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Oxygen availability and strain combination modulate yeast growth dynamics in mixed culture fermentations of grape must with *Starmerella bacillaris* and *Saccharomyces cerevisiae*

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ABSTRACT

Starmerella bacillaris (synonym *Candida zemplinina*) is a non-*Saccharomyces* yeast that has been proposed as a co-inoculant of selected *Saccharomyces cerevisiae* strains in mixed culture fermentations to enhance the analytical composition of the wines. In order to acquire further knowledge on the metabolic interactions between these two species, in this study we investigated the impact of oxygen addition and combination of *Starm. bacillaris* with *S. cerevisiae* strains on the microbial growth and metabolite production. Fermentations were carried out under two different conditions of oxygen availability. Oxygen availability and strain combination clearly influenced the population dynamics throughout the fermentation. Oxygen concentration increased the survival time of *Starm. bacillaris* and decreased the growth rate of *S. cerevisiae* strains in mixed culture fermentations, whereas it did not affect the growth of the latter in pure culture fermentations. This study reveals new knowledge about the influence of oxygen availability on the successional evolution of yeast species during wine fermentation.

1. Introduction

Ethanol levels in wines have been rising over the last decade in many wine-producing countries, as a consequence of the high sugar content of the grapes currently used in wine production. This trend has often been attributed to global warming and the consumer preferences for well structured and full bodied wines produced from fully matured grapes (Mira de Orduña, 2010). The excessive sugar in the musts affects the fermentation process. High ethanol levels produced during the fermentation process may be toxic for the yeast cell by altering its membrane fluidity and this in turn may lead to arrested or sluggish sugar-to-ethanol conversion (Henderson and Block, 2014). Similarly, malolactic fermentation (MLF) a secondary bacterial fermentation occurring in red wines, during which *Oenococcus oeni* and other lactic acid bacteria (LAB) deacidify wine by conversion of malic to lactic acid, may be negatively affected (Zapparoli et al., 2009). Furthermore, ethanol can create sensory imbalance in the wine by increasing the perception of bitterness and hotness, as well as decreasing the perception of some wine aromas and flavour attributes (Goldner et al., 2009). From a commercial point of view, it can lead to an increase of the consumer's costs in countries where taxes are levied according to alcohol concentration (Sharma et al., 2014). Lastly, wine consumers are increasingly concerned with high ethanol content because of its harmful effect on human health (both physical and mental). Therefore, there is growing interest in reducing ethanol concentration in wine.

To this end, several techniques are being developed, targeting various steps of the winemaking process, starting from the vineyard to the winery, including grapevine and clonal selection, pre-fermentation, fermentation and post-fermentation strategies (Longo et al., 2016; Pickering, 2000; Varela et al., 2015). Among the available strategies, the choice should be economically relevant and at the same time, should not compromise organoleptic balance and other sensory characteristics of wine (Varela et al., 2015). The selection of yeasts able to convert glucose and fructose towards multiple secondary metabolites rather than ethanol, seems to be best suited for this purpose, since they do not require specific

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equipment (Tilloy et al., 2015). Indigenously isolated *Saccharomyces cerevisiae* strains exhibit similar ethanol yield values and as a consequence the research is focusing on developing *S. cerevisiae* and isolating non-*Saccharomyces* strains with improved phenotypes, able to divert carbon away from ethanol production (Ciani et al., 2016; Tilloy et al., 2015). Non-*Saccharomyces* yeasts are an integral part of the indigenous mycobiota present on grapes and at least at the initial stages of most spontaneous or inoculated grape must fermentations (Cravero et al., 2016; Varela and Borneman, 2016a; Varela, 2016b). In pure culture fermentations, these species are generally characterized by low fermentation efficiency (inability of completing alcoholic fermentation) and as a result the inoculation of the same must with selected *S. cerevisiae* strains, results fundamental in order to ensure complete fermentation of sugars (Andorrà et al., 2012; Tofalo et al., 2016). This can be achieved simultaneously or sequentially (Ciani et al., 2010). Conducting mixed culture wine fermentations, by controlled inoculation of selected non-*Saccharomyces* and *S. cerevisiae* strains is a strategy that takes advantage of the unique features of the former yeast group (Varela, 2016b).

Mixed fermentations and the employment of non-*Saccharomyces* species have received growing attention over the recent years from the winemaking community. They reflect yeast biodiversity of indigenous wine microbiota and modulate the production of specific chemical compounds, as a consequence of the early growth of non-*Saccharomyces* species (Ciani et al., 2010; Fleet, 2008; Jolly et al., 2014). Their efficiency is associated with the promotion of the growth and metabolic activity of the selected non-*Saccharomyces* yeasts by outcompeting or reducing the activity of the *S. cerevisiae* strain (Varela, 2016b). To this end, numerous winemaking variables could be manipulated to encourage non-*Saccharomyces* growth rate and contribution to the chemical composition and sensory quality of the wine. These variables, include sugar concentration, fermentation temperature, inoculum density, nitrogen and oxygen availability, inhibitory or stimulatory substances produced by the growth of yeasts or bacteria, fungicide residues from the grapes and sulphur dioxide (SO_2) addition (Fleet and Heard, 1993).

The application of non-*Saccharomyces* yeasts, in co-inoculation or sequential inoculation with *S. cerevisiae* has been investigated in recent years for reducing the ethanol yield (Bely et al., 2013; Canonico et al., 2016; Contreras et al., 2015a, 2015b; Giaramida et al., 2013; Quirós et al., 2014; Varela et al., 2016c). Among them, *Starmerella bacillaris* (synonym *Candida zemplinina*) is known as a high glycerol and low ethanol producer (Englezos et al., 2015; Masneuf-Pomarede et al., 2015; Tofalo et al., 2012). We recently reported a microbiological approach for reducing the ethanol content in wines based on mixed culture fermentations of *Starm. bacillaris* and *S. cerevisiae* (Englezos et al., 2016a). In this approach, *S. cerevisiae* was sequentially inoculated 48 h after *Starm. bacillaris*, leading to a marked decrease in the ethanol content up to 0.5–0.7% (v/v), compared to *S. cerevisiae* in pure culture fermentation. An important question still open after this study was if strain compatibility and environmental factors could affect microbial growth and as a consequence metabolites production. In this context, oxygen availability and strain compatibility were considered to have great influence on fermentation speed as they impact on yeast metabolism and growth during fermentation (Hansen et al., 2001; Jolly et al., 2014). As a proof of concept, the objective of the present study was to acquire further knowledge about the impact of these parameters on mixed fermentation performance, carried out using conventional and evolutionary engineered (optimized for glycerol production/ethanol reduction) *S. cerevisiae* strains as partners of *Starm. bacillaris* stains.

2. Materials and methods

2.1. Strains

In the present study two *Starm. bacillaris* and two *S. cerevisiae* strains were used as starters. The *S. cerevisiae* strains were the commercial strains Uvaferm BC® and IONYS WF®, both from Lallemand Inc. (Montreal, Canada). The *Starm. bacillaris* strains used in this study were FC54 (yeast culture collection of DISAFA, Dipartimento di Scienze Agrarie, Forestali e Alimentari, University of Torino, Italy) and MUT 5705 (Mycotheca Universitatis Taurinensis-MUT, DBIOS, University of Torino, Italy), called CBE4 in previous studies (Englezos et al., 2015). All strains were selected for their enological traits in laboratory scale fermentations (Englezos et al., 2015, 2016a; Tilloy et al., 2014).

2.2. Fermentation trials

Fermentations were carried out in red must, without skins and seeds from Barbera grapes, which is the most planted red grape variety in Piedmont region (Northwest Italy). Barbera must contained 246.4 g/L sugars, pH 3.0, total acidity 10.0 g/L (expressed as g/L of tartaric acid) and 130 mg/L of yeast assimilable nitrogen (YAN) composed by 60 mg/L of inorganic nitrogen and 70 mg/L of organic nitrogen. The must was supplemented with 50 mg/L of organic nitrogen using the commercial product Fermaid O® (Lallemand Inc.) to achieve an initial YAN concentration of 180 mg/L. Before fermentation the must was pasteurized at 60 °C for 1 h, as previously described by Englezos et al. (2016b) and the absence of viable yeast populations was checked by plate counting on wallerstein laboratory nutrient (WLN) medium (Biogenetics, Milan, Italy).

Two sets of inoculation protocols were performed: a pure culture fermentation with *S. cerevisiae* strains and a mixed culture fermentation where *S. cerevisiae* strains were inoculated 48 h after *Starm. bacillaris* inoculation. Mixed fermentations were carried out using the 4 different combinations of *Starm. bacillaris* and *S. cerevisiae* strains (FC54 and Uvaferm BC®, MUT 5705 and Uvaferm BC®, FC54 and IONYS WF®, MUT 5705 and IONYS WF®). All strains were inoculated as active dry yeast (ADY) and rehydrated according to manufacturer's instructions, except for strain MUT 5705 which was preadapted in the same must for 48 h at 25 °C. Prior to inoculation, yeast cells were counted by a Thoma hemocytometer chamber using methylene blue dye as a marker of cell viability. Then, appropriate amounts of inoculum were used to reach an initial cell population of about 5.0×10^6 cells/mL, that corresponds to a dose of 25 g/hL of ADY.

Triplicate fermentations were performed without and with the addition of oxygen (condition I and II respectively) in 1000 mL sterile glass bottles containing 800 mL Barbera grape must at 25 °C without agitation. After inoculation the bottles were closed with air locks containing sterile paraffin oil, to allow only the CO_2 to escape from the fermenting medium and prevent external contamination. For oxygen addition, the fermenting musts were saturated (about 7 mg/L of O_2) with pure oxygen (Rivoira, Milan, Italy) 24 and 48 h after yeast inoculation. To estimate the dissolution of oxygen during fermentation, another grape must sample (inoculated with Uvaferm BC®) was micro-oxygenated and the oxygen content was controlled using a Nomasense oxygen analyzer (Nomacorc, SA). In order to improve O_2 solubility, the must was maintained in medium/high agitation (about 150 rev min^{-1}) on a rotary shaker (Velp Scientifica, Monza and Brianza, Italy) during oxygen addition. Samples were micro-oxygenated with Ox-evolution and ceramic diffuser (Intec, Pramaggiore, VE, Italy) with 10 mg/min oxygen flow rate for 10 min.