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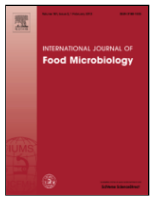

Torulaspora delbrueckii contribution in mixed brewing fermentations with different *Saccharomyces cerevisiae* strains

Laura Canonico, Francesca Comitini, Maurizio Ciani*

Dipartimento Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy

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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

* Corresponding author.
Email address: m.ciani@univpm.it (M. Ciani)

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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, 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 fer-

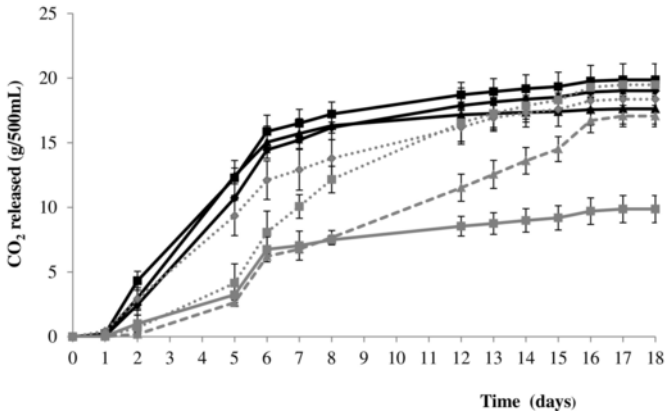

Fig. 1. Fermentation kinetics of the pure and mixed fermentations. *S. cerevisiae* pure fermentations: US-05 (—●—); WB-06 (—■—); Belgian wheat (—▲—). *T. delbrueckii* pure fermentation (—■—). Mixed fermentations: US-05 + *T. delbrueckii* (···●···); WB-06 + *T. delbrueckii* (···■···); Belgian wheat + *T. delbrueckii* (···▲···).

mentation 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* pure fermentations showed the slowest fermentation kinetics, with lower wort attenuation (Table 1).

3.2. Population dynamics

The *S. cerevisiae* US-05 pure fermentation achieved a cell concentration > 10^7 CFU/mL after four days of fermentation, and maintained this until the end of the process (Fig. 2a). In the mixed fermentations, US-05 reached a biomass of 6.1×10^6 CFU/mL at day 4 and maintained this viable cell level (with a slight reduction) until the end of the fermentation process. Here, the growth kinetics of *T. delbrueckii* showed population dynamics comparable to those of the *T. delbrueckii* pure fermentation. These data indicate that at this 20:1 inoculation ratio with US-05, *T. delbrueckii* dominated the process. The same population dynamics were observed in the case of WB-06 and *T. delbrueckii* mixed fermentations. However, in this last case, the cell concentrations at the end of the fermentation process were about 10^6 CFU/mL for both *S. cerevisiae* and *T. delbrueckii*, which indicated that the *T. delbrueckii* growth was affected by the WB-06 strain (Fig. 2b). For the Belgian Wheat pure fermentation, this showed slower growth, to reach a biomass of just 6.7×10^6 CFU/mL, maintaining this tendency until the end of the fermentation (Fig. 2c). However, in the mixed fermentation for the Belgian Wheat strain, the biomass reached 6.4×10^6 CFU/mL, which was very close to the pure fermentation, and which indicated that the Belgian Wheat's performance was not so affected by the presence of *T. delbrueckii*. Finally, in mixed fermentations *T. delbrueckii* showed lower viable cells at the end of the process, which was indicative of the progressive competition with the *S. cerevisiae* strains, even under a 20-fold inoculation condition.

3.3. Main analytical characteristics

The data for the main characters of the beers obtained at the end of the primary fermentations are given in Table 1. The mixed fermentations showed ethanol contents varying from 4.65% to 4.89% (v/v), which did not differ significantly from the ethanol content of *S. cerevisiae* pure fermentations. As expected, the *T. delbrueckii* pure fermentation showed a significantly lower ethanol content, of 2.62% (v/v), which was associated with a consistent maltose residue. This low ethanol content with the *T. delbrueckii* pure fermentation was confirmed by the low wort gravity attenuation (6.11 °P, vs. mixed fermentations of 1.38–1.81 °P), with the real attenuation of 38%, which was significantly lower than that in the mixed fermentations (66.88%–72.07%). For the volatile acidity, the *T. delbrueckii* pure fermentation produced beers with an acetic acid content (0.41 g/L) that was significantly higher than that for the *S. cerevisiae* pure fermentations and the mixed fermentations, with the only exception being the WB-06 pure fermentation and the Belgian wheat/*T. delbrueckii* mixed fermentation. For the yeast-assimilable nitrogen content, higher nitrogen consumption was observed in the pure fermentations with the strains US-05 in comparison with the other two *S. cerevisiae*. In the mixed fermentations there was a different trend among the trials, which indicated different behaviors for these two yeast species. For pH, in comparison with the *S. cerevisiae* pure fermentations, *T. delbrueckii* alone showed a lower final pH of the beer (pH 4.29), although only the mixed fermentation with US-05 was significantly lower than its pure fermentation (pH 4.32).

3.4. Main volatile compounds

Data regarding the volatile compounds concentrations are reported in Table 2. US-05 showed significantly lower ethyl butyrate content in comparison to the other pure fermentations. Also, in the US-05 and WB-06 mixed fermentations with *T. delbrueckii*, the contents of this aroma compound were higher, as compared to the respective pure fermentations, while the Belgian Wheat mixed fermentation did not show any variation in the ethyl butyrate content from its pure fermentation.

The data show significantly higher isoamyl acetate contents for WB-06 and Belgian Wheat mixed fermentations, as compared to their respective *S. cerevisiae* pure fermentations. The ethyl acetate content, which is the ester responsible for the fruity or solvent aroma of beer (Pires et al., 2014), was significantly higher in all of the mixed fermentations, as compared to the respective *S. cerevisiae* pure fermentations. For the contents of the other esters, such as ethyl hexanoate (i.e., apple, fruit flavor), ethyl octanoate (i.e., apple, aniseed flavor), and phenyl ethyl acetate (i.e., floral, honey, sweet) (Pires et al., 2014), and for butyric acid, the data showed opposite trends to those of the ethyl butyrate and isoamyl acetate contents. Indeed, in the mixed fermentations, the contents of these volatile compounds were significantly lower, compared to their respective *S. cerevisiae* pure fermentations. Significantly lower levels were also seen in the mixed fermentations for diethyl succinate (US-05 = 0.008, WB06 = 0.015, Belgian wheat = 0.016 mg/L) and β-phenyl ethanol contents (US-05 = 15.45, WB06 = 37.72, Belgian Wheat = 18.25 mg/L), compared to their respective *S. cerevisiae* pure fermentations.

There was also a significant enhancement of the higher alcohol contents in all of the mixed fermentations, as compared to their respective *S. cerevisiae* pure fermentations, with the exception of Belgian wheat/*T. delbrueckii*, which did not show any significant variations for isobutanol and n-propanol contents. Furthermore, the acetaldehyde contents were significantly higher in all of the mixed fermentations, as compared to the respective *S. cerevisiae* pure fermentations, apart from Belgian wheat/*T. delbrueckii*, where this was similar to that of the Belgian Wheat pure fermentation.

To assess the overall effects of *T. delbrueckii* in these mixed fermentations with the different *S. cerevisiae* starter strains, data regarding all the volatile compounds studied were analyzed by PCA (Fig. 3). The biplots of the analyzed parameters confirmed the effects of *T. delbrueckii* in these mixed fermentations. Indeed, the mixed fermentations fell into

Table 1
The main analytical characteristics of the beers produced in the pure and mixed fermentations.

Fermentation	Residual sugar (mg/L)			Yeast-assimilable nitrogen[a]	pH	Wort gravity attenuation[b]	Apparent attenuation	Real attenuation	Ethanol	Volatile acidity
	Sucrose	Glucose	Maltose	(mg/L)		(°P)	(%)	(%)	(% v v^{-1})	(g/L)
US-05	0.00 ± 0.00^{d}	0.03 ± 0.00^{c}	0.05 ± 0.01^{b}	136.08 ± 0.17^{d}	4.73 ± 0.01^{a}	1.72 ± 0.30^{b}	85.81 ± 2.45^{a}	69.76 ± 2.00^{a}	4.70 ± 0.22^{a}	0.24 ± 0.03^{d}
WB-06	0.07 ± 0.02^{c}	0.09 ± 0.01^{a}	0.41 ± 0.00^{b}	157.61 ± 2.50^{b}	4.40 ± 0.10^{c}	1.64 ± 0.30^{b}	87.23 ± 2.12^{a}	70.92 ± 1.73^{a}	4.64 ± 0.12^{a}	0.38 ± 0.03^{ab}
Belgian Wheat	0.02 ± 0.00^{d}	0.08 ± 0.00^{a}	0.42 ± 0.00^{b}	144.74 ± 2.76^{c}	4.63 ± 0.10^{ab}	2.15 ± 0.15^{b}	82.26 ± 1.22^{a}	66.88 ± 1.00^{a}	4.72 ± 0.51^{a}	0.25 ± 0.01^{d}
US-05/*T. delbrueckii*	0.15 ± 0.00^{a}	0.00 ± 0.00^{d}	0.51 ± 0.01^{b}	153.71 ± 2.15^{b}	4.32 ± 0.03^{cd}	1.81 ± 0.01^{b}	85.10 ± 0.01^{a}	69.19 ± 0.01^{a}	4.65 ± 0.11^{a}	0.33 ± 0.03^{c}
WB-06/*T.delbrueckii*	0.11 ± 0.01^{b}	0.05 ± 0.01^{b}	0.52 ± 0.02^{b}	136.14 ± 3.02^{d}	4.41 ± 0.03^{c}	1.38 ± 0.15^{b}	88.65 ± 1.23^{a}	72.07 ± 1.00^{a}	4.78 ± 0.34^{a}	0.32 ± 0.02^{c}
Belgian Wheat/*T. delbrueckii*	0.00 ± 0.00^{d}	0.04 ± 0.00^{bc}	0.49 ± 0.00^{b}	147.48 ± 1.81^{c}	4.57 ± 0.02^{b}	1.46 ± 0.40^{b}	82.94 ± 4.25^{a}	71.49 ± 3.5^{a}	4.89 ± 0.20^{a}	0.38 ± 0.03^{ab}
T. delbrueckii	0.00 ± 0.00^{d}	0.000 ± 0.00^{d}	24.02 ± 1.45^{a}	166.51 ± 3.88^{a}	4.29 ± 0.02^{d}	6.11 ± 0.43^{a}	46.80 ± 0.01^{b}	38.05 ± 0.01^{b}	2.62 ± 0.12^{b}	0.41 ± 0.01^{a}

Data are means ± standard deviations.
Data with different superscript letters (^{a,b,c,d}) within each column are significantly different (Duncan tests; $P < 0.05$).
[a] Starting yeast-assimilable nitrogen, 263 mg N/L.
[b] Starting wort gravity, 12.3°P.

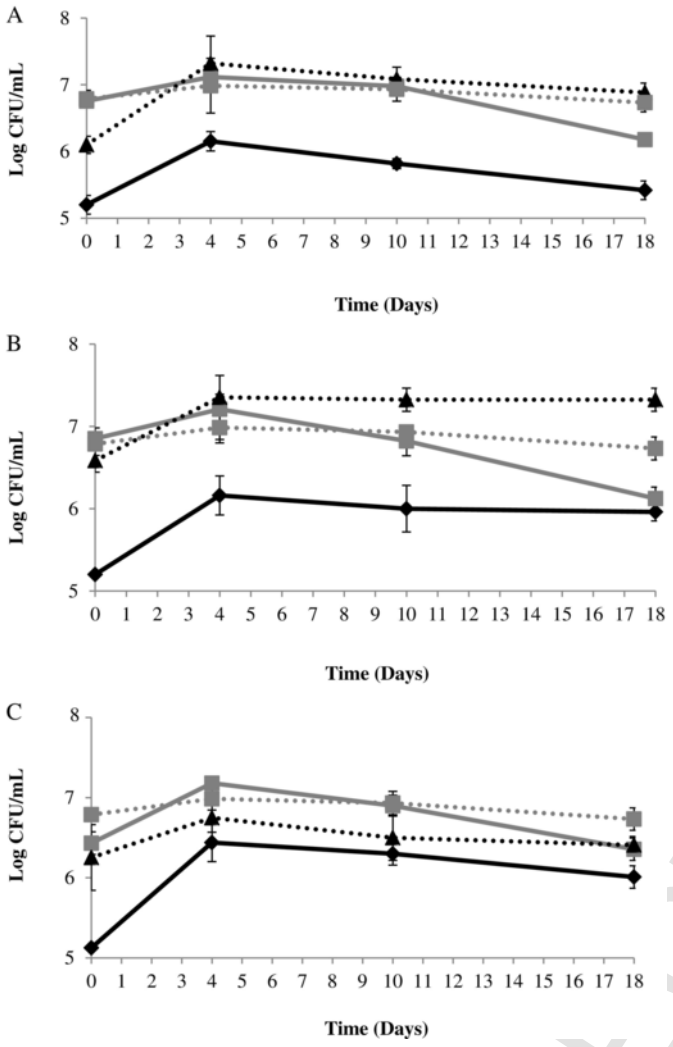

Fig. 2. Growth kinetics of the pure and mixed fermentations. *S. cerevisiae* population in pure fermentations (···▲···) and *T. delbrueckii* population in pure fermentation (···■···), *S. cerevisiae* (—●—) and *T. delbrueckii* (—■—) populations in mixed fermentations. (A) US-05. (B) WB-06. (C) Belgian Wheat.

the upper half of the biplot (PC 2 component), due to the isoamylic alcohol, ethyl acetate, isoamyl acetate, and amylic alcohol contents that, in the increased amounts detected, positively influenced the analytical profile of the beers. *T. delbrueckii* pure fermentation is mainly distinguished by acetaldehyde isobutanol and ethyl butyrate (bottom left of the biplot). *S. cerevisiae* fermentation trials (mainly for WB-06 strain) are characterized by the production of β-phenyl ethanol (bottom right of biplot). Moreover, the *T. delbrueckii* pure fermentation showed a different profile from both the *S. cerevisiae* pure commercial strains and the mixed fermentations, which indicated synergistic interactions in the mixed fermentations for the production of some of the aroma compounds. In this regard, *T. delbrueckii* had a stronger influence in the mixed fermentation with US-05, which is considered a neutral strain on the aromatic contribution (Fig. 3, upper left quadrant).

4. Discussion

The use of *T. delbrueckii* in mixed fermentations with *S. cerevisiae* starter strains has been studied previously in winemaking (Azzolini et al., 2015; Comitini et al., 2011; Cordero-Bueso et al., 2013; Sadoudi et al., 2012) and recently the use of this non-conventional yeast was proposed for the brewing process (Canonico et al., 2016; Michel et al., 2016, 2017; Petruzzi et al., 2016). The most abundant aroma compounds present in beer are the higher alcohols, such as n-propanol, isobutanol, amyl alcohol, and isoamyl alcohol, which define the warm 'mouthfeel', and the fruit notes, and β-phenyl ethanol, which imparts a rose-like and floral aroma to the beer (Hughes, 2009; Pires et al., 2014).

In winemaking, the use of *T. delbrueckii* in such mixed fermentations with *S. cerevisiae* demonstrated generally higher β-phenyl ethanol contents (Azzolini et al., 2015; Comitini et al., 2011; Cordero-Bueso et al., 2013; Sadoudi et al., 2012). In contrast, with the *S. cerevisiae* strains in the present study on beer fermentation, the addition of *T. delbrueckii* in the mixed fermentations showed an opposite trend. Indeed, significantly lower β-phenyl ethanol content in beer has also been reported previously (Canonico et al., 2016), which was thus confirmed here. These studies highlight the different behaviors and interactions in mixed fermentation with *S. cerevisiae* and *T. delbrueckii* both in winemaking and brewing processes.

On the contrary, these mixed fermentations showed general superior contents of the other higher alcohols in the beers, with the exception of the Belgian Wheat mixed fermentation, suggesting a synergistic interaction among the agents involved. In this context, the competition for the use of yeast assimilable nitrogen in mixed fermentations might have an important role in the formation of higher alcohols (He et al., 2014). Indeed, brewers have already been trying to increase the synthesis of higher alcohols during the fermentation process through the use of commercial proteases, or by modifications to the processing conditions, such as mashing temperature and pH (Igyor et al., 2001; Piddocke et al., 2011). In this context, the use of such *S. cerevisiae/T. delbrueckii* mixed fermentations in brewing might be a suitable way to enhance the higher alcohols content in beer.

The other important fermentation-derived flavor-active compounds include esters, such as ethyl butyrate, isoamyl acetate, and ethyl acetate, which are responsible for the fruity, floral, and solvent aromas of beers (Boulton and Quain, 2006; Casey, 2007). Also for these compounds, the present study highlights synergistic effects between *S. cerevisiae* and *T. delbrueckii* in these mixed fermentations. Indeed, the contents of ethyl acetate (i.e., fruity) (Pires et al., 2014) and isoamyl acetate (i.e., banana) were significantly higher in all of these mixed fermentations. On the contrary, the ethyl hexanoate and ethyl octanoate contents were lower, as compared to the respective *S. cerevisiae* starter strains, here showing opposite trends in these mixed fermentations compared with previous data (Canonico et al., 2016). This behavior could be due to the use of different wort and hop varieties. Indeed, in the previous study, the wort used was for production of an American Amber Ale beer style, which is different from the wort used in the present study. In beer fermentation, *T. delbrueckii* is able to provide a distinct, uppermost ester content in comparison to wine fermentation (Padilla et al., 2016). A possible explanation for this behavior relates to the different initial substrates (sugars, aminoacid composition, flavor-active compounds precursors, and others).

The results indicate that these mixed fermentations at the ratio proposed in this study show a constant influence of *T. delbrueckii* irrespective of the *S. cerevisiae* strain used. On the other hand, these final products were influenced by the *S. cerevisiae* starter strains studied in our assays. The same behavior had already been observed in the case of Verdicchio wine, where *S. cerevisiae/T. delbrueckii* mixed fermentations influenced the analytical and aroma profiles of the wines according to the *S. cerevisiae* starter strain used (Canonico et al., 2015).

In conclusion, the use of *T. delbrueckii* in mixed fermentations with *S. cerevisiae* is a suitable strategy to control flavor production during beer fermentation, and thus to obtain products with aroma compounds that are different from those for beers brewed using pure *S. cerevisiae* starter strains. These data confirm that the brewing yeast used can modulate the production of the aroma compounds in the final beers

Table 2
The main by-products and volatile compounds in the beers produced in the pure and mixed fermentations.

Main by-product/volatile	Fermentation						
(mg/L)	US-05	WB-06	Belgian wheat	US-05/*T. delbrueckii*	WB-06/*T. delbrueckii*	Belgian wheat/*T. delbrueckii*	*T. delbrueckii*
Esters							
Ethyl butyrate	0.165 ± 0.001^{d}	0.313 ± 0.002^{ab}	0.319 ± 0.006^{bc}	0.287 ± 0.011^{c}	0.413 ± 0.037^{a}	0.287 ± 0.031^{bc}	0.275 ± 0.003^{bc}
Ethyl acetate	4.33 ± 0.70^{e}	9.53 ± 2.03^{d}	13.11 ± 1.62^{c}	20.38 ± 2.28^{b}	24.87 ± 1.22^{a}	18.15 ± 2.10^{b}	4.09 ± 0.50^{e}
Phenyl ethyl acetate	0.006 ± 0.001^{f}	0.037 ± 0.001^{c}	0.040 ± 0.004^{b}	0.010 ± 0.001^{e}	0.030 ± 0.001^{d}	0.044 ± 0.001^{a}	0.004 ± 0.002^{f}
Ethyl hexanoate	0.047 ± 0.015^{a}	0.035 ± 0.001^{b}	0.016 ± 0.004^{bcd}	0.018 ± 0.003^{bc}	0.011 ± 0.001^{cd}	0.002 ± 0.004^{d}	0.017 ± 0.005^{bcd}
Ethyl octanoate	0.013 ± 0.001^{d}	0.057 ± 0.002^{a}	0.030 ± 0.001^{c}	0.011 ± 0.002^{e}	0.047 ± 0.001^{b}	0.010 ± 0.001^{e}	ND
Isoamyl acetate	0.144 ± 0.001^{e}	0.383 ± 0.021^{c}	0.354 ± 0.001^{d}	0.163 ± 0.020^{e}	0.423 ± 0.013^{b}	0.482 ± 0.017^{a}	0.169 ± 0.015^{e}
Alcohols							
n-Propanol	12.94 ± 0.16^{ef}	27.81 ± 2.47^{b}	16.04 ± 0.35^{d}	24.63 ± 1.88^{c}	40.42 ± 1.71^{a}	15.26 ± 0.59^{de}	12.2 ± 0.75^{f}
Isobutanol	16.66 ± 1.10^{c}	18.38 ± 0.73^{c}	18.19 ± 2.40^{c}	28.06 ± 1.70^{b}	35.23 ± 4.21^{a}	14.69 ± 0.55^{c}	18.82 ± 0.20^{c}
Amylic alcohol	3.07 ± 0.51^{e}	7.75 ± 0.68^{d}	8.07 ± 1.04^{d}	13.36 ± 2.04^{b}	18.65 ± 1.11^{a}	11.12 ± 0.41^{c}	2.27 ± 0.63^{f}
Isoamylic alcohol	19.03 ± 0.58^{g}	39.22 ± 2.1^{e}	43.5 ± 1.02^{d}	70.74 ± 4.4^{a}	64.41 ± 1.93^{b}	49.14 ± 0.80^{c}	23.17 ± 1.80^{f}
β-Phenyl ethanol	20.99 ± 1.45^{c}	51.15 ± 0.93^{a}	36.35 ± 2.81^{b}	15.45 ± 0.46^{c}	37.72 ± 0.93^{b}	18.25 ± 0.76^{c}	5.53 ± 0.60^{d}
Carbonyl compounds							
Acetaldehyde	24.49 ± 2.01^{c}	19.11 ± 1.06^{e}	20.00 ± 0.76^{de}	43.69 ± 1.57^{a}	29.14 ± 2.38^{b}	17.61 ± 2.01^{e}	22.59 ± 1.30^{cd}
Acetoin	ND	ND	ND	ND	ND	ND	ND
Carboxylic acids							
Butyric acid	0.054 ± 0.001^{d}	0.154 ± 0.010^{a}	0.100 ± 0.008^{b}	0.073 ± 0.006^{c}	0.024 ± 0.014^{e}	0.018 ± 0.004^{e}	0.048 ± 0.012^{d}
Diethyl succinate	0.049 ± 0.010^{a}	0.045 ± 0.001^{a}	0.033 ± 0.024^{ab}	0.008 ± 0.001^{c}	0.015 ± 0.006^{c}	0.016 ± 0.003^{bc}	0.022 ± 0.006^{bc}

Data are means ± standard deviation.
Data with different superscript letters (^{a,b,c,d,e,f}) within each row are significantly different (Duncan tests; $P < 0.05$).
ND, not detected.

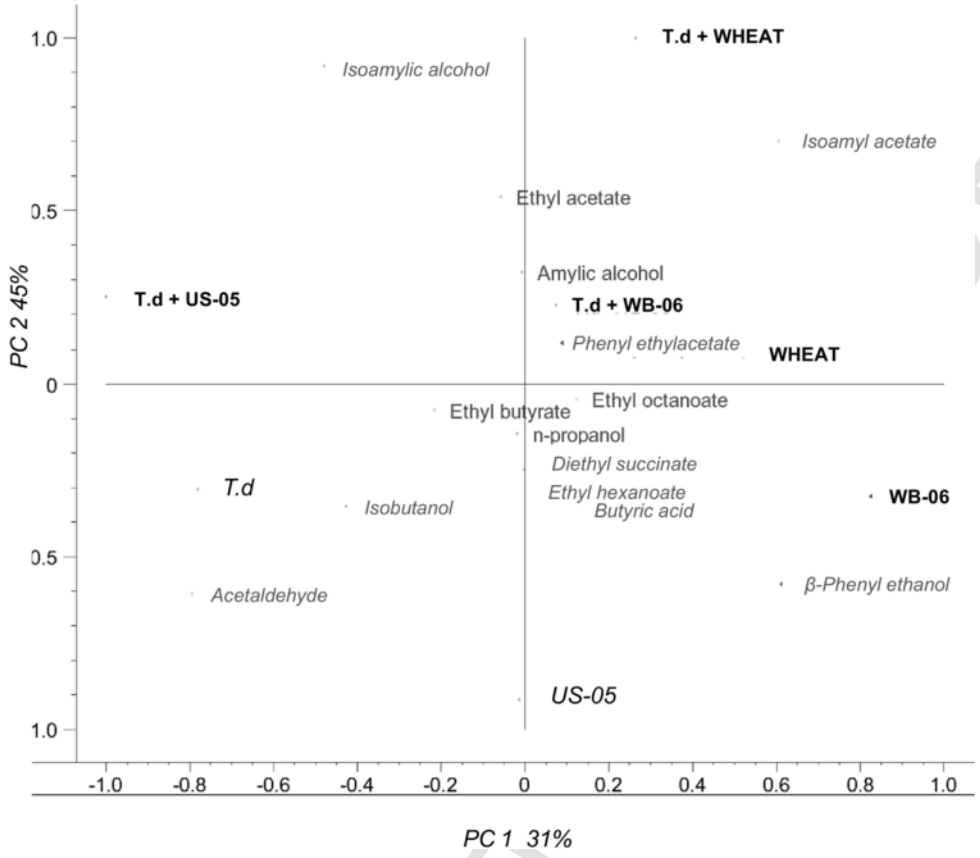

Fig. 3. Principal component analysis for the esters, alcohols, carbonyl compounds and carboxylic acids of the pure *S. cerevisiae* fermentations (US-05, WB-06, WHEAT [Belgian Wheat]) and their mixed fermentations with *T. delbrueckii* (T.d.). The variance explained by PCA analysis is 86% (PC 1 31% X-axis and PC 2 45% Y-axis).

also in relation to the wort composition (Saerens et al., 2008; Verstrepen et al., 2003; Younis and Stewart, 1998).

Uncited references

Boulton, 2006
Polaina, 2002

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