



# Assessment of non-conventional yeasts with potential probiotic for protein-fortified craft beer production

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## ABSTRACT

The use of non-conventional yeasts could be an interesting source of biodiversity for developing innovative fermented beverages. Here, 43 wild yeast strains belonging to different genera such as *Lachancea*, *Kluyveromyces*, *Torulaspora*, *Metschnikowia*, *Kazachstania*, *Brettanomyces*, *Pichia*, *Candida*, *Hanseniaspora*, *Rhodotorula*, *Rodospiridobolus* and *Saccharomyces*, previously evaluated for their probiotic traits, have been tested for craft beer production. Different experimental lines were carried out to develop a new beverage which could combine increased aromatic characteristics and improved nutritional properties: i) beers produced from pils wort (PW); ii) beers from pils wort enriched with lentil (PLW) or chickpea flour extracts (PCW).

PW beer trials were characterized by a low ethanol content. The PCW results in beer with an unpleasant aromatic taste, while the presence of lentil (PLW) confers effective fermentative characters and pleasant aromatic notes to the final beers.

The selected strains *Lachancea thermotolerans*, *Kazachstania unispora* and *Saccharomyces cerevisiae* determined a significantly increase in the main aromatic compounds such as ethyl acetate, isoamyl acetate and higher alcohols in PLW. The sensorial profile indicated that the beers were characterized with emphasized aromatic attributes. The yeasts selected here, could contribute to obtain a premium craft beer, with highly nutritional and functional characteristics, with a distinctive aromatic character.

## 1. Introduction

Beer is one of the most popular and consumed beverages in the world (Bokulich, Bamforth, & Mills, 2012). *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* are the two species commonly used for beer production (Lodolo, Kock, Axcell, & Brooks, 2008). In Europe, in response to an increase of global market, the production of “industrial” beer in 2016 was 400.2 million hectolitres. This amount lead Europe to be the second largest producer in the world, exceeding the production of the United States and Brazil. Nowadays, consumers are increasingly opting to experiment with locally produced premium and international beer varieties, that attract greater attention from consumers and proving to be a crucial competitor to the more traditional beer brands. The craft beer industry is one of the growing segments in the beverage industry and its increasing diffusion also affects individuals’ commercial beer preferences and consumption trends, in Europe but also worldwide (Aquilani, Laureti, Poponi, & Secondi, 2015). The microbreweries pay attention on quality of raw materials to obtain craft beer characterized

and diversified by peculiar aromatic and nutritional profiles. These craft beers differ significantly from the industrialized one where the main goal is to deliver a standardized quality product recognized by the consumer. Craft beers have often a unique taste resulting from specific technological processes and the selection of raw materials, often sourced locally (Cipollaro, Sottini, & Fabbrizzi, 2018; Schnell & Reese, 2003). Probably, this is the main reason why craft beers appeal to consumers who are seeking for a “taste revolution” (Alfeo, Todaro, Migliore, Borsellino, & Schimmenti, 2019). This latter highlights the need for developing craft beers that combines high aromatic quality and improved nutritional value. On such regards, consumers are seeking for healthy and innovative products (Senkarcinova, Dias, Nespor, & Branjik, 2019). For these reasons, the beverage market push for the development of specialty beers such as low alcohol, low calorie, gluten free, novel flavoured and health promoting beers (Yeo & Liu, 2014). Generally, beer represents a source of minerals, vitamins, polyphenols and fibre. Among functional beers, it is noticed xanthohumulol beer, oestrogenic beer and probiotic beer (Yeo & Liu, 2014). Probiotic beer is

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obtained using probiotic microorganism during fermentation process. A probiotic has been defined as “cell preparations or components of microbial cells that have a beneficial effect on the health and wellbeing of the host” (Salminen, 1999). Generally, the most known probiotic microorganisms are lactic bacteria, especially *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium*, *Enterococcus*, or *Streptococcus*. Only *S. cerevisiae* var. *boulardii* is the unique commercial yeast used as a probiotic for its properties such as the survival body temperature (37 °C), the resistance to stomach acids and bile acids and inhibits the growth of a number of microbial pathogens (Czerucka, Piche, & Rampal, 2007; Kelesidis & Pothoulakis, 2012; McFarland, 2010). Capece et al. (2018) and Mulero-Cerezo, Briz-Redón, and Serrano-Aroca (2019) aimed to produce craft beer using probiotic fermenting yeast characterized by higher antioxidant activity, lower alcohol content and positive sensory attributes. Another goal in the craft beer production is the use of legume materials. Legumes are indispensable for human diet in respect to their valuable and nutritive bioactive molecules. Legumes and derivative foodstuffs are rich in fiber, proteins, vitamins and some valuable phytochemicals, which exhibit important biological activities (Patil, Brennan, Mason, & Brennan, 2016). These aspects were not been combined before in the development of innovative beverages; therefore, a low alcohol protein enriched drink fermented with functional microorganisms and with an enhanced aromatic footprint has been proposed.

In the present study, the fermentative performances of some previously characterized wild probiotic yeasts (Agarbati et al., 2020), listed in Table 1 suppl., were evaluated in wort individually enriched with hydrolysed lentil and chickpea flour. In particular, the use of non-conventional yeasts with potential probiotic properties has been proposed to produce a premium craft beer, where 20% of the initial wort was replaced to hydrolysed lentil or chickpea flour supernatant, as protein sources, with the aim to placing on the market a high-nutritious drink with beneficial effects on consumer health. Especially hybrid beverages, containing cereal and legume proteins, promise a balanced amino acid composition and an upgraded nutritional value of a new craft beer product.

## 2. Materials and methods

### 2.1. Yeast strains

Forty-three strains used in this study belong to the genera *Lachancea*, *Torulaspota*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Rhodotorula*, *Candida*, *Kazachstania*, and *Hanseniaspora* were obtained from the Yeast Collection of (DiSVA) of the Polytechnic University of Marche (Italy) (Table 1). The strains coming from different un-anthropized environments or spontaneously fermented foods had been isolated, identified and characterized as probiotic and/or functional strains (Agarbati et al., 2020). All the strains were identified through the sequencing of the D1/D2 domains of the 26S rDNA gene, as reported by Comitini et al. (2011) and Solieri, Landi, De Vero, and Giudici (2006). The *S. cerevisiae* commercial strain US-05 (Fermentis, Lesaffre, France) and commercial probiotic *S. cerevisiae* var. *boulardii* (Codex, Zambon, Italy) were used as control strains. All the yeast strains were maintained on yeast extract (10 g/L), peptone (20 g/L), dextrose (20 g/L) (YPD) agar (18 g/L) (Oxoid, Basingstoke, UK) 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. Lentil and chickpea wort preparation

Hitempase STXL, a heat-stable  $\alpha$ -amylase (*Bacillus licheniformis*) was sourced from Kerry Group (Tralee, Ireland). It is an endo-amylase which randomly hydrolyses the  $\alpha$ -1,4-glycosidic linkages in amylose and amylopectin, resulting in the production of dextrins. Bioprotease P1, a proteolytic enzyme, was also sourced from Kerry Group (Tralee, Ireland). It is a complex enzyme system derived from selected microbial strain and plant species and is used in sorghum brewing to ensure adequate levels of free  $\alpha$ -amino nitrogen in the wort. A mixture of 70% of lentil flour and

**Table 1**

Fermentation kinetics parameters of different yeast strains on malt extract. Isolation source of these strains are reported by Agarbati et al., 2020.

Strains	Code	Total CO <sub>2</sub> evolved (12 Days)	Fermentation rate (g CO <sub>2</sub> /Day) 6 days
<i>Lachancea thermotolerans</i>	104	0.44 ± 0.05	0.06 ± 0.02
<i>Lachancea thermotolerans</i>	105	0.51 ± 0.04	0.07 ± 0.01
<i>Lachancea thermotolerans</i>	B13	1.56 ± 0.36	0.23 ± 0.04
<i>Lachancea waltii</i>	B8	0.42 ± 0.09	0.06 ± 0.01
<i>Kluyveromyces marxianus</i>	Md	0.27 ± 0.02	0.04 ± 0.01
<i>Kluyveromyces marxianus</i>	MF	0.41 ± 0.06	0.05 ± 0.00
<i>Torulaspota delbrueckii</i>	19.2t2	0.49 ± 0.12	0.06 ± 0.01
<i>Torulaspota delbrueckii</i>	19.3 t2	0.39 ± 0.09	0.06 ± 0.00
<i>Torulaspota delbrueckii</i>	3H	0.37 ± 0.04	0.05 ± 0.01
<i>Torulaspota delbrueckii</i>	38	0.46 ± 0.01	0.07 ± 0.03
<i>Torulaspota delbrueckii</i>	5D	0.43 ± 0.02	0.06 ± 0.00
<i>Torulaspota delbrueckii</i>	2A	0.39 ± 0.03	0.06 ± 0.00
<i>Torulaspota delbrueckii</i>	C7.4	1.40 ± 0.10	0.15 ± 0.05
<i>Torulaspota delbrueckii</i>	1.1T2	1.30 ± 0.16	0.13 ± 0.04
<i>Torulaspota delbrueckii</i>	J401	0.40 ± 0.03	0.06 ± 0.01
<i>Torulaspota delbrueckii</i>	40	0.47 ± 0.05	0.07 ± 0.00
<i>Torulaspota delbrueckii</i>	39	0.39 ± 0.03	0.06 ± 0.00
<i>Torulaspota delbrueckii</i>	TD vcs	0.41 ± 0.03	0.06 ± 0.00
	FF		
<i>Metschnikowia pulcherrima</i>	B33	0.31 ± 0.06	0.04 ± 0.00
<i>Metschnikowia</i> sp.	F14	0.45 ± 0.20	0.06 ± 0.00
<i>Metschnikowia pulcherrima</i>	Apple	0.34 ± 0.12	0.05 ± 0.00
	G3		
<i>Metschnikowia pulcherrima</i>	B42	0.38 ± 0.06	0.06 ± 0.00
<i>Kazachstania unispora</i>	M3-5	0.39 ± 0.10	0.04 ± 0.00
<i>Kazachstania unispora</i>	M3B3	0.60 ± 0.06	0.05 ± 0.01
<i>Brettanomyces bruxellensis</i>	G2	1.73 ± 0.53	0.12 ± 0.08
<i>Pichia fermentans</i>	Mg	0.51 ± 0.09	0.07 ± 0.00
<i>Pichia fermentans</i>	MA	0.43 ± 0.14	0.05 ± 0.00
<i>Pichia anomala</i>	18	0.33 ± 0.06	0.04 ± 0.00
<i>Candida homilentoma</i>	28	0.37 ± 0.04	0.05 ± 0.00
<i>Candida</i> spp.	B29	0.31 ± 0.00	0.03 ± 0.01
<i>Hanseniaspora uvarum</i>	Mb	0.31 ± 0.02	0.04 ± 0.00
<i>Hanseniaspora</i> spp.	LV12	0.32 ± 0.02	0.04 ± 0.00
<i>Rhodotorula mucilaginosa</i>	B5	0.10 ± 0.00	0.00 ± 0.00
<i>Rhodospordiobolus</i> spp.	B14	0.31 ± 0.00	0.01 ± 0.00
<i>Saccharomyces cerevisiae</i>	6	1.63 ± 0.23	0.26 ± 0.04
<i>Saccharomyces cerevisiae</i>	10C	1.57 ± 0.38	0.23 ± 0.04
<i>Saccharomyces cerevisiae</i>	B6	1.59 ± 0.25	0.23 ± 0.03
<i>Saccharomyces cerevisiae</i>	M2-3	1.69 ± 0.39	0.27 ± 0.03
<i>Saccharomyces cerevisiae</i>	1 PV	1.62 ± 0.41	0.20 ± 0.04
<i>Saccharomyces cerevisiae</i>	M1-7	1.72 ± 0.37	0.27 ± 0.05
<i>Saccharomyces cerevisiae</i>	2 PV	1.56 ± 0.21	0.19 ± 0.06
<i>Saccharomyces cerevisiae</i>	M1-3	1.77 ± 0.05	0.27 ± 0.03
<i>Saccharomyces cerevisiae</i>	7	1.70 ± 0.29	0.26 ± 0.06
<i>Saccharomyces cerevisiae</i>	CODEX	1.90 ± 0.03	0.30 ± 0.00
<i>Saccharomyces cerevisiae</i>	var. <i>boulardii</i>		
<i>Saccharomyces cerevisiae</i>	US05	1.70 ± 0.09	0.27 ± 0.03

Data are the means ± standard deviations. CO<sub>2</sub> g evolved after 12 days of fermentation in 70 mL of 10% malt extract. Fermentation rate: CO<sub>2</sub> g/day (over the first 6 days of fermentation).

water has been heated up to 45 °C and then calcium chloride (1.3 g/L) was added to stabilize the enzyme Hitempase (0.5 mL/L). Subsequently, Bioprotease P1 (0.5 mL/L) was added and the mixture was incubated up to 75 °C for 1 h. At the end of mashing, starch negativity was checked by iodine test. After that, the lentil and chickpea suspensions were boiled and centrifuge obtaining, thus, the resulting worts.

### 2.3. Preliminary screening

The ability of the selected yeast strains to ferment maltose as carbon source was assessed by microfermentations in 100-ml flasks containing 70 mL of malt extract (10%v/v) (substrate that mimics the wort) under sterile conditions as reported by Canonico, Galli, Comitini, and Ciani (2019) in comparison with *S. cerevisiae* commercial strain US-05 commonly used in beer production. The fermentation kinetics after incubation at 20 °C and the ethanol content of the products were assayed.

#### 2.4. Fermentation trials

A batch of 1500 l of malted barley wort to produce Pilsner beer was used in this study. Its main analytical characters were pH 5.4, specific gravity 12.2 °Plato, and 20 IBU. The fermentation potential of selected strain was evaluated in two set of fermentation trials.

#### 2.5. First set of trials

From preliminary screening, thirteen strains belonging to different genera, that exhibited the best fermentation performance were selected and used in pure fermentations. In the first set of fermentation trials it was carried out: i) on pils wort (PW); ii) enriched pils wort where 20% of wort was replaced with 20% of hydrolysed chickpea wort (PCW); iii) enriched pils wort where 20% of wort was replaced with 20% of hydrolysed lentil wort (PLW). All fermentations were carried out at 20 °C in flask containing 250 mL wort under sterile conditions. Pre-cultures were grown in 10% malt extract at 20 °C for 48h. The biomass was collected by centrifugation, washed three times with sterile distilled water and inoculated into wort to obtain an initial concentration of approximately  $1 \times 10^6$  cell/mL for each yeast. The flasks were locked with a Müller valve containing sulphuric acid, to allow only the CO<sub>2</sub> to escape from the system.

The fermentation kinetics were monitored by measuring the weight loss of the flasks due to the CO<sub>2</sub> evolution which was followed to the end of the fermentation (i.e., constant weight for 3 consecutive days). The ethanol content was determined using the procedure of Official European Union Methods (EC, 2000) sugar residual and protein content were evaluated following the protocols of Canonico, Comitini, and Ciani (2018).

#### 2.6. Second set of trials

From the results of the first set of fermentations six strains were selected and used to carried out trials on pils wort and pils wort enriched with 20% of lentil wort. The experiments were carried out in flask containing 500 mL of wort in triplicate in the same fermentation conditions and analyses (fermentations kinetics, ethanol production, residual sugar, protein content) reported above for the preliminary screening.

#### 2.7. Analytical determinations of volatile compounds

Acetaldehyde, ethyl acetate, n-propanol, isobutanol, amyl, and isoamyl alcohols, were quantified by direct injection into a gas-liquid chromatography system (GC-2014; Shimadzu, Kyoto, Japan). The samples were injected into a 2 m × 2 mm i.d. glass column, packed with 80/100 Carbowax C/0.2% Carbowax 1500 (Supelco, Sigma Aldrich, Milan, Italy), with an internal standard of 3-methyl-2-butanol. Nitrogen was used as the carrier gas. A Shimadzu gas chromatograph (Japan) equipped with a flame ionization detector was used. The oven temperature ranged from 45 to 160 °C. The temperature of the injector and the detector was 220 °C as reported by Canonico, Comitini, and Ciani (2015).

The solid-phase microextraction (HS-SPME) method was used to determine the concentration of the volatile compounds. Five ml of beer was placed in a vial containing 1 g NaCl closed with a septum-type cap. HS-SPME was carried out with magnetic stirring for 10 min at 25 °C. After this period, the internal standard (3-octanol) at a concentration of 1.6 mg/L was added, and the sample was heated to 40 °C. Divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre (Sigma-Aldrich) was inserted into the vial headspace for 30 min. The compounds were desorbed by inserting the fibre into a Shimadzu gas chromatograph GC injector for 5 min. A glass capillary column was used: 0.25 µm Supelcowax 10 (length, 60 m; internal diameter, 0.32 mm). The fibre was inserted in the split-splitless mode as reported by Canonico et al. (2015). The compounds were identified and quantified by comparisons with external calibration curves for each compound. Specific enzymatic kits (Megazyme, Ireland) were used to determine the

concentrations of glucose sucrose, maltose (kit k-masug) according to the manufacturer instructions. The Lowry method was used to measure the protein content in final beers (Bensadoun & Weinstein, 1976).

#### 2.8. Sensory analysis

At the end of the fermentation process, the beers obtained unfiltered were transferred into 330-ml bottles, adding 5.5 g/L of sucrose. The secondary fermentation in the bottle was carried out at 18–20 °C for 7–10 days. After this period, the beers were stored at 4 °C and underwent sensory analysis using a scale from 1 to 10 (Analytica EBC, 1997). This was carried out by a group of 10 trained testers, that evaluated the main aromatic notes regarding the gustatory perception and structural features. The data were elaborated with statistical analyses to obtain information about the contribution of each descriptor on the organoleptic quality of beer.

#### 2.9. Yeast vitality assay after 3 months of bottling

The vitality of the strains after 3 months of bottling was carried out using viable cell counts on WL Nutrient Agar (Oxoid, Hampshire, UK) and Lysine Agar (Oxoid, Hampshire, UK). Lysine Agar is a medium unable to support the growth of *S. cerevisiae* (Lin, 1975) for the differentiation of non-*Saccharomyces* yeast from *S. cerevisiae* strain. The media were incubated at 25 °C for 2–3 days.

#### 2.10. Statistical analysis

Analysis of variance (ANOVA) was applied to the experimental data for the main characteristics of the beers. The means were analysed using the STATISTICA 7 software (Stat Soft, Inc, Tulsa, OK, USA). The significant differences were determined by the means of Duncan tests, and the results were considered significant if the associated P values were <0.05. The results of the sensory analysis were also subjected to Fisher ANOVA, to determine the significant differences with a p value < 0.05. Principal component analysis (PCA) was applied to discriminate between the means of the contents of volatile compounds and was carried out using the statistical software package JMP 11®. The mean data were normalised to neutralize any influence from hidden factors.

### 3. Results

#### 3.1. Preliminary screening

Initial screening was carried out on forty-three yeasts strains belonging to different species coming from un-anthropized or spontaneously processed foods selected for their functional and probiotic traits (Agarbati et al., 2020). Their ability to ferment maltose, the most abundant fermentable sugar in the brewing wort, was tested for their possible use as starter in beer production (Table 1). The results of the fermentation capacity showed that all the strains tested exhibited the ability to ferment maltose, although even at different grades. Out of forty-three strains, thirteen yeast belonging to the species *L. thermotolerans* (B13), *T. delbrueckii* (C7.4; 1.1T2), *K. unisporea* (M3B3) and *S. cerevisiae* (6, 10C, B6, M2-3, 1 PV, M1-7, 2 PV, M1-3 and 7) were selected since they showed the best fermentative performance with the highest final g CO<sub>2</sub> produced and fermentation rate in comparison with *S. cerevisiae* US-05. For these reasons, these strains were selected and used subsequently in micro-fermentation trials on different wort.

#### 3.2. Fermentation trials on wort: first set

Results obtained by fermentations on PW and PLW and PCW were reported in Table 2. As expected, protein content was significant increased in all fermentations carried out with all strains tested in PLW and PCW. All *S. cerevisiae* strains showed a significant increase in fermentation rate in PW when compared with PLW and PCW. Among the

**Table 2**

Fermentation parameters, ethanol, residual maltose g/L, and protein content on i) pils wort; ii) pils wort added with 20% lentil wort; iii) pils wort added with 20% chickpea wort.

Strains	Fermentation parameters	PW	PLW	PCW
B13 <i>L. thermotolerans</i>	Fermentation rate (g CO <sub>2</sub> /Day)	0.34 ± 0.09 <sup>b</sup>	1.23 ± 0.02 <sup>a</sup>	0.34 ± 0.04 <sup>b</sup>
	Ethanol (% v/v)	3.14 ± 0.58 <sup>a</sup>	3.05 ± 0.23 <sup>a</sup>	3.72 ± 0.14 <sup>a</sup>
	Residual maltose g/L	11.67 ± 0.50 <sup>a</sup>	9.89 ± 1.89 <sup>a</sup>	13.02 ± 2.01 <sup>a</sup>
	Protein content g/L	19.7 ± 0.25 <sup>c</sup>	41.50 ± 0.51 <sup>a</sup>	38.38 ± 0.30 <sup>b</sup>
C 7.4 <i>T. delbrueckii</i>	Fermentation rate (g CO <sub>2</sub> /Day)	0.21 ± 0.01 <sup>b</sup>	0.41 ± 0.03 <sup>a</sup>	0.22 ± 0.01 <sup>b</sup>
	Ethanol (% v/v)	2.83 ± 0.02 <sup>c</sup>	3.89 ± 0.26 <sup>a</sup>	3.02 ± 0.32 <sup>b</sup>
	Residual maltose g/L	16.30 ± 0.74 <sup>c</sup>	24.23 ± 2.4 <sup>b</sup>	33.02 ± 3.25 <sup>a</sup>
	Protein content g/L	19.07 ± 0.23 <sup>b</sup>	54.81 ± 0.59 <sup>a</sup>	51.93 ± 0.87 <sup>a</sup>
1.1 T2 <i>T. delbrueckii</i>	Fermentation rate (g CO <sub>2</sub> /Day)	0.29 ± 0.03 <sup>b</sup>	0.42 ± 0.01 <sup>a</sup>	0.19 ± 0.05 <sup>b</sup>
	Ethanol (% v/v)	0.94 ± 0.00 <sup>b</sup>	2.61 ± 0.21 <sup>a</sup>	2.06 ± 0.11 <sup>a</sup>
	Residual maltose g/L	9.03 ± 0.84 <sup>b</sup>	23.06 ± 1.58 <sup>a</sup>	21.03 ± 1.96 <sup>a</sup>
	Protein content g/L	17.05 ± 1.43 <sup>c</sup>	41.84 ± 1.98 <sup>a</sup>	34.35 ± 1.25 <sup>b</sup>
M3B3 <i>Kazachstania unisporea</i>	Fermentation rate (g CO <sub>2</sub> /Day)	0.10 ± 0.01 <sup>b</sup>	0.18 ± 0.00 <sup>a</sup>	0.13 ± 0.03 <sup>b</sup>
	Ethanol (% v/v)	0.33 ± 0.36 <sup>a</sup>	0.7 ± 0.02 <sup>a</sup>	0.52 ± 0.06 <sup>a</sup>
	Residual maltose g/L	44.77 ± 1.09 <sup>c</sup>	58.65 ± 1.75 <sup>b</sup>	63.21 ± 2.14 <sup>a</sup>
	Protein content g/L	25.99 ± 2.21 <sup>b</sup>	39.53 ± 2.25 <sup>a</sup>	29.73 ± 2.36 <sup>b</sup>
6 <i>S. cerevisiae</i>	Fermentation rate (g CO <sub>2</sub> /Day)	1.08 ± 0.03 <sup>a</sup>	0.72 ± 0.03 <sup>b</sup>	0.74 ± 0.06 <sup>b</sup>
	Ethanol (% v/v)	3.08 ± 0.09 <sup>a</sup>	3.22 ± 0.36 <sup>a</sup>	3.44 ± 0.14 <sup>a</sup>
	Residual maltose g/L	3.98 ± 0.20 <sup>a</sup>	1.56 ± 0.23 <sup>b</sup>	1.86 ± 0.22 <sup>b</sup>
	Protein content g/L	15.61 ± b	67.49 ± 2.36 <sup>a</sup>	19.93 ± 2.20 <sup>b</sup>
10C <i>S. cerevisiae</i>	Fermentation rate (g CO <sub>2</sub> /Day)	1.24 ± 0.15 <sup>a</sup>	0.82 ± 0.03 <sup>b</sup>	0.64 ± 0.06 <sup>b</sup>
	Ethanol (% v/v)	3.37 ± 0.66 <sup>a</sup>	4.22 ± 0.36 <sup>a</sup>	3.44 ± 0.14 <sup>a</sup>
	Residual maltose g/L	1.56 ± 0.22 <sup>b</sup>	3.77 ± 0.69 <sup>b</sup>	14.30 ± 2.36 <sup>a</sup>
	Protein content g/L	18.46 ± 0.99 <sup>b</sup>	46.16 ± 1.78 <sup>a</sup>	49.33 ± 1.25 <sup>a</sup>
B6 <i>S. cerevisiae</i>	Fermentation rate (g CO <sub>2</sub> /Day)	0.99 ± 0.42 <sup>b</sup>	0.25 ± 0.06 <sup>b</sup>	0.26 ± 0.03 <sup>b</sup>
	Ethanol (% v/v)	3.36 ± 0.15 <sup>a</sup>	3.35 ± 0.13 <sup>a</sup>	3.45 ± 0.25 <sup>a</sup>
	Residual maltose g/L	3.84 ± 0.07 <sup>a</sup>	5.52 ± 0.18 <sup>b</sup>	6.58 ± 0.36 <sup>b</sup>
	Protein content g/L	21.37 ± 1.26 <sup>b</sup>	56.23 ± 2.35 <sup>a</sup>	58.69 ± .25 <sup>a</sup>
M2-3 <i>S. cerevisiae</i>	Fermentation rate (g CO <sub>2</sub> /Day)	1.32 ± 0.02 <sup>a</sup>	0.91 ± 0.12 <sup>ab</sup>	0.83 ± 0.36 <sup>b</sup>
	Ethanol (% v/v)	3.32 ± 0.09 <sup>b</sup>	4.12 ± 0.17 <sup>a</sup>	4.44 ± 0.19 <sup>a</sup>
	Residual maltose g/L	6.61 ± 0.87 <sup>b</sup>	7.04 ± 1.03 <sup>b</sup>	10.67 ± 2.01 <sup>a</sup>
	Protein content g/L	21.09 ± 0.85 <sup>b</sup>	67.49 ± 2.01 <sup>a</sup>	19.93 ± 1.28 <sup>b</sup>
1 PV <i>S. cerevisiae</i>	Fermentation rate (g CO <sub>2</sub> /Day)	0.79 ± 0.01 <sup>a</sup>	0.56 ± 0.10 <sup>ab</sup>	0.36 ± 0.09 <sup>b</sup>
	Ethanol (% v/v)	3.71 ± 0.12 <sup>a</sup>	3.63 ± 0.12 <sup>a</sup>	3.59 ± 0.14 <sup>a</sup>
	Residual maltose g/L	11.31 ± 0.73 <sup>a</sup>	12.36 ± 0.85 <sup>a</sup>	12.59 ± 0.78 <sup>a</sup>
	Protein content g/L	44.72 ± 0.96 <sup>b</sup>	59 ± 1.26 <sup>a</sup>	63 ± 2.38 <sup>a</sup>
M1-7 <i>S. cerevisiae</i>	Fermentation rate (g CO <sub>2</sub> /Day)	1.58 ± 0.19 <sup>a</sup>	0.90 ± 0.36 <sup>b</sup>	0.73 ± 0.13 <sup>b</sup>
	Ethanol (% v/v)	3.34 ± 0.32 <sup>a</sup>	3.4 ± 0.14 <sup>a</sup>	3.9 ± 0.36 <sup>a</sup>
	Residual maltose g/L	2.34 ± 1.01 <sup>b</sup>	6.69 ± 1.22 <sup>a</sup>	5.19 ± 1.06 <sup>a</sup>
	Protein content g/L	16.76 ± 1.24 <sup>c</sup>	42.13 ± 1.39 <sup>a</sup>	27.72 ± 2.33 <sup>b</sup>
2 PV <i>S. cerevisiae</i>	Fermentation rate (g CO <sub>2</sub> /Day)	0.60 ± 0.04 <sup>a</sup>	0.81 ± 0.03 <sup>a</sup>	0.20 ± 0.03 <sup>b</sup>
	Ethanol (% v/v)	3.10 ± 0.09 <sup>b</sup>	3.84 ± 0.32 <sup>a</sup>	3.6 ± 0.14 <sup>b</sup>
	Residual maltose g/L	4.98 ± 0.64 <sup>b</sup>	10.46 ± 1.98 <sup>a</sup>	4.48 ± 1.00 <sup>b</sup>
	Protein content g/L	8.69 ± 0.98 <sup>b</sup>	41.84 ± 2.04 <sup>a</sup>	40.4 ± 1.98 <sup>a</sup>
M1-3 <i>S. cerevisiae</i>	Fermentation rate (g CO <sub>2</sub> /Day)	1.49 ± 0.03 <sup>a</sup>	0.81 ± 0.23 <sup>b</sup>	0.42 ± 0.14 <sup>b</sup>
	Ethanol (% v/v)	3.25 ± 0.03 <sup>b</sup>	3.45 ± 0.21 <sup>a</sup>	3.36 ± 0.05 <sup>a</sup>
	Residual maltose g/L	0.14 ± 0.16 <sup>b</sup>	2.36 ± 0.56 <sup>a</sup>	2.56 ± 0.21 <sup>a</sup>
	Protein content g/L	25.99 ± 2.14 <sup>a</sup>	27.32 ± 1.22 <sup>a</sup>	26.33 ± 0.21 <sup>a</sup>
7 <i>S. cerevisiae</i>	Fermentation rate (g CO <sub>2</sub> /Day)	1.23 ± 0.49 <sup>a</sup>	0.85 ± 0.24 <sup>b</sup>	0.74 ± 0.28 <sup>b</sup>
	Ethanol (% v/v)	3.27 ± 0.13 <sup>a</sup>	3.9 ± 0.31 <sup>a</sup>	3.59 ± 0.21 <sup>a</sup>
	Residual maltose g/L	0 ± 0	11.17 ± 1.25 <sup>a</sup>	5.97 ± 1.36 <sup>b</sup>
	Protein content g/L	17.34 ± 0.21 <sup>b</sup>	47.78 ± 2.25 <sup>a</sup>	15.73 ± 2.39 <sup>b</sup>
Codex <i>S. cerevisiae</i> var. <i>boulardi</i>	Fermentation rate (g CO <sub>2</sub> /Day)	0.82 ± 0.04 <sup>a</sup>	0.75 ± 0.21 <sup>a</sup>	0.68 ± 0.26 <sup>a</sup>
	Ethanol (% v/v)	3.30 ± 0.33 <sup>b</sup>	4.05 ± 0.28 <sup>ab</sup>	0.5 ± 0.09 <sup>a</sup>
	Residual maltose g/L	3.63 ± 0.077 <sup>b</sup>	4.62 ± 0.87 <sup>b</sup>	10.24 ± 0.99 <sup>a</sup>
	Protein content g/L	22.8 ± 2.02 <sup>b</sup>	42.42 ± 3.24 <sup>a</sup>	28.58 ± 1.25 <sup>b</sup>
US-05 <i>S. cerevisiae</i>	Fermentation rate (g CO <sub>2</sub> /Day)	1.12 ± 0.02 <sup>a</sup>	0.85 ± 0.23 <sup>b</sup>	0.74 ± 0.25 <sup>b</sup>
	Ethanol (% v/v)	3.97 ± 0.02 <sup>a</sup>	3.9 ± 0.31 <sup>a</sup>	3.59 ± 0.21 <sup>a</sup>
	Residual maltose g/L	6.97 ± 0.16 <sup>b</sup>	11.17 ± 1.25 <sup>a</sup>	5.97 ± 1.36 <sup>b</sup>
	Protein content g/L	11.29 ± 1.24 <sup>c</sup>	67.78 ± 2.36 <sup>a</sup>	25.73 ± 1.52 <sup>b</sup>

Data are the means ± standard deviations. Data with different superscript letters (<sup>a,b,c</sup>) within each row between the same strain tested on different wort (Duncan tests; p < 0.05).

PW: Pils Wort; PLW: Pils + lentil wort; PCW: Pils + chickpea wort.

Fermentation rate: CO<sub>2</sub> g/day (over the first 3 days of fermentation).

The initial composition of the sugars in pils wort were: Sucrose 6.4 g/L; glucose 7.5 g/L; Maltose 55.73 g/L. Protein content: 10.42 g/L.

The initial composition of the sugars in pils wort added with lentil were: Sucrose 34.82 g/L; glucose 11.24 g/L; Maltose 88.26 g/L. Protein content: 37.23 g/L.

The initial composition of the sugars in pils wort added with chickpea were: Sucrose 24.19 g/L; glucose 1.49 g/L; Maltose 71.18 g/L. Protein content: 22.24 g/L.



legume-enriched worts, PCW was showing a lower fermentation rate and fermentation capacity as confirmed by a high residual maltose. On the other hand, all *S. cerevisiae* strains tested showed a lower residual maltose in all substrates in comparison with non-*Saccharomyces* species. The ethanol content was significantly higher in *S. cerevisiae* M2-3 and M1-3 in PLW and PCW, while *S. cerevisiae* 2 PV exhibited a higher ethanol content only in PLW. *L. thermotolerans* B13, *T. delbrueckii* C7.4 e 1.1 T2, *K. unispora* M3B3 exhibited a higher fermentation rate on PLW when compared with PW and PCW.

Among non-*Saccharomyces* strains, *K. unispora* M3B3 was the only strain that showed a limited ability to metabolize the substrate, as showed by the high residual maltose and protein and by the limited amount of ethanol produced in all samples (Table 2). Regarding the ethanol content, the strains showed a different trend on the three substrates tested. The two strains of *T. delbrueckii*, showed a similar fermentation rates in the three substrates, highlighting a significant increase of fermentative performance in PLW. Moreover, beer obtained by *T. delbrueckii* C7.4 exhibited a higher protein content in this wort in comparison with *T. delbrueckii* 1.1 T2. Likewise, *L. thermotolerans* B13, showed a significant increase in fermentation rate and protein content in PLW when compared with the other two substrates tested.

### 3.3. Fermentation trials on wort: second set

The second set of fermentation trials was carried out on a selection of yeast strains and substrate of fermentation. In this regard, the fermentation on PCW was excluded since the chickpea wort negatively influenced the fermentation performance of the tested strains and the sensory profile of the end-products (data not shown). The strains selection was carried out based on the fermentative performance of first set of fermentation trials and considering also their functional and probiotic traits previously evaluated (Agarbati et al., 2020) (Table 1 supp material). The main trait that were previously evaluated and here confirmed were yeast growth at 37 °C, acid pH and presence of bile salts, antioxidant activity and the antagonistic effect against pathogenic bacteria. The six selected strains, *L. thermotolerans* B13, *T. delbrueckii* C7.4 and *K. unispora* M3B3, *S. cerevisiae* 2 PV, B6 and M1-7), were further tested in PLW against PW, used as control, for evaluating the fermentation kinetics, the analytical characters and sensorial profile. As reference strains, *S. cerevisiae* commercial strain US-05 (Fermentis, Lesaffre, France) and commercial probiotic *S. cerevisiae* var. *bouardii* (Codex, Zambon, Italy) were used.

### 3.4. Fermentation kinetics

The data of the fermentation kinetics were reported in Fig. 1. The results showed different fermentation behaviour among the yeast strains tested significantly influenced by the substrates. On PW (Fig. 1A) *S. cerevisiae* M1-7 and 2 PV strains, showed a CO<sub>2</sub> production comparable to *S. cerevisiae* US-05 starter strain, but a slower fermentation kinetics. The non-*Saccharomyces* strains *L. thermotolerans* B13, *T. delbrueckii* C7.4 and *K. unispora* M3B3, showed a lower fermentation performance in comparison with the other strains, indicating that they did not achieved the complete wort attenuation. The same trend was also exhibited on PLW (Fig. 1B) with a higher total CO<sub>2</sub> released compared to PW, mainly due to a higher initial sugar content provided by the lentil wort addition.

### 3.5. Main analytical profile and vitality assay after 3 months

The results regarding the analytical profile of beer samples are reported in Table 3. All strains tested showed a good vitality after 3 months of bottling exhibiting a viable cell count c.a.  $\geq 6.5$  log CFU/mL in both substrates. In comparison with the initial c.a. 6 log CFU/mL, the viability evaluated after three months did not show significant difference in wort with or without lentil addition.

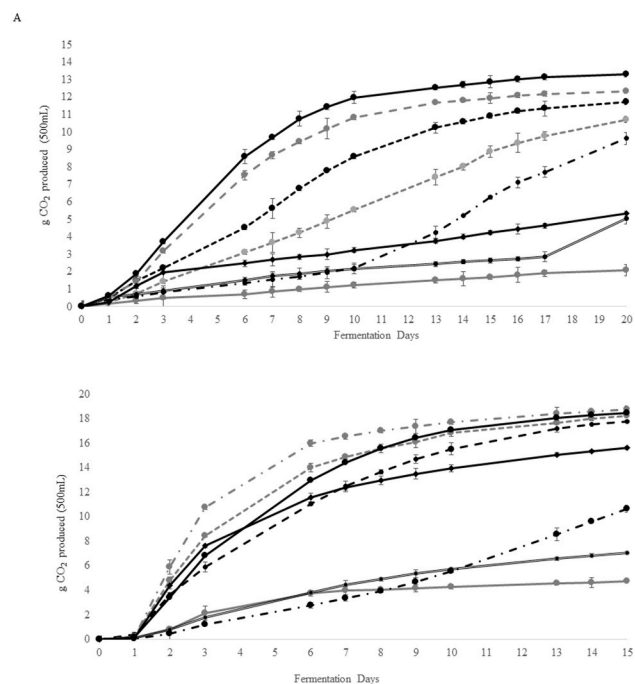


Fig. 1. Fermentation kinetics of different strains on pils wort (A) and pils wort added lentil (B). —●— (M3B3); —■— (2 PV); —○— (B6); —▲— (C7.4); —●— (B13); —●— (M1-7); —●— (US-05); —●— (Codex).

All fermentation trials carried out on PW associated with a higher sugar residual level showed a significant lower ethanol content. Moreover, the fermentation carried out on PLW showed a significant increase in final protein content of 50%, probably small due to the peptides or free aminoacids obtained after lytic enzyme treatment, in *L. thermotolerans* B13, and c.a.  $\geq 50\%$  with *K. unispora* M3B3 and *S. cerevisiae* 2PV. pH values were comparable between all trials except for *L. thermotolerans* B13, due to the production of lactic acid typical characteristics of this yeast specie.

### 3.6. By-products and volatile compounds

The data of the main by-products and volatile compounds of beers are reported in Table 4. The results highlighted a significant difference when the strains were tested on PW and PLW.

The acetaldehyde content showed a significant increase in all fermentation trials with lentil even if its content was under the threshold value. An opposite trend was exhibited by *T. delbrueckii* C7.4, that in PLW decres significantly in comparison with PW.

The ethyl acetate content, responsible of fruits notes, significantly increase only in *S. cerevisiae* B6, 2 PV and M1-7 and *L. thermotolerans* B13 fermented on PLW. *K. unispora* M3B3 and *T. delbrueckii* C7.4 did not show significant differences in two substrates tested.

Ethyl hexanoate content fruity esters associated with apple flavour, showed a significant decrease in *S. cerevisiae* 2 PV, *T. delbrueckii* C7.4 and *K. unispora* M3B3 in PLW.

Regarding to the higher alcohols (n-propanol, isobutanol, isoamyl alcohol and amylic alcohol) which define the warm 'mouthfeel', a generally increase for all strains was observed, with different trend for each strain tested on PLW with the only exception of *T. delbrueckii* C7.4, that did not show significant difference. Another important higher alcohol is  $\beta$ -phenyl ethanol, rose aroma that showed significant increase on PLW fermented with *S. cerevisiae* M1-7, 2 PV and *K. unispora* M3B3. An opposite trend of  $\beta$ -phenyl ethanol was exhibited by *T. delbrueckii*

**Table 3**

The main fermentation parameters and vitality of beers produced by different strains on pils wort (PW) and pils wort added lentil (PLW).

Strains	Fermentation parameters	PW	PLW
B13 <i>L. thermotolerans</i>	Vitality log CFU/ml	6.98 ± 0.16 <sup>b</sup>	7.90 ± 0.12 <sup>a</sup>
	Ethanol (% v/v)	2.80 ± 0.00 <sup>b</sup>	3.51 ± 0.00 <sup>a</sup>
	pH	3.75 ± 0.00 <sup>a</sup>	3.81 ± 0.00 <sup>a</sup>
	Residual maltose g/L	57.05 ± 4.12 <sup>a</sup>	31.10 ± 1.23 <sup>b</sup>
	Protein content g/L	46.06 ± 4.67 <sup>b</sup>	71.23 ± 0.40 <sup>a</sup>
C 7.4 <i>T. delbrueckii</i>	Vitality log CFU/mL	6.65 ± 0.40 <sup>a</sup>	7.60 ± 0.20 <sup>a</sup>
	Ethanol (% v/v)	0.84 ± 0.00 <sup>b</sup>	1.63 ± 0.00 <sup>a</sup>
	pH	4.78 ± 0.00 <sup>a</sup>	4.60 ± 0.00 <sup>a</sup>
	Residual maltose g/L	38.04 ± 0.74 <sup>b</sup>	97.83 ± 8.10 <sup>a</sup>
	Protein content g/L	32.33 ± 0.81 <sup>b</sup>	77.29 ± 9.37 <sup>a</sup>
M3B3 <i>K. unisporea</i>	Vitality log CFU/mL	7.16 ± 0.12 <sup>a</sup>	7.24 ± 0.16 <sup>a</sup>
	Ethanol (% v/v)	0.10 ± 0.00 <sup>b</sup>	0.53 ± 0.00 <sup>a</sup>
	pH	4.82 ± 0.00 <sup>a</sup>	4.80 ± 0.00 <sup>a</sup>
	Residual maltose g/L	43.66 ± 0.55 <sup>b</sup>	84.27 ± 8.10 <sup>a</sup>
	Protein content g/L	46.16 ± 14.67 <sup>b</sup>	97.18 ± 1.22 <sup>a</sup>
M1-7 <i>S. cerevisiae</i>	Vitality log CFU/mL	6.91 ± 0.09 <sup>a</sup>	6.89 ± 0.06 <sup>a</sup>
	Ethanol (% v/v)	2.87 ± 0.00 <sup>b</sup>	4.75 ± 0.00 <sup>a</sup>
	pH	4.78 ± 0.00 <sup>a</sup>	4.75 ± 0.02 <sup>a</sup>
	Residual maltose g/L	28.29 ± 3.27 <sup>a</sup>	4.05 ± 1.40 <sup>b</sup>
	Protein content g/L	64.04 ± 4.07 <sup>b</sup>	86.37 ± 3.46 <sup>a</sup>
B6 <i>S. cerevisiae</i>	Vitality log CFU/mL	7.2 ± 0.23 <sup>a</sup>	7.45 ± 0.03 <sup>a</sup>
	Ethanol (% v/v)	2.93 ± 0.00 <sup>b</sup>	5.11 ± 0.02 <sup>a</sup>
	pH	4.65 ± 0.01 <sup>a</sup>	4.65 ± 0.00 <sup>a</sup>
	Residual maltose g/L	25.18 ± 3.08 <sup>b</sup>	2.06 ± 1.71 <sup>a</sup>
	Protein content g/L	63.04 ± 4.07 <sup>b</sup>	82.37 ± 3.46 <sup>a</sup>
2 PV <i>S. cerevisiae</i>	Vitality log CFU/mL	7.23 ± 0.36 <sup>a</sup>	6.80 ± 0.11 <sup>a</sup>
	Ethanol (% v/v)	2.86 ± 0.00 <sup>b</sup>	4.71 ± 0.00 <sup>a</sup>
	pH	4.73 ± 0.00 <sup>a</sup>	4.73 ± 0.00 <sup>a</sup>
	Residual maltose g/L	14.93 ± 20.85 <sup>a</sup>	6.02 ± 4.47 <sup>a</sup>
	Protein content g/L	33.19 ± 3.26 <sup>b</sup>	83.92 ± 3.66 <sup>a</sup>
Codex <i>S. cerevisiae</i> var. <i>boulardi</i>	Vitality log CFU/mL	7.93 ± 0.33 <sup>a</sup>	6.81 ± 0.12 <sup>a</sup>
	Ethanol (% v/v)	1.42 ± 0.00 <sup>b</sup>	4.26 ± 0.00 <sup>a</sup>
	pH	4.68 ± 0.02 <sup>a</sup>	4.65 ± 0.01 <sup>a</sup>
	Residual maltose g/L	36.26 ± 0.55 <sup>a</sup>	7.29 ± 0.05 <sup>b</sup>
	Protein content g/L	28.72 ± 2.64 <sup>b</sup>	73.84 ± 6.93 <sup>a</sup>
US-05 <i>S. cerevisiae</i>	Vitality log CFU/mL	6.42 ± 0.11 <sup>a</sup>	5.09 ± 0.31 <sup>a</sup>
	Ethanol (% v/v)	2.99 ± 0.00 <sup>b</sup>	4.64 ± 0.00 <sup>a</sup>
	pH	4.62 ± 0.02 <sup>a</sup>	4.64 ± 0.00 <sup>a</sup>
	Residual maltose g/L	14.05 ± 1.45 <sup>a</sup>	1.31 ± 0.45 <sup>b</sup>
	Protein content g/L	22.67 ± 5.50 <sup>b</sup>	54.09 ± 7.94 <sup>a</sup>

Data are the means ± standard deviations. Data with different superscript letters (<sup>a,b</sup>) within each row between the same strain tested on different wort (Duncan tests;  $p < 0.05$ ).

The initial composition of the sugars in pils wort were: Sucrose 5.34 g/l; glucose 6.5 g/L; Maltose 56.96 g/L. Protein content: 9.56 g/L pH 5.4.

The initial composition of the sugars in pils wort added with lentil were: Sucrose 29.63 g/l; glucose 10.28 g/L; Maltose 82.16 g/L. Protein content: 35.29 g/L pH 5.63.

C7.4, *S. cerevisiae* B6 that increase in fermentation carried out on PW.

Ethyl butyrate content did not show a significant difference among the strains and substrates with the only exception of *S. cerevisiae* B6 on PLW. Moreover, the isoamyl acetate content significant increase *S. cerevisiae* 2 PV, M1-7 and *K. unisporea* M3B3 in PLW. The linalool and diethyl succinate compounds analysed did not show significant difference between two fermentations substrates.

To assess the overall effects of different yeast strains and different wort used, data regarding the by-products and the volatile compounds were analysed by PCA (Fig. 2). The PCA analysis showed that the all *S. cerevisiae* fermentation carried out on PLW, were located in the right quadrants and were mainly affected by higher alcohols production strains with the exception of 2 PV strain. Interesting, PLW did not affect the non-*Saccharomyces* behavior. Indeed, the graphical representation showed that both trials on PW and PLW were closely related: *K unisporea* M3B3P and M3B3L) in upper left plot, and *T. delbrueckii* C7.4 (C7.4 P and C7.4 L) in down left plot. *L. thermotolerans* B13 showed and intermediate result.

### 3.7. Sensory analysis

The beers obtained in pure cultures on PW and PLW were characterised by sensory analysis, with the results illustrated in Fig. 3. For the main sensorial descriptors, the data showed that within each strains the PW beers were significantly different from the PLW one. In particular, PLW beers brewed with 2 PV (*S. cerevisiae*), B6 (*S. cerevisiae*) and B13 (*L. thermotolerans*) strains the fruity/ester notes were emphasized, while cereal attribute was emphasized for 2 PV (*S. cerevisiae*). Two non-conventional yeasts C7.4 (*T. delbrueckii*) and B13 (*L. thermotolerans*) produced in PLW beers with an acid character and a strong astringency. *L. thermotolerans*, *K. unisporea*, led beers featured by aromatic notes enclosed to wort (DMS and sulphide) related to a low fermentative attitude of this strains. Moreover, each beer tested showed a different aroma profile in comparison with the products obtained with *S. cerevisiae* starter strain US-05. In addition, the starter strain US-05 showed a different aroma profile on PLW with an emphasized perception of alcohol and toasted. Only the fruity/citric notes increased in PW beers brewed with US-05 respect to PLW.

**Table 4**  
The main by-products and volatile compounds (mg/L) in beers produced by different strains on pils wort and pils wort added lentil.

Strains	Fermentation trials	Volatile compounds											
		Ethyl butyrate	Ethyl acetate	Linalool	Diethyl succinate	Ethyl hexanoate	Isoamyl acetate	n-propanol	Isobutanol	Amylic alcohol	Isoamyl alcohol	$\beta$ -phenyl ethanol	Acetaldehyde
<b>M3B3</b>	<i>K. unispora</i>	0.15 $\pm$ 0.03 <sup>a</sup>	2.83 $\pm$ 1.32 <sup>a</sup>	0.07 $\pm$ 0.09 <sup>a</sup>	0.002 $\pm$ 0.00 <sup>a</sup>	0.13 $\pm$ 0.02 <sup>a</sup>	0.41 $\pm$ 0.06 <sup>b</sup>	10.88 $\pm$ 0.12 <sup>a</sup>	ND	1.33 $\pm$ 0.62 <sup>a</sup>	9.36 $\pm$ 1.12 <sup>b</sup>	0.78 $\pm$ 0.4 <sup>b</sup>	5.79 $\pm$ 0.35 <sup>b</sup>
	<i>Pils</i>												
	<i>K. unispora Pils + lentil</i>	0.07 $\pm$ 0.03 <sup>a</sup>	2.80 $\pm$ 1.65 <sup>a</sup>	0.21 $\pm$ 0.01 <sup>a</sup>	ND	0.04 $\pm$ 0.01 <sup>b</sup>	1.54 $\pm$ 0.22 <sup>a</sup>	21.28 $\pm$ 0.41 <sup>a</sup>	4.90 $\pm$ 0.27 <sup>a</sup>	2.66 $\pm$ 0.27 <sup>a</sup>	16.85 $\pm$ 1.87 <sup>a</sup>	5.44 $\pm$ 0.4 <sup>a</sup>	12.93 $\pm$ 1.40 <sup>a</sup>
<b>2 PV</b>	<i>S. cerevisiae</i>	0.04 $\pm$ 0.01 <sup>a</sup>	0.38 $\pm$ 0.54 <sup>b</sup>	0.12 $\pm$ 0.02 <sup>a</sup>	ND	0.21 $\pm$ 0.01 <sup>a</sup>	0.71 $\pm$ 0.01 <sup>b</sup>	18.90 $\pm$ 1.01 <sup>b</sup>	9.70 $\pm$ 0.55 <sup>a</sup>	7.16 $\pm$ 0.93 <sup>a</sup>	41.52 $\pm$ 4.27 <sup>a</sup>	39.02 $\pm$ 5.98 <sup>a</sup>	26.33 $\pm$ 1.48 <sup>b</sup>
	<i>Pils</i>												
	<i>S. cerevisiae Pils + lentil</i>	0.14 $\pm$ 0.05 <sup>a</sup>	10.94 $\pm$ 1.02 <sup>a</sup>	0.05 $\pm$ 0.06 <sup>a</sup>	0.016 $\pm$ 0.00 <sup>a</sup>	0.05 $\pm$ 0.00 <sup>b</sup>	3.50 $\pm$ 1.13 <sup>a</sup>	26.29 $\pm$ 0.46 <sup>a</sup>	11.68 $\pm$ 0.50 <sup>a</sup>	9.40 $\pm$ 0.17 <sup>a</sup>	53.26 $\pm$ 0.17 <sup>a</sup>	10.30 $\pm$ 1.90 <sup>b</sup>	59.63 $\pm$ 2.54 <sup>a</sup>
<b>B6</b>	<i>S. cerevisiae</i>	0.03 $\pm$ 0.01 <sup>b</sup>	3.11 $\pm$ 1.16 <sup>b</sup>	0.07 $\pm$ 0.01 <sup>a</sup>	ND <sup>b</sup>	0.10 $\pm$ 0.11 <sup>a</sup>	1.05 $\pm$ 0.14 <sup>a</sup>	16.16 $\pm$ 1.62 <sup>b</sup>	8.61 $\pm$ 0.89 <sup>b</sup>	6.65 $\pm$ 0.91 <sup>a</sup>	28.27 $\pm$ 4.52 <sup>b</sup>	15.61 $\pm$ 1.13 <sup>a</sup>	11.24 $\pm$ 0.73 <sup>b</sup>
	<i>Pils</i>												
	<i>S. cerevisiae Pils + lentil</i>	0.15 $\pm$ 0.02 <sup>a</sup>	14.74 $\pm$ 1.41 <sup>a</sup>	0.08 $\pm$ 0.00 <sup>a</sup>	0.05 $\pm$ 0.01 <sup>a</sup>	0.034 $\pm$ 0.01 <sup>a</sup>	1.84 $\pm$ 0.0 <sup>a</sup>	34.30 $\pm$ 2.33	16.51 $\pm$ 0.84 <sup>a</sup>	6.52 $\pm$ 0.64 <sup>a</sup>	45.50 $\pm$ 6.55 <sup>a</sup>	0.10 $\pm$ 0.05 <sup>b</sup>	60.48 $\pm$ 3.08 <sup>a</sup>
<b>C 7.4</b>	<i>T. delbrueckii</i>	0.21 $\pm$ 0.07 <sup>a</sup>	3.05 $\pm$ 0.58 <sup>a</sup>	0.11 $\pm$ 0.013 <sup>a</sup>	ND	0.17 $\pm$ 0.01 <sup>a</sup>	0.30 $\pm$ 0.08 <sup>a</sup>	11.41 $\pm$ 0.25 <sup>a</sup>	5.45 $\pm$ 1.32 <sup>a</sup>	3.74 $\pm$ 1.17 <sup>a</sup>	22.49 $\pm$ 7.29 <sup>a</sup>	6.21 $\pm$ 3.42 <sup>a</sup>	44.37 $\pm$ 17.14 <sup>a</sup>
	<i>Pils</i>												
	<i>T. delbrueckii Pils + lentil</i>	0.17 $\pm$ 0.11 <sup>a</sup>	3.19 $\pm$ 0.06 <sup>a</sup>	0.10 $\pm$ 0.03 <sup>a</sup>	0.004 $\pm$ 0.00 <sup>a</sup>	0.03 $\pm$ 0.02 <sup>b</sup>	0.81 $\pm$ 0.24 <sup>a</sup>	11.80 $\pm$ 0.07 <sup>a</sup>	5.81 $\pm$ 0.07 <sup>a</sup>	1.15 $\pm$ 0.98 <sup>a</sup>	18.91 $\pm$ 1.10 <sup>a</sup>	0.16 $\pm$ 0.06 <sup>b</sup>	26.59 $\pm$ 12.94 <sup>b</sup>
<b>B13</b>	<i>L. thermotolerans Pils</i>	0.01 $\pm$ 0.01 <sup>a</sup>	1.47 $\pm$ 0.90 <sup>b</sup>	0.09 $\pm$ 0.03 <sup>a</sup>	0.037 $\pm$ 0.05 <sup>a</sup>	0.32 $\pm$ 0.01 <sup>a</sup>	1.04 $\pm$ 0.01 <sup>a</sup>	12.62 $\pm$ 0.49 <sup>a</sup>	5.35 $\pm$ 0.08 <sup>b</sup>	2.92 $\pm$ 0.66 <sup>b</sup>	23.27 $\pm$ 0.85 <sup>b</sup>	17.97 $\pm$ 1.74 <sup>a</sup>	7.37 $\pm$ 1.22 <sup>b</sup>
	<i>L. thermotolerans Pils + lentil</i>	0.25 $\pm$ 0.21 <sup>a</sup>	8.55 $\pm$ 0.02 <sup>a</sup>	0.099 $\pm$ 0.02 <sup>a</sup>	0.003 $\pm$ 0.00 <sup>a</sup>	0.25 $\pm$ 0.07 <sup>a</sup>	0.52 $\pm$ 0.03 <sup>b</sup>	14.48 $\pm$ 0.87 <sup>a</sup>	9.82 $\pm$ 0.12 <sup>a</sup>	6.32 $\pm$ 0.87 <sup>a</sup>	37.8 $\pm$ 2.36 <sup>a</sup>	2.53 $\pm$ 0.15 <sup>b</sup>	27.87 $\pm$ 1.2 <sup>a</sup>
	<i>S. cerevisiae Pils</i>	0.08 $\pm$ 0.04 <sup>a</sup>	1.98 $\pm$ 0.03 <sup>b</sup>	0.06 $\pm$ 0.02 <sup>a</sup>	ND	0.03 $\pm$ 0.04 <sup>a</sup>	2.79 $\pm$ 0.83 <sup>b</sup>	13.40 $\pm$ 0.66 <sup>a</sup>	7.08 $\pm$ 0.35 <sup>b</sup>	5.58 $\pm$ 0.31 <sup>b</sup>	29.44 $\pm$ 0.00 <sup>b</sup>	3.79 $\pm$ 0.35 <sup>b</sup>	22.61 $\pm$ 0.01 <sup>b</sup>
<b>US05</b>	<i>S. cerevisiae</i>	0.00 $\pm$ 0.00 <sup>a</sup>	17.94 $\pm$ 0.68 <sup>a</sup>	0.11 $\pm$ 0.01 <sup>a</sup>	ND	0.08 $\pm$ 0.02 <sup>a</sup>	6.97 $\pm$ 0.11 <sup>a</sup>	16.41 $\pm$ 2.09 <sup>a</sup>	15.11 $\pm$ 0.56 <sup>a</sup>	14.64 $\pm$ 0.67 <sup>a</sup>	51.54 $\pm$ 1.80 <sup>a</sup>	11.16 $\pm$ 1.53 <sup>a</sup>	113.20 $\pm$ 6.58 <sup>a</sup>
	<i>Pils</i>												
	<i>S. cerevisiae Pils + lentil</i>	0.20 $\pm$ 0.11 <sup>a</sup>	2.43 $\pm$ 0.41 <sup>b</sup>	0.19 $\pm$ 0.15 <sup>a</sup>	0.024 $\pm$ 0.00 <sup>a</sup>	0.012 $\pm$ 0.01 <sup>a</sup>	0.88 $\pm$ 0.36 <sup>b</sup>	14.93 $\pm$ 1.25 <sup>a</sup>	6.81 $\pm$ 0.09 <sup>b</sup>	6.13 $\pm$ 0.73 <sup>b</sup>	26.50 $\pm$ 1.27 <sup>b</sup>	10.40 $\pm$ 1.62 <sup>a</sup>	13.49 $\pm$ 0.59 <sup>b</sup>
<b>Codex</b>	<i>S. cerevisiae</i>	0.10 $\pm$ 0.00 <sup>a</sup>	9.93 $\pm$ 2.61 <sup>a</sup>	0.10 $\pm$ 0.01 <sup>a</sup>	ND	0.02 $\pm$ 0.00 <sup>a</sup>	3.20 $\pm$ 0.14 <sup>a</sup>	19.91 $\pm$ 2.64 <sup>a</sup>	13.71 $\pm$ 1.16 <sup>a</sup>	12.73 $\pm$ 1.87 <sup>a</sup>	43.32 $\pm$ 9.41 <sup>a</sup>	10.06 $\pm$ 1.32 <sup>a</sup>	76.63 $\pm$ 16.86 <sup>a</sup>
	<i>Pils</i>												
	<i>S. cerevisiae var. boulardii</i>	0.00 $\pm$ 0.00 <sup>a</sup>	2.68 $\pm$ 0.088 <sup>b</sup>	0.08 $\pm$ 0.01 <sup>a</sup>	ND	0.27 $\pm$ 0.10 <sup>a</sup>	0.38 $\pm$ 0.02 <sup>b</sup>	11.90 $\pm$ 0.29 <sup>b</sup>	0 $\pm$ 0 <sup>b</sup>	2.47 $\pm$ 0.89 <sup>b</sup>	12.86 $\pm$ 2.34 <sup>b</sup>	2.41 $\pm$ 1.20 <sup>b</sup>	14.09 $\pm$ 1.91 <sup>b</sup>
<b>Codex</b>	<i>S. cerevisiae var. boulardii</i>	0.02 $\pm$ 0.01 <sup>a</sup>	11.18 $\pm$ 0.98 <sup>a</sup>	0.07 $\pm$ 0.01 <sup>a</sup>	0.57 $\pm$ 0.01 <sup>a</sup>	0.16 $\pm$ 0.03 <sup>a</sup>	1.24 $\pm$ 0.02 <sup>a</sup>	31.11 $\pm$ 0.74 <sup>a</sup>	14.09 $\pm$ 0.28 <sup>a</sup>	10.78 $\pm$ 2.33 <sup>a</sup>	41.95 $\pm$ 2.60 <sup>a</sup>	4.00 $\pm$ 0.36 <sup>a</sup>	55.43 $\pm$ 4.35 <sup>a</sup>
	<i>Pils + lentil</i>												

Data are means  $\pm$  standard deviations from three independent experiments. Data with different superscript letters (<sup>a,b</sup>) within each column between the same strain tested on different wort, are different homogeneous groups according to Duncan tests (0.05%). ND (not detected).

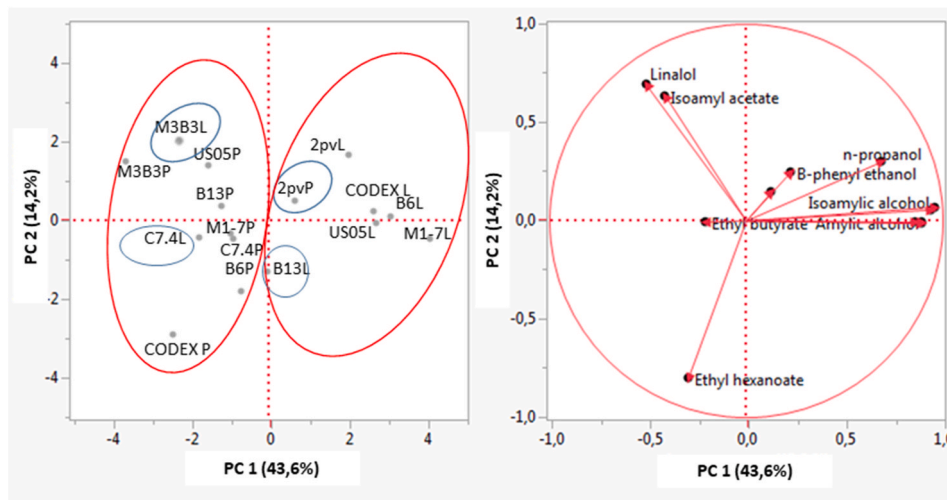


Fig. 2. Principal component analysis for the main by-products and volatile compounds of craft beer obtained by different yeast strains in pils wort (P) and pils wort added lentil (L). The variance explained by principle component analysis (PCA) analysis is PC 1 43.6% X-axis and PC 2 14.2% Y-axis. PC 1: principal component.

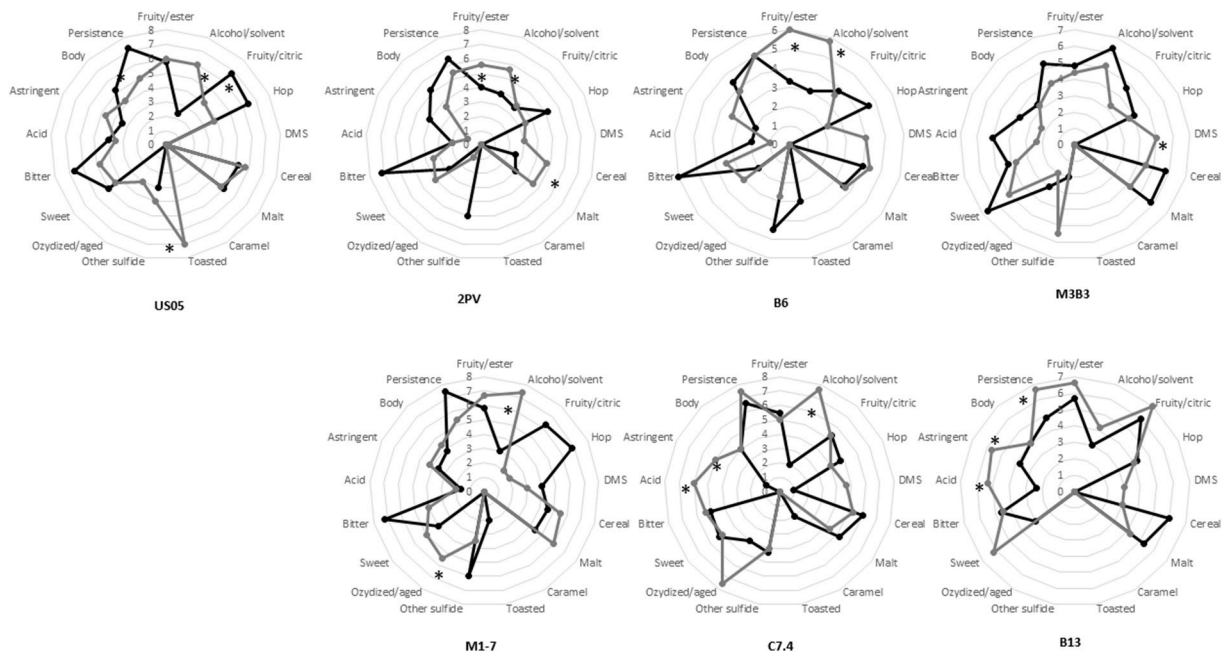


Fig. 3. Sensory analysis of the beer produced by different strains on pils wort — and pils wort added lentil —. \*, significantly different (Fisher ANOVA; p-value 0.05). DMS, Dimethyl sulphide. Score 0: absence of the descriptor analysed.

4. Discussion

In the recent years, consumption of fermented foods and beverages has substantially increased around the world, since they play a central role in the daily diet of several cultures because of their multiple health benefits such as antimicrobial, antidiabetic, anti-atherosclerotic, antioxidant and anti-inflammatory activities (Şanlıer, Gökçen, & Sezgin, 2019). Consequently, fermenting microorganisms, fermentation process and its products attract scientific interest in applied microbiology field.

Nowadays, several ingredients, such as wheat, corn, rice and fruits, have been tested in beer, to improve the phenolic profiles of several commercialized product, with the aim to obtain a functional beverage (Ambra, Pastore, & Lucchetti, 2021). On the other hand, the use of legumes to fortified foods are extensively tested in bakery products (Foschia, Horstmann, Arendt, & Zannini, 2017), but never were proposed to combine the use of legumes for functional beer production.

In this context, the aim of this study was to evaluate of the performance of non-conventional yeasts with potential probiotic aptitude previously characterized by Agarbati et al., 2020 and listed in Table 1 supplementary materials, to produced premium craft beer with added nutritional properties and strengthened sensory profile.

Although most of the published investigations on probiotics focused on bacteria, especially lactic acid bacteria used in dairy foods and beverages, in recent years, potential probiotic yeasts and their fermented products have gathered high scientific and commercial interests (Shakibzadeh et al., 2011). In parallel, with increasing vegetarianism and veganism, there is also collective demand for non-dairy fermented probiotic products.

In this regard, scientific studies focused on yeasts as potential probiotics are emerging, due to their excellent fermentative performances both in food and drinks (Hatoum, Labrie, & Fliss, 2012; Moslehi-Jenabian, Lindegaard, & Jespersen, 2010; Perricone, Bevilacqua, Corbo, &



Sinigaglia, 2014). Indeed, beneficial treats of yeasts are related with the improvement of bioavailability of minerals by phytate hydrolysis (Regon, Chowra, Awasthi, Borgohain, & Panda, 2019), anti-inflammatory effect (Mumy, Chen, Kelly, & McCormick, 2008) and the ability to produce natural antioxidants such as carotenoids, citric acid, ascorbic acid, glutathione that boost the host health, retarding the lipid oxidative degeneration (Arroyo-López et al., 2012).

While the addition of hydrolysed chickpea flour compromises the aromatic traits, the addition of 20% of lentil showed a promising analytical and sensorial composition of the final beers. Lentil (*Lens culinaris*), a grain legume principally grown in Turkey and Canada, represent an important source of protein (25–30%). Although the Italy is not a one of the major producing country in terms of quantity, some lentil variety are excellent product such as lentil of Colfiorito and Castelluccio di Norcia, a small area of Central Italy with an ideal microclimate (Micioni Di Bonaventura et al., 2017).

Although in the past, beer was considered a beverage formulated to replace other alcoholic and more expensive beverages nowadays, the unfiltered and unpasteurized craft beer has proven higher quality than industrial analogues, and the use of non-conventional yeast in brewing process was widely investigated for their contribution improve aromatic taste (Canonico, Agarbati, Comitini, & Ciani, 2016; Canonico, Ciani, Galli, Comitini, & Ciani, 2020; Canonico et al., 2019; Domizio, House, Joseph, Bisson, & Bamforth, 2016; Holt, Mukherjee, Lievens, Verstrepen, & Thevelein, 2018; Osburn et al., 2018) and functional properties.

In the present research, *L. thermotolerans* previously isolated in a natural un-anthropized environment (oak moss in wood) led an effective sour notes and fruity/ester character and an increase of aromatic notes. Moreover, the low ethanol content exhibited by *L. thermotolerans* pure cultures was also relevant to produce low alcohol beer.

The wild *S. cerevisiae* strain 2 PV with demonstrated probiotic traits (isolated from a winery where never commercial starter cultures were used) exhibited a fermentative behaviour comparable to that exhibited by *S. cerevisiae* starter strain US05, conferring to the beers a favourable aroma profile.

*K. unispora*, formerly *Saccharomyces unisporus* (Bhattacharya, Yan, & Shankar, 2013), isolated by artisanal sourdough exhibited an effective and peculiar aroma potential, although its fermentation rate is very low. This trend could be useful for obtaining a craft beer marked by low ethanol content and at the same time with distinctive aromatic notes. Another relevant aspect in PLW is the increased fermentative performance of yeasts where the lentil wort act as a “nutritional supplement” increasing, on one side, some volatile compounds such as higher alcohols and allowing, on the other side, the increase of the enzymatic activity in wort could be a positive advantage in production costs (higher extract) in brewing process.

The promising fermentation capacity of a small number of selected yeast strains with previously defined probiotic aptitude and the use of protein-rich lentil wort, could be a valuable biotechnological approach to produce an innovative beer with low alcohol content with increased nutritional value. Although these results are promising, further investigations are necessary to assay the drink's effectiveness during pilot scale production, and to carry out a consumer survey in which the final product must be challenged to verify the degree of acceptability.

## 5. Conclusion

In this study, the selected yeast strains showed a better fermentation capacity than to conventional *S. cerevisiae* commercial starter strain. These strains could be an effective alternative to the only commercially available probiotic yeast *S. cerevisiae* var. *bouardii*, during fortified craft beer production. Moreover, the non-*Saccharomyces* yeasts selected here, could be a suitable strategy to manage a premium craft beer fermentation with promising sensory profile with the future prospective to introduce in the market an improved product. Indeed, the conjunction of

a legume fortified beer (with increased phytochemicals isoflavones, saponins, alkaloids) and the presence of probiotic yeasts could represent a great opportunity to place an innovative product on the market.

The other phytochemicals (isoflavones, saponins, alkaloids) in legumes have been reported to have potential benefit in human health. It is reported that isoflavones are largely isolated from the Fabaceae family (Leguminosae) (Rochfort & Panozzo, 2007). Isoflavones have been shown to have several biological activities including reduction in osteoporosis, prevention of cancer and cardiovascular disease.

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## CRedit authorship contribution statement

**Laura Canonico:** contributed equally to this manuscript, carried out the experimental part of the work, carried out the analysis of the data and wrote the manuscript. **Emanuele Zannini:** contributed equally to this manuscript, carried out the analysis of the data and wrote the manuscript. **Maurizio Ciani:** contributed equally to this manuscript, carried out the analysis of the data and wrote the manuscript. **Francesca Comitini:** contributed equally to this manuscript, carried out the analysis of the data and wrote the manuscript, All authors participated in the design and discussion of the research, All authors have read and approved the final manuscript.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2021.111361>.

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