.272 ± 0.185[b]	0.279 ± 0.063[b]	*	Fruity
Nerolidyl acetate	1971	0.068 ± 0.002[b]	0.039 ± 0.008[a]	0.029 ± 0.010[a]	0.040 ± 0.006[a]	***	Floral, woody
2-Phenylethyl acetate	1815	1.128 ± 0.150[b]	0.519 ± 0.116[a]	1.118 ± 0.441[b]	1.398 ± 0.409[b]	**	Floral, rose, sweet, honey,
Σ Esters		50.609 ± 7.226	52.236 ± 11.261	66.894 ± 21.566	69.507 ± 12.556	NS	
Fatty acids							
Decanoic acid	2138	0.527 ± 0.013	0.385 ± 0.037	0.736 ± 0.335	0.495 ± 0.180	NS	Fatty, rancid
Dodecanoic acid	2266	0.014 ± 0.006[a]	0.014 ± 0.006[a]	0.082 ± 0.05[b]	0.039 ± 0.026[ab]	**	Fatty
Hexanoic acid	1838	0.205 ± 0.006	0.197 ± 0.006	0.256 ± 0.076	0.243 ± 0.029	NS	Cheese, sweaty, fatty
Octanoic acid	1986	0.580 ± 0.002	0.493 ± 0.010	0.66 ± 0.253	0.525 ± 0.139	NS	Fatty, rancid, cheese
Σ Fatty acids		1.326 ± 0.013	1.091 ± 0.043	1.733 ± 0.689	1.300 ± 0.358	NS	
Aldehydes and ketones							
Decanal	1506	0.008 ± 0.005	0.006 ± 0.003	0.008 ± 0.002	0.008 ± 0.003	NS	Soap, orange, peel, tallow

4-Methyl-benzaldehyde	1653	0.011 ± 0.003[a]	0.035 ± 0.01[b]	0.034 ± 0.01[b]	0.047 ± 0.018[b]	**	Almond
3-Methyl butanone	nd	0.111 ± 0.045	0.174 ± 0.089	0.188 ± 0.079	0.205 ± 0.045	NS	Camphor
2-Nonanone	1395	0.014 ± 0.005[a]	0.024 ± 0.008[ab]	0.042 ± 0.013[c]	0.034 ± 0.01[bc]	**	Green fruity, soap,
Σ Aldehydes and ketones		0.145 ± 0.047[a]	0.240 ± 0.079[ab]	0.272 ± 0.072[b]	0.293 ± 0.055[b]	**	
Terpenes and C13-norisoprenoid							
Linalool	1556	0.021 ± 0.001[a]	0.024 ± 0.002[a]	0.024 ± 0.003[ab]	0.027 ± 0.003[b]	**	Flower, lavender
β-Damascenone	1820	0.003 ± 0.003[a]	0.006 ± 0.002[b]	0.008 ± 0.002[b]	0.008 ± 0.002[b]	**	Apple, rose, honey
Σ Terpenes and C13-norisoprenoid		0.024 ± 0.003[a]	0.031 ± 0.003[b]	0.032 ± 0.003[bc]	0.035 ± 0.003[c]	**	
Sulphur compounds							
Benzothiazole	1916	0.01 ± 0.002[a]	0.077 ± 0.014[c]	0.039 ± 0.013[b]	0.048 ± 0.016[b]	***	Gasoline, rubber
3-(Methylthio)-1-propanol	1727	0.058 ± 0.018[c]	0.021 ± 0.003[a]	0.035 ± 0.008[ab]	0.039 ± 0.006[b]	***	Cauliflower, cabbage
Σ Sulphur compounds		0.066 ± 0.021[a]	0.098 ± 0.018[b]	0.072 ± 0.013[ab]	0.087 ± 0.019[ab]	*	
Lactones							
γ-Butyrolactone	1633	0.116 ± 0.014[b]	0.072 ± 0.006[a]	0.122 ± 0.021[ab]	0.092 ± 0.018[b]	**	Caramel, sweet

All data are expressed as average value ± standard deviation (n = 4 for *S. cerevisiae*, n= 8 for *Starm. bacillaris*, n=16 for co-inoculated and sequentially inoculated fermentations). Different Latin letters within the same row indicate significant differences among the applied inoculation protocols, according to the Tukey-b test ($p < 0.05$). nd: not determinable. [1]Sig: *, **, *** and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant respectively. [2]Odor descriptions were taken from http://www.flavornet.com and http://www.thegoodscentscompany.com

The main objective of this study was to evaluate how the inoculation strategy and strain selection could modulate the production of volatile aroma compounds during fermentation. The results are shown in Table 2, as an average value for each inoculation protocol applied in this study. Chromatographic analysis allowed the identification of 42 volatiles compounds (Fig1. Supplementary material) belonging to seven chemical families, including 9 alcohols, 19 esters, 4 fatty acids, 4 aldehydes and ketones, 2 terpenes and C13-norisoprenoids, 2 sulfur compounds and 1 lactone.

By comparing these secondary aroma compounds, all the fermentation protocols produced the same levels of alcohols, in concentrations that could enhance the desirable complexity in the wines (Rapp & Versini, 1991). The total alcohol concentration in the mixed fermentations was found to be very similar to this occurring in pure fermentations with *S. cerevisiae*, mainly due to the contribution of the main aromatic alcohols 2-phenylethanol and isoamyl alcohol, in accordance with the results reported by Sadoudi et al. (2012). On the other hand, aliphatic alcohols (1-propanol, 2-methyl-1-propanol, hexanol and 2-ethyl-hexanol) were detected in significantly higher concentrations, in the fermentations in which *Starm. bacillaris* was involved (Andorrà et al., 2010; Zara et al., 2014).

Total esters concentration was not affected by the presence of *Starm. bacillaris* and all the wines showed the same esters production pattern. Ethyl acetate, the most significant ester present in the wines (Swiegers et al., 2005), was generally produced in relative low quantitates, well below the spoilage and threshold values of 150 and 12 mg/L, respectively, reported in the literature for the red wines (Corison, Ough, Berg, & Nelson, 1979). In this context, low production of this ester (below 70 mg/L) is considered positive for the wine aroma and complexity, since it is associated with fruity, solvent and balsamic descriptors (Rapp, Pretorius, & Kugler, 1992). Both mixed and pure fermentations with *S. cerevisiae* produced wines with significant increased concentrations of 2-phenyl acetate (Andorrà et al., 2010), which contribute to the overall flavour of the young wines and thus the wines could be characterized by higher complexity, in accordance with Lambrechts & Pretorius (2000). The concentration of some pleasant esters, such as hexyl acetate, ethyl hexanoate, ethyl heptanoate, ethyl dodecanoate and ethyl butanoate was significantly higher in the mixed fermentations

(Fig. 3), highlighting an important positive interaction between the two species, as previously reported by Andorrà et al. (2010) in Macabeo must.

The concentration of specific aldehydes and ketones (2-nonanone and 4-methyl-benzaldehyde) was also found to be significantly higher in the wines produced by mixed fermentations and due to the low threshold values of these compounds they could enhance the overall aroma and bouquet of the wines (Lambrechts & Pretorius, 2000). The production of free terpenes and C13-norisoprenoids was also found to be significantly higher in the mixed fermentations compared to the pure *S. cerevisiae* fermentations, as already reported in Sadoudi et al. (2012) and Whitener et al. (2016). Regarding the fatty acids production, no significant differences were observed in the wines, with only exception of the dodecanoic acid, which was produced in higher quantities in the co-inoculated trials. Finally, the concentration sulphur compounds and lactones increased significantly in pure culture fermentations with *Starm. bacillaris* and *S. cerevisiae* respectively.

The aroma values of 30 compounds (Table 2), which differences among the inoculation protocols were significant were analysed using a Principal Component Analysis (PCA), in order to evaluate the correlation among samples and aroma compounds. The first two components obtained explained the 63 % of the total variance, while the replicates were clustered quite well indicating a high experimental reproducibility (Fig. 3). The first principal component (PC1) was correlated positively with the most important esters, isoamyl alcohol and 2-phenylethanol. The second principal component (PC2) was correlated positively to diethyl succinate, 2-phenyl ethyl acetate and negatively to hexanol, 2-ethyl hexanol, β-damascenone and 1-propanol.

As it can be seen from the PCA output, the samples were classified into two groups (Fig. 3, Panel B). One group clustered mixed fermentations (both co-inoculated and sequentially inoculated) and pure fermentations conducted by *Starm. bacillaris* strains, while the other group included the pure fermentations performed by *S. cerevisiae* strains. Wines produced from mixed fermentations, showed a homogeneous distribution in the PCA plot independent the couple of strains used, with only exception a major part of wines produced from sequentially inoculated cultures which were grouped closer to pure fermented wines with *Starm. bacillaris*. Based on these results, it

can be speculated that *Starm. bacillaris* is effective in impacting and modulating the aroma profiles of the wines produced from the mixed fermentations, in agreement with general observations that non-*Saccharomyces* yeasts could enhance the organoleptic complexity of the wines (Jolly et al., 2013).

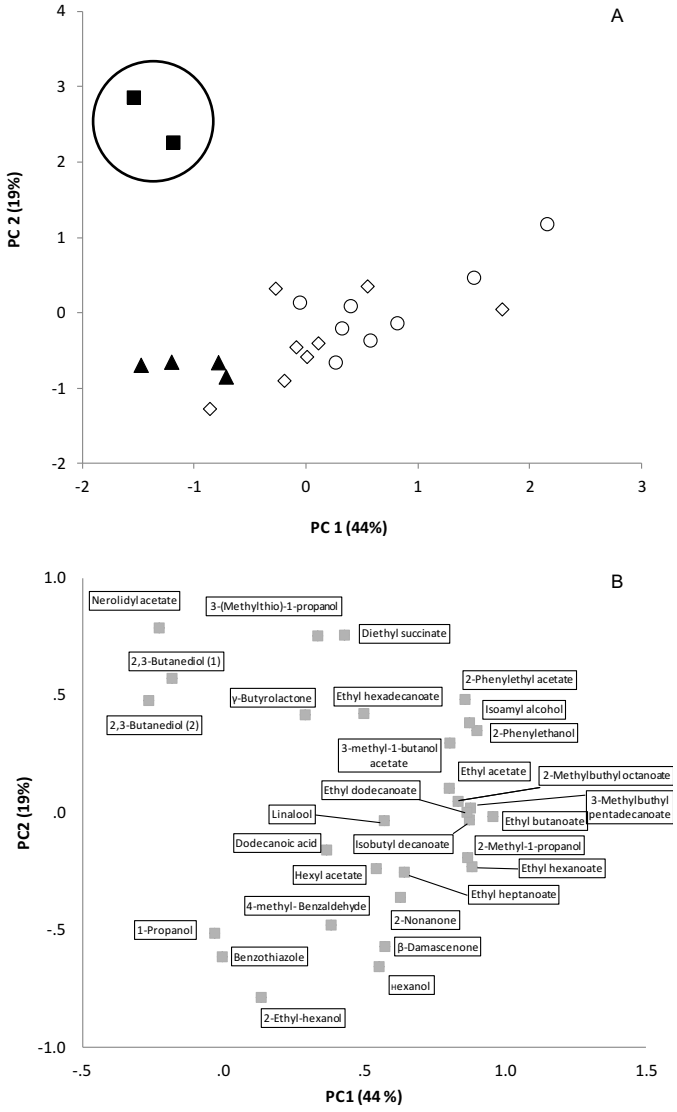

Fig. 3. Score plot (A) and loading plot (B) of the first and second principal components after analysis of the volatile aroma compounds produced from the pure and mixed culture fermentations. Inoculation protocols were represented as: *S. cerevisiae* (*black square*), *Starm. bacillaris* (*black triangle*) co-inoculation (*white circle*) and sequential inoculated fermentations (*white diamond*).

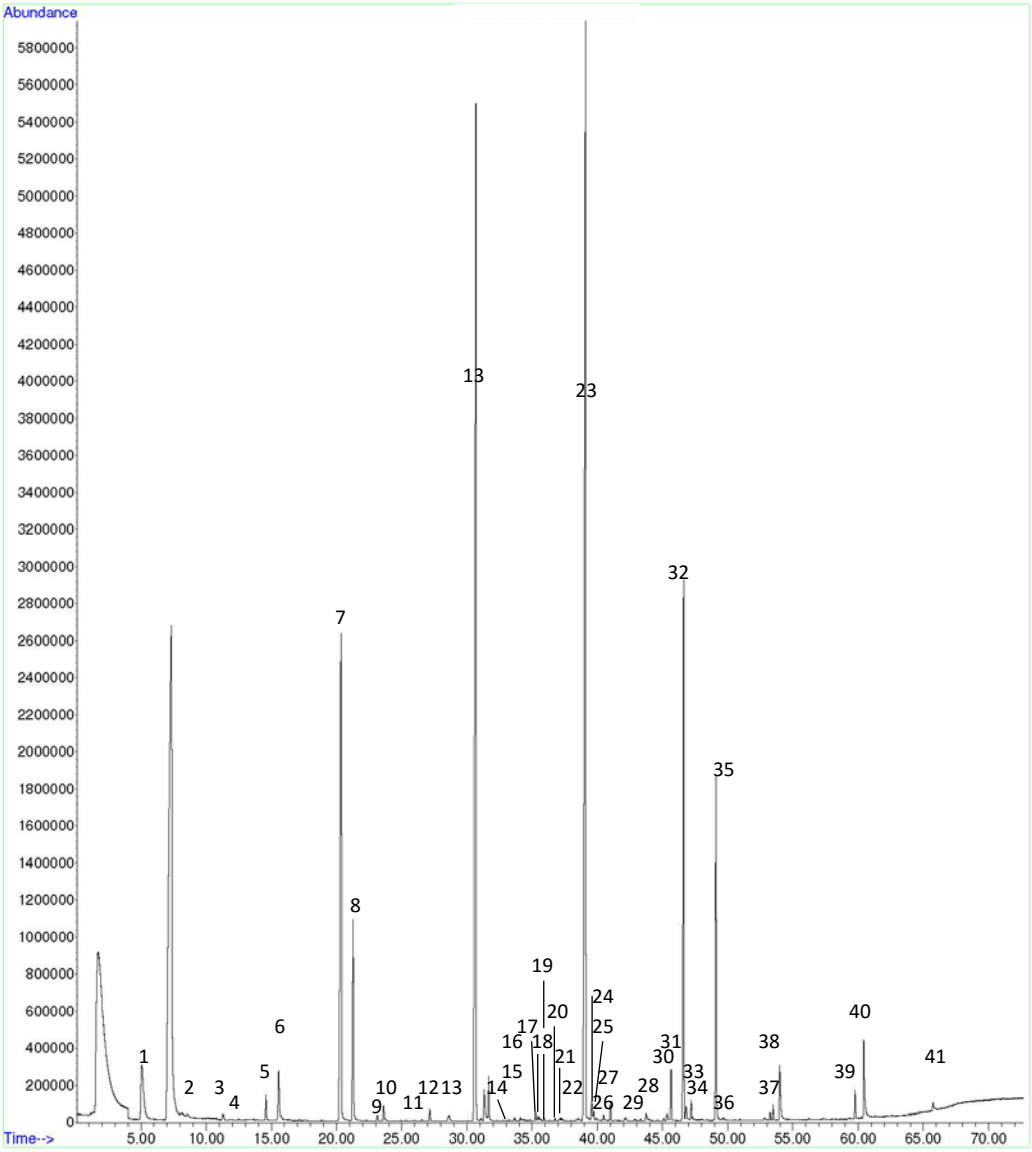

Fig. 1. Supplementary data. Chromatogram of fermented wine: 1 Ethyl acetate, 2 3-Methyl butanone, 3 Ethyl butanoate, 4 1-Propanol, 5 2-Methyl-1-propanol, 6 3-Methyl-1-butyl acetate, 7 Isoamyl alcohol, 8 Ethyl hexanoate, 9 Hexyl acetate, 10 Ethyl heptanoate, 11 Hexanol, 12 2-Nonanone, 13 Ethyl octanoate, 14 2-Ethyl-hexanol, 15 Decanal, 16 Ethyl nonanoate, 17 (R,R)-2,3-Butanediol, 18 Linalool, 19 1-Octanol, 20 (R,S-meso)-2,3-Butanediol, 21 Methyl decanoate, 22 γ-Butyrolactone, 23 Ethyl decanoate, 24 4-Methyl-benzaldehyde, 25 2-Methylbuthyl octanoate, 26 Diethyl succinate, 27 Ethyl 9-decenoate, 28 3-(Methylthio)-1-propanol, 29 Isobutyl decanoate, 30 2-Phenylethyl acetate, 31 β-Damascenone, 32 Ethyl dodecanoate, 33 Hexanoic acid, 34 3-Methylbutyl pentadecanoate, 35 2-Phenylethanol, 36 Benzothiazole, 37 Nerolidyl acetate, 38 Octanoic acid, 39 Ethyl hexadecanoate, 40 Decanoic acid, 41 Dodecanoic acid.

Analytical profiles of the wines produced by the pure and mixed cultures

The chemical composition of the wines produced from pure and mixed cultures are shown in Table 3. Pure culture fermentations of the *Starm. bacillaris* strains exhibited a clear fructophilic pattern, leaving only glucose in the medium (29.3 – 35.9 g/L) confirming the clear fructophilic character of this species (Rantsiou et al., 2012). Concerning, ethanol production all the wines reached significant values ranging from 11.8 to 12.2 (% v/v). On the other hand, *S. cerevisiae* strains exhibited more complete utilization of sugars and produced less glycerol (7.1 – 7.3 g/L) than the wines fermented by *Starm. bacillaris* in pure culture, in agreement with previous studies (Englezos et al., 2015; Suzzi et al., 2012). Concerning acetic acid production all the strains gave values not greater than 0.50 g/L.

Fermentations conducted by using a combination of *Starm. bacillaris* and *S. cerevisiae,* independent from the inoculation strategy applied, produced partially fermented wines (86 – 97 % total sugar consumption) (Table 4). Compared to sequential inoculated fermentations, co-inoculated fermentations produced wines with less residual sugars. This data was in accordance with the plate count results, since *Starm. bacillaris* in sequential fermentations affected *S. cerevisiae* growth and subsequently its metabolic activity. The inhibitory effect observed in these fermentations, validate the observations by Englezos et al. (2016, accepted manuscript). Comparing residual sugar composition, co-inoculated fermentations fermented fructose at a lower rate, leaving a higher residual fructose concentration, compared to sequential inoculated wines, probably due to the competitive ability of *S. cerevisiae* over *Starm. bacillaris* yeast cells. Chemical analyses values for the co-inoculated wines did not vary substantially from those of *S. cerevisiae* strains in pure cultures, in agreement with Englezos et al. (2016, accepted manuscript) and Soden et al. (2000). On the contrary, sequential inoculated wines were very similar in composition to the wines produced by *Starm. bacillaris* in pure culture. Glycerol concentration slightly increased (8.8 – 9.8 g/L), when compared to the pure culture *S. cerevisiae* wines (Romboli, Mangani, Buscioni, Granchi, & Vincenzini, 2015; Suzzi et al. 2012). The acetic acid production ranged from moderate amounts to values up to 0.4 g/L and fermentation purities were also very low (0.02 – 0.03).

Conclusion

Here, new information about the influence of *Starm. bacillaris* and *S. cerevisiae* mixed fermentations on the formation of volatile aroma compounds was presented. The results obtained revealed significant differences between the two yeast species and high similarities among the two inoculation protocols investigated in this study. As shown, mixed culture fermentations resulted in greater complexity due to the higher production of volatile compounds, independently of the couple tested. A better knowledge of the environmental factors (such as nitrogen composition and concentration), which modulate the yeast growth, will allow a greater understanding and management of the production of specific metabolites during the alcoholic fermentation.

Acknowledgements

The conducted research received funding from the EU (FP7/2007-2013), under grant agreement no. 315065- WILDWINE (www.wildwine.eu). The information in this document only reflects the authors' views and the Community is not liable for any use that may be made of the information contained herein.

References

Alexandre, H., and Charpentier, C. (1998). Biochemical aspects of stuck and sluggish fermentation in grape must. *Journal of Industrial Microbiology and Biotechnology* 20, 20-27.

Andorrà, I., Berradre, M., Mas, A., Esteve-Zarzoso, B., Guillamón, J. M. (2012). Effect of mixed culture fermentations on yeast populations and aroma profile. *LWT - Food Science and Technology*, 49, 8-13.

Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J. M., Esteve-Zarzoso, B. (2010). Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *European Food Research and Technology* 23, 215-224.

Bruwer, J., Saliba, A., Miller, B. (2011). Consumer behaviour and sensory preference differences: implications for wine product marketing. *Journal of Consumer Marketing* 28, 5-18.

Bokulich, N., Swadener, M., Sakamoto, K., Mills, D. A., Bisson, L. F. (2015). Sulfur dioxide treatment alters wine microbial diversity and fermentation progression in a dose-dependent fashion *American Journal of* Enology and Viticulture 66, 73-79.

Boulton, R. B., Singleton, V. L., Bisson, L. F., Kunkee, R.E. (1996). Principles and practices of winemaking. New York: Chapman & Hall, (Chapter 12).

Ciani, M. and Maccarelli, F. (1998). Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology* 14, 199-203.

Ciani, M. and Comitini, F. (2015). Yeast interactions in multi-starter wine fermentation. *Current Opinion in Food Science* 1, 1–6.

Cocolin, L. and Mills D. A. (2003). Wine yeast inhibition by sulfur dioxide: a comparison of culture-dependent and independent methods. *American Journal of Enology and Viticulture* 54, 125-130.

Corison, C. A., Ough, C. S., Berg, H. W., Nelson, K. E. (1979). Must acetic acid and ethyl acetate as mold rot indicators in grapes. *American Journal of Enology and Viticulture* 30, 130-134.

Cramer, A.C., Vlassides, S., Block, D. E. (2002). Kinetic model for nitrogen-limited wine fermentations. *Biotechnology and Bioengineering* 77, 49-60.

Delfini, C., Cocito, C., Bonino, M., Schellino, R., Gaia, P., Baiocchi, C. (2001). Definitive evidence for the actual contribution of yeast in the transformation of neutral precursors of grape aromas. *Journal of Agricultural and Food Chemistry 49,* 5397-5408.

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., Cocolin L. (2015). Exploitation of the non-Saccharomyces yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: physiological and molecular characterization. *International Journal of Food Microbiology* 199, 33-40.

Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Ortiz-Julien, A., Gerbi V., Rolle, L., Cocolin, L. (2016). *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. *Applied Microbiology and Biotechnology* 100, 5515-5526.

Fleet, G.H. (2003). Yeasts interactions and flavour. *International Journal of Food microbiology 86*, 11-22.

Fleet, G.H. (2008). Wine yeast for the future. *FEMS Yeast Research* 8, 979-995.

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M. (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. *Food Microbiology* 33, 271-281.

González-Barreiro, C., Rial-Ortero, R., Cancho-Grande, B., Simal-Gándara, J. (2015). Wine aroma compounds in grapes: a critical review. *Critical reviews in food science and nutrition* 55, 202-218.

Jolly, N.P., Varela, C. and Pretorius, I.S. (2013). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Research* 14, 215-237.

Lambrechts, M. G., and Pretorius, I. S. (2000). Yeasts and its importance to wine aroma. *South African Journal of Enology and Viticulture*, *21*, 97-129.

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., et al. (2012). *Candida zemplinina* can reduce acetic acid production by *Saccharomyces cerevisiae* in sweet wine fermentations. *Applied and Environmental Microbiology*, *78*, 1987-1994.

Rapp, A. and Mandery, H. (1986). Wine aroma. *Experientia* 42, 873-884.

Rapp, A. and Versini, G. (1991). Influence of nitrogen compounds in grapes on aroma compounds in wine. Proceedings of the International Symposium on Nitrogen in Grapes and Wine, Seattle, USA (American Society of Enology and Viticulture: Davis, CA), 156–164.

Rapp, A. Pretorius, P. Kugler, D. (1992). Foreign an undesirable flavours in wine. *Developments in Science* 28, 485–522.

Romano, P., Suzzi, G., Comi, G. Zironi, R. (1993). Higher alcohol and acetic acid production by apiculate wine yeasts. *Journal of Applied Bacteriology* 73, 126-130.

Romano, P., Fiore, C., Paraggio, M., Caruso, M., Capece, A. (2003). Function of yeast species and strains in wine flavour. *International Journal of Food Microbiology* 86, 169-180.

Rolle, L., Giordano, M., Giacosa, S., Vincenzi, S., Río Segade, S., Torchio, F., Perrone, B., Gerbi, V. (2012). CIEL*a*b* parameters of white dehydrated grapes as quality markers according to chemical composition, volatile profile and mechanical properties. *Analytica Chimica Acta* 732, 105-112.

Rolle, L., Torchio, F., Giacosa, S., Río Segade, S. (2015). Berry density and size as factors related to the physicochemical characteristics of Muscat Hamburg table grapes (Vitis vinifera L.). *Food Chemistry* 173, 105-113.

Romboli, Y., Mangani, S., Buscioni, G., Granchi, L., Vincenzini, M. (2015). Effect of *Saccharomyces cerevisiae* and *Candida zemplinina* on quercetin, vitisin A and hydroxytyrosol contents in Sangiovese wines. *World Journal of Microbiology and Biotechnology* 31, 1137-1145.

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J., Ballester, J., Vichi, S., Guérin-Schneider, R., Caixach, J., Alexandre, H. (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiology* 32, 243-253

Sun, S.Y., Che, C.Y., Sun, T.F., Lv, Z.Z., He, S.X., Gu, H.N., Shen, W.J., Chi da, C., Gao, Y. (2013). Evaluation of sequential inoculation of *Saccharomyces cerevisiae* and *Oenococcus oeni* strains on the chemical and aromatic profiles of cherry wines. *Food Chemistry* 138, 2233–2241.

Suzzi, G., Schirone, M., Sergi, M., Marianella, R.M., Fasoli, G., Aguzzi, I., Tofalo, R. (2012). Multistarter from organic viticulture for red wine Montepulciano d' Abruzzo production. Frontiers in Microbiology 3, 1-10.

Soden, A., Francis, I. L., Oakey, H., Henschke, P. A. (2000). Effects of co-fermentation with *Candida stellata* and *Saccharomyces* cerevisiae on the aroma and composition of Chardonnay wine. *Australian Journal of Grape and Wine Research* 6, 21-30.

Swiegers, J.H., Bartowksy, E.J., Henschke, P.A., Pretorius I.S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Australian Journal of Grape and Wine Research* 11, 127-138.

Swiegers, J.H., Francis, I.L., Herderich, M.J., Pretorius, I.S. (2006). Meeting consumer expectations through management in vineyard and winery: the choice of yeast for fermentation offers great potential to adjust the aroma of Sauvignon Blanc wine. *Australian and New Zealand Wine Industry Journal* 21, 34-42.

Whitener, M.E.B., Carlin, S., Jacobson, D., Weighill, D., Divol, B., Conterno, L., Du Toit, M., Vrhovsek, U. (2015). Early fermentation volatile metabolite profile of non-*Saccharomyces* yeasts in red and white grape must: A targeted approach. *LWT - Food Science and Technology* 64, 412-422.

Whitener, M.E.B., Stanstrup, J., Panzeri, V., Carlin, S., Divol, B., Du Toit, M., Vrhovsek, U. (2016). Untangling the wine metabolome by combining untargeted SPME–GCxGC-TOF-MS and sensory analysis to profile Sauvignon blanc co-fermented with seven different yeasts. *Metabolomics* 12*,* 1-25.

Zara, G., Mannazzu, I., Del Caro, A., Budroni, M., Pinna, M. B., Murru, M., Farris, G.A., Zara, S. (2014). Wine quality improvement through the combined utilisation of yeast hulls *and Candida zemplinina*/*Saccharomyces cerevisiae* mixed starter cultures. *Australian Journal of Wine and Grape Research* 20, 199–207.

Conclusions and Future perspectives

With this series of studies an improved knowledge of phenotypic and genotypic biodiversity of *Starm. bacillaris* was obtained, moreover the impact of *Starm. bacillaris* and *S. cerevisiae* mixed fermentations on wine composition was described in detail. The studies were grouped in two main parts and each one of them has shown important advancements with respect to the state of the art.

In order to access the phenotypic and genotypic biodiversity of *Starm. bacillaris*, a collection of sixty-three isolates from different Italian regions was subjected to physiological and molecular characterizations with emphasis on parameters of oenological interest. The results obtained from the analysis of these isolates, demonstrated the potential production of extracellular enzymes, in agreement with general observations, that non-*Saccharomyces* yeasts are more probable to possess these enzymes than *S. cerevisiae* strains (Strauss et al., 2001). The ability of *Starm. bacillaris* to grow at different concentrations of ethanol and SO_2 was also evaluated. The results, uncovered that ethanol concentration affected the lag phase of the isolates, by increasing its length, while 50 mg/L of SO2 are sufficient to inhibit its growth. On the other hand, molecular characterization, revealed a high level of genetic similarity between isolates of *Starm. bacillaris* coming from different isolation origins. 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. Its strong fructophilic character and its ability to produce low quantities of ethanol and acetic acid with high amounts of glycerol were confirmed in accordance with previous findings (Tofalo et al., 2012). To our knowledge, this is the first time that a large number of *Starm. bacillaris* isolates were subjected to physiological and molecular characterizations.

Taking in consideration the interesting phenotypic characteristics, a partnership with selected *S. cerevisiae* strains was proposed in order to improve wine quality by enhancing or reducing the production of target metabolites. The impact of *Starm. bacillaris* in mixed fermentations with *S. cerevisiae* can be more definitive when specific metabolites are targeted, such as reducing the ethanol and increasing the glycerol and aroma complexity in the wines. Indeed, the inoculation timing and combination of strains is critical to achieve these objectives, without reducing wines quality.

In this context, the importance of the inoculation time of *S. cerevisiae* on wine

composition in mixed fermentations with *Starm. bacillaris* was investigated in Barbera grape musts. Sequential fermentations with 48h delay, which enables *Starm. bacillaris* growth, led to the production of wines with significant reductions in ethanol and an increase in glycerol, while co-inoculated fermentations produced wines with a chemical composition very close to those produced by *S. cerevisiae* strains in pure cultures. The poor performance of co-inoculated fermentation could be partially explained by the competitive ability of *S. cerevisiae* over non-*Saccharomyces* yeast cells, probably due to cell-to-cell contact mediated mechanisms and depletion of nutrients (Albergaria and Arneborg, 2016; Ciani and Comitini, 2015). Coupling the right strains of *Starm. bacillaris* and *S. cerevisiae* in a sequential fermentation has an impact to the wine quality, in particular on residual sugars content. Particularly, *S. cerevisiae* commercial strain showed a better pairing with different *Starm. bacillaris* strains in sequential fermentations, while another indigenous *S. cerevisiae* strain left a significantly higher residual sugar content in wines.

Concerning the aroma profile of the wines, significant differences were found between the two yeast species and high similarities between co-inoculated and sequentially inoculated wines with 48 hours delay. Wines produced from mixed culture fermentations contained higher levels of aldehydes and ketones, as well as terpenes and C-13 norisoprenoids compared to those produced by *S. cerevisiae* in pure culture fermentations. No significant differences were observed for esters and fatty acids production. The results highlighted the important contribution of this non-*Saccharomyces* yeast on the chemical profile of the wines and support further application in mixed fermentations with *S. cerevisiae.*

At the moment, mixed starter fermentations with selected *Starm. bacillaris* and *S. cerevisiae* strains is considered as a state of art strategy to increase or decrease the production of target metabolites. A better knowledge of the fermentation conditions in which the yeast species are subjected, such as strain compatibility, physicochemical and nutrition parameters, are little understood and require further investigation.

References

Albergaria, H., Arneborg, N. (2016). Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation processes: role of physiological fitness and microbial interactions. *Applied Microbiology and Biotechnology* 100, 2035-2046.

Ciani, M., Comitini, F. (2015). Yeast interactions in multi-starter wine fermentation. *Current Opinion in Food Science and Technology* 1, 1-6.

Strauss, M.L.A., Jolly, N.P., Lambrechts, M.G., van Resemburg, P. (2001). Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. *Journal of Applied Microbiology* 91, 182-190.

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., Suzzi, G., 2012. Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiology* 29, 18-26.

Annex: Research products

The underline in the Author field means first author publication or first author-equivalent contribution to the study.

ISI indexed journal articles

Alessandria, V., Marengo, F., Englezos, V., Gerbi, V., Rantsiou, K., Cocolin, L. (2015). Mycobiota of Barbera grapes from Piedmont region from a single vintage year. *American Journal of Enology and Viticulture* 66, 244-250.

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., Cocolin, L. (2015). Exploitation of the non-Saccharomyces yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: physiological and molecular characterizations. *International Journal of Food Microbiology* 199, 33-40.

Masneuf-Pomarede, I., Juquin, E., Miot-Sertier, C., Renault, P., Laizet, Y., Salin, F., Alexandre, H., Capozzi, V., Cocolin, L., Colonna-Ceccaldi, B., Englezos, V., Girard, P., Gonzalez, B., Lucas, P., Mas, A., Nisiotou, A., Sipiczki, M., Spano, G., Tassou, C., Bely, M., Albertin, W. (2015) The yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) shows high genetic diversity in winemaking environments. *FEMS Yeast Research* 15, fov045. doi: 10.1093/femsyr/fov045.

Laureano, J., Giacosa, S., Río Segade, S, Torchio, F., Cravero, F., Gerbi, V., Englezos, V., Carboni, C., Cocolin, L., Rantsiou, K., Faroni, L. D. R., Rolle, L. (2016). Effect of continuous exposure to ozone gas and electrolyzed water on the skin hardness of table and wine grape varieties. *Journal of Texture Studies* 47, 40-48.

Cravero, F., Englezos, V., Torchio, F., Giacosa, S., Río Segade, S., Gerbi, Rantsiou, K., Rolle, L., Cocolin, L. (2016). Post-harvest control of wine grape mycobiota using electrolyzed water. *Innovative Food Science and Emerging Technologies* 35, 21-28.

Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Ortiz-Julien, A., Gerbi, V., Rolle, L., Cocolin, L. (2016). *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. *Applied microbiology and biotechnology* 12, 5515-5526.

Cravero, F., Englezos, V., Rantsiou, K., Torchio, F., Giacosa, S., Río Segade, S., Gerbi, Rolle, L., Cocolin, L. (2016). Ozone treatments of post harvested wine grapes: Impact on fermentative yeasts and wine chemical properties. *Food Research International* 87, 134-141.

Englezos, V., Torchio, F., Cravero, F., Marengo, F., Giacosa S., Gerbi, V., Rantsiou, K., Rolle, L., Cocolin, L. (2016). Aroma profile and composition of Barbera wines obtained by mixed fermentations of *Starmerella bacillaris* (synonym *Candida zemplinina*) and *Saccharomyces cerevisiae*. *LWT - Food Science and Technology* 73, 567-575.

Paissoni, M.A., Río Segade, S., Giacosa, S., Torchio, F., Cravero, F., Englezos, V., Rantsiou, K., Carboni C., Gerbi, V., Teissedre, P.L., Rolle L. (2016). Impact of post-harvest ozone treatments on the skin phenolic extractability of red wine grapes cv Barbera and Nebbiolo (Vitis vinifera L.). *Food Research International*, 98, 68-78.

Rantsiou, K., Englezos, V., Torchio, F., Risse, P.A., Cravero, F., Gerbi, V., Rolle, L., Cocolin, L. (2017). Modelling of the fermentation behavior of *Starmerella bacillaris*. *American Journal of Enology and Viticulture*, 68, 378-385.

Rantsiou, K., Marengo, F., Englezos, V., Torchio, F., Giacosa, S., Rolle, L., Gerbi, V., Cocolin, L. (2017). *Saccharomyces cerevisiae* biodiversity in Monferrato, North West Italy and selection of indigenous starter cultures for Barbera wine production. *Italian Journal of Food Science*, 29, 518-536.

Rolle, L., Englezos, V., Torchio, F., Cravero, F., Río Segade, S., Rantsiou, K., Giacosa, S., Gambuti, A., Gerbi, Cocolin, L. (2018). Alcohol reduction in red wines by technological and microbiological approaches: a comparative study. *Australian Journal of Grape and Wine Research*, in press.

Englezos, V., Giacosa, S., Rantsiou, K., Rolle, L., Cocolin, L. (2017). *Starmerella bacillaris* in winemaking: opportunities and risks. Current Opinion in Food Science, in press.

Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Ortiz-Julien, A., Gerbi, V., Rolle, L., Cocolin, L. (2018). Oxygen availability and strain combination modulate yeast growth dynamics in mixed culture fermentations of grape must with *Starmerella bacillaris* and *Saccharomyces cerevisiae*. *Food Microbiology*, 69, 179-188.

Non-ISI indexed journal articles

Englezos, V., Rantsiou, K., Cocolin, L. (2017). Nuove strategie nella riduzione del grado alcoolico dei vini. Le potenzialità di *Starmerella bacillaris*. *Corriere viticolo* 1, 22-23.

In-volume contributions

Rolle L., Carboni C., Gerbi V., Giacosa S., Torchio F., Rio Segade S., Paissoni M., Ossola C., Englezos V., Cravero F., Rantsiou K., Cocolin L. Esperienze di trattamenti dell'uva da vino in post-raccolta con ozono gassoso e acqua ozonizzata. Vitenda 2017, Pagine. Ed. Vit. En., Calosso (Asti, IT). ISBN 978-88-86055-34-5. www.viten.net.

Oral presentations at congresses

Englezos, V., Rantsiou, K., Ortiz-Julien, A., Torchio, F., Cravero, F., Rolle, L., Cocolin, L. Mixed fermentations with *Starmerella bacillaris* (synonym *Candida zemplinina*) and *Saccharomyces cerevisiae* for the reduction of the alcohol in the wine. Oeno 2015 Bordeaux (France). 27 June-01 July 2015.

Proceedings at congresses

Englezos, V., Rantsiou, K., Cocolin, L. *C. zemplinina*: genetic biodiversity and extracellular hydrolytic enzymes production. MD2013, Torino (Italy), 23-25 October 2013.

Marengo, F., Englezos, V., Alessandria, V., Rantsiou, K., Cocolin, L. Yeast ecology of twelve vineyards of Barbera within Monferrato wine area and selection of

autochthonous *Saccharomyces cerevisiae* strains. MD2013, Torino (Italy), 23-25 October 2013.

Albertin, W., Chasseriaud, L., Miot-Sertier, C., Capozzi, V., Cocolin, L., Englezos, V., Gonzalez, B., Mas, A., Nisiotou, A., Sipiczki, M., Spano, G., Bely, M., Masneuf-Pomarede, I. Development of microsatellite markers for the rapid and reliable genotyping of *Candida zemplinina* at strain level. Ecologie, Genomique, Genetique des populations, Approches globales, 2014.

Granchi, L., Ganucci, D., Englezos, V., Mangani, S., Vincenzini, M. Behaviour of *C. zemplinina* as affected by the pumping over operation. Environmental sustainability and food security, Potenza (Italy), 2014.

Alessandria, V., Marengo, F., Englezos, V., Rantsiou, K., Cocolin, L. Study of the microbial ecology of Barbera grapes. Food Micro 2014 (France), 1-4 September 2014.

Cravero, F., Englezos, V., Torchio, F., Rantsiou, K., Giacosa, S., Río Segade, S., Carboni, C., Rolle, L., Cocolin, L. Use of ozone for Clean-in-Place (CIP) in the winery. Enoforum, Vicenza (Italy), 5-7 May 2015.

Englezos, V., Rantsiou, K., Ortiz-Julien, A., Torchio, F., Cravero, F., Rolle, L., Cocolin, L. An innovative tool to reduce the production of alcohol in the wine: mixed fermentations with *Starmerella bacillaris* (synonym *Candida zemplinina*) and *Saccharomyces cerevisiae*. Enoforum, Vicenza (Italy), 5-7 May 2015.

Rantsiou, K., Englezos, V., Risse, P. A., Cravero, F., Torchio, F., Rolle, L., Cocolin, L. Fermentative behavior of *Candida zemplinina*. Enoforum, Vicenza (Italy), 5-7 May 2015.

Englezos, V., Torchio, F., Giacosa, S., Cravero, F., Río Segade, S., Laureano, J., Rantsiou, K., Carboni, C., Rolle, L., Gerbi, V., Cocolin, L. Effect of ozone treatments on wine grape microbiota, skin mechanical properties and phenolic compounds extractability. 22nd World Congress & Exhibition – Ozone and advanced oxidation leading-edge science and technologies (Spain). 28 June-3 July 2015.

Cravero, F., Rantsiou, K., Englezos, V., Torchio, F., Giacosa, S., Rio Segade, S., Rolle, L., Cocolin, L. Low environmental impact sanitization treatments: effect of electrolyzed water and ozone treatments on grape and wine microbiota. Oeno 2015 (France). 27 June – 01 July, 2015.

Cocolin, L., Englezos, V., Torchio, F., Cravero, F., Ortiz-Julien, A., Gerbi, V., Rolle, L., Rantsiou, K. Use of *Candida zemplinina* in wine making: phenotypic diversity. 8th International Symposium Oenoviti International network "Non-conventional microorganism in wine making". Padova, Italy. 27-28 October, 2015

Paissoni, M.A., Río Segade, S., Giacosa, S., Torchio, F., Cravero, F., Englezos, V., Rantsiou, K., Cocolin, L., Carboni C., Teissedre, P.L., Gerbi, V., Rolle L.(2016). Effect of post-harvest ozone treatments on the skin phenolic composition and extractability of red wine grapes cv Nebbiolo and Barbera. Macrowine (Switzerland). 27 – 30 June, 2016.

Peer review activities

Peer reviewer for International Journal of Food Science and Technology.

Master thesis co-supervision

Identificazione molecolare e caratterizzazione fisiologica di ceppi di *Candida zemplinina* ad interesse enologico (2013). Student: Mattia Roà. Corso di Laurea Magistrale Interateneo in Scienze Viticole ed Enologiche, Università degli Studi di Torino.

Modellizzazione del comportamento fermentativo di *Candida zemplinina* (2014). Student: Paul-Andre' Risse. Corso di Laurea Magistrale Interateneo in Scienze Viticole ed Enologiche, Università degli Studi di Torino.

Saccharomyces cerevisiae-Starmerella bacillaris fermentazioni con inocula sequenziale per la produzione di vini bianchi (2016). Student: Atte Sukki. Corso di Laurea Magistrale Interateneo in Scienze Viticole ed Enologiche, Università degli Studi di Torino.

Utilizzo di *Starmerella bacillaris* in mosti da diverse varietà di uve rosse ed impatto della macro-ossigenazione in fermentazione (2016). Student: Federico Ugolini. Corso di Laurea Magistrale Interateneo in Scienze Viticole ed Enologiche, Università degli Studi di Torino.

Interazioni microbiche tra *Starmerella bacillaris* e *Saccharomyces cerevisiae* in fermentazioni miste di mosto da uve Nebbiolo (2017). Studente: Giulia Porello. Corso di Laurea Magistrale in Scienze e Tecnologie Alimetari, Università degli Studi di Torino.

Acknowledgements

First of all, I would like to thank my supervisor Luca Cocolin for the guidance constant encouragement and patience. I am very grateful, for all helpful discussions we have had during my time as PhD student and there is no way to repay what I have learnt from him. I am also very thankful to Kalliopi Rantsiou, for her experience and critical view, was always ready with a solution for solving problems during my experiments and suggesting future directions. Thank you to Luca Rolle and Vincenzo Gerbi, every time spent together was an occasion to increase my scientific knowledge. A special thought goes to Francesco and Fabrizio for all discussions and jokes we have shared: it has been great being in the lab with them. I could not have wished better friends and colleagues. Thanks goes to Simone, Maria, Martina, Antonio, Simona, Anna, Susana, Stefano, Enzo, Valentina, Paola and Chiara and all of the members of DISAFA for making my PhD time such a great experience. Thank you to everyone who has come and gone in the microbiology lab in Alba and Grugliasco over the last years, making the time in the lab more fun. Thank you to everyone, who has been part of my research experience in France, at the National Institute of Agricultural Research (INRA). Special thanks to Carole, Sylvie for always having the door open for my questions and Jean-Luc Legras for the interesting discussions. Finally, I would like to thank my family and friends for supporting me and being part of my life. Thank you to everyone else for having been part of this journey!