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. debrueckii 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-1) and ethyl octaonate (0.014 mg l-1) 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 β-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, "***Torulaspora 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.

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. debrueckii* 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^{-1}) and ethyl octaonate (0.014 mg l^{-1}) 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 β-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.

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

1. Introduction

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

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

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

2. Materials and methods

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_2 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*

From preliminary screening *T. delbrueckii* DiSVA 254 was selected and used in the pure and mixed fermentations with the *S. cerevisiae* US-05 starter strain at different *S. cerevisiae* to *T. delbrueckii* ratios (i.e., 1:1, 1:10, 1:20, respectively). A batch of 1,500 l of malted barley wort for the production of American Amber Ale was used in this study. Its main analytical characters were: pH 5.47; specific gravity 12.7 °Plato. The fermentation potential of the selected strain was evaluated in fermentation trials carried out at 20 °C in flasks containing 500 ml wort under sterile conditions. The flasks were locked with a Müller valve containing sulphuric acid, to allow only CO_2 to escape from the system.

Pre-cultures were grown in 10% malt extract at 20 °C for 48 h. The fermentation kinetics were monitored by measuring the weight loss of the flasks due to the CO_2 evolution, which was followed to the end of the fermentation (i.e., constant weight for 3 consecutive days). The growth kinetics were monitored using viable cell counts on WL Nutrient Agar (Oxoid, Hampshire, UK) and Lysine Agar (Oxoid, Hampshire, UK) a selective medium unable to support the growth of *S. cerevisiae* (Lin, 1975) , for differentiation of the *T. delbrueckii* yeast from the *S. cerevisiae* starter strain. The fermentations were carried out in duplicate trials under static conditions.

2.4. Analytical determinations

The specific gravity was measured using a DA-300 specific gravity meter (Kyoto Instruments). The volatile acidity and pH determinations were performed according to the Official European Union Methods (EC, 2000). Ethanol was measured according to the Association of Official Analytical Chemists (1990). Acetaldehyde, ethyl acetate, higher alcohols, and volatile compounds were determined by direct injection into a gas–liquid 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^{-1}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.

3. Results

3.1. Sugar fermentation and fermentative performance in the wort

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

These eight strains of *T. delbrueckii* that fermented maltose were evaluated for their fermentative performance on the brewing wort. The parameters of the fermentation kinetics are reported in Table 2. As expected, none of these eight strains showed fermentation parameters comparable to those of the commercial *S. cerevisiae* starter strain. This behaviour is important since the strains with lower fermentation performances in the wort are generally of interest for low-alcohol beer production. However, among the strains tested, *T. delbrueckii* strain DiSVA 254 showed a good fermentative performance, with the highest fermentation rate and final CO_2 production. . For this reason, *T. debrueckii* DiSVA 254 was selected for the subsequent trials in pure and mixed fermentations with the *S. cerevisiae* starter strain US-05, to evaluate the influence of *T. debrueckii* DiSVA 254 (henceforth: *T. debrueckii*) on the analytical and aromatic profiles of the beers produced.

3.2. Fermentation trials with the selected T. delbrueckii strain

3.2.1. Fermentation kinetics

T. delbrueckii pure cultures and the three *S. cerevisiae* US-05 to *T. delbrueckii* inoculum ratios of 1:1, 1:10 and 1:20, respectively, were investigated, with *S. cerevisiae* US-05 pure cultures as the control. These mixed cultures of *S. cerevisiae* US-05 and *T. delbrueckii* showed CO_2 production that was comparable to that of *S. cerevisiae* US-05 alone, irrespective of the different inoculation ratios (Fig. 1).

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

3.2.2. Biomass evolution

The growth kinetics of the *S. cerevisiae* US-05 pure cultures achieved *ca*. 10^8 CFU ml^{-1} at 7 days of fermentation, and maintained 10^7 CFU ml^{-1} until the end of fermentation (Fig. 2). In the mixed cultures with the inoculum ratio of 1:1, *S. cerevisiae* US-05 reached a lower biomass (*ca*. 10^7 CFU ml^{-1}; Fig 2A), while for the inoculum ratios of 1:10 and 1:20, *S. cerevisiae* US-05 remained at cell concentrations $<10^6$ CFU ml^{-1}(Fig. 2B, C). In these trials, the growth kinetics of *T. delbrueckii* showed biomass evolution compared with the *T. delbrueckii* pure culture indicating that at these inoculation ratios *S. cerevisiae* was not competitive with *T delbrueckii*, while at the 1:1 inoculum ratio, the *S. cerevisie* competition occurred since the *T. delbrueckii* growth kinetics were much slower. Therefore, these data 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 7th
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^{-1}). 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

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

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

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

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

3.2.5. Sensory analysis

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

4. Discussion

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

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

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

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

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

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

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

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

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

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340 **References**

341

342 Analytica EBC 13.10/1997. European Brewery Convention.
343 http://www.europeanbreweryconvention.org/.

344 AOAC, 1990. Official Methods of Analyses, 15th ed., Vol. 2, p. 700. Association of Official
345 Analytical Chemists, VA.

346 Azzolini, M., Tosi, E., Lorenzini, M., Finato, F., Zapparoli, G., 2015. Contribution to the
347 aroma of white wines by controlled *Torulaspora delbrueckii* cultures min association
348 with *Saccharomyces cereviasiae*. World J. Microbiol. Biotechnol. 31, 277-293.

349 Bely, M., Stoeckle, P., Masneuf-Pomarède, I., Dubourdieu, D., 2008. Impact of mixed
350 *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation.
351 Int. J. Food Microbiol. 122, 312-320.

352 Benito, S., Morata, A., Palomero, F., Gonzalez, M.C., Suarez-Lepe, J.A., 2011. Formation of
353 vinylphenolic pyranoanthocyanins by *Saccharomyces cerevisiae* and *Pichia*
354 *guillermondii* in red wines produced following different fermentation strategies. Food
355 Chem. 124, 15-23.

356 Bokulich, N.A., Bamforth, C.W., Mills, D.A., 2012. Brewhouse-Resident Microbiota Are
357 Responsible forMulti-Stage Fermentation of American Coolship Ale. PLoS ONE 7(4):
358 e35507. doi:10.1371/journal.pone.0035507.

359 Canonico, L., Comitini, F., Ciani, M., 2014. Dominance and influence of selected
360 *Saccharomyces cerevisiae* strains on the analytical profile of craft beer refermentation J.
361 Inst. Brew. 120, 262–267.

362 Carlquist, M., Gibson, B., Karagul Yuceer , Y., Paraskevopoulou, A., Sandell, M., Angelov,
363 A.I., Gotcheva, V., Angelov, A.D., Etschmann, M., de Billerbeck, G.M., Lidén, G., 2015.
364 Process engineering for bioflavour production with metabolically active yeasts – a mini-
365 review. Yeast. 32, 123–143.

366 Carrau, F., Gaggero, C., Aguilar, P. S., 2015. Yeast diversity and native vigor for flavor
367 phenotype. Trends Biotechnol. 33, 148-154.

368 Cheetham, P.S.J., 1993. The use of biotransformation for the production of flavours and
369 fragrances. Trends Biotechnol. 11, 478–488.

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

373 Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M.,
374 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations
375 with *Saccharomyces cerevisiae*. Food Microbiol. 28, 873–888.

376 Contreras, A., Hidalgo, C., Henschke, P.A., Chambers, P.J., Curtin, C., Varela, C., 2014.
377 Evaluation of non-*Saccharomyces* Yeasts for the Reduction of Alcohol Content in Wine.
378 Appl. Environ. Microbiol. 80, 1670-1678.

379 De Francesco, G., Turchetti, B., Sileoni, V., Marconi, O., Perretti, G., 2015. Screening of new
380 strains of *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* to produce low-
381 alcohol beer. J. Inst. Brew. 121 (1), 113-121.

382 Dequin, S., 2001. The potential of genetic engineering for improving brewing, wine-making
383 and baking yeasts. Appl. Microbiol. Biotechnol. 56, 577-88.

384 Dukes, B.C., and Butzke, C.E., 1998. Rapid determination of primary amino acids in grape
385 juice using an *o*-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay. Am.
386 J. Enol. Vitic. 49, 125-134.
387 EC, December 19, 2000. Community reference methods for the analysis of spirits drinks, Off.
388 J. Eur. Commun. Reg. EC No2870/00. Brussels.
389 Harrison, M.A., 2009. Beer/Brewing, Encyclopedia of Microbiology, 3rd Edition, Elsevier
390 Inc.
391 Hernàndez-Orte, P., Ibarz, M.J., Cacho, J., Ferreira, V., 2005. Effect of the addition of
392 ammonium and amino acids to musts of Airen variety on aromatic composition and
393 sensory properties of the obtained wine. Food Chem. 89, 163–174.
394 Huige, N.J., Sanchez, G.W., Leidig, A.R., 1990. Process for preparing a nonalcoholic (less the
395 0.5 volume percent alcohol) malt beverage, US Patent 4,970,082 A.
396 Igyor, M.A., Ogbonna, A.C., Palmer, G.H., 2001. Effect of malting temperature and mashing
397 methods on sorghum wort composition and beer flavour. Process Biochem. 36, 1039–
398 1044
399 Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast: non-*Saccharomyces*
400 yeasts in wine production uncovered. FEMS Yeast Res. 14, 215–237.
401 King, A., Dickinson, J.R., 2000. Biotransformation of monoterpene alcohols by
402 *Saccharomyces cerevisiae*, *Torulspora delbrueckii* and *Kluyveromyces lactis*. *Yeast* **16**,
403 499–506.
404 King, A., Dickinson, J.R., 2003. Biotransformation of hop aroma terpenoids by ale and lager
405 yeasts. FEMS Yeast Res. 3, 53-62.
406

Kurtzman, C.P., Fell, J.W., 1998. The Yeasts, A Taxonomic Study, 4th ed. Amsterdam: Elsevier.

Kyselová, L., Brányik, T., 2014. Quality improvement and fermentation control in beer. Advances in fermented foods and beverages, *In* Holzapfel W. (ed), pp 471-500. Woodhead Publishing.

Lin, Y., 1975 Detection of wild yeasts in brewery. Efficiency of differential media. J. Inst. Brew. 81, 410-417.

Lodolo, E., Kock, J.L.F., Axcell, B.C., Brooks, M., 2008. The yeast *Saccharomyces cerevisiae* – the main character in beer brewing. FEMS Yeast Res. 8, 1018–1036.

Loira, I., Morata, A., González, C., Suárez-Lepe, J.A., 2012. Selection of glycolytically inefficient yeasts for reducing the alcohol content of wines from hot regions. Food Bioprocess Tech. 5, 2787–2796.

Meilgaard, M.C., 1975. Flavor chemistry of beer: Part II: flavor and threshold of 239 aroma volatiles. MBAA Techn. Qu. 12, 151–168.

Mertens, S., Steensels, J., Saels, V., De Rouck, G., Aerts G., Verstrepen, K.J., 2015. A large set of newly created interspecific Saccharomyces hybrids increases aroma diversity in lager beers. Appl. Environ. Microbiol. 81: In press. doi: 10.1128/AEM.02464-15

Morata, A., Benito, S., Loira, I., Palomero, F., Gonzalez, M.C., Suarez-Lepe, J.A., 2012. Formation of pyranoanthocyanins by *Schizosaccharomyces pombe* during the fermentation of red must. Int. J. Food Microbiol. 159, 47-53.

Narziss, L., Miedaner, H., Kern, E., Leibhard, M., 1992. Technology and composition of non-alcoholic beers. Processes using arrested fermentation. Brauwelt Int. 4, 396–410.

N'Guessan, F. K., N'Dri, D. Y., Camara, F., Dje, M. K., 2010. *Saccharomyces cerevisiae* and *Candida tropicalis* as starter cultures for the alcoholic fermentation of tchapalo, a traditional sorghum beer. World J. Microbiol. Biotechnol. 26,693–699.

Piddocke, M.P., Fazio, A., Vongsangnak, W., Wong, M.L., Heldt-Hansen, H.P., Workman, C., Nielsen, J., Olsson, L., 2011. Revealing the beneficial effect of protease supplementation to high gravity beer fermentations using '-omics' techniques. Microb. Cell Fact. 10, 27.

Pires, E.J., Teixeira, J.A., Brányik, T., Vincente, A.A., 2014. Yeast: the soul of beer's aroma – a review of flavour-active esters and higher alcohols produced by the brewing yeast. Appl. Microbiol. Biotechnol. 98,1937–1949.

Priefert, H., Rabenhorst, J., Steinbuchel, A., 2001. Biotechnological production of vanillin. Appl. Microbiol. Biotechnol. 56, 296-314.

Quirós, M., Rojas, V., Gonzalez, R., Morales, P., 2014. Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. Int. J. Food Microbiol. 181, 85–91.

Ramachandra Rao, S., Ravishankar, G.A., 2000. Review Vanilla flavour: production by conventional and biotechnological routes. J. Sci. Food Agric. 80, 289-304.

Remize, F., Roustan, J., Sablayrolles, J., Barre, P., Dequin, S., 1999. Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in by-product formation and to a stimulation of fermentation rate in stationary phase. Appl. Environ. Microbiol. 65, 143–149.

Sadoudi, M., Tourdot-Marechal, R., Rousseaux, S., Steyer, D., Gallardo-Chacon, J.J., Ballester, J., Vichi, S., Guerin-Schneider, R., Caixach, J., Alexandre, H., 2012. Yeast-

452 yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine
453 fermented by single or co-culture of non- *Saccharomyces* and *Saccharomyces* yeasts.
454 Food Microbiol. 32, 243–253.

455 Solieri, L., Landi, S., De Vero, L., Giudici, P., 2006. Molecular assessment of indigenous
456 yeast population from traditional balsamic vinegar. J. Appl. Microbiol. 101, 63-71.

457 Spitaels, F., Wieme, A.D., Janssens, M., Aerts, M., Daniel, H. Van Landschoot, A., De Vuyst,
458 L., Vandamme, P. 2014. The microbial diversity of traditional spontaneously fermented
459 lambic beer. PLoS ONE 9, e95384. doi:10.1371/journal.pone.0095384

460 Tataridis, P., Kanelis, A., Logotetis, S., and Nerancis, E., 2013. Use of non-*Saccharomyces*
461 *Torulaspora delbrueckii* yeast strains in winemaking and brewing. *Zb. Matitse Srp. Prir.*
462 *Nauke.* 124, 415–426.

463 Treimo, J., Aspmo, S.I., Eijsink, V.G.H., Horn, S.J., 2008. Enzymatic solubilization of
464 proteins in brewer's spent grain. J. Agr. Food Chem. 56, 5359–5365.

465 Vanderhaegen, B., Neven, H., Coghe, S., Verstrepen, K.J., Derdelinckx, G., Verachtert, H.,
466 2003. Bioflavoring and beer refermentation. Appl. Microbiol. Biotechnol. 62,140–150.

467 Verstrepen, K.J., Derdelinckx, G., Verachtert, H., Delvaux, F.R., 2003. Yeast flocculation:
468 what brewers should know. Appl. Microbiol. Biotechnol. 61, 197–205.

469 Vriesekoop, F., Krahl, M., Hucker, B., Menz, G. 2012. 125th Anniversary Review: Bacteria
470 in brewing: The good, the bad and the ugly. J. Inst. Brew.118, 335–345.

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Table 1. *Torulaspora delbrueckii* strains used in this study.

Source of isolation	Geographic origin	Strain code[a]
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

[a] Accession number of DiSVA Collection (Department of Life and Environmental Sciences).

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

DiSVA code	Total CO_2 evolved (g [30 days]$^{-1}$)[a]	Fermentation rate (g CO_2 day^{-1})[b]
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
S. cerevisiae[c]	20.97 ±0.58	3.34 ±0.04

Data are means ±standard deviations

[a]CO_2 g evolved after 30 days of fermentation (in 500 ml wort)

[b]Fermentation rate: CO_2 g/day (over the first 6 days of fermentation)

[c]Commercial strain: *S. cerevisiae* US-05 (Fermentis, Lesaffre, France)

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

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

[a]The initial composition of the sugars in wort were: Sucrose 6.4 g l^{-1}; glucose 8.5g l^{-1}; Maltose 64.06 g l^{-1}

[b]The initial yeast assimilable nitrogen (YAN) were 251 mg l^{-1}

[c]The wort gravity at the start was 12.7 °P.

Data are means± standard deviation

Data with different superscript letters (^{a,b,c,d}) 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^{-1})**					
		Acetaldehyde	**Ethyl acetate**	**n-propanol**	**Isobutanol**	**Amylic alcohol**	**Isoamylic alcohol**
S. cerevisiae US-05	Pure	5.80 ±0.45^{d}	17.57 ±0.24^{a}	30.56 ±0.29^{a}	24.38 ±0.31^{a}	12.69 ±0.22^{a}	68.53 ±0.08^{a}
T. delbrueckii DiSVA254	Pure	7.50 ±0.40^{c}	3.46 ±0.40^{d}	15.41 ±0.04^{e}	7.98 ±0.10^{d}	3.82 ±0.04^{c}	32.79 ±0.42^{e}
S. cerevisiae US-05 +	1:1	10.72 ±0.13^{b}	16.75 ±0.06^{b}	26.36 ±0.29^{b}	18.45±0.32^{b}	10.60 ±0.13^{b}	58.69 ±0.35^{c}
T. delbrueckii DiSVA254	1:10	30.48 ±0.22^{a}	14.65 ±0.04^{c}	20.14 ±0.21^{d}	16.04 ±0.34^{c}	10.61 ±0.60^{b}	55.65 ± 0.47^{d}
	1:20	5.56 ±0.15^{d}	16.25 ±0.16^{b}	22.59 ±0.06^{c}	16.15 ±0.24^{c}	11.29 ±0.19^{b}	60.05 ±0.06^{b}

Data are means ± standard deviations

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

Data are means ±standard deviations

Data with different superscript letters (^{a,b,c,d}) within each column are significantly different (Duncan tests; $P < 0.05$).

ND : not detected

Figure captions

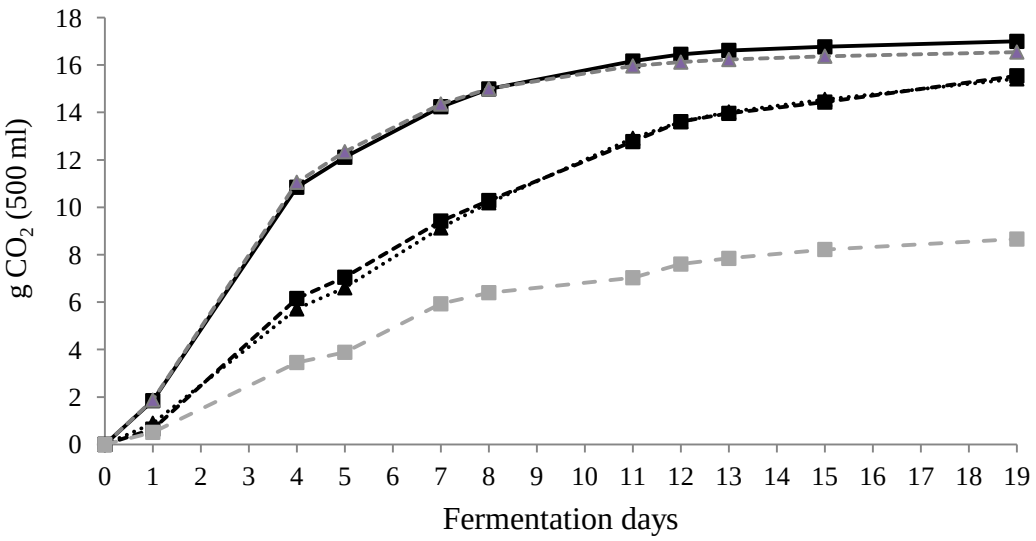
Figure 1. Fermentation kinetics of the pure and mixed fermentations. Pure cultures of *S. cerevisiae* ( ) and *T. delbrueckii* ( ), and mixed cultures of *S. cerevisiae* + *T. delbrueckii* at 1:1 ( ), 1:10 ( ) and 1:20 ( ).

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.

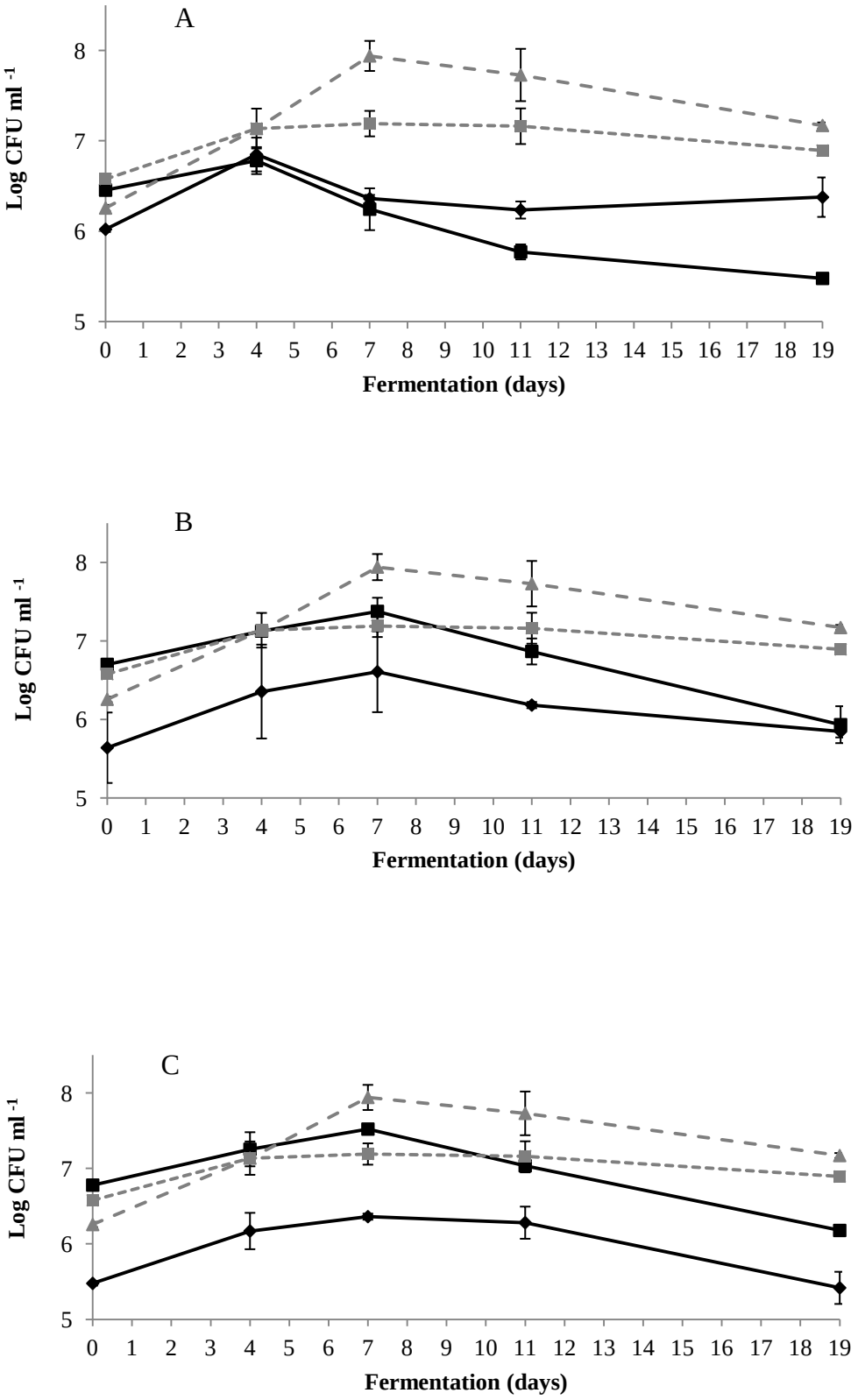
Figure

FIG 1


g CO_2 (500 ml)
0
2
4
6
8
10
12
14
16
18
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Fermentation days

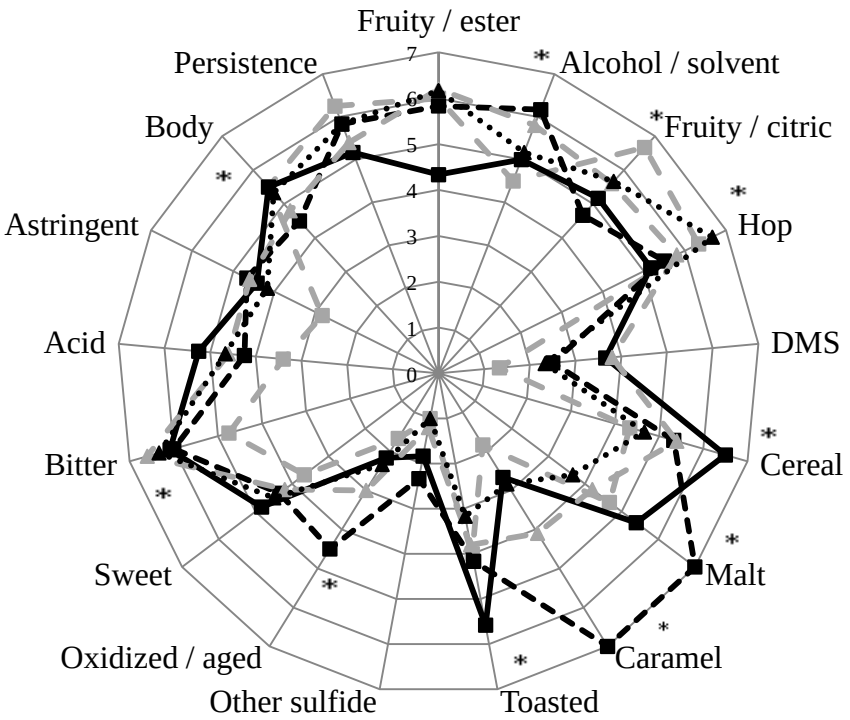
Figure

FIG 2


A
B
C
Log CFU ml -1
Fermentation (days)
5
6
7
8
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure

FIG 3


Fruity / ester
Alcohol / solvent
Fruity / citric
Hop
DMS
Cereal
Malt
Caramel
Toasted
Other sulfide
Oxidized / aged
Sweet
Bitter
Acid
Astringent
Body
Persistence
0
1
2
3
4
5
6
7
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