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New Approaches for the Fermentation of Beer: Non-*Saccharomyces* Yeasts from Wine

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Abstract: Non-*Saccharomyces* yeasts represent a very attractive alternative for the production of beers with superior sensory quality since they are able to enhance the flavour of beer. Furthermore, they can produce beers with low ethanol content due to the weak fermentative capacity of a large percentage of non-*Saccharomyces* species. The objective of this study was to evaluate the ability of 34 non-*Saccharomyces* yeast strains isolated from Madrilenian agriculture to produce a novel ale beer. The non-*Saccharomyces* yeast strains were screened at two scales in the laboratory. In the first screening, those with undesirable aromas were discarded and the selected strains were analysed. Thirty-three volatile compounds were analysed by GC, as well as melatonin production by HPLC, for the selected strains. Thirteen strains were then fermented at a higher scale in the laboratory for sensory evaluation. Only yeast strains of the species *Schizosaccharomyces pombe* and *Lachancea thermotolerans* were able to complete fermentation. Species such as *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Wickerhamomyces anomalus*, *Hanseniaspora vineae*, and *Hanseniaspora guilliermondii* could be used both for production of low ethanol beers and co-fermentation with a *Saccharomyces* yeast to improve the organoleptic characteristics of the beer. In addition, for these strains, the levels of melatonin obtained were higher than the concentrations found for *Saccharomyces* strains subjected to the same study conditions. The selected strains can be used in future trials to further determine their viability under different conditions and for different purposes.

Keywords: non-*Saccharomyces*; ale yeast; volatile compounds; melatonin

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1. Introduction

Traditionally, the role of non-*Saccharomyces* yeasts species in the brewing processes has been viewed negatively on the basis that their use could present problems associated with beer turbidity, filterability, viscosity, phenolic off-flavours (POF), acidity, and other changes in the flavour profile [1,2]. However, the proper application and selection of non-conventional yeasts in brewing can enable favourable characteristics to be obtained. For this reason, in recent years, starter strains of non-*Saccharomyces* species that have been isolated and characterised as contributing to a distinctive flavour and aromatic components have been selected [3–5]. Wine yeasts involved in wine fermentation have been extensively studied. Their use in beer fermentation is becoming more widespread, as they are considered to be of particular value in the search for yeasts that produce outstanding aromas [6–8]. Similarly, ale yeasts (top fermentation, 16–24 °C) are now used as they have been found to result in beers with fruitier flavours than those fermented with lager yeasts (bottom fermentation, 6–15 °C), where the aromas are more neutral [9].

Non-*Saccharomyces* yeasts represent a great source of biodiversity for the production of new beer styles, since they can be used in different industrial areas, as pure culture starters, in co-fermentation with *Saccharomyces*, and in spontaneous fermentation (i.e., lambic and

gueuze production, with the main contribution of *Brettanomyces* yeast). Pure culture fermentations mainly focus on the production, on the one hand, of beers with differentiated characteristics, involving either a higher production of esters (e.g., *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* species) [5,10,11] or higher production of certain compounds, such as lactic acid in the case of *Lachancea* yeasts [12], and, on the other hand, the production of low ethanol beers using maltose-negative yeast. The production of low ethanol beers using non-*Saccharomyces* yeasts is an increasingly popular method as it avoids the loss of aromas (mainly of higher alcohols and esters) and body that occurs when alcohol is mechanically removed from the beer [13]. Beers brewed in this way can be more malty, sweeter, and fruitier than alcoholic beers brewed in the traditional way or by physical methods. Several studies have shown the potential of yeasts such as *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Saccharomycodes ludwigii*, and *Zygosaccharomyces rouxii* for the production of these beers [5,11,14,15]. Moreover, their low ethanol production makes them suitable for co-fermentation with *Saccharomyces* yeasts, as they can enhance flavours, as shown in previous studies [6,8,16].

Compared to conventional brewing yeasts, these species, which have been isolated from different foods or environments, offer various functional benefits, not only in terms of aroma production and low ethanol content, but also with respect to the production of compounds such as melatonin [17,18]. Melatonin in beer can provide antioxidant [19,20], anti-ageing [21,22], anti-inflammatory [23,24], antitumor [25,26] and metabolic [27,28] properties. Furthermore, melatonin has been functionally linked to the regulation of circadian and seasonal rhythms [29]. Beer, in moderate consumption, can, therefore, be considered as a functional beverage, as its constituents can contribute to its overall therapeutic characteristics [6].

The present study aimed to evaluate the fermentative capacity of 34 non-*Saccharomyces* yeast strains isolated from Madrid agriculture with respect to their use as starters for craft ale beer production and their ability to produce potentially functional beers. The volatile profiles, and the main parameters of beer (i.e., residual fermentable sugars, ethanol content, colour, bitterness, lactic acid) were determined and sensory analysis of beer samples was undertaken to produce a novel beer.

2. Materials and Methods

2.1. Yeast Collection

This study was carried out using the Madrid Institute for Rural, Food and Agriculture Research and Development (IMIDRA, Madrid, Spain) collection of native wine yeast from D.O. "Vinos de Madrid" [30]. These yeasts strains were isolated from wine, grapes, must, vineyard and cellars in Madrid, identified and then cryogenically preserved at −80 °C (YPD broth supplemented with 40% (*w/v*) glycerol) as in previous studies [30]. The *Saccharomyces cerevisiae* commercial strain SafAle S-04 (Fermentis, Lesaffre, France) was used as the control during the fermentation trials.

Thirty-four non-*Saccharomyces* yeast strains, from 15 species and belonging to seven different genera, were studied.

2.2. Preliminary Screening

Non-*Saccharomyces* yeasts were first evaluated based on their ability to assimilate maltose and their production of hydrogen sulphide (H_2S). Maltose fermentation was tested using the Durham test [31] with 10 mL of a medium composed of yeast extract (10 g L^{-1}), bacteriological peptone (20 g L^{-1}) (Condalab, Madrid, Spain), and maltose (20 g L^{-1}) (Serva, Heidelberg, Germany). The production level of H_2S was evaluated by inoculating the yeasts on bismuth-containing indicator medium BIGGY agar (Oxoid) and incubating plates at 28 °C for 2 days [32]. A colour scale was used (1–4) based on the browning of yeast colonies in medium BiGGY agar:

- Type I (white/creme): low-null production H_2S
- Type II (light brown): moderate production H_2S

- Type III (brown): high production H_2S
- Type IV (dark brown/black): very high production H_2S

2.3. Laboratory Scale Fermentations: 100 mL and 1 L

Pale hopped wort produced at La Cibeles microbrewery was employed to carry out the different fermentations throughout the study. The characteristics of the wort were: gravity 1045 ± 0.00 g cm^{-3} and 11.1 ± 0.16 °P, pH 5.54 ± 0.14, free amino nitrogen 232.89 ± 11.36 mg L^{-1}, starch 0.61 ± 0.07 g L^{-1}, 32.77 ± 2.58 IBU and 76.33 ± 8.96 g L^{-1} of total sugars expressed in maltose.

Small scale fermentations were performed in small bottles of 150 mL containing 100 mL of wort and sealed with airlocks, allowing the CO_2 to escape. Fermentations were conducted for each strain in triplicate, at 20 °C and with rotary shaking at 120 rpm. Yeast pre-cultures were grown in liquid YPD media (10 g L^{-1} yeast extract, 20 g L^{-1} bacteriology peptone and 20 g L^{-1} glucose) (Condalab, Madrid, Spain) at 28 °C and 120 rpm overnight. The yeast strains were inoculated at a concentration of 10^6 cells mL^{-1}. The weight loss of the bottles due to CO_2 production was measured daily until their stabilization (three consecutive days of the same weight). After stabilisation of the fermentation, the selected fermented samples were centrifuged and stored at −30 °C until analysis (for volatile compounds and melatonin). The strains that did not show suitable characteristics by direct sensorial olfaction (i.e., off-flavours) for beer were discarded. The selected strains were tested at the next scale in 1 L vessels stoppered with glass fermentation traps and containing 900 mL of sterilized wort. The process and conditions were the same as in the previous test (18 °C, 120 rpm, daily lost weight and 10^6 cells mL^{-1} of inoculum). Starter cultures were prepared in the same conditions as for the fermentations in 100 mL (24 h culture in YPD). After the fermentation was finished (three consecutive days with no variation in weight), 7 g L^{-1} of glucose (sterilized under ultraviolet light) was added to the beers, which were then bottled and stored at 20 °C for one week for bottled conditioning and one month at 4 °C for maturation. Two bottles were bottled for each fermenter. Yeast viability was measured before bottling by a direct microscopic method using a Thoma counting chamber and methylene blue procedure [33]. All the fermentations were carried out in triplicate and used the *Saccharomyces cerevisiae* commercial yeast strain S-04 (Fermentis, Lesaffre, France) as reference.

2.4. Beer Analysis

Five different parameters, colour (range 1–100 EBC—European Brewing Convention), bitterness (range 5–100 IBU—International Bitterness Unit), fermentable sugars (sum of glucose, fructose and maltose, range 15–200 g L^{-1}), lactic acid (range 150–3500 ppm) and alcohol (range 1–10% *v/v*) were analysed with CDR FoodLab, BeerLab software [34] and verified by the international reference analysis laboratory Campden BRI [35]. All analyses were based on enzymatic reactions and spectrophotometry.

As recommended by the manufacturer, beer samples were degassed through a cellulose filter of grade 2 V (Whatman, Maidstone, UK) prior to analysis.

2.5. Determination of Major Aromatic Compounds

Major aromatic compounds (comprising 33 compounds, divided into different families: higher alcohols, esters, carboxylic acids, acetaldehydes-ketones, lactones and phenols) were determined according to the method of Ortega et al. [36], based on liquid-phase microextraction with dichloromethane (DCM, Panreac, Barcelona, Spain), and then identified with gas chromatography. A gas chromatograph 6850 (GC-FID, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a flame ionization detector was utilized for analysis. The conditions were as follows: column: DB-WAX (60 m × 0.32 mm i.d. and 0.5 µm film); oven temperature: 5 min at 40 °C, followed by 3 °C min^{-1} increase up to 200 °C; injector and detector temperatures: 200 °C; the carrier gas was helium at a flow rate of 2 mL min^{-1} with splitless injection.

The samples were extracted in triplicate. Four internal standards (2-butanol, 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone, 2-octanol) were used for the determination of the major aromatic compounds. Levels of the different compounds were determined by calibration lines for each compound (R^2 = 0.9861–0.9969).

2.6. Determination of Melatonin

Melatonin in the brewed beers was extracted by solid-phase extraction (SPE) with RP-18 standard PP-tubes (Agilent Technologies, Inc., Santa Clara, CA, USA). The extraction columns used were conditioned with 2 mL of methanol (Scharlab, Barcelona, Spain) and 5 mL of ultrapure water (type 1), loaded with 500 µL of beer, and impurities washed (2 mL ultrapure water) before elution with 2 mL of methanol [37]. The extracted samples were dried into a thermoblock at 80 °C under a nitrogen stream, and, finally, they were reconstituted with 300 µL of methanol and 700 µL of mobile phase (formic acid (0.1%)/Acetonitrile (95:5)) (HPLC grade; Carlo Erba, Italia/Panreac-Applichem, Barcelona, Spain). Before injection, the reconstituted extract was filtered through a 0.22 µm syringe filter (13 mm hydrophilic PVDF syringe filter).

A Waters 600 HPLC controller system, connected to an autosampler Waters 717 plus and a Waters 2475 multifluorescence detector, was used. The chromatographic separations were performed on a ZORBAX Eclipse Plus C18 column (Agilent Technologies, Inc., Santa Clara, CA, USA) using a mixture of 0.1% formic acid in water and acetonitrile (95:5) at a flow rate of 1 mL min^{-1} at 30 °C. The mobile phase was first degassed in an ultrasonic bath. The injected volume was 10 µL. The fluorescence detector recorded wavelengths of 270 nm for excitation and 372 nm for emission. The concentration of melatonin was measured using a linear calibration curve (R^2 > 0.9856) [38–40].

2.7. Sensory Analysis

To evaluate the flavour of beers fermented by different strains on a 1 L scale, a panel of ten trained and experienced beer tasters (five male, five female) judged the beers using different descriptors. The panel was trained to identify various beer flavours (i.e., diacetyl, DMS, acetaldehyde, bitter, butyric acid, isovaleric acid, lactic acid, earthy, H_2S, geraniol, clove, grainy, papery, indole, light-struck) (Siebel Institute of Technology, Chicago, IL, USA) according to EBC method 13 [41–43]. A based beer spiked with the different parameters was used for the training. The samples were initially spiked with concentrations three times the threshold found in beer and gradually reduced to the corresponding threshold.

The evaluation took place in a special tasting room, with room temperature from 24 °C to 25 °C [44]. Fresh samples of three bottles were tasted, one from each fermenter, matured for a minimum of one month after bottling.

This test performed a formal and structured quantitative descriptive analysis to obtain a flavour profile of the beers, assessing the intensity of each attribute. The overall quality scores were also used to rate the overall quality of the beers (EBC method 13.10) [45]. The sensory analysis was divided into three phases: visual (colour, foam retention), aroma (esters, alcohols), taste (alcohol, sweetness, saltiness, acidity, bitterness, astringency, effervescence, warmth, slickness, body), and, finally, a general impression of each beer was provided. Every category was judged from 0, meaning not noticeable, to 5, meaning extremely noticeable. A radar chart was elaborated showing the average values of the different attributes for each beer.

2.8. Statistical Analysis

The data for the triplicates were presented in terms of mean and standard deviation. A one-way ANOVA with Tukey post hoc test with a significance level of $p < 0.05$ was carried out to detect significant differences between the variables analysed, depending on the yeast strain inoculated. Identification of significant data correlation was performed with a Pearson test. Principal component analysis (PCA) was carried out to highlight differences between the obtained results, both for the aromas and all the overall parameters analysed.

The program used was R Studio 4.1 (Integrated Development for R. RStudio, PBC, Boston, MA, USA).

3. Results and Discussion

3.1. Preliminary Screening of Non-Saccharomyces Strains

A preliminary screening was carried out with 34 non-*Saccharomyces* strains to determine those that were suitable for fermenting beer. The ability to ferment maltose and the production level of H_2S were evaluated (Table 1). Maltose is the most abundant fermentable sugar in the brewing wort and it is assimilated by yeast after glucose and fructose uptake [46]. There were only seven yeast strains found to be maltose positive by CO_2 production with Durham test (CLI 1, CLI 190, CLI 920, CLI 650, CLI 996, CLI 1232, 9-6C), while 28 were maltose negative. With respect to H_2S production, 47% of the strains showed medium H_2S production (Type II), while 38.2% showed high H_2S production (Type III), and only 14.7% of the strains showed low or no H_2S production (Type I). The yeast strains that assimilated the maltose sugars showed moderate and high production of H_2S (Type II and III). Only two strains corresponded to H_2S production type IV; these were discarded as they presented undesirable aromas in subsequent assessment by olfactory analysis. The two strains discarded belonged to the species *Zygosaccharomyces bailii*, and *Candida sorbose*. Qualitative H_2S production was shown to be strain-dependent, as differences between strains of the same species were observed.

H_2S is a volatile compound that imparts a rotten-egg smell, thus masking other desired aromas in beer [47]. Its concentration changes during the fermentation process due to the depletion of fermentable sugars with a rapid decrease when the assimilation rate drops below 0.05 *w/w* % h^{-1}. However, it can also vary during the final stage of fermentation depending on the effect of yeasts taking up H_2S in green beer [48].

To further explore the results, the yeast strains were tested for their fermentative performance on the brewing wort (100 mL) in order to understand more about their behaviour in beer. Some of the strains that were able to ferment maltose were those that produced higher levels of CO_2, with fermentation kinetics that took five/six days (CLI 996, CLI 1232, 9-6C, *Lachancea thermotolerans* and CLI 650, *Schizosaccharomyces pombe*). For the other yeasts, fermentation stabilised in three days (the same weight for three consecutive days), with lower CO_2 production because they were not able to ferment maltose, as evidenced by the Durham test. However, strains CLI 1, CLI 190 and CLI 920 were maltose positive in the test but did not finish fermentation. This may have been due, according to some studies [49–55], to the ability of glucose to control maltose metabolism, as it can repress the synthesis of maltose transporters and the α-glucosidases (maltases) that hydrolyse this sugar inside the cell.

Based on these results, as well as on direct olfactory analysis, 18 strains were preselected (strains marked in bold in Table 1). A total of 16 strains were discarded as they showed accentuated off-aromas that are not desired in a pale ale beer style, including diacetyl, sulphur-containing compounds, with phenolic off-flavours (POFs) [56]. These produce beers with a strong medicinal, clove-like aroma, but are viewed as an essential flavour in Belgian white beers, German Weizen and Rauch beers [55].

The green beers containing the preselected yeasts were stored at −30 °C until their analysis.

Table 1. Maltose metabolism and H_2S production of the 34 non-*Saccharomyces* yeast strains studied.

Yeast Strain	Species	Maltose Assimilation	H_2S Production Type	Yeast Strain	Species	Maltose Assimilation	H_2S Production Type
CLI 1	*Wickerhamomyces anomalus*	+	I	**CLI 650**	***Schizosaccharomyces pombe***	**+**	**II**
CLI 3	***Hanseniaspora vineae***	**-**	**III**	CLI 679	*Pichia membranefaciens*	-	II
CLI 64	*Torulaspora delbrueckii*	-	II	CLI 691	*Zygosaccharomyces bailii*	-	I
CLI 68	*Metschnikowia pulcherrima*	-	I	**CLI 894**	***Lachancea thermotolerans***	**-**	**II**
CLI 72	*Kloeckera* spp.	-	II	CLI 900	*Torulaspora delbrueckii*	-	II
CLI 101	*Metschnikowia pulcherrima*	-	I	**CLI 902**	***Torulaspora delbrueckii***	**-**	**III**
CLI 120	*Torulaspora delbrueckii*	-	II	**CLI 918**	***Torulaspora delbrueckii***	**-**	**III**
CLI 186	*Torulaspora delbrueckii*	-	II	**CLI 920**	***Candida stellata***	**+**	**II**
CLI 190	***Hanseniaspora guilliermondii***	**+**	**I**	**CLI 921**	***Hanseniaspora guilliermondii***	**-**	**II**
CLI 194	***Hanseniaspora valbyensis***	**-**	**III**	**CLI 996**	***Lachancea thermotolerans***	**+**	**II**
CLI 219	*Metschnikowia pulcherrima*	-	I	**CLI 1028**	***Wickerhamomyces anomalus***	**-**	**II**
CLI 225	***Hanseniaspora guilliermondii***	**-**	**III**	**CLI 1232**	***Lachancea thermotolerans***	**+**	**III**
CLI 330	*Torulaspora delbrueckii*	-	III	**6-5A**	***Wickerhamomyces anomalus***	**-**	**II**
CLI 417	*Hanseniaspora valbyensis*	-	II	**9-6C**	***Lachancea thermotolerans***	**+**	**III**
CLI 457	***Metschnikowia pulcherrima***	**-**	**II**	**7A-3A**	***Torulaspora delbrueckii***	**-**	**III**
CLI 512	***Hanseniaspora guilliermondii***	**-**	**II**	19A-10B	*Torulaspora delbrueckii*	-	III
CLI 622	*Zygosaccharomyces bailii*	-	IV	25A-2A	*Candida sorbosa*	-	IV
CLI 623	*Zygosaccharomyces bailii*	-	I	17B-10A	*Candida stellata*	-	III

Yeast strains able to assimilate maltose are represented by "+", while yeast strains not able to assimilate maltose are represented by "-". Maltose assimilation is indicated as: Type I for low-null production; Type II for moderate production; Type III for high production; and Type IV for very high production. Strains marked in bold were preselected after the qualitative analysis of H_2S, maltose metabolism and fermentative behaviour in 100 mL.

3.2. Beer Analysis

The data for the different analytical parameters detected in the fermented beers and analysed with CDR FoodLab are reported in Table 2 with yeast strain S-04 as reference.

Table 2. Parameters analysed in 18 fermented beers in 100 mL with CDR.

Yeast Strain	Residual Sugars (g L^{-1})	Lactic Acid (ppm)	Colour (EBC)	Bitterness (IBU)	Alcohol (% *v*/*v*)
CLI 3	74.50 ± 3.50 ^{ab}	176.50 ± 26.50 ^{ef}	12.50 ± 1.53 ^{defg}	24.90 ± 1.30 ^{abcdef}	≤1.00 ^{d}
CLI 190	63.00 ± 0.00 ^{de}	152.50 ± 0.58 ^{f}	12.50 ± 1.53 ^{defg}	27.75 ± 0.75^{ab}	≤1.00 ^{d}
CLI 194	66.00 ± 3.00 ^{cde}	177.00 ± 6.00 ^{ef}	13.50 ± 1.53 ^{bcdef}	23.75 ± 1.25 ^{bcdef}	≤1.00 ^{d}
CLI 225	69.50 ± 4.51 ^{bcd}	176.00 ± 26.00 ^{ef}	12.00 ± 1.00 ^{efg}	24.95 ± 1.55 ^{abcde}	≤1.00 ^{d}
CLI 457	60.00 ± 0.00 ^{e}	175.50 ± 25.50 ^{ef}	16.00 ± 0.00 ^{bc}	21.50 ± 1.60 ^{def}	≤1.00 ^{d}
CLI 512	68.50 ± 0.58 ^{bcd}	193.00 ± 17.00 ^{def}	13.00 ± 0.00 ^{cdef}	20.35 ± 1.15 ^{f}	1.1 ± 0.10 ^{d}
CLI 650	≤15.00 ^{f}	274.00 ± 39.00 ^{c}	15.50 ± 1.53 ^{bcd}	22.80 ± 1.00 ^{cdef}	4.95 ± 0.15 ^{b}
CLI 894	78.00 ± 3.00 ^{a}	230.5 ± 21.50 ^{cde}	15.50 ± 0.58 ^{bcd}	25.75 ± 1.24 ^{abcd}	1.10 ± 0.10 ^{d}
CLI 902	79.00 ± 0.00 ^{a}	166.50 ± 16.50 ^{ef}	16.00 ± 0.00 ^{bc}	22.30 ± 1.90 ^{cdef}	1.35 ± 0.15 ^{d}
CLI 918	64.50 ± 0.58 ^{cde}	150.00 ± 0.00 ^{f}	22.00 ± 0.00 ^{a}	24.00 ± 0.90 ^{bcdef}	≤1.00 ^{d}
CLI 920	60.00 ± 0.00 ^{e}	190.00 ± 0.00 ^{ef}	21.50 ± 0.58 ^{a}	23.25 ± 0.05 ^{bcdef}	≤1.00 ^{d}
CLI 921	67.00 ± 4.00 ^{bcde}	263.00 ± 37.00 ^{cd}	12.50 ± 0.58 ^{defg}	24.65 ± 0.85 ^{bcdef}	1.02 ± 0.20 ^{d}
CLI 996	17.50 ± 1.53 ^{f}	277.00 ± 17.00 ^{c}	10.50 ± 1.53 ^{fg}	26.50 ± 1.60 ^{abc}	5.600 ± 0.70 ^{ab}
CLI 1028	69.00 ± 7.00 ^{bcd}	196.00 ± 0.00 ^{def}	11.50 ± 0.58 ^{efg}	29.30 ± 2.10 ^{a}	≤1.0 ^{d}
CLI 1232	15.50 ± 0.58 ^{f}	3,283.50 ± 23.50 ^{a}	9.50 ± 1.53 ^{g}	13.50 ± 0.30 ^{g}	5.70 ± 0.40 ^{a}
6-5A	72.00 ± 4.00 ^{abc}	151.50 ± 1.53 ^{f}	13.50 ± 1.53 ^{bcdef}	23.40 ± 1.40 ^{cdef}	1.10 ± 0.10 ^{d}
9-6C	≤15.00 ^{f}	2,720.50 ± 43.50 ^{b}	16.50 ± 0.58 ^{b}	15.50 ± 0.80 ^{g}	3.85 ± 0.05 ^{c}
7A-3A	64.00 ± 0.00 ^{cde}	172.00 ± 22.00 ^{ef}	12.00 ± 1.00 ^{efg}	24.30 ± 1.80 ^{bcdef}	≤1.00 ^{d}
S-04	≤15.00 ^{f}	263.33 ± 37.29 ^{cd}	14.30 ± 1.53 ^{bcde}	21.10 ± 3.32 ^{ef}	6.32 ± 0.58 ^{a}

Data are means ± standard deviations of three replicates. Data with different superscript letters within each column are significantly different (Tukey tests: $p < 0.05$). For those values ≤15.00 and ≤1.00, the statistical analysis was carried out taking as reference the values of 15 and 1, respectively.

One of the main quality characteristics of beer is the appearance [51] and colour was analysed to assess this characteristic. Measurements of colour were 9.5–22 EBC. This variation could have been due to the Maillard reaction which is an important contributor to the flavour and colour of malt and beer. Maillard reactions occur at different stages of the process: malting, wort boiling and fermentation [52]. For this study, the wort recipe was always the same; thus, the main parameter that produced variations in beer colour could be fermentation with different yeast strains. During fermentation, yeast metabolism can affect haze formation, foam stability and head retention, as well as the colour of beers, in a negative manner. Beer haze is mainly produced by organic components, such as proteins, polyphenols and carbohydrates (e.g., α-glucans, β-glucans) [53]. Polyphenols can be oxidised during the fermentation process, influencing the colour of the beer [54]. Certain variants of *S. cerevisiae* yeasts, such as var. *boulardii*, have been shown to produce high amounts of polyphenols [55]. Another compound produced by yeasts that may contribute to beer colour is riboflavin [54]. Further in-depth studies could be performed to analyse the presence of these compounds in beer. The effect on beer colour might be strain-dependent, as observed in the results obtained in this study. The lowest values were found in strains CLI 996 and CLI 1232, which correspond to *L. thermotolerans*. This has also been observed in other studies, such that of Marek Zdaniewicz et al. [56], with lower values observed in beer fermented with *L. thermotolerans*. However, this was not observed for all *L. thermotolerans* strains (such as 9-6C), and thus is considered a strain-dependent parameter.

In terms of lactic acid production, the concentration of this compound was greater in *L. thermotolerans* strains CLI 1232 and 9-6C with concentrations of 3283.5 and 2720.5 ppm observed, respectively, while CLI 894 and CLI 996 showed similar values to other species that finished the fermentation (230.5 and 277 ppm, respectively). *Lachancea* is unique among

yeast genera in its ability to produce lactic acid, which can affect both flavour and mouthfeel. Acidification can, for instance, lower the risk of protein haze formation and other microbial contamination. Other benefits are finer foam bubbles and stable, longer-lasting foam, fresher mouth-feel, smoother bitterness and fuller flavour profile [57]. However not all *L. thermotolerans* strains are able to produce higher concentrations of lactic acid as described by Zdaniewicz et al. [56] and as we found in our research. The low lactic acid production may have been due to variability in lactate dehydrogenase activity, which can have a lactic acid production capacity of 0.02 g L^{-1} to 6.15 g L^{-1} [58]. Strains producing high levels of lactic acid can potentially be used for sour beer production without bacterial intervention, the presence of which would complicate cleaning in the brewery and pose a potential risk of cross-contamination within the brewery. For the other strains tested, values were from 150 to 274 ppm and 263.3 ppm for the commercial strain S-04. The production of lactic acid by strain CLI 921 (*H. guilliermondii*) is noteworthy, as, despite not fermenting maltose, it had values similar to those obtained by strains that fermented maltose.

Bitterness values found in this study ranged from 29.3 (for strain CLI 1028, *W. anomalus*) to 13.5 IBU (for strain CLI 1232, *L. thermotolerans*). Some studies suggest that α-acid molecules from hops may bind to the cell walls of the yeast, which would be deposited at the bottom of the fermenter, thus reducing the bitterness of the beer [59–61]. Initial values obtained for bitterness were 32.77 ± 2.58 IBU. A reduction in bitterness caused by the different non-*Saccharomyces* strains during fermentation of between 58.8 and 10.6% was then observed, while for the commercial strain S-04, bitterness was only reduced by 35.6%. These values were similar to those found in other studies with both *Saccharomyces* and non-*Saccharomyces* yeast strains [59,61,62]. It should also be noted that fermentations carried out with two *L. thermotolerans* yeast strains (CLI 1232, 9-6C) showed a greater reduction in the bitterness content of the beer, as previously described, due to higher lactic acid content. The relationship between lactic acid and bitterness parameters showed a negative correlation of r = 0.81, $p < 0.01$.

Regarding the residual fermentable sugars, a clear difference was observed between the yeast strains that fermented (approximately 15 g L^{-1} residual maltose) and the yeast strains that were not able to ferment all sugars present in the beer (60–79 g L^{-1} residual maltose). Four strains from two different species finished the fermentation, including one *S. pombe* strain (CLI 650) and three *L. thermotolerans* strains (CLI 996, CLI 1232 and 9-6C), which showed maltose fermentation capacity in the Durham test. On the other hand, the yeast strains CLI 190 (*H. guilliermondii*) and CLI 920 (*Candida stellata*) also showed the ability to hydrolyse maltose but were not able to finish the fermentation, with a consequent increase in residual sugars. Yeasts metabolise the wort sugar content in the following order, glucose, fructose, maltose and maltotriose, maltose being the most abundant in the wort. *Saccharomyces* yeasts are generally capable of fermenting glucose, fructose, maltose and some species can ferment maltotriose, but not all non-*Saccharomyces* species are able to consume the four sugars [63]. Additionally, the other strains analysed in this study, corresponding to the species *H. vineae*, *H. valbyensis*, *H. guilliermondii*, *C. stellata* and *M. pulcherrima*, have not been described as fermenters of maltose, as the Durham test showed, although *H. vineae* and *M. pulcherrima* are able to assimilate them [64–66]. Similarly, *T. delbrueckii* and *W. anomalus* have a variable ability to ferment and assimilate maltose [67,68]. This is relevant from a sensory point of view, as the incomplete fermentation of maltose can lead to sweet beers with more viscosity, contributing to the body and mouthfeel [54,69]. Finally, the four *L. thermotolerans* strains showed different fermentative behaviours, as they have variable maltose fermentative capacity [70]. Thus, strains CLI 996, CLI 1232 and 9-6C were able to complete fermentation, while strain CLI 894 was not.

The S-04 commercial strain showed the best performance regarding ethanol production (6.3% *v/v*). However, the strains studied were grouped into those that did not complete fermentation, thus leaving residual sugars, and those that did. The group characterised by ethanol content ranging from 5.7 to 3.9% *v/v* consisted of strains CLI 996,

CLI 1232 and 9-6C (belonging to *L. thermotolerans* species) and strain CLI 650 (*S. pombe*), while the remaining strains belonged to the group whose ethanol content was around 1% *v/v*. The *L. thermotolerans* species showed variability in maltose fermentation, depending on the study strain [12]. For this reason, only three of the four strains were able to produce alcohol levels similar to the control strain, while the remaining strain (CLI 894, 1.1% *v/v*) could be used for the production of low ethanol beer [71]. *S. pombe* has been described as having a high fermentative capacity, the alcohol obtained in the present study being 5% (*v/v*) [72]. The other strains tested (i.e., *H. vineae*, *H. valbyensis*, *H. guilliermondii*, *T. delbrueckii*, *W. anomalus*, *C. stellata* and *M. pulcherrima*) showed an ethanol production of around 1% (*v/v*), which makes them good candidates for low ethanol or mixed-culture brewing [6,8,15,62,73–76].

3.3. Aromatic Profiles in 100 mL Fermentations

The use of non-*Saccharomyces* yeasts opens up new possibilities to improve and create innovations in the sensory profile of beers [6]. This would meet the expectations of the modern consumer, satisfying consumer demand for beers with improved and differentiated flavours, as well as for products without chemical additives. Therefore, the 18 selected beers obtained in the 100 mL fermentations were analysed for their content of the main volatile compounds (Tables 3 and 4).

Higher alcohols are the most abundant organoleptic compounds present in beer and are generally regarded as desirable. Concentrations below 300 mg L^{-1} impart refreshing, floral and pleasant notes, as well as desirable warm character compounds, which add complexity to the beer [77]. The total higher alcohols produced by the different strains did not exceed this concentration in any instance, with the commercial strain producing the highest concentrations (119.55 mg L^{-1}). The two most important higher alcohols in beers are isobutanol and isoamyl alcohol, but no strain exceeded their sensory thresholds in any of the fermentations (200 mg L^{-1} and 70 mg L^{-1}, respectively) [78]. The results suggested that *Hanseniaspora* species (22.51 to 43.43 mg L^{-1}), *M. pulcherrima* (22.79 mg L^{-1}) and *W. anomalus* (30.01 to 45.67 mg L^{-1}) were poor/medium producers of higher alcohols compared to *S. cerevisiae* (119.55 mg L^{-1}), which has previously been described as a good producer in wine and beer fermentation [16,79,80]. The rest of the strains studied showed an average production (from 47.03 to 82.24 mg L^{-1}) compared to strain S-04, although the production of strains CLI 894 (*L. thermotolerans*), CLI 920 (*C. stellata*), CLI 902, CLI 918 and 7A-3A (*T. delbrueckii*) should be highlighted, as, despite not having finished fermentation, they produced similar levels to those strains that fermented glucose, fructose, and maltose sugars. This has been observed in other studies where non-conventional yeasts were used to produce low ethanol beer [8].

Esters, together with higher alcohols, are the most abundant families of aromatic compounds that can be found in beer. Esters play an important role in the fruity notes of beer [81]. They are formed during fermentation by yeast via an enzyme-catalysed reaction between acyl-CoA and higher alcohols. The results obtained showed important differences in the levels of some esters among the strains tested. According to these results, *L. thermotolerans* strains CLI 1232 and 9-6C had the highest ester content, mostly attributed to higher levels of ethyl lactate (butter and fruit aroma), which is a component found in sour beers [82]. Only some yeast strains exceeded the perception threshold for some esters, such as ethyl hexanoate (fruity "apple" flavour, 0.21 mg L^{-1}) and ethyl butyrate (fruity flavour, 0.4 mg L^{-1}) [78]. Ethyl hexanoate is an important flavouring compound, with levels above threshold in ale beers [77]; however, in our study, only five strains showed such values, corresponding to *L. thermotolerans* strains that finished the fermentation (CLI 996, CLI 1232, 9-6C) and two *T. delbrueckii* (CLI 902, CLI 918) strains. In contrast, the strains that exceeded the ethyl butyrate threshold were CLI 194 (*H. valbyensis*) and CLI 1232 (*L. thermotolerans*), in addition to the commercial strain. It should be noted that its production by strain CLI 1232 was three times higher than that of the commercial strain S-04. Ethyl butyrate was positively correlated with β-phenylethanol ($r = 0.65$, $p < 0.01$).

In terms of 2-phenylethyl acetate (rose, floral flavour), *Hanseniaspora* strains characteristically produce high levels, which could be observed in the study strains [83], though the values were quite small. Isoamyl acetate was only detected in five strains (CLI 512, CLI 650 and CLI 996, 9-6C and S-04), including the commercial strain although under the perception threshold (1.2 mg L^{-1}) [78]. Isoamyl acetate showed a non-direct correlation with its precursor alcohol (isoamyl alcohol). Ester production correlated positively with ethanol production ($r = 0.64$, $p < 0.01$), suggesting that ester production capacity is in some way linked to alcoholic fermentation capacity.

Most samples contained fatty acids, which, at high concentrations, can contribute to sour and salty flavours in beer, as well as to the presence of off-flavours, such as cheesy and sweaty [84]. However, only butyric acid exceeded the perception threshold (2 mg L^{-1}) [85] for some strains (CLI 194, CLI 225, CLI 894, CLI 902 and 6-5A). Based on direct smelling, however, no significant off-note was perceived. Hexanoic acid, octanoic acid and decanoic acid impart the so-called caprylic flavour which introduces unpleasant flavours to beer when they are above their threshold levels (8, 15 and 100 mg L^{-1}, respectively) [86]. Strains CLI 512 and CLI 894 were outstanding in terms of hexanoic acid production, but always below the perception threshold. The obtained levels of octanoic and decanoic acid were below those obtained by the control strain S-04, except for strain CLI 996, which produced similar levels of octanoic acid. Decanoic acid showed a positively correlation with β-phenylethanol ($r = 0.72$, $p < 0.01$). Differences in the fatty acids produced during fermentation by the different yeasts studied can be influenced by several factors, including the extent of intracellular retention, acylation and esterification or alcoholysis to form esters [87].

Regarding aldehyde and ketone production, different concentrations were found depending on the strain; however, none were above their threshold levels. Acetoin is formed from the reduction of diacetyl, being a less flavour-active compound with a high threshold (50 mg L^{-1}). *H. guilliermondii* (CLI 190, CLI 225, CLI 512), *H. vineae* (CLI 3), and the *L. thermotolerans strain* CLI 894, showed higher production than the commercial strain. On the other hand, *H. valbyensis* (CLI 194), *T. delbrueckii* (CLI 902, CLI 918, 7A-3A), *M. pulcherrima* (CLI 457), *S. pombe* (CLI 650), *C. stellata* (CLI 920) and the other *L. thermotolerans* strains (CLI 996, CLI 1232, 9-6C), produced lower (or null) concentrations than S-04. *Hanseniaspora* species are described as high acetoin producers, while *T. delbrueckii* is described as a low acetoin producer [88], which is consistent with the data obtained. γ- Butyrolactone imparts a sweet and worty flavour to beer. Some strains of the genus *Hanseniaspora* (CLI 3, CLI 194, CLI 225, CLI 512) produced the lowest concentrations of γ- Butyrolactone together with *C. stellata* (CLI 920) and the *T. delbrueckii* strain 7A-3A, compared to strain S-04. These results are contrary to those obtained in other wine studies [89]. *T. delbrueckii* and *L. thermotolerans* species, on the other hand, are described as major producers of γ- butyrolactone [90,91].

Non-conventional yeasts have great potential for flavour production, being outstanding in their phenolic profile [8]. In this case, guaiacol, with a very low threshold (3.88 ppb) and smoky flavour [92], was studied. Two *H. guilliermondii* strains (CLI 225 and CLI 512) were found to be the major producers, with concentrations five times higher than the S-04 strain.

Some species, such as *H. guilliermondii, L. thermotolerans, T. delbrueckii* and *W. anomalus* showed large variations in the aromatic profile produced by the different strains of the same species. This variation in aromatic profiles could have been due to specific mutations in the genomes, possibly because of adaptations to the environment from which these strains were isolated [93].

Table 3. The main volatile compounds (higher alcohols and esters) in the green beer produced by the different yeast strains (mg L^{-1}).

Yeast Strain	Higher Alcohols				Esters					
	Isobutanol	**Isoamyl Alcohol**	**1-Hexanol**	**β-Phenylethanol**	**Ethyl Butyrate**	**Ethyl Isovalerate**	**Isoamyl Acetate**	**Ethyl Hexanoate**	**Ethyl Lactate**	**2-Phenethyl Acetate**
CLI 3	4.06 ± 0.99 ^{gh}	19.34 ± 1.04 ^{efg}	nd	5.25 ± 0.45 ^{ghi}	0.32 ± 0.04 ^{bc}	0.03 ± 0.03 ^{c}	nd	0.10 ± 0.00 ^{c}	nd	nd
CLI 190	5.15 ± 1.67 ^{fgh}	20.75 ± 0.65 ^{efg}	1.20 ± 0.30 ^{a}	9.70 ± 0.70 ^{efghi}	0.01 ± 0.00 ^{f}	0.03 ± 0.03 ^{c}	nd	0.10 ± 0.04 ^{c}	nd	0.03 ± 0.01 ^{a}
CLI 194	5.99 ± 0.45 ^{efg}	16.44 ± 1.23 ^{fg}	nd	20.73 ± 1.55 ^{cd}	**0.47 ± 0.04 ^{b}**	0.06 ± 0.05 ^{a}	nd	0.01 ± 0.00 ^{c}	nd	nd
CLI 225	4.60 ± 2.40 ^{fgh}	13.64 ± 1.44 ^{fg}	1.20 ± 0.20 ^{a}	3.82 ± 0.82 ^{hi}	0.04 ± 0.00 ^{f}	0.03 ± 0.03 ^{bc}	nd	0.04 ± 0.01 ^{c}	nd	0.00 ±0.00 ^{c}
CLI 457	8.79 ± 0.09 ^{de}	13.39 ± 0.13 ^{fg}	nd	0.61 ± 0.01 ^{i}	nd	0.06 ± 0.00 ^{bc}	nd	0.10 ± 0.00 ^{c}	nd	nd
CLI 512	4.40 ± 0.04 ^{fgh}	19.28 ± 0.19 ^{efg}	nd	7.32 ± 0.07 ^{fghi}	0.03 ± 0.00 ^{f}	nd	0.04 ± 0.00 ^{c}	0.04 ± 0.00 ^{c}	nd	nd
CLI 650	8.02 ± 0.87 ^{def}	42.01 ± 2.51 ^{bcd}	nd	13.98 ± 0.98 ^{defg}	0.24 ± 0.05 ^{cde}	0.05 ± 0.05 ^{bc}	0.16 ± 0.00 ^{c}	0.18 ± 0.15 ^{bc}	nd	0.00 ± 0.00 ^{c}
CLI 894	10.44 ± 0.20 ^{cd}	35.69 ± 1.69 ^{bcde}	1.22 ± 0.22 ^{a}	14.46 ± 1.46 ^{defg}	0.11 ± 0.01 ^{def}	0.03 ± 0.03 ^{c}	nd	0.04 ± 0.00 ^{c}	nd	nd
CLI 902	4.27 ± 1.45 ^{gh}	46.10 ± 1.09 ^{abc}	nd	20.90 ± 0.48 ^{cd}	0.04 ± 0.01 ^{f}	0.04 ± 0.04 ^{bc}	nd	**0.34 ± 0.31 ^{abc}**	nd	0.00 ± 0.00 ^{c}
CLI 918	5.39 ± 0.05 ^{efgh}	40.83 ± 0.40 ^{bcd}	nd	18.43 ± 0.19 ^{cde}	nd	0.08 ± 0.00 ^{bc}	nd	**0.74 ± 0.01 ^{a}**	nd	0.00 ± 0.00 ^{bc}
CLI 920	22.56 ± 0.22 ^{a}	25.47 ± 0.25 ^{defg}	nd	3.52 ± 0.03 ^{hi}	nd	nd	nd	0.03 ± 0.00 ^{c}	nd	nd
CLI 921	1.86 ± 0.92 ^{h}	13.45 ± 1.46 ^{fg}	1.16 ± 0.26 ^{a}	5.81 ± 0.81 ^{ghi}	0.17 ± 0.07 ^{cdef}	0.04 ± 0.04^{bc}	nd	0.10 ± 0.00 ^{c}	nd	0.00 ± 0.00 ^{c}
CLI 996	10.04 ± 1.11 ^{cd}	36.07 ± 12.48 ^{bcde}	nd	27.07 ± 0.40 ^{bc}	0.29 ± 0.09 ^{bcd}	0.12 ± 0.02 ^{b}	0.39 ± 0.19 ^{a}	**0.60 ± 0.57 ^{ab}**	nd	0.01 ±0.00 ^{b}
CLI 1028	4.55 ± 0.56 ^{fgh}	24.33 ± 1.33 ^{defg}	nd	16.77 ± 1.77 ^{def}	0.06 ± 0.01 ^{ef}	0.03 ± 0.03 ^{bc}	nd	0.17 ± 0.10 ^{bc}	nd	nd
CLI 1232	15.59 ± 0.03 ^{b}	9.44 ± 0.56 ^{g}	nd	35.82 ± 1.82 ^{ab}	**1.22 ± 0.23 ^{a}**	0.06 ± 0.02 ^{bc}	nd	**0.23 ± 0.04 ^{bc}**	13.85 ± 1.15 ^{b}	nd
6-5A	3.54 ± 0.95 ^{gh}	15.86 ± 0.86 ^{fg}	nd	10.60 ± 0.60 ^{efgh}	nd	0.08 ± 0.00 ^{bc}	nd	0.14 ± 0.13 ^{bc}	nd	0.00 ± 0.00 ^{c}
9-6C	19.29 ± 1.10 ^{a}	53.67 ± 3.02 ^{ab}	nd	8.52 ± 0.54 ^{fghi}	nd	0.08 ± 0.02^{bc}	0.05 ± 0.00 ^{c}	**0.27 ± 0.07 ^{abc}**	20.51 ± 0.51 ^{a}	nd
7A-3A	1.85 ± 0.15 ^{h}	28.09 ± 1.97 ^{cdef}	1.20 ± 0.20 ^{a}	15.81 ± 0.81 ^{def}	0.01 ± 0.01 ^{f}	0.03 ± 0.03 ^{bc}	nd	0.02 ± 0.01 ^{c}	nd	0.00 ± 0.00 ^{c}
S-04	12.25 ± 2.60 ^{bc}	60.66 ± 18.16 ^{a}	1.45 ± 0.03 ^{a}	44.72 ± 10.75 ^{a}	**0.45 ± 0.11 ^{b}**	nd	0.39 ± 0.19 ^{b}	0.06 ± 0.04 ^{c}	0.58 ± 0.83 ^{c}	nd

Data are means ± standard deviations of three replicates. Compounds above their threshold levels are marked in bold. Data with different superscript letters within each column are significantly different (Tukey tests: $p < 0.05$); nd: not detected.

Table 4. The main volatile compounds (fatty acids, aldehydes/ketones and guaiacol) in the green beer produced by the different yeast strains.

Yeast Strain	Fatty Acids				Aldehydes/Ketones				Guaiacol
	Butyric Acid	Hexanoic Acid	Octanoic Acid	Decanoic Acid	Acetoin	Benzaldehyde	Phenyl Acetaldehyde	γ- Butyrolactone	
CLI 3	0.01 ± 0.00 ^{c}	0.18 ± 0.08 ^{d}	0.02 ± 0.00 ^{b}	0.01 ± 0.00 ^{b}	8.24 ± 0.74 ^{ab}	0.04 ± 0.01 ^{cd}	0.01 ± 0.00 ^{b}	nd	nd
CLI 190	nd	0.25 ± 0.04 ^{cd}	0.02 ± 0.02 ^{b}	0.01 ± 0.01 ^{b}	10.03 ± 1.03 ^{a}	0.57 ± 0.07 ^{a}	0.03 ± 0.01 ^{a}	3.75 ± 2.25 ^{ab}	**0.10 ± 0.01 ^{cd}**
CLI 194	**3.89 ± 0.29 ^{ab}**	0.16 ± 0.01 ^{d}	0.06 ± 0.00 ^{b}	0.02 ± 0.00 ^{b}	nd	nd	nd	0.58 ± 0.04 ^{fgh}	**0.07 ± 0.01 ^{de}**
CLI 225	**4.67 ± 0.67 ^{a}**	0.31 ± 0.06 ^{cd}	0.03 ± 0.03 ^{b}	0.01 ± 0.00 ^{b}	7.27 ± 1.07 ^{bc}	0.57 ± 0.07 ^{a}	0.00 ± 0.00 ^{c}	0.36 ± 0.06 ^{gh}	**0.36 ± 0.06 ^{a}**
CLI 457	0.05 ± 0.00 ^{c}	0.24 ± 0.00 ^{cd}	0.04 ± 0.00 ^{b}	0.01 ± 0.00 ^{b}	3.59 ± 0.04 ^{ef}	nd	nd	2.64 ± 0.03 ^{bcd}	nd
CLI 512	nd	2.61 ± 0.02 ^{a}	nd	nd	9.43 ± 0.09 ^{a}	nd	nd	0.12 ± 0.00 ^{h}	**0.32 ± 0.00 ^{a}**
CLI 650	0.03 ± 0.03 ^{c}	0.65 ± 0.01 ^{bcd}	0.10 ± 0.10 ^{b}	0.02 ± 0.02 ^{b}	1.49 ± 0.49 ^{hi}	0.10 ± 0.00 ^{bc}	nd	1.81 ± 0.81 ^{cdef}	**0.02 ± 0.02 ^{e}**
CLI 894	**4.99 ± 0.99 ^{a}**	1.01 ± 0.21 ^{b}	0.02 ± 0.02 ^{b}	0.01 ± 0.01 ^{b}	8.83 ± 0.83 ^{ab}	nd	0.00 ± 0.00 ^{c}	1.81 ± 0.81 ^{cdef}	**0.16 ± 0.06 ^{bc}**
CLI 902	**2.46 ± 2.46 ^{b}**	0.61 ± 0.14 ^{bcd}	0.02 ± 0.02 ^{b}	0.02 ± 0.02 ^{b}	5.66 ± 0.66 ^{cd}	nd	nd	2.06 ± 0.56 ^{cde}	**0.01 ± 0.00 ^{e}**
CLI 918	0.05 ± 0.00 ^{c}	0.31 ± 0.00 ^{cd}	0.02 ± 0.00 ^{b}	0.01 ± 0.00 ^{b}	5.22 ± 0.05 ^{de}	nd	nd	2.65 ± 0.03 ^{bcd}	nd
CLI 920	nd	0.15 ± 0.00 ^{d}	0.02 ± 0.00 ^{b}	0.01 ± 0.00 ^{b}	1.52 ± 0.02 ^{ghi}	nd	nd	nd	nd
CLI 921	0.03 ± 0.03 ^{c}	0.28 ± 0.16 ^{cd}	0.08 ± 0.08 ^{b}	0.03 ± 0.03 ^{b}	4.03 ± 1.03 ^{def}	0.13 ± 0.03 ^{b}	0.01 ± 0.00 ^{b}	1.21 ± 0.21 ^{efgh}	**0.19 ± 0.04 ^{b}**
CLI 996	0.04 ± 0.04 ^{c}	0.73 ± 0.40 ^{bc}	1.05 ± 0.23 ^{a}	0.04 ± 0.04 ^{b}	5.91 ± 0.35 ^{cd}	0.01 ± 0.01 ^{d}	nd	2.06 ± 0.56 ^{cde}	**0.03 ± 0.01 ^{de}**
CLI 1028	0.02 ± 0.02 ^{c}	0.37 ± 0.07 ^{cd}	0.03 ± 0.03 ^{b}	0.01 ± 0.01 ^{b}	nd	0.11 ± 0.01 ^{bc}	0.00 ± 0.00 ^{c}	1.64 ± 0.98 ^{defg}	**0.02 ± 0.01 ^{de}**
CLI 1232	nd	0.60 ± 0.10 ^{bcd}	0.05 ± 0.05 ^{b}	0.07 ± 0.07 ^{b}	nd	0.13 ± 0.03 ^{b}	nd	3.13 ± 0.63 ^{abc}	nd
6-5A	0.06 ± 0.00 ^{c}	0.26 ± 0.11 ^{cd}	0.06 ± 0.00 ^{b}	0.07 ± 0.00 ^{b}	2.74 ± 1.24 ^{fgh}	nd	nd	4.18 ± 0.18 ^{a}	nd
9-6C	**3.78 ± 0.78 ^{ab}**	0.61 ± 0.17 ^{bcd}	0.03 ± 0.03 ^{b}	0.01 ± 0.01 ^{b}	nd	nd	nd	1.81 ± 0.81 ^{cdef}	nd
7A-3A	0.03 ± 0.03 ^{c}	0.21 ± 0.09 ^{d}	0.03 ± 0.03 ^{b}	0.01 ± 0.01 ^{b}	3.53 ± 0.54 ^{efg}	nd	nd	nd	**0.04 ± 0.02 ^{de}**
S-04	nd	0.62 ± 0.39 ^{bcd}	1.08 ± 0.54 ^{a}	0.31 ± 0.07 ^{a}	5.42 ± 0.98 ^{cde}	nd	0.00 ± 0.00 ^{c}	0.57 ± 0.01 ^{fgh}	**0.06 ± 0.05 ^{de}**

Data are means ± standard deviations of three replicates. Compounds above their threshold levels are marked in bold. Data with different superscript letters within each column are significantly different (Tukey tests: $p < 0.05$); nd: not detected.

3.4. Melatonin Production

Figure 1 shows the melatonin content of the 18 different green beers obtained in 100 mL. Melatonin is a sleep-regulating hormone that can modulate circadian and seasonal rhythms, has antioxidant properties and can be produced by yeasts during beer fermentation [17]. The values obtained ranged between 6.69 and 102.98 ng mL^{-1}, the highest concentration being found for the strain 9-6C (*L. thermotolerans*), whereas the lowest was observed for the CLI 921 (*H. guilliermondii*) strain, in contrast to the *S. cerevisiae* commercial yeast strain (S-04) with 25.32 ng mL^{-1}. Melatonin was not detected in three strains (CLI 190, CLI 1232 and 7A-3A), which may have been due to its isolation origins and/or its use, and, therefore, to the different mechanisms of adaptation to the fermentation environment that different strains possess [94,95]. In these studies, with lager beers, melatonin was analysed with ELISA methods, and the concentrations found were lower than in our study. In relation to other foods analysed by liquid chromatography (e.g., bread, tomato, black tea), the concentrations found were higher than average [38]. It is also worth noting that the melatonin concentrations obtained for the non-*Saccharomyces* yeasts were higher than those obtained in previous studies with *Saccharomyces* yeasts isolated from the same environments [96]. Administration of exogenous melatonin can change body rhythms, including sleep, core body temperature, endogenous melatonin and cortisol [97]. Doses of melatonin that can influence these changes in the body range from 0.5 to 10 mg [98]. In our study, the maximum melatonin level found was 102.98 ng mL^{-1}, which is lower than the level that can cause an effect on the body. However, beer is not the only food that can provide exogenous melatonin to the body; foods such as bread, coffee and tomatoes also provide melatonin. Thus, moderate consumption of beer, together with a balanced diet, can increase the daily intake of this compound. For this reason beers are considered to be functional foods, since, with moderate consumption, they can provide health benefits, such as vitamins, minerals, and polyphenols, as well as melatonin [6].

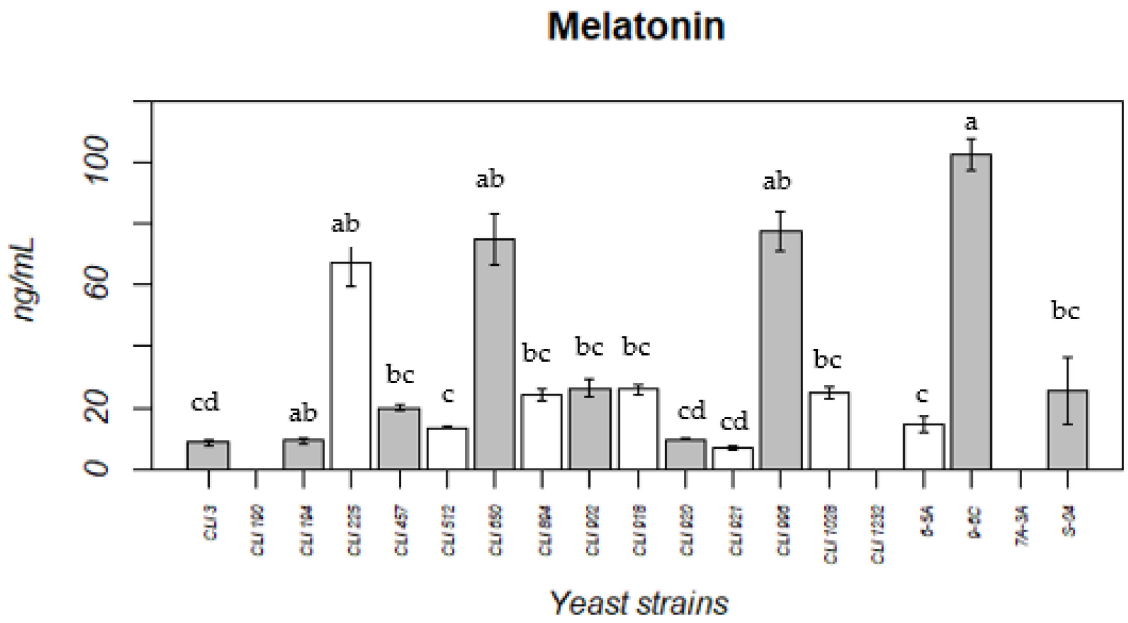

Figure 1. Melatonin content in the 18 selected yeast strains and S-04. Each value is the mean of three replicates expressed in ng mL^{-1}. Data with different superscript letters are significantly different (Tukey tests: $p < 0.05$).

3.5. Statistical Analysis

A multivariate analysis was conducted to determine which strains and species clustered together according to the different volatile compounds, as well as to the different parameters analysed in the 100 mL beers (Figures 2 and 3).

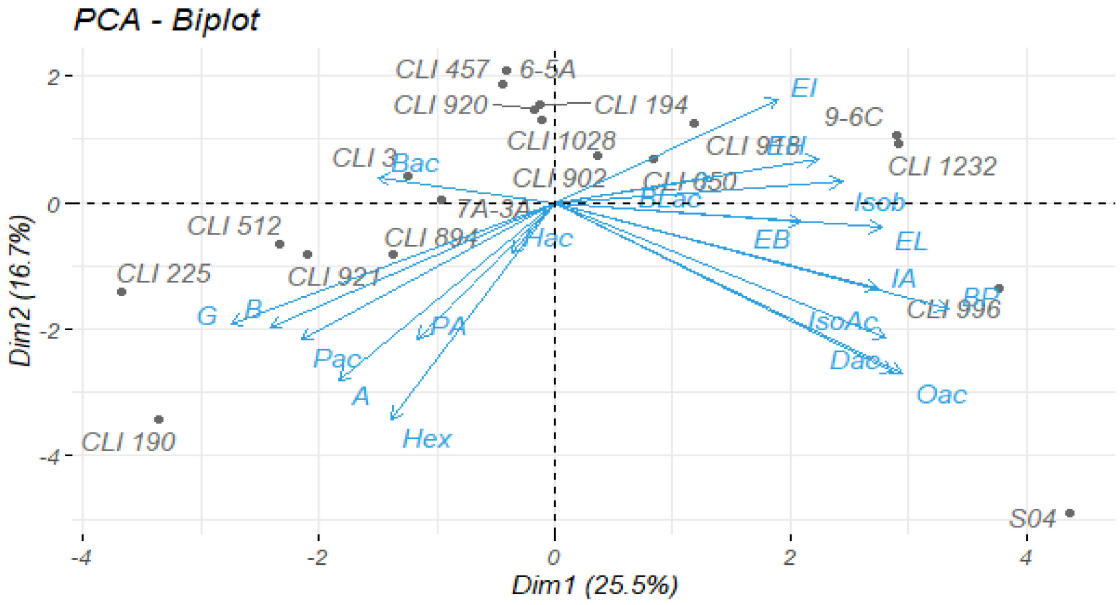

Figure 2. Projection of the beers on the axes formed by the principal components 1 and 2. Each object is the average of the three corresponding experimental beers. Isob, isobutanol; IA, isoamyl alcohol; Hex, 1-hexanol; BP, β-phenylethanol; EB, ethyl butyrate; EI, ethyl isovalerate; IsoAc, isoamyl acetate; EH, ethyl hexanoate; EL, ethyl lactate; PA, 2-phenethyl acetate; Bac, butyric acid; Hac, hexanoic acid; Oac, octanoic acid; Dac, decanoic acid; A, acetoin; B, benzaldehyde; Pac, phenyl acetaldehyde; BLac, γ-butyrolactone; G, guaiacol.

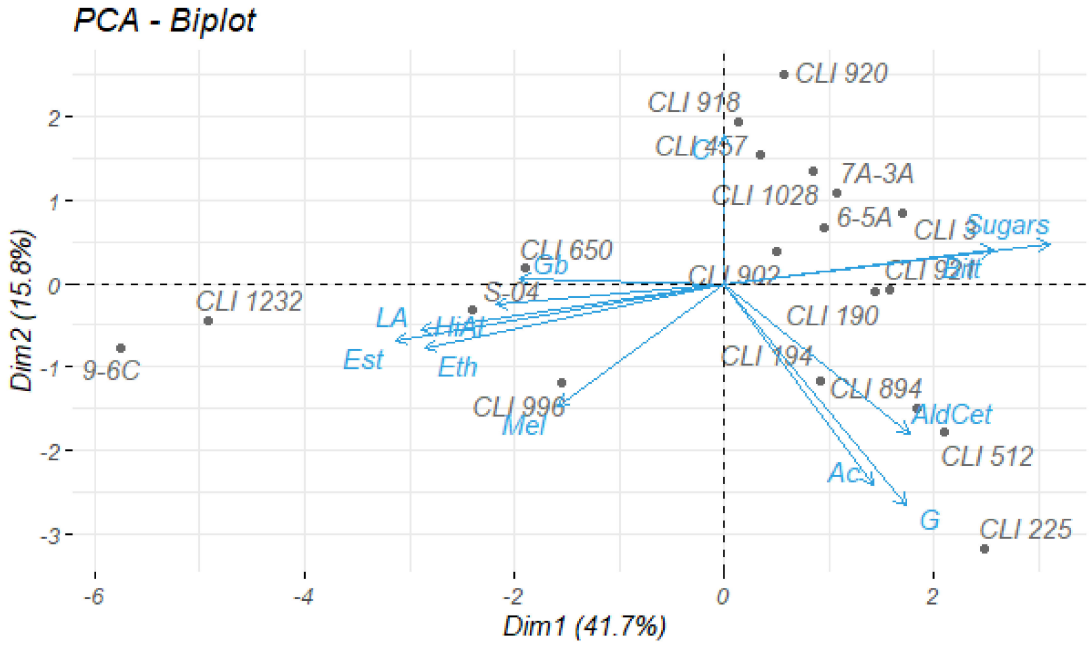

Figure 3. Projection of the beers on the axes formed by the principal components 1 and 2. Each object is the average of the three corresponding experimental beers. Eth, ethanol; LA, lactic acid; Bitt, bitterness; C, colour; HiAl, higher alcohols; Est, esters; Ac, acids; AldCet, aldehydes/ketones; Gb, γ-butyrolactone; G, guaiacol; Mel, melatonin.

Regarding volatile compounds (Figure 2), principal components (PC) 1 and 2 (Dim1 and Dim2) explained 42.2% of the system variance. The strains of *H. guilliermondii* species were grouped into negative values of components 1 and 2, which were associated with aldehydes/ketones (benzaldehyde, phenyl acetaldehyde, acetoin), guaiacol, 2-phenethyl acetate and 1-hexanol. On the other hand, *T. delbrueckii*, *W. anomalus*, *C. stellata*, *M. pulcherrima*, *H. valbyensis*, *H. vineae*, and *S. pombe* strains were situated on the positive side of component 2, near to butyric acid, ethyl lactate, ethyl hexanoate, γ- butyrolactone and isobutanol volatile compounds. Only two strains of *L. thermotolerans* (CLI 1232 and 9-6C) were found together on the positive side of components 1 and 2, while the other two strains were found on the negative side (CLI 996 and CLI 894). This could have been due to the fermentation kinetics of each strain and their different aromatic compound production. Finally, the commercial strain S-04 did not show a clear trend and did not cluster closely to any group of compounds, probably influenced by its fermentation performance and by belonging to the *Saccharomyces* species.

Figure 3 shows the scatterplot of the beers obtained by the strains with the main parameters (biplot). PC1 explained up to 41.7% of the total variance and PC2 explained another 15.8%. The strains that completed fermentation were found in the negative values of components 1 and 2 and were therefore associated with the production of ethanol, lactic acid, higher alcohols, esters and melatonin. Esters were positively correlated with lactic acid ($r = 0.88$, $p < 0.01$) and ethanol ($r = 0.64$, $p < 0.01$), and negatively with sugars ($r = 0.71$, $p < 0.01$) and bitterness ($r = 0.77$, $p < 0.01$). *Hanseniaspora* species, except *H. vineae*, were projected on the positive side of component 1, reflecting their high content of acids, guaiacol and aldehydes/ketones.

3.6. Fermentation Kinetics in 1 L

The second part of the screening was performed by scaling up the fermentation process. Firstly, based on the results obtained, 13 strains were evaluated in 1 L and evaluated by sensory analysis. Those species that completed fermentation (CLI 650, CLI 996, CLI 1232, 9-6C), with high production of compounds such as lactic acid (CLI 1232, 9-6C), as well as those with outstanding aromatic and/or melatonin production (CLI 3, CLI 190, CLI 225, CLI 457, CLI 650, CLI 918, CLI 921, CLI 996, CLI 1028, CLI 1232, 6-5A, 9-6C, 7A-3A) compared to the reference *Saccharomyces* strain, were initially selected. The parameters of the fermentation kinetics are reported in Figure 4.

The strains showed similar fermentation kinetics as for 100 mL fermentation. All the strains studied, with the exception of CLI 996, CLI 1232 and 9-6C belonging to the species *L. thermotolerans*, CLI 650 (*S. pombe*) and the commercial strain S-04 (*S. cerevisiae*), showed weak fermentation kinetics, with a fermentation rate ranging between 2.6 and 14.8 g CO_2 lost per 900 mL, in agreement with other studies [11,62,71,76]. Therefore, they could be promising yeasts to produce low-alcohol beer. As expected, the best fermentation performance corresponded to the commercial *S. cerevisiae* strain; however, it also corresponded to strain CLI 650 (*S. pombe*), since, despite being less vigorous initially, the fermentation process ultimately reached similar levels in terms of CO_2 loss as S-04. *L. thermotolerans* (CLI 996, CLI 1232, 9-6C) showed the best results for non-*Saccharomyces* yeasts. However, their fermentative capacity was below S-04.

3.7. Sensory Analysis of Beers

Figure 5A–C represents a spider graph employing key descriptors considered in the sensory tests for the 13 beers obtained in 1 L fermentation.

H. vineae, *T. delbrueckii*, *L. thermotolerans* and *W. anomalus* strains (CLI 3, CLI 918, 7A-3A, CLI 1232 and 9-6C) showed marked fruity/ester notes, while for the rest of the strains these were poorly pronounced. *Lachancea* strains CLI 1232 and 6-5A were notable for their fruity apple cider notes, while the remainder showed fruity aromas in general. *H. vineae*, *Torulaspora* and *Wickerhamomyces* species were characterised by fruity flavours [5,10,99].

Almost all strains showed the presence of phenolic aromas in the sensory analysis, with the strains CLI 918, CLI 921, CLI 1028, 6-5A and 9-6C, as well as the control strain S-04, having a more marked profile. However, the phenolic aroma was integrated with the other aromas, without standing out above them. The phenolic aroma (phenolic off-flavours, POFs) is associated with a clove-like aroma, which is an essential flavour of some wheat and blond beer styles and is therefore considered to be a positive characteristic [100].

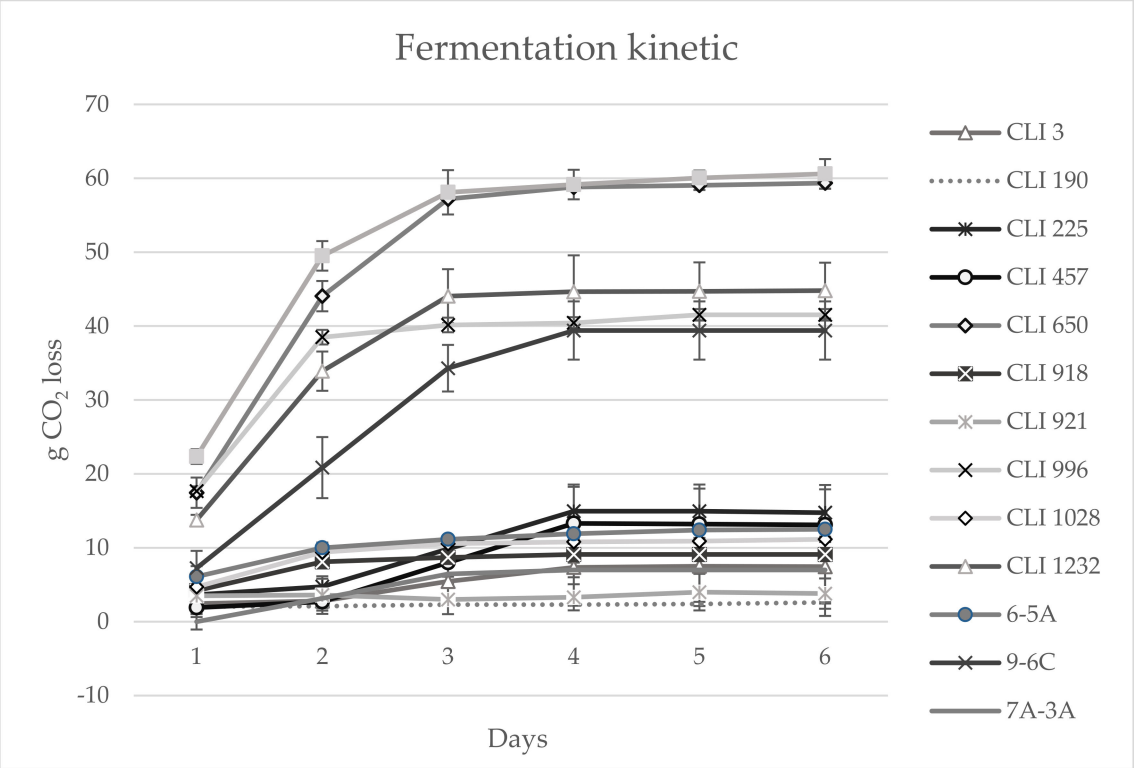

Figure 4. Fermentation kinetics in 1 L fermentation for 13 studied strains. Each value is the mean of three trials expressed in g CO_2 lost per day.

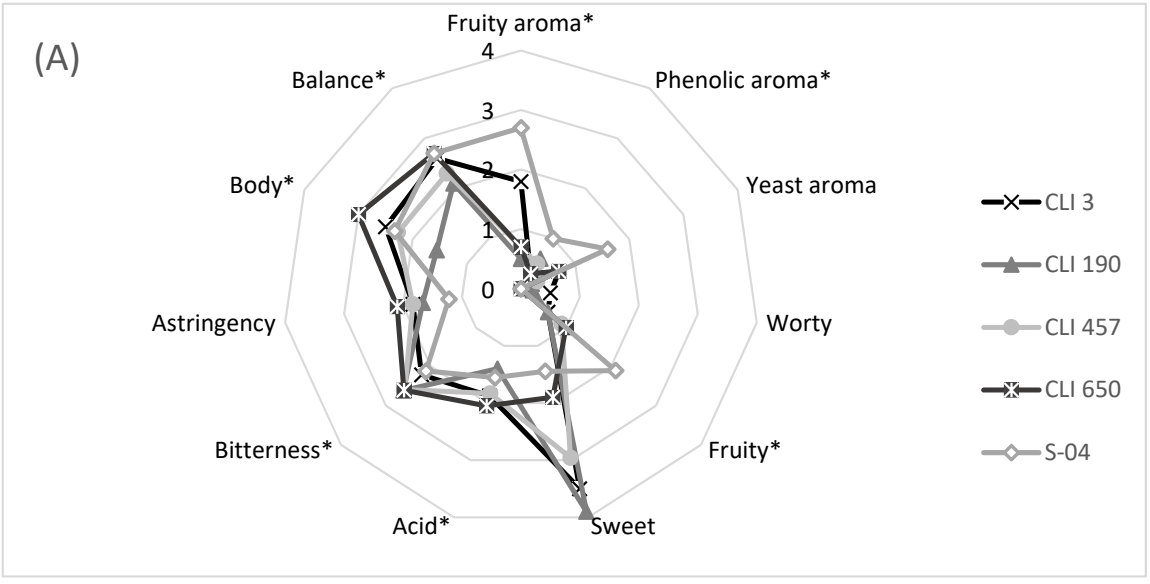

Figure 5. *Cont.*

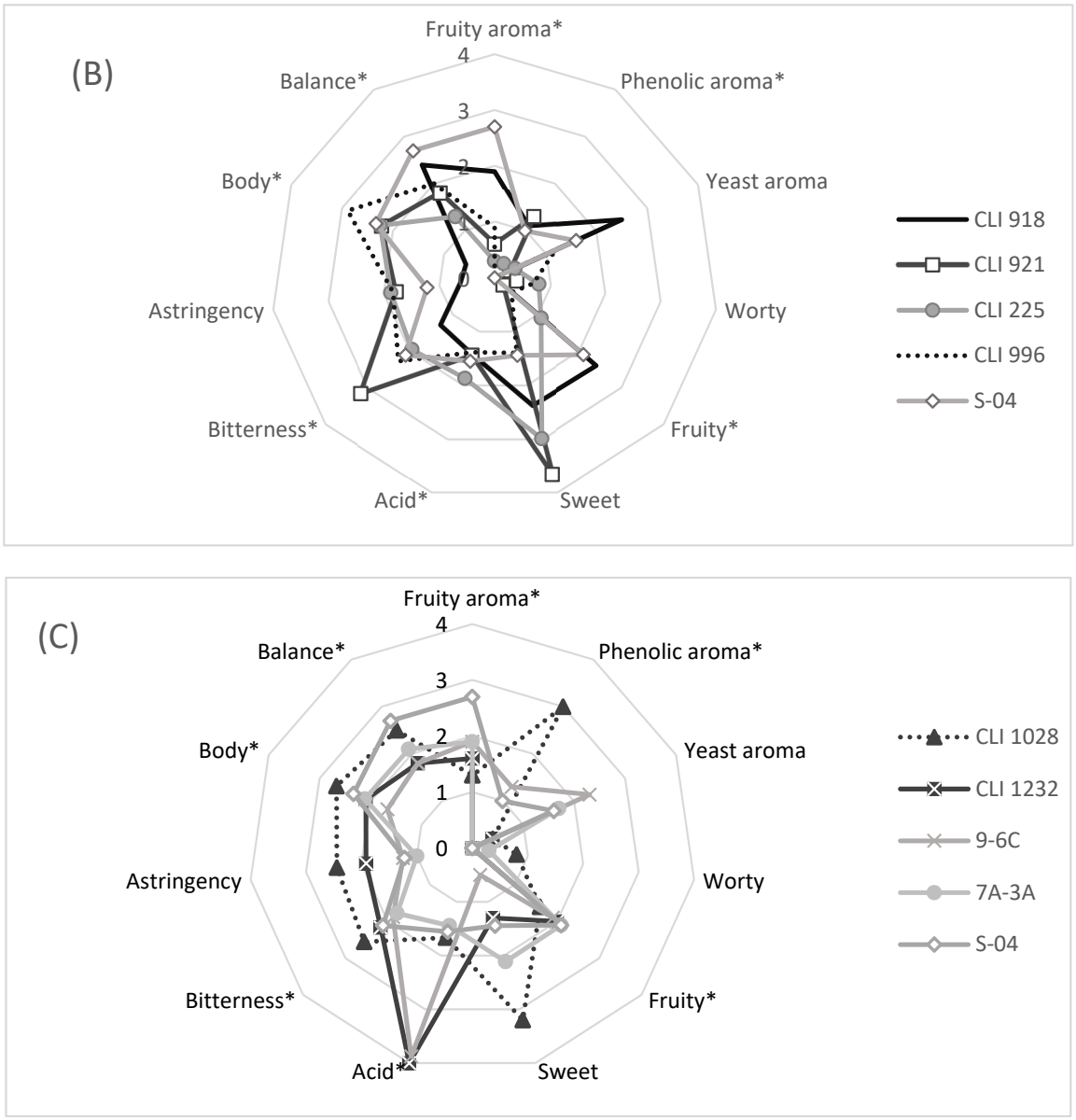

Figure 5. Sensory analysis of the 13 beers studied. Aroma attributes: fruity aroma, phenolic aroma, yeast aroma; flavour attributes: worty, fruity, sweet, acid, bitter; overall attributes: astringency, body, balance. * Significantly different (ANOVA; $p < 0.05$). (**A**) sensory analysis for strains CLI 3, CLI 190, CLI 457, CLI 650, S-04; (**B**) CLI 918, CLI 921, CLI 225, CLI 996, S-04; (**C**) sensory analysis for strains CLI 1028, CLI 1232, 9-6C, 7A-3A y S-04.

The beers that showed a sweet flavour profile, as expected, were those that did not finish fermentation. This observation has been confirmed by different authors, whereby those yeasts with a low or null capacity to ferment maltose tend to increase the sensory sweetness in the beer, due to its high residual maltose content [72]. Despite this, these beers were not assessed negatively, as they did not have any worty aromas or flavours during tasting.

The aromatic profiles obtained in beers fermented with *Hanseniaspora* and *Wickerhamomyces* species appeared similar, with the predominant descriptor being sweetness. As has been found in other studies [15], this is a common characteristic of low alcohol beers obtained by maltose-negative yeasts; they also tend to stand out for their wort and cereal aromas, although this was not observed in our study.

4. Conclusions

One of the key factors for successful innovation in beer production is yeast selection. The use of non-*Saccharomyces* yeasts can enable the production of beers with distinctive characteristics, and at the same time, satisfy new consumer trends. Non-*Saccharomyces* yeasts isolated from agricultural and viticultural production in Madrid can be used in beer fermentation, but for different purposes, mainly due to their fermentative capacity and sensory brewing potential. Although the original wort was similar in all the fermentation experiments conducted, the beers produced with *H. vineae*, *H. guilliermondii*, *H. valbyensis*, *S. pombe*, *L. thermotolerans*, *T. delbrueckii*, *C. stellata* and *M. pulcherrima* species showed markedly different fermentation performances and sensory profiles, even within the species. Wort fermented with *Hanseniaspora* spp. (CLI 3, CLI 190, CLI 194, CLI 225, CLI 512, CLI 921), *T. delbrueckii* (CLI 902, CLI 918, 7A-3A), *C. stellata* (CLI 920), *M. pulcherrima* (CLI 457) and *L. thermotolerans* (CLI 894) yeast strains resulted in increased residual maltose sugar concentrations and contributed to enhanced sweetness in the beer. As these strains were not able to ferment maltose, the alcohol levels produced did not exceed 1.4% *v/v*, which is why they could be considered for brewing low ethanol beer. They could also be used in co-fermentations with a *Saccharomyces* yeast, since *H. vineae, T. delbrueckii* and *W. anomalus* species showed more fruity notes, thus enhancing the organoleptic characteristics of the beers. Further studies would therefore be needed to develop these beers. On the other hand, strains CLI 1232 and 9-6C (*L. thermotolerans*) could be used for production of sour beer due to the derived lactic acid content. The non-*Saccharomyces* yeasts studied showed higher melatonin production than *Saccharomyces* yeasts, making them good candidates to obtain potentially functional beers.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflict of interest.

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