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Torulaspora delbrueckii contribution in mixed brewing fermentations with different *Saccharomyces cerevisiae* strains

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ABSTRACT

In recent years, there has been growing demand for distinctive high quality beer. Fermentation management has a fundamental role in beer quality and the levels of aroma compounds. Use of non-conventional yeast has been proposed to enhance beer bioflavor. In the present work we investigated mixed fermentations using three commercial *Saccharomyces cerevisiae* strains, without and with addition of a selected *Torulaspora delbrueckii* strain evaluating their interactions, as well as the aroma profiles. At the *S. cerevisiae/T. delbrueckii* co-inoculation ratio of 1:20, viable cell counts indicated that *T. delbrueckii* dominated all of the three combinations. In the mixed fermentations, *T. delbrueckii* provided higher levels of higher alcohols (excepting of β-phenyl ethanol), in contrast to data obtained in winemaking, where higher alcohols had lower levels. Moreover, mixed fermentations showed significantly higher ethyl acetate (from 5 to 16 mg/L) and isoamyl acetate (from 0.019 to 0.128 mg/L), and were generally lower in ethyl hexanoate and ethyl octanoate. Therefore, irrespective of *S. cerevisiae* strain, *T. delbrueckii* influenced on all mixed fermentations. On the other hand, the mixed fermentations were also affected by each of the three *S. cerevisiae* strains, which resulted in beers with distinctive flavors.

1. Introduction

In recent years, consumers have begun to appreciate more the beers that are characterized by distinctive sensory characteristics. Brewers have tried to achieve this through their selection of hop varieties, malts, and yeast, and through fermentation management (Chen and Xu, 2013; De Keukeleire et al., 2010; King and Dickinson, 2003; He et al., 2014; Pires et al., 2014; Stewart, 2016; Vanderhaegen et al., 2003).

The choice of the yeast in the brewing process is also crucial to achieve a product with the distinctive flavors expected by consumers. Indeed, yeasts produce distinctive fermentative aroma profiles and transform precursors of feedstock into more flavor-active compounds, which thus contribute to the final aroma of the beer. On the other hand, yeast strains used in fermentation are mainly selected for flocculation, wort fermentation ability, and ethanol tolerance (De Keukeleire et al., 2010; Stewart, 2016; Vanderhaegen et al., 2003). Recently, with the aim to obtain beers with more complex aroma profiles, researchers have focused their attention on non-conventional yeasts (Basso et al., 2016; Varela, 2016). Indeed, the impact of non-conventional yeasts used in pure and mixed fermentations with *S. cerevisiae* on the flavor profile of other fermented and distilled spirit beverages, has been revaluated (Ciani and Comitini, 2015; Comitini et al., 2011; Varela, 2016; Jolly et al., 2014).

Recent genetic investigations have also been focused on methods to enhance the fermentation efficiency and aromatic profile of selected *S. cerevisiae* (Krogerus et al., 2017; Saerens et al., 2010; Steensels et al., 2012; Steensels and Verstrepen, 2014). At the same time, the isolation of new starter yeasts from natural matrices (Marongiu et al., 2015; Mascia et al., 2015), and the selection of wine yeast strains (Canonico et al., 2014) have also been proposed. Other studies have focused on beer obtained by spontaneous fermentation, such as the Belgian lambic beers, gueuze, American coolship ale, Berlin wheat beers, and some Belgian trappist beers (Bokulich et al., 2012; Crauwels et al., 2015; Martens et al., 1997; Spitaels et al., 2014; Steensels and Verstrepen, 2014).

Recently, among the non-conventional yeasts used in brewing, *Torulaspora delbrueckii* has received attention due to its ability to ferment maltose, produce ester compounds, and biotransform the monoterpenoid flavor compounds of hops (Canonico et al., 2016; King and Dickinson, 2000; Michel et al., 2016; Tataridis et al., 2013). In particular, *T. delbrueckii* can produce different fruity aromas, such as from β-phenylethanol ('rose' flavors), n-propanol, iso-butanol, amyl alcohol ('solvent brandy' aroma), and ethyl acetate (Basso et al., 2016; Etschmann et al., 2015; Pires et al., 2014). Beer produced by pure cultures of *T. delbrueckii* and by mixed fermentations of *S. cerevisiae/T. delbrueckii* were characterized by 'fruit/citric' and 'fruity/ester' notes,

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and had 'full-bodied' attributes (Canonico et al., 2016; Michel et al., 2016).

The aim of the present study was to investigate the influence of a selected *T. delbrueckii* strain used in mixed fermentation with three commercial *S. cerevisiae* strains on the analytical composition and aromatic profile of the final beers. The interactions between these two yeasts during the mixed fermentation and the contribution of the *S. cerevisiae* starter strain were also evaluated.

2. Materials and methods

2.1. Yeast strains

Three different commercial *S. cerevisiae* strains were used, in pure fermentations as controls, and each in the mixed fermentations: Safale US-05 (Fermentis, Lesaffre, France); Safbrew WB-06 (Fermentis, Lesaffre, France); and Belgian Wheat 3942 (Wyeast Laboratories, Richardson, USA). The US-05 and WB-06 dry yeasts were rehydrated following the manufacture instructions, while liquid Belgian Wheat 3942 was plated on YPD agar medium at 25 °C, by spreading 0.1 mL yeast suspension onto the surface of the medium. In addition, the presence of lactic acid bacteria was determined using MRS agar (Oxoid, Basingstoke, UK) supplemented with 0.005% cycloheximide (Sigma-Aldrich, St. Louis, MO, USA), to suppress the growth of yeast, and incubated anaerobically in jars, at 30 °C for 3–8 days.

The selected *T. delbrueckii* strain used in this study was DiSVA 254, also in both pure and mixed fermentations, which was obtained from the Yeast Collection of the Department of Life and Environmental Sciences (DiSVA) of the Polytechnic University of Marche (Italy). This was originally isolated from Papaya leaves from Cameroon (Africa), and has been used previously for beer production (Canonico et al., 2016). All of the yeast strains were maintained at 4 °C for short-term storage, and in YPD broth (without agar) supplemented with 80% (w/v) glycerol at − 80 °C for long-term storage.

2.2. Wort production

The wort used for the microfermentation trials was produced at Birra dell'Eremo Microbrewery (Assisi, Italy) from a batch of 1500 L. The wort was made with pilsner malt (100%) and the Cascade hop variety. The main analytical characters of this wort were: pH 5.47; specific gravity 12.3 °Plato; Free Assimilable Nitrogen, 263 mg N/L, and 20 IBU. The wort was produced according to the following scheme: 53 °C for 10 min; 67 °C for 70 min, and 76 °C for 10 min; with boiling for 60 min.

2.3. Fermentation trials

The three different *S. cerevisiae* starter strains were used in pure and mixed fermentations with *T. delbrueckii* DiSVA 254, each at the *S. cerevisiae* to *T. delbrueckii* ratio of 1:20, on the basis of previous work (Canonico et al., 2016). The trials carried out at inoculum ratio 1:20 increase the production of fruity esters and showed fermentation kinetics comparable to the *S. cerevisiae* starter strain.

The fermentation potential of the selected yeast strains and their interactions in the wort were evaluated in fermentation trials carried out at 19 ± 1 °C in flasks containing 500 mL wort under sterile conditions. The flasks were sealed with a Müller valve containing sulfuric acid, to allow the CO_2 produced to escape from the system. Pre-cultures were grown in 10% malt extract at 19 ± 1 °C for 48 h (*S. cerevisiae*) and 72 h (*T. delbrueckii*),obtaining an inoculum of approximately 5×10^6 cell/mL. The fermentation kinetics were monitored by measuring the weight loss of the flasks due to the CO_2 evolved, until the end of the fermentation (i.e., constant weight for three consecutive days). The growth kinetics were monitored by colony forming unit (CFU) counts on both WL Nutrient Agar (Oxoid, Hampshire, UK) and Lysine Agar (Oxoid, Hampshire, UK). This last is a selective medium that does not support the growth of *S. cerevisiae* (Lin, 1975), thus providing differentiation of the *T. delbrueckii* colonies from *S. cerevisiae* in the mixed fermentation. The fermentations were carried out in triplicate under static conditions. At the end of each fermentation, the beer with the remaining yeast (1×10^5 cell/mL) was transferred into 330-mL bottles after primary fermentation, to which sucrose was added at 5 g/L. The secondary fermentation in the bottle was carried out at 19 ± 1 °C for 10 days.

2.4. Analytical determinations

Specific gravity was measured using a specific gravity meter (DA-300; Kyoto Instruments). All of the specific gravity measurements were converted to densities and then to degrees Plato, according to Brown and Hammond (2003).

The volatile acidity and pH determinations were performed according to the Official European Union Methods (EC, 2000). Ethanol content was measured according to the Association of Official Analytical Chemists (AOAC, 1990). The contents of acetaldehyde, ethyl acetate, higher alcohols (n-propanol, isobutanol, amilic alcohol, isoamilic alcohol) were determined by direct injection into a gas–liquid chromatography system. The other volatile compounds were extracted using an ether-hexane (1:1) extraction technique, and evaluated using a capillary gas chromatography system (GC-2014; Shimadzu, Kyoto, Japan), as reported by Canonico et al. (2014). The free amino nitrogen content was determined following a procedure described previously by Dukes and Butzke (1998). The contents of glucose, sucrose, maltose, and ammonia were determinated using enzymatic kits (k-masug, k-amiar kits, respectively; Megazyme, Ireland), according to the manufacturer's instructions.

2.5. Statistical analysis

Analysis of variance (ANOVA) was applied to the experimental data for the main characteristics of the beers. The means were analyzed using the STATISTICA 7 software. Significant differences were determined by the means of Duncan tests, and the results were considered significant if the associated P values were < 0.05. Principal component analysis (PCA) was applied to discriminate among the means of the contents of esters, higher alcohols, and carbonyl compounds in the beers from the pure and mixed fermentations. PCA was carried out using the Unscrambler 7.5 software (CAMO ASA, Oslo, Norway), and the data are presented as biplot graphs. The mean data were normalized, to neutralize any influence of hidden factors. The PCA provides a graphical representation of the overall differences due to *T. delbreuckii* in terms of the fermentation by-products of the final beers.

3. Results

3.1. Fermentation kinetics

Fig. 1 shows the fermentation kinetics of the three *S. cerevisiae* starter strains in pure and mixed fermentation with the *T. delbrueckii* strain.

All of these fermentations (pure or mixed) showed similar fermentation kinetics. However, the mixed fermentations showed slower fermentation kinetics in comparison to the respective *S. cerevisiae* pure fermentations. Here, the high inoculation level of *T. delbrueckii* had different influences on the fermentation kinetics of these three *S. cerevisiae* strains. The US-05 mixed fermentation showed slight slower fermentation kinetics in comparison to the corresponding pure fermentation. In contrast, the Belgian Wheat and WB-06 mixed fermentations showed overlapping fermentation kinetics with the *T. delbrueckii* pure fermentation for up to six days of fermentation. After this, they showed progressively faster fermentation kinetics. The *T. delbrueckii*