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Torulaspora delbrueckii in the brewing process: A new approach to enhance bioflavour and to reduce ethanol content

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Abstract: Nowadays, consumers require fermented alcoholic beverages with particular and enhanced flavour profiles while avoiding the health concerns due to high ethanol content. Here, the use of *Torulaspora delbrueckii* was evaluated for beer production, in both pure and in mixed cultures with a *Saccharomyces cerevisiae* starter strain (US-05). The yeast interactions were also evaluated. In mixed fermentations with *S. cerevisiae*, the main analytical characters from *T. delbrueckii* were comparable with those of the *S. cerevisiae* starter strain, but the beers were characterized by a distinctive overall analytical and aromatic profile. Indeed, there were interactions between *S. cerevisiae* and *T. delbrueckii*, with enhanced ethyl hexanoate (0.048 mg l<sup>-1</sup>) and ethyl octanoate (0.014 mg l<sup>-1</sup>) levels at the 1:20 and 1:10 inoculation ratios, respectively; while phenyl ethyl acetate increased in all mix combinations. The presence of *T. delbrueckii* resulted in reduced  $\beta$ -phenyl ethanol and isoamyl acetate levels, which are responsible for floral and fruity aromas, respectively. Beer produced with *T. delbrueckii* pure cultures had a low alcohol content (2.66%; v/v), while also showing a particularly analytical and aromatic profile.

Dear Editor,

We would be glad if you would reconsider the enclosed revised manuscript, entitled, “***Torulaspota delbrueckii* in the brewing process: a new approach to enhance bioflavour and to reduce ethanol content**”, for inclusion in *Food Microbiology*.

We carefully revised the manuscript following the suggestions of the reviewer 2.

Yours faithfully

Prof. Maurizio Ciani

## \*Detailed Response to Reviewers

Response to reviewers

Reviewers' comments:

Reviewer #2: In the highlights the genus names should be written in full:  
Torulaspora delbrueckii and Saccharomyces cerevisiae

Corrected in the highlights

Line 67: However, the Belgian lambic beer is obtained from the fermentation of  
Change to: However, the Belgian lambic beers are obtained from the spontaneous fermentation ...

Corrected in the text

Line 136: bottles, adding ...  
Change to: bottles, adding

we suppose Line 140 the space was already present in previous R2 version

Line 202: ...showing very closed cell ...  
Change to: ...showing very similar cell ...

we suppose line 206 "similar" at place of "closed" already corrected in the previous version R2

Line 211: ...together with maltose residue.  
Change to: ...associated with higher residual maltose levels.

Changed in the text

Lines 213 and 214: use absolute numbers for attenuation (no decimals)

Corrected in the text

Line 216: ...an higher consumption ...  
Change to: ...a higher consumption ... (as already mentioned before!)

we suppose line 220 already corrected in the R2 previous version

Line 218: a competitive interactions in mixed fermentation even if a large amounts  
... Change to: a competitive interaction in mixed fermentation even if large amounts  
...

Corrected in the text

Lines 297-298: not clear  
Do you mean: ...that in mixed fermentations (...) exhibiting a higher consumption of YAN this could be related to the production of aromatic compounds (..)?

Yes, we corrected in the text following your suggestion.

Reviewer #3: This paper presents an interesting study about the application of non-Saccharomyces yeasts in brewing. In particular, the paper studies the influence of *Torulaspora delbrueckii* on analytical and aromatic profile.

All the comments indicated by previous reviewers have been resolved, and this has significantly improved the paper, specially the introduction.

## Highlights

The potential use of a selected *Torulaspora delbrueckii* strain in the brewing process was evaluated.

Mixed fermentation *Torulaspora delbrueckii*/*Saccharomyces cerevisiae* produced beer with distinctive analytical and aromatic profile.

*Torulaspora delbrueckii* may be proposed to produce a low alcohol beer.

1 ***Torulaspota delbrueckii* in the brewing process: a new approach to enhance bioflavour**  
2 **and to reduce ethanol content**

3

4

5

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19 **Abstract**

20

21 Nowadays, consumers require fermented alcoholic beverages with particular and enhanced  
22 flavour profiles while avoiding the health concerns due to high ethanol content. Here, the use  
23 of *Torulaspora delbrueckii* was evaluated for beer production, in both pure and in mixed  
24 cultures with a *Saccharomyces cerevisiae* starter strain (US-05). The yeast interactions were  
25 also evaluated. In mixed fermentations with *S. cerevisiae*, the main analytical characters from  
26 *T. delbrueckii* were comparable with those of the *S. cerevisiae* starter strain, but the beers were  
27 characterized by a distinctive overall analytical and aromatic profile. Indeed, there were  
28 interactions between *S. cerevisiae* and *T. delbrueckii*, with enhanced ethyl hexanoate (0.048  
29 mg l<sup>-1</sup>) and ethyl octanoate (0.014 mg l<sup>-1</sup>) levels at the 1:20 and 1:10 inoculation ratios,  
30 respectively; while phenyl ethyl acetate increased in all mix combinations. The presence of *T.*  
31 *delbrueckii* resulted in reduced β-phenyl ethanol and isoamyl acetate levels, which are  
32 responsible for floral and fruity aromas, respectively. Beer produced with *T. delbrueckii* pure  
33 cultures had a low alcohol content (2.66%; v/v), while also showing a particularly analytical  
34 and aromatic profile.

35

36 **Keywords:** beer, *Torulaspora delbrueckii*, mixed fermentation, bioflavour, low-alcohol  
37 content

38

39

## 40 **1. Introduction**

41

42 Over the years, brewers have always tried to find yeast strains that can improve the quality of  
43 their beer and provide it with specific sensory notes. The various aroma compounds that  
44 characterize different beers styles come from the raw materials of barley, malt and hops.  
45 However, yeast has a central role in the brewing process, metabolizing of sugars in the beer  
46 wort into ethanol, carbon dioxide, and several aroma compounds, including esters, higher  
47 alcohols, aldehydes and organic acids (Kyselová and Brányik, 2014; Lodolo et al., 2008; Pires  
48 et al., 2014). In particular, in the beer industry, the goal of the use of inoculated yeast is to  
49 increase the fermentation efficiency, to develop new beers, and especially to enhance the  
50 sensory complexity of the final beer produced (Harrison, 2009). The production of aroma  
51 compounds through biological methods exploits the metabolic pathways of the yeast, for the  
52 promotion of the so-called bioflavour (Cheetham, 1993; Vanderhaegen et al., 2003). This  
53 approach can include microbial bioconversion of the flavour precursors, use of strains that  
54 produce the required compounds, and genetic modification of the yeast (Dequin, 2001;  
55 Priefert et al., 2001; Ramachandra and Ravishankar, 2000; Mertens et al., 2015).

56 In winemaking, there has been a re-evaluation of the role of non-*Saccharomyces* yeast and  
57 their use in mixed fermentations, with the aim to enhance the analytical and aromatic profile  
58 of the final wine and to reduce the alcohol content (Benito et al., 2011; Ciani et al., 2010;  
59 Comitini et al., 2011; Contreras et al., 2014; Morata et al., 2012; Quirós et al., 2014; Sadoudi  
60 et al., 2012). Within the non-*Saccharomyces* yeast species, attention has been focused on  
61 *Torulaspora delbrueckii*, as this yeast has shown a positive impact in terms of low production  
62 of undesirable compounds, such as acetaldehyde, acetoin and acetic acid, and concomitant  
63 enhancement of other desired compounds (Azzolini et al., 2015; Bely et al., 2008; Comitini et

64 al., 2011; Jolly et al., 2014; Loira et al., 2012). The use of non-*Saccharomyces* yeast has been  
65 less investigated in the brewing industry, where most beers are brewed with the use of a  
66 single yeast strain. However, the Belgian lambic beers are obtained from the spontaneous  
67 fermentation of *Saccharomyces* and *Brettanomyces* yeasts, with the contribution of lactic acid  
68 bacteria and acetic acid bacteria (Bokulich et al., 2012; Spitaels et al., 2014; Vanderhaegen et  
69 al., 2003). These mixed fermentations were also used in the production of some weissbier  
70 German style beer (Vriesekoop et al., 2012). During the maturation of acidic ale beers,  
71 different yeasts belonging to *Candida*, *Torulopsis*, *Pichia*, *Hansenula* and *Cryptococcus*  
72 genera were isolated, but their contribution on the aroma composition was not investigated  
73 (Vanderhaegen et al., 2003). Other non conventional beers such as Tchapalo, are brewed  
74 using *Candida tropicalis* and *S. cerevisiae* cultures selected for their ability to ferment  
75 sorghum wort (N'Guessan et al., 2010). Regarding to the use of *T. delbrueckii* strains in  
76 brewing process, only a few studies have been conducted. King and Dickinson (2000, 2003)  
77 reported that *T. delbrueckii* has the ability to transform hop aroma terpenoids, influencing the  
78 aroma profile of the final beer. More recently, Tataridis and co-workers (2013) carried out a  
79 preliminary study on the use of *T. delbrueckii* strains in the production of “wheat” style beers.  
80 These authors found that this species was able to consume maltose more slowly than *S.*  
81 *cerevisiae* commercial starter strain, giving more intensity and complexity to the product. In  
82 the present study, after a preliminary screening, we evaluated the use of a selected strain of *T.*  
83 *delbrueckii* in wort fermentation in pure and mixed cultures. The influence on the analytical  
84 and aromatic profile of beer, as well as the potential of producing a low-alcohol beer with *T.*  
85 *delbrueckii* was evaluated.

## 86 **2. Materials and methods**

87

88 *2.1. Yeast strains*

89 The 28 yeast strains used in this study belong to the species *T. delbrueckii* and were obtained  
90 from the Yeast Collection of the Department of Life and Environmental Sciences (DiSVA) of  
91 the Polytechnic University of Marche (Italy). These had been isolated from natural matrices  
92 from different environments and in different geographical areas (i.e., Italy, Cameroons)  
93 (Table 1). All of the *T. delbrueckii* strains were identified through 5.8S internal transcribed  
94 spacer rDNA polymerase chain reaction restriction fragment length polymorphism analysis,  
95 and sequencing of the D1/D2 domains of the 26S rDNA gene, as reported by Comitini et al.  
96 (2011) and Solieri et al. (2006). The *S. cerevisiae* commercial strain US-05 (Fermentis,  
97 Lesaffre, France) was used as the control.

98 For short-term storage, all of the yeast strains were maintained on YPD medium (1% yeast  
99 extract, 2% peptone, 2% glucose, 1.8% agar; all w/v) (Oxoid, Basingstoke, UK) at 4 °C, and  
100 for long-term storage, in YPD broth supplemented with 80% (w/v) glycerol at -80 °C.

101

102 *2.2. Preliminary screening*

103 The fermentation of glucose, maltose and sucrose by these 28 *T. delbrueckii* strains was  
104 assessed using the Durham test, according to Kurtzman and Fell (1998). The fermentative  
105 performance of eight of these *T. delbrueckii* strains that fermented maltose were determined at  
106 20 °C in flasks that contained 500 ml malted barley wort under sterile conditions. The main  
107 parameters of the fermentation kinetics (fermentation rate, total CO<sub>2</sub> evolved) of these *T.*  
108 *delbrueckii* strains and of the *S. cerevisiae* starter strain on the wort were assayed.

109

110 *2.3. Fermentation trials*

111 From preliminary screening *T. delbrueckii* DiSVA 254 was selected and used in the pure and  
112 mixed fermentations with the *S. cerevisiae* US-05 starter strain at different *S. cerevisiae* to *T.*  
113 *delbrueckii* ratios (i.e., 1:1, 1:10, 1:20, respectively). A batch of 1,500 l of malted barley wort  
114 for the production of American Amber Ale was used in this study. Its main analytical  
115 characters were: pH 5.47; specific gravity 12.7 °Plato. The fermentation potential of the  
116 selected strain was evaluated in fermentation trials carried out at 20 °C in flasks containing  
117 500 ml wort under sterile conditions. The flasks were locked with a Müller valve containing  
118 sulphuric acid, to allow only CO<sub>2</sub> to escape from the system.

119 Pre-cultures were grown in 10% malt extract at 20 °C for 48 h. The fermentation kinetics  
120 were monitored by measuring the weight loss of the flasks due to the CO<sub>2</sub> evolution, which  
121 was followed to the end of the fermentation (i.e., constant weight for 3 consecutive days). The  
122 growth kinetics were monitored using viable cell counts on WL Nutrient Agar (Oxoid,  
123 Hampshire, UK) and Lysine Agar (Oxoid, Hampshire, UK) a selective medium unable to  
124 support the growth of *S. cerevisiae* (Lin, 1975) , for differentiation of the *T. delbrueckii* yeast  
125 from the *S. cerevisiae* starter strain. The fermentations were carried out in duplicate trials  
126 under static conditions.

127

#### 128 *2.4. Analytical determinations*

129 The specific gravity was measured using a DA-300 specific gravity meter (Kyoto  
130 Instruments). The volatile acidity and pH determinations were performed according to the  
131 Official European Union Methods (EC, 2000). Ethanol was measured according to the  
132 Association of Official Analytical Chemists (1990). Acetaldehyde, ethyl acetate, higher  
133 alcohols, and volatile compounds were determined by direct injection into a gas-liquid  
134 chromatography system, as reported by Canonico et al. (2014). The free amino nitrogen was

135 determined following a procedure described previously by Dukes and Butzke (1998). Specific  
136 enzymatic kits (Megazyme, Ireland) were used to determine the concentrations of glucose  
137 sucrose, maltose (kit k-masug) and ammonia (kit k-amiar) according to the manufacturer  
138 instructions.

### 139 *2.5. Sensory analysis*

140 At the end of the fermentation process, the beers obtained were transferred into 500-ml  
141 bottles, adding 5 g l<sup>-1</sup> sucrose. The secondary fermentation in the bottle was carried out at 18-  
142 20 °C for 7-10 days. After this period, the beers underwent sensory analysis (Analytica EBC,  
143 1997) on the basis of a list of descriptors related to both the aromatic notes (e.g., floral, fruity,  
144 toasty) and the main structural features (e.g., sweet, acidity, flavour, astringency, bitterness,  
145 olfactory persistence). A group of six trained tasters were asked to rate each sensory category  
146 using a scale from 1 to 10. The results were combined, and the means were subjected to  
147 statistical analysis. The data processed in this way were used to construct the Figures to  
148 provide information on both the contribution of each descriptor to the overall organoleptic  
149 quality of the beers, and the significant differences between the beers in relation to each  
150 descriptor.

151

### 152 *2.6. Statistical analysis*

153 Analysis of variance (ANOVA) was applied to the experimental data for the main  
154 characteristics of the beers. The means were analyzed using the STATISTICA 7 software.  
155 The significant differences were determined by the means of Duncan tests, and the results  
156 were considered significant if the associated *P* values were <0.05. The results of the sensory  
157 analysis were also subjected to Fisher ANOVA, to determine the significant differences with  
158 a p-value <0.05.

### 159 3. Results

160

#### 161 3.1. Sugar fermentation and fermentative performance in the wort

162 Initial screening of the 28 *T. delbrueckii* strains was carried out to determine whether they  
163 fermented maltose, the most abundant fermentable sugar in the brewing wort (at 50%-60%),  
164 to select for their potential use in beer production. The data from this screening with Durham  
165 tubes indicated that out of the 28 strains tested, only eight cultures fermented maltose: DiSVA  
166 254, DiSVA 602, DiSVA 603, DiSVA 343, DiSVA 399, DiSVA 413, DiSVA 419 and  
167 DiSVA 426. All of the 28 *T. delbrueckii* strains fermented glucose and sucrose.

168 These eight strains of *T. delbrueckii* that fermented maltose were evaluated for their  
169 fermentative performance on the brewing wort. The parameters of the fermentation kinetics  
170 are reported in Table 2. As expected, none of these eight strains showed fermentation  
171 parameters comparable to those of the commercial *S. cerevisiae* starter strain. This behaviour  
172 is important since the strains with lower fermentation performances in the wort are generally  
173 of interest for low-alcohol beer production. However, among the strains tested, *T. delbrueckii*  
174 strain DiSVA 254 showed a good fermentative performance, with the highest fermentation  
175 rate and final CO<sub>2</sub> production. . For this reason, *T. delbrueckii* DiSVA 254 was selected for the  
176 subsequent trials in pure and mixed fermentations with the *S. cerevisiae* starter strain US-05,  
177 to evaluate the influence of *T. delbrueckii* DiSVA 254 (henceforth: *T. delbrueckii*) on the  
178 analytical and aromatic profiles of the beers produced.

179

#### 180 3.2. Fermentation trials with the selected *T. delbrueckii* strain

181

182 3.2.1. Fermentation kinetics

183 *T. delbrueckii* pure cultures and the three *S. cerevisiae* US-05 to *T. delbrueckii* inoculum  
184 ratios of 1:1, 1:10 and 1:20, respectively, were investigated, with *S. cerevisiae* US-05 pure  
185 cultures as the control. These mixed cultures of *S. cerevisiae* US-05 and *T. delbrueckii*  
186 showed CO<sub>2</sub> production that was comparable to that of *S. cerevisiae* US-05 alone, irrespective  
187 of the different inoculation ratios (Fig. 1).

188 The fermentation evolution of the trials inoculated at the 1:1 ratio were consistent with the *S.*  
189 *cerevisiae* US-05 pure cultures. These data revealed that the inoculation of *T. delbrueckii* at  
190 the same concentration as *S. cerevisiae* US-05 did not affect the fermentation performance of  
191 the starter strain. In contrast, the fermentations carried out with the 1:10 and 1:20 ratios  
192 showed slower fermentation kinetics in comparison with *S. cerevisiae* US-05 alone. The *T.*  
193 *delbrueckii* pure culture also showed slow kinetics, which indicated that it did not provide  
194 complete wort attenuation.

195 3.2.2. Biomass evolution

196 The growth kinetics of the *S. cerevisiae* US-05 pure cultures achieved *ca.* 10<sup>8</sup> CFU ml<sup>-1</sup> at 7  
197 days of fermentation, and maintained 10<sup>7</sup> CFU ml<sup>-1</sup> until the end of fermentation (Fig. 2). In  
198 the mixed cultures with the inoculum ratio of 1:1, *S. cerevisiae* US-05 reached a lower  
199 biomass (*ca.* 10<sup>7</sup> CFU ml<sup>-1</sup>; Fig 2A), while for the inoculum ratios of 1:10 and 1:20, *S.*  
200 *cerevisiae* US-05 remained at cell concentrations <10<sup>6</sup> CFU ml<sup>-1</sup>(Fig. 2B, C). In these trials,  
201 the growth kinetics of *T. delbrueckii* showed biomass evolution compared with the *T.*  
202 *delbrueckii* pure culture indicating that at these inoculation ratios *S. cerevisiae* was not  
203 competitive with *T. delbrueckii*, while at the 1:1 inoculum ratio, the *S. cerevisiae* competition  
204 occurred since the *T. delbrueckii* growth kinetics were much slower. Therefore, these data  
205 indicated that *T. delbrueckii* at high concentrations (10-fold and 20-fold higher, vs. *S.*

206 *cerevisiae* US-05) dominated the process (Fig. 2). With 1:1 inoculation ratio both species  
207 suffered of the presence of each other showing very similar cell concentrations until the 7<sup>th</sup>  
208 day of fermentation. This behavior indicates a high level of competitiveness of *T. delbrueckii*  
209 towards *S. cerevisiae* commercial strain in wort.

210

### 211 3.2.3. Main analytical profiles

212 The data for the analytical compositions of the beers produced are reported in Table 3. The  
213 mixed cultures with *S. cerevisiae* US-05 and *T. delbrueckii* produced beers with ethanol levels  
214 that were significantly lower, although essentially comparable, to those of the *S. cerevisiae*  
215 US-05 control (4.51%-4.85%). On the other hand, the inoculations with *T. delbrueckii* pure  
216 cultures showed a large reduction in the ethanol level (2.66%) associated with higher residual  
217 maltose levels. Consequently, *T. delbrueckii* pure cultures showed a low attenuation (7.51 vs.  
218 2.84-2.96 °P) with the real attenuation of 37%, which was significantly lower than that for the  
219 other trials (63%-64%). For the volatile acidity, the mixed cultures produced beers with acetic  
220 acid levels that were significantly higher than for the *T. delbrueckii* pure cultures (0.15 vs.  
221 0.22-0.29 g L<sup>-1</sup>). Regarding to assimilable nitrogen (YAN), a higher consumption in mixed  
222 fermentations in comparison with pure cultures was showed. This behaviour indicated a  
223 competitive interaction in mixed fermentation even if a large amounts of YAN remained.

224

### 225 3.2.4. By-products and volatile compounds

226 The data for the main by-products are reported in Table 4. The *T. delbrueckii* pure culture  
227 showed significant reductions for the levels of all of the by-products except for acetaldehyde,  
228 in comparison with the *S. cerevisiae* US-05 control. In particular, ethyl acetate is responsible  
229 for the fruity and solvent notes in the beer, and this was slightly, but significantly, reduced in

230 the mixed fermentations compared with *S. cerevisiae* US-05 alone (14.65-16.25 vs. 17.57 mg  
231 l<sup>-1</sup>), with a significantly greater reduction for the pure *T. delbrueckii* culture (3.46 mg l<sup>-1</sup>). The  
232 acetaldehyde level was slightly, but significantly, higher in the beers produced with *T.*  
233 *delbrueckii* alone compared to *S. cerevisiae* US-05 alone (7.50 vs. 5.80 mg l<sup>-1</sup>), and even  
234 further enhanced at the 1:1 (10.72 mg l<sup>-1</sup>) and 1:10 (30.48 mg l<sup>-1</sup>) fermentation ratios.

235 For the other analyzed by-products in the mixed fermentations, all of the beers produced by  
236 the association of *S. cerevisiae* US-05 and *T. delbrueckii* showed different profiles to those  
237 produced from the *S. cerevisiae* US-05 and *T. delbrueckii* pure fermentations. Indeed, all  
238 three mixed fermentations (i.e., 1:1, 1:10, 1:20) showed lower n-propanol levels in  
239 comparison with *S. cerevisiae* US-05 (20.14-26.36 vs. 30.56 mg l<sup>-1</sup>). For isobutanol, amylic  
240 and isomylic alcohols, *S. cerevisiae* US-05 showed higher levels than the *T. delbrueckii* pure  
241 cultures (24.38 vs. 7.98 mg l<sup>-1</sup>), while the mixed fermentations showed intermediate levels  
242 (16.04-18.45 mg l<sup>-1</sup>).

243 The effects of *T. delbrueckii* on the beers were particularly evident for the main volatile  
244 compounds (Table 5). Indeed, the contribution of this non-*Saccharomyces* yeast was clear for  
245 the ethyl butyrate and  $\beta$ -phenyl ethanol levels, where the trend was strictly linked to the  
246 inoculum ratios. The mixed fermentation at the inoculum ratio of 1:20 showed ethyl butyrate  
247 levels comparable to that for the *T. delbrueckii* pure cultures (0.185 vs. 0.168 mg l<sup>-1</sup>), while  
248 for the inoculation ratio of 1:1 the ethyl butyrate levels were comparable to *S. cerevisiae* US-  
249 05 pure culture (0.339 vs. 0.319 mg l<sup>-1</sup>). This evolution was also particularly evident for  $\beta$ -  
250 phenyl ethanol. Indeed, the increasing *T. delbrueckii* in the mixed fermentations resulted in  
251 volatile  $\beta$ -phenyl ethanol levels that were lower or higher when compared with the pure  
252 cultures of *S. cerevisiae* US-05 or *T. delbrueckii*, respectively. As regards the phenyl ethyl  
253 acetate, the results showed an increase in mixed fermentations, particularly in 1:1 inoculum  
254 ratio. Different behaviors were seen for ethyl hexanoate and ethyl octanoate. Indeed, there

255 were generally higher levels of these compounds in the mixed fermentations, and particularly  
256 with the high inoculation ratio (i.e., 1:20).

### 257 3.2.5. Sensory analysis

258 The beers obtained by these pure and mixed fermentations underwent sensory analysis, with  
259 the data illustrated in Figure 3. All of the beers analyzed showed significant differences for  
260 their main aromatic notes. In particular, for the main sensorial descriptors, the data showed  
261 that the beer obtained with all of the mixed fermentations and for the *T. delbrueckii* pure  
262 cultures were significantly different from those of the *S. cerevisiae* US-05 starter strain for a  
263 variety of the sensorial characteristics. Within the mixed fermentations, with the 1:1 inoculum  
264 ratio, there was a bouquet with notes that particularly emphasized the cereal, toasted and full-  
265 bodied sensorial attributes, while the fruity/ ester notes were poorly pronounced. At the 1:10  
266 inoculum ratio, there was a high perception of the alcohol/ solvent, malt, caramel and  
267 oxidized/ aged attributes, while those for fruit/ citric were lower and watery. Then for the 1:20  
268 inoculum ratio, the fruity/ester and hop attributes were enhanced. For the *T. delbrueckii* pure  
269 cultures, these were characterized by fruit/ citric notes and the full-bodied attributes, while the  
270 alcohol/ solvent, cereal, caramel, oxidized/ aged and astringency attributes were little  
271 expressed. In addition, the beers produced by the *T. delbrueckii* pure cultures and with the  
272 inoculum ratio of 1:20 were characterized by a pale yellow colour, clarity, and persistent and  
273 compact foam, which are very important features in the assessment of the quality of a beer  
274 (data not shown).

275

## 276 4. Discussion

277

278 In the present study, the use of non-conventional yeast to produce beers with distinctive  
279 bioflavours and reduced alcohol contents was investigated. Fermented beverages with  
280 improved flavours can be profitably obtained through the yeast during the fermentation  
281 process (Carlquist et al., 2015). Indeed, among the volatile compounds produced by yeast, the  
282 higher alcohols and esters are crucial to the definition of the final quality of a beer (Pires et  
283 al., 2014).

284 Of particular interest here was the production of the three esters phenyl ethyl acetate (i.e.,  
285 floreal, honey, sweet) ethyl hexanoate (i.e., apple, fruit flavours) and ethyl octanoate (i.e.,  
286 apple, aniseed flavours), which showed increases for the mixed fermentations, possibly  
287 related to the increase in consumption of YAN. These data were only in part consistent with  
288 the results obtained by sensory analysis. Indeed, the panel tests showed that the beers made  
289 with *T. delbrueckii* showed fruity/esters comparable with those obtained with *S. cerevisiae*  
290 US-05 while they were characterized for malt, caramel and hop attributes. This might be  
291 explained considering that some esters that are characterized by a powerful odour can act in  
292 synergy with other compounds, thus influencing the final beer flavor at concentrations below  
293 the threshold of perception (Meilgaard, 1975).

294 The formation of  $\beta$ -phenyl ethanol, diethyl succinate, ethyl butyrate and isoamyl acetate,  
295 relates directly to the inoculation levels, and thus to the consequent metabolic expression  
296 levels of each of the two strains in the mixed fermentations. These results clearly showed that  
297 *T. delbrueckii* more significant affects the analytical and aromatic profile of beers when the  
298 inoculation ratios for *S. cerevisiae* US-05 to *T. delbrueckii* were at 1:10 or 1:20.

299 Over the years, several studies have shown that the levels of the aroma compounds can be  
300 increased or decreased through modifications to the constituents of the wort, such as the  
301 nitrogen content (Hernández-Orte et al., 2005, Igyor et al., 2001). In this context, we found

302 that in mixed fermentations (*S. cerevisiae*/*T. delbrueckii*) exhibiting a higher consumption of  
303 YAN this could be related to the production of aromatic compounds (Carrau et al., 2015).  
304 Also the use of commercial enzyme preparations, such as proteases that act on certain  
305 proteins in the malt can promote increased contents of the higher alcohols (Pidcocke et al.,  
306 2011; Treimo et al., 2008). However, the results of the present study show that modifications  
307 of the volatile profile of beers can be easily obtained without any modifications to the  
308 brewing process or the use of enzymes, but instead through the use of non-conventional yeast,  
309 such as *T. delbrueckii*.

310 Another important feature of the use of *T. delbrueckii* in this brewing process might be the  
311 production of beer with a low alcohol content. Indeed, nowadays, as well as considering the  
312 aromatic profile of a beer, consumers pay more attention to health-related problems. The  
313 biological approaches that have been proposed to reduce the ethanol content in beer mainly  
314 rely on the selection of strains with particular properties or on the modification of the brewing  
315 yeast through genetic engineering (Remize et al., 1999; Verstrepen et al., 2003). Taking  
316 advantage of fermentative performance, some practices are based on the use of *S. cerevisiae*  
317 which is also generally used in winemaking.

318 Another yeast species that has been proposed for the production of beer with a low alcohol  
319 content on an industrial scale is *Saccharomyces ludwigii* (Huige et al., 1990). In this regard,  
320 however, conflicting data on the volatile profiles of the beers have been reported (De  
321 Francesco et al., 2015; Narziss et al., 1992). In the present study, we have shown instead that  
322 *T. delbrueckii* used in mixed fermentations had little or no effect on the ethanol content in  
323 comparison with the *S. cerevisiae* US-05 pure cultures. In contrast, *T. delbrueckii* in pure  
324 cultures showed a substantial, almost 50%, reduction in the ethanol content that was  
325 accompanied by a pleasant and valuable sensorial profile. In this regard, other *T. delbrueckii*

326 strains initially selected in this study as maltose fermenters might be of interest for low-  
327 alcohol beer production.

328 In conclusion, beers produced with *T. delbrueckii* in mixed fermentations and in pure cultures  
329 show important features that are relevant for brewing beer. In particular, the beer obtained  
330 using *T. delbrueckii* pure cultures showed a low alcohol content while maintaining at the  
331 same time a pleasant and aromatic taste, a lighter colour, and a compact and persistent foam.  
332 On the other hand, *T. delbrueckii* in mixed fermentations with *S. cerevisiae* US-05 can fully  
333 convert the fermentable sugars (63- 64% of real attenuation), thus resulting in a final ethanol  
334 content that is comparable to that of the control *S. cerevisiae* US-05 starter strain, while  
335 showing distinctive analytical and aromatic profiles.

336

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481 **Table 1.** *Torulaspota delbrueckii* strains used in this study.

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<b>Source of isolation</b>	<b>Geographic origin</b>	<b>Strain code<sup>a</sup></b>
Winery environment	Sardinia, Italy	315, 130
Soil	Italy	55
Papaya leaves	Cameroon	254, 419, 343, 413, 255
Sugar cane juice	Cameroon	426, 430, 431, 432, 363, 603
Grapes	Italy	258, 259, 260, 261, 313, 606, 607, 608, 609
Fig fruit	Italy	604, 605
Coconut palm	Cameroon	445
Corrosol fruit	Cameroon	602, 399

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484 <sup>a</sup> Accession number of DiSVA Collection (Department of Life and Environmental Sciences).

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489 **Table 2.** Fermentation kinetics parameters of the eight *T. delbrueckii* strains and the *S.*  
 490 *cerevisiae* US-05 starter strain for the wort.

DiSVA code	Total CO <sub>2</sub> evolved (g [30 days] <sup>-1</sup> ) <sup>a</sup>	Fermentation rate (g CO <sub>2</sub> day <sup>-1</sup> ) <sup>b</sup>
254	13.99 ±0.68	0.96 ±0.05
602	5.87 ±1.04	0.68 ±0.10
603	4.92 ±0.37	0.61 ±0.04
343	5.94 ±0.80	0.68 ±0.11
399	4.24 ±0.20	0.51 ±0.03
413	4.69 ±0.38	0.59 ±0.01
419	4.62 ±0.08	0.55 ±0.02
426	4.48 ±0.40	0.61 ±0.06
<i>S. cerevisiae</i> <sup>c</sup>	20.97 ±0.58	3.34 ±0.04

491

492 Data are means ±standard deviations

493 <sup>a</sup>CO<sub>2</sub> g evolved after 30 days of fermentation (in 500 ml wort)

494 <sup>b</sup>Fermentation rate: CO<sub>2</sub> g/day (over the first 6 days of fermentation)

495 <sup>c</sup>Commercial strain: *S. cerevisiae* US-05 (Fermentis, Lesaffre, France)

496

**Table 3.** The main analytical characteristics of the beer produced by the pure and mixed fermentations.

Fermentation	Ratio	Residual sugar (g l <sup>-1</sup> ) <sup>a</sup>				Analytical characteristic					
		Sucrose	Glucose	Maltose	YAN (mg l <sup>-1</sup> ) <sup>b</sup>	pH	Ethanol (% v/v)	Wort gravity attenuation (°P) <sup>c</sup>	Apparent attenuation (%)	Real attenuation (%)	Volatile acidity (g l <sup>-1</sup> )
<i>S. cerevisiae</i> US-05	Pure	0.02±0.01	0.01 ±0.00	0.05 ±0.01	193.84 ±0.39	4.80 ±0.03 <sup>a</sup>	4.85 ±0.00 <sup>a</sup>	2.84 ±0.00 <sup>b</sup>	78.40 ±0.00 <sup>a</sup>	63.73 ±0.00 <sup>a</sup>	0.22 ±0.00 <sup>c</sup>
<i>T. delbrueckii</i> DiSVA254	Pure	0.42 ±0.01	0.00 ±0.00	35.43 ±0.31	174.58 ±3.57	4.56 ±0.01 <sup>c</sup>	2.66 ±0.05 <sup>d</sup>	7.51 ±0.00 <sup>a</sup>	45.09 ±0.00 <sup>b</sup>	36.65 ±0.00 <sup>b</sup>	0.15 ±0.02 <sup>d</sup>
<i>S. cerevisiae</i> US-05 + <i>T. delbrueckii</i> DiSVA254	1:1	0.02 ±0.01	0.02 ±0.01	0.33 ±0.00	141.79±1.96	4.61 ±0.01 <sup>b</sup>	4.51 ±0.00 <sup>c</sup>	2.96 ±0.17 <sup>b</sup>	77.44 ±0.01 <sup>a</sup>	62.76 ±0.01 <sup>a</sup>	0.23 ±0.01 <sup>c</sup>
	1:10	0.08 ±0.00	0.03 ±0.00	0.75 ±0.05	144.32 ±2.41	4.43 ±0.01 <sup>d</sup>	4.68 ±0.03 <sup>b</sup>	2.84 ±0.00 <sup>b</sup>	78.40 ±0.00 <sup>a</sup>	63.73 ±0.00 <sup>a</sup>	0.26 ±0.00 <sup>b</sup>
	1:20	0.10 ±0.01	0.04 ±0.02	1.65 ±0.10	142.87 ±2.41	4.38 ±0.00 <sup>d</sup>	4.59 ±0.07 <sup>bc</sup>	2.96 ±0.17 <sup>b</sup>	77.44 ±0.01 <sup>a</sup>	62.76 ±0.01 <sup>a</sup>	0.29 ±0.00 <sup>a</sup>

<sup>a</sup>The initial composition of the sugars in wort were: Sucrose 6.4 g l<sup>-1</sup>; glucose 8.5g l<sup>-1</sup>; Maltose 64.06 g l<sup>-1</sup>

<sup>b</sup>The initial yeast assimilable nitrogen (YAN) were 251 mg l<sup>-1</sup>

<sup>c</sup>The wort gravity at the start was 12.7 °P.

Data are means± standard deviation

Data with different superscript letters (<sup>a,b,c,d</sup>) within each column are significantly different (Duncan tests; P <0.05).

**Table 4.** The main by-products in the beer produced by the pure and mixed fermentations.

Fermentation	Ratio	By-products (mg l <sup>-1</sup> )					
		Acetaldehyde	Ethyl acetate	n-propanol	Isobutanol	Amylic alcohol	Isoamylic alcohol
<i>S. cerevisiae</i> US-05	Pure	5.80 ±0.45 <sup>d</sup>	17.57 ±0.24 <sup>a</sup>	30.56 ±0.29 <sup>a</sup>	24.38 ±0.31 <sup>a</sup>	12.69 ±0.22 <sup>a</sup>	68.53 ±0.08 <sup>a</sup>
<i>T. delbrueckii</i> DiSVA254	Pure	7.50 ±0.40 <sup>c</sup>	3.46 ±0.40 <sup>d</sup>	15.41 ±0.04 <sup>c</sup>	7.98 ±0.10 <sup>d</sup>	3.82 ±0.04 <sup>c</sup>	32.79 ±0.42 <sup>e</sup>
<i>S. cerevisiae</i> US-05 +	1:1	10.72 ±0.13 <sup>b</sup>	16.75 ±0.06 <sup>b</sup>	26.36 ±0.29 <sup>b</sup>	18.45±0.32 <sup>b</sup>	10.60 ±0.13 <sup>b</sup>	58.69 ±0.35 <sup>c</sup>
<i>T. delbrueckii</i> DiSVA254	1:10	30.48 ±0.22 <sup>a</sup>	14.65 ±0.04 <sup>c</sup>	20.14 ±0.21 <sup>d</sup>	16.04 ±0.34 <sup>c</sup>	10.61 ±0.60 <sup>b</sup>	55.65 ± 0.47 <sup>d</sup>
	1:20	5.56 ±0.15 <sup>d</sup>	16.25 ±0.16 <sup>b</sup>	22.59 ±0.06 <sup>c</sup>	16.15 ±0.24 <sup>c</sup>	11.29 ±0.19 <sup>b</sup>	60.05 ±0.06 <sup>b</sup>

Data are means ± standard deviations

Data with different superscript letters (<sup>a,b,c,d,e</sup>) within each column are significantly different (Duncan tests; P <0.05).

**Table 5.** The main volatile compounds in the beer produced by the pure and mixed fermentations.

Fermentation	Ratio	Main volatile compounds (mg l <sup>-1</sup> )							
		Ethyl butyrate	Isoamyl acetate	Ethyl hexanoate	Ethyl octanoate	Butyric acid	Diethyl succinate	phenyl ethyl acetate	β-phenyl ethanol
<i>S. cerevisiae</i> US-05	Pure	0.319±0.01 <sup>a</sup>	0.346±0.045 <sup>a</sup>	0.037±0.010 <sup>b</sup>	0.007±0.001 <sup>b</sup>	0.158±0.033 <sup>ab</sup>	0.016±0.004 <sup>ab</sup>	0.001±0.001 <sup>b</sup>	40.77±0.444 <sup>a</sup>
<i>T. delbrueckii</i> DiSVA254	Pure	0.168±0.00 <sup>c</sup>	0.134±0.005 <sup>d</sup>	0.031±0.013 <sup>b</sup>	0.006±0.003 <sup>b</sup>	0.074±0.028 <sup>bc</sup>	ND	ND	6.52±0.038 <sup>c</sup>
<i>S. cerevisiae</i> US-05 +	1:1	0.339±0.018 <sup>a</sup>	0.321±0.001 <sup>ab</sup>	0.023±0.011 <sup>c</sup>	0.009±0.003 <sup>ab</sup>	0.184±0.058 <sup>a</sup>	0.039±0.022 <sup>a</sup>	0.008±0.129 <sup>a</sup>	30.69±0.018 <sup>ab</sup>
<i>T. delbrueckii</i> DiSVA254	1:10	0.26±0.03 <sup>b</sup>	0.258±0.017 <sup>bc</sup>	0.019±0.004 <sup>c</sup>	0.014±0.001 <sup>a</sup>	0.119±0.021 <sup>abc</sup>	0.023±0.010 <sup>ab</sup>	0.004±0.001 <sup>a</sup>	27.53±0.035 <sup>c</sup>
	1:20	0.185±0.00 <sup>c</sup>	0.227±0.030 <sup>c</sup>	0.048±0.011 <sup>a</sup>	0.009±0.001 <sup>b</sup>	0.047±0.022 <sup>c</sup>	0.010±0.007 <sup>b</sup>	0.003±0.001 <sup>a</sup>	15.48±0.115 <sup>d</sup>

Data are means ±standard deviations

Data with different superscript letters (<sup>a,b,c,d</sup>) within each column are significantly different (Duncan tests; P <0.05).

ND : not detected

## Figure captions

**Figure 1.** Fermentation kinetics of the pure and mixed fermentations. Pure cultures of *S. cerevisiae* ( - ▲ - ) and *T. delbrueckii* ( --■-- ), and mixed cultures of *S. cerevisiae* + *T. delbrueckii* at 1:1 ( —■— ), 1:10 ( --■-- ) and 1:20 ( ...▲... ).

**Figure 2.** Growth kinetics of the pure and mixed fermentations. Pure cultures of *S. cerevisiae* ( - ▲ - ) and *T. delbrueckii* ( --■-- ) (A-C), and of the mixed fermentation with *S. cerevisiae* ( —▲— ) and *T. delbrueckii* ( —■— ) individually for the mixed cultures at 1:1 (A), 1:10 (B) and 1:20 (C).

**Figure 3.** Sensory analysis of the beer produced by the mixed fermentations. From pure cultures of *S. cerevisiae* ( - ▲ - ) and *T. delbrueckii* ( --■-- ), and mixed cultures of *S. cerevisiae* + *T. delbrueckii* at 1:1 ( —■— ), 1:10 ( --■-- ) and 1:20 ( ...▲... ). \*, Significantly different (Fisher ANOVA; p-value 0.05). DMS, Dimethyl sulfide.

FIG 1

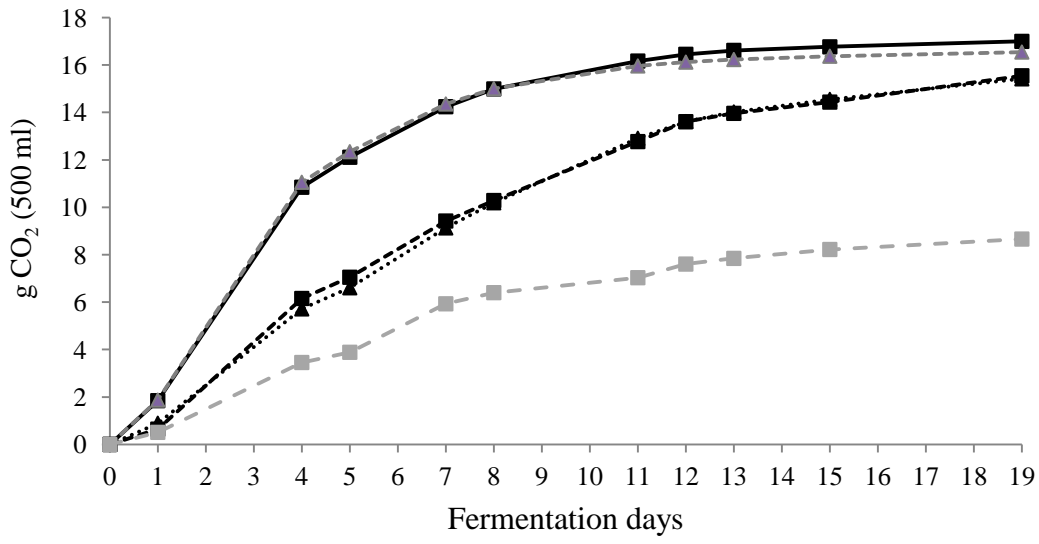


FIG 2

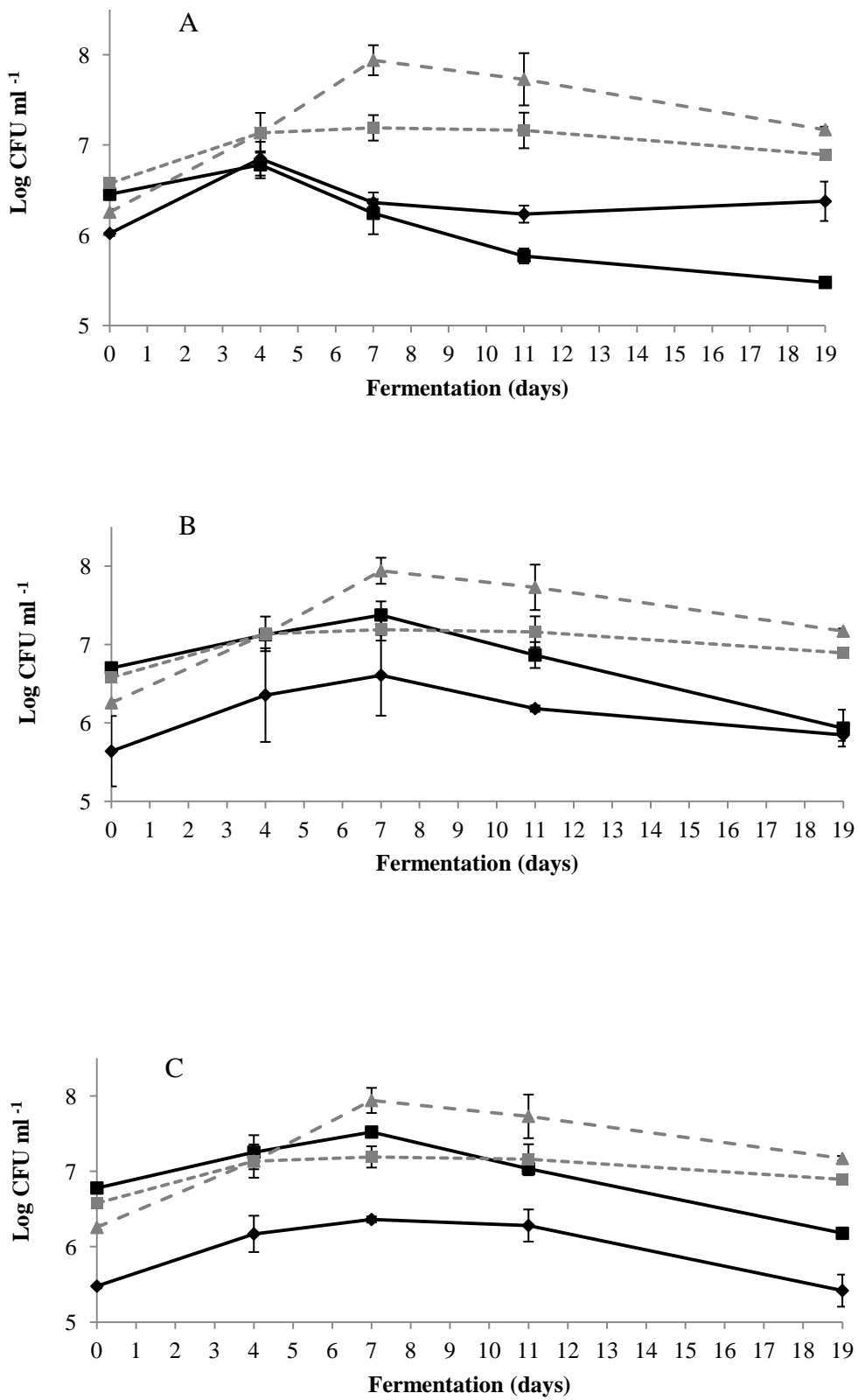


FIG 3

