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Biomodulation of Physicochemical Parameters, Aromas, and Sensory Profile of Craft Beers by Using Non-*Saccharomyces* Yeasts

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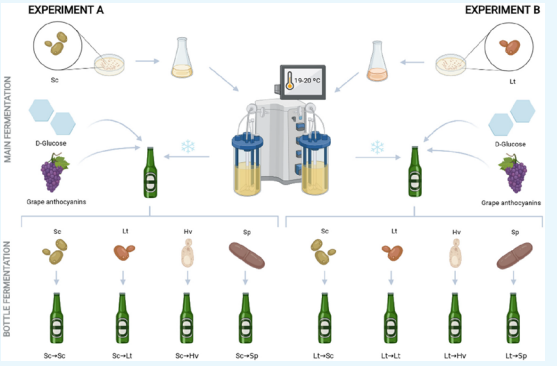
ABSTRACT: Beer is an alcoholic beverage produced by the metabolism of yeasts and made from water, malt, and hops. In recent years, the interest in craft beers has increased considerably due to the demand for new beverages and the consumer's willingness to pay higher prices. This article explores the sensorial changes produced in craft beers by using different *Saccharomyces* and non-*Saccharomyces* yeasts with several instrumental and sensory analyses performed. After a primary fermentation process with *Saccharomyces cerevisiae* or *Lachancea thermotolerans*, it was observed that green beer brewed with *L. thermotolerans* had a lower pH (3.41) due to the significant production of L-lactic acid (3.98 g/L) compared to that brewed with *S. cerevisiae*. Following, the bottle conditioning was carried out with a culture of *S. cerevisiae*, *L. thermotolerans*, *Hanseniaspora vineae*, or *Schizosaccharomyces pombe*. Of note is the increased production of aromatic esters, including 2-phenylethyl acetate in the *H. vineae* conditioning, which is associated with a high aromatic quality, as well as ethyl lactate in all samples, whose main fermentation was carried out with *L. thermotolerans*. Although this research is at an early stage, future complementary studies may shed more light on this topic.



1. INTRODUCTION

Beer is an alcoholic beverage fermented from four basic ingredients: water, malt (usually barley), hops, and yeast,[1] plus other ingredients specific to each brewmaster and geographical area.[2] The increased volume of beer production in Europe is accompanied by a wide range of varieties, due to the richness and traditions of beer culture in each country.[3] This diversity creates an additional value for consumers who demand the existence of new beers such as radlers and nonalcohol and low alcohol beers (NABLAB).[4] In fact, consumption of craft beers has increased due to consumers' willingness to pay higher prices for a high-value product.[3] To boost this sector, one of the most interesting biotechnological strategies is the use of new yeast species, from non-*Saccharomyces* genera. They are able to generate desirable metabolites in beers, and with diverse fermentative capabilities, which can facilitate the production of beers with no or low alcohol content.[5,6]

Up to 99% of beer produced worldwide is made using *Saccharomyces* spp. yeasts as the sole inoculum isolate. Meanwhile, the use of non-*Saccharomyces* yeasts has traditionally been linked to spontaneous fermentations.[7] The exclusive use of *Saccharomyces* spp. for decades is based on three fundamental characteristics such as their efficiency to produce ethanol, the use of fermentation as the main metabolic pathway, favored by the Crabtree effect, and finally, their tolerance to environmental stress caused by ethanol (cell-toxic compound) or other metabolites.[8,9] The added value of brewing beers with non-*Saccharomyces* yeasts lies in the good and different fermentative performances, but also in the generation of aromatic and taste compounds through their metabolisms.[10,11] The yeasts employed in this research were *Saccharomyces cerevisiae*, *Lachancea thermotolerans*, *Hanseniaspora vineae*, and *Schizosaccharomyces pombe*.

S. cerevisiae is a globular yeast and is widely used in food fermentation (bread, wine, beer) thanks to its ability to ferment both monosaccharides (glucose and fructose), disaccharides (sucrose, galactose, mannose, maltose), and trisaccharides (raffinose).[12] Its fermentative power is between 12 and 18% v/v ethanol, reaching the maximum alcoholic strength in wines. In the case of the nitrogen source necessary for its growth, it uses urea, ammonium, and amino acids, while as micronutrients it needs phosphate and biotin, among others. In addition to ethanol, the volatile compounds generated include higher alcohols and esters.[13,14]

L. thermotolerans is a globular yeast and similar in size to *S. cerevisiae* (~7 μm). Its fermentative power is medium and stands at 10% v/v ethanol.[15] It is characterized by its ability to ferment sugars such as glucose, fructose, and galactose and is also variably

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able to metabolize maltose.[16] Its fermentative metabolism of sugars leads to the production of L-lactic acid, reaching concentrations of up to 16 g/L, which gives a sour taste.[17] It is positioned as a yeast suitable to produce beers in a single fermentation step and without the use of lactic acid bacteria (LAB).[18] Its volatile acidity is low (<0.5 g/L), so it is used to control acetic acid levels in sequential inoculations with *S. cerevisiae* or other non-*Saccharomyces* species.[19] It produces controlled levels of acetaldehyde and higher alcohols, while it is characterized by a high production of both glycerol, giving it osmophilic characteristics, and aromatic esters such as 2-phenylethyl acetate and ethyl lactate.[19,20]

H. vineae is an apiculate yeast.[21] It is characterized by its medium fermentative power, reaching up to 9% v/v ethanol, and for this purpose the carbon sources it uses are glucose and fructose, being unable to assimilate other sugars such as maltose,[22] so it may be unable to complete alcoholic fermentation on its own. For this reason, it is not usually used for the main fermentation in the brewing process, but rather in bottle conditioning, giving high levels of attenuation after 2 weeks.[18] It is noted for its positive aromatic contribution through the production of fruity and floral volatile compounds[23] such as 2-phenylethyl acetate and benzyl acetate.[24]

S. pombe is a rod-shaped yeast with dimensions of 3−4 μm in diameter and 7−20 μm in length.[14] It has a high fermentative power reaching up to 10−13% v/v under anaerobic conditions.[25,26] However, its growth rate is low due to its high vitamin requirement.[27] As a carbon source it is able to use glucose, fructose, sucrose, maltose, and even raffinose and glycerol.[28] The generation of higher concentrations of pyruvate as an intermediate product highlights its oenological interest in red wine, as it favors the formation of vitisin A, by condensation of pyruvate and anthocyanins.[29] Finally, it is worth mentioning its favorable impact in terms of food safety, on the one hand, because it has low assimilable nitrogen requirements compared to *S. cerevisiae*, which minimizes the formation of biogenic amines[27] and, on the other hand, because of the reduction of urea content and, consequently, of ethylcarbamate through its urease activity.[25]

Beer is a complex beverage composed mainly of ethanol, CO_2, glycerol, and carbohydrates not fermentable by yeasts, in a ratio of more than 1 g/L. Its complexity lies in more than 800 organic compounds produced by yeasts, most of which are involved in the aromas and flavor of beer (higher alcohols, organic acids, esters, aldehydes, ketones, and sulfur compounds).[5] However, a number of factors are involved in the aromatic quality of this alcoholic beverage: ingredients such as hop variety, malt roasting, and wort boiling, the yeast's own secondary metabolism during fermentation, microbiological contamination as well as beer storage conditions (exposure to light and oxygen).[30,31] Yeasts use sugars, nitrogen compounds, and sulfur compounds for the synthesis of components for their growth, that is, amino acids, proteins, lipids, or nucleic acids among others. Aromatic compounds are a catabolic product of metabolizing the must, among which we can find aliphatic and aromatic alcohols, esters, aldehydes, organic acids, carbonyl compounds and terpenic substances. Non-*Saccharomyces* yeasts are characterized by a shift in metabolism toward the production of secondary metabolites as opposed to the biomass and ethanol production of the classical *Saccharomyces* spp.[10]

At last, anthocyanins have been added previous second fermentation in bottle to change the color of beer. Anthocyanins are phenolic compounds, belonging to the flavonoid type, which have the following rings: benzopyrillium, flavilium cation (B) and pyrillium cation. The color of wines depends both on the pH and on the hydroxylation or methoxylation patterns of the B-ring, which is responsible for the absorption of the visible spectrum.[32,33] In the wine fermentation process, anthocyanins are transformed into derived pigments, called pyranoanthocyanins, which are more stable with respect to color, pH variations, or SO_2 bleaching, as they increase the resonant forms due to the double pyrilium ring.[34,35] The formation of pyranoanthocyaninins is a consequence of a condensation reactions between the anthocyanins themselves or with metabolites generated during yeast fermentation. The first is a completely chemical reaction, whereby condensation occurs between hydroxycinnamic acids and anthocyanin molecules.[36] Whereas the second strategy occurs through the intervention of the enzyme hydroxycinnamate decarboxylase (HCDC) for the transformation of hydroxycinnamic acids into vinylphenol adducts,[37] which are highly reactive and will condense with the anthocyanins to generate vinylphenolic-pyranoanthocyanins.[38]

The general objective of this project is to modulate the sensory profile of craft beers thanks to biotechnology, that is, using non-*Saccharomyces* yeast species. In particular, the aim is to (i) obtain beers with specific characteristics according to the type of yeast used, being sour with *L. thermotolerans*, aromatic with *H. vineae*, and with a high alcoholic rate with *S. pombe*; (ii) compare the evolution of sensorial characteristics after bottle conditioning for up to 8 weeks from two green craft fermented beers; and finally, (iii) study the effect of natural coloring agents (anthocyanins) from red grape skins on the beer.

2. MATERIALS AND METHODS

2.1. Malt: Milling and Characterization. The cereal used for brewing the beers was Pilsen malt (MD Mouterij Dingemans NV. Stabroek, Belgium). A sample of 5500 g was milled using a two-roll hand mill (Brouwland, Belgium), which was set with six turns of the screw. Of the total malt milled, 500 g was used to characterize the degree of milling of the grain using a Plasfinter, four sieves of different pore diameters (ø = 3 mm > 1 mm > 0.50 mm > 0.3 mm) and a balance for weighing the different flour fractions. Meanwhile, the rest of the ground malt was used for brewing wort.

2.2. Wort Brewing: Malt Mashing, Mash Filtering, and Wort Boiling. The malt mashing phase was carried out in three stages in order to maintain maximum enzyme activity. The first stage at 52 °C for 10 min (protein rest) favors the release of proteases for the degradation of the amino acids that make up the proteins (45−55 °C) and, consequently, facilitates the development of yeasts during fermentation. The second stage was carried out at 62 °C for 45 min (maltose release rest), involving dextrinases (60−63 °C at pH 5.4−5.5) for the degradation of high molecular weight starch into fermentable sugars and also β-amylases (60−65 °C at pH 5.0−5.4) that act on the nonreducing ends of starch resulting in the release of glucose, maltose, and maltotriose. Finally, the third stage was carried out at 72 °C for 15 min (saccharification rest) and involves α-amylases (67−75 °C at pH 5.2−5.5) that favor the release of small dextrins by attacking 1−4 bonds inside the starch chains. The pH and density were determined at 20 °C after each maceration stage and before continuing with the next one, in order to verify that the parameters obtained are correct. At the end of the last stage, it was checked if there were still intact starch chains by means of the iodine test; if the sample turns blue, the last maceration stage should be prolonged before continuing.

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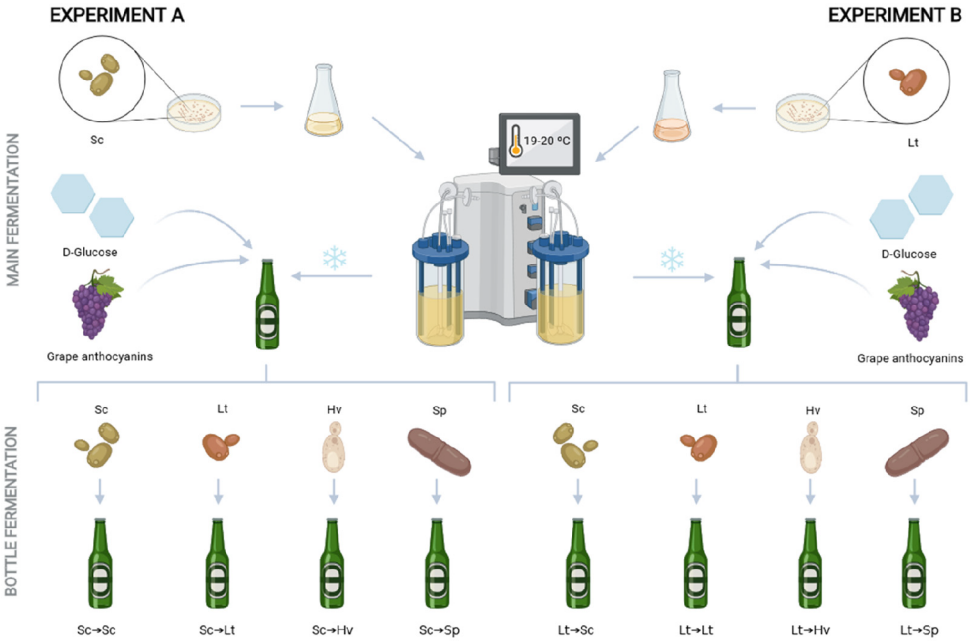

Figure 1. Experimental design of craft beers brewery with different *Saccharomyces* and non-*Saccharomyces yeasts*: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

The lautering, recirculation, and washing of the mash wort took place in the tank with the filter bed. It was necessary to use 12 L of tap water dechlorinated at 80 °C. As for the wort boiling phase (90 min), *Nugget* hop pellets (with high bitterness and medium/high myrcene oil content that brings out a hint of wood) were added at different times and amounts (6 g at 0 min, 12.5 g at 30 min, and 6.5 g at 60 min of boiling). In the last 15 min of the vigorous boiling, *Irish moss*, a coagulant from a moss/algae that grows abundantly on the Irish coast, was added in dehydrated form for protein aggregation to facilitate protein separation in the beer wort. Finally, the beer wort was cooled in a coil through which cold tap water is recirculated to produce heat exchange and reduce the temperature to a range suitable for yeast inoculation.

2.3. Density and pH Determinations. Two density meters (Proton, Barcelona, Spain) were used to determine the density in the beer wort. The range of the density meters was 1000−1050 kg/m^3 and 1050−1100 kg/m^3, and both were calibrated at 20 °C. The pH of the different samples was measured with a Crison micropH 2000 pH meter (Hach Lange, Barcelona, Spain) at 20 °C.

2.4. Saccharomyces and non-Saccharomyces Yeasts. The yeasts used in this project are part of the own culture collection of microorganisms of the Department of Chemistry and Food Technology of the Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas (ETSIAAB) of the Universidad Politécnica de Madrid (UPM, Spain):

- *Saccharomyces cerevisiae* (7VA) belongs to the yeast collection of the Department of Chemistry and Food Technology (ETSIAAB) of the UPM. In this manuscript it is referred to by the abbreviation Sc.
- *Lachancea thermotolerans* (L3.1) was isolated from the Ribera del Duero region (Spain) by the EnotecUPM group of the Department of Chemistry and Food Technology of the UPM (Spain). In this manuscript it is referred to by the abbreviation Lt.
- *Hanseniaspora vineae* was isolated by Prof. Francisco Carrau (Faculty of Chemistry, University of the Republic, Montevideo, Uruguay) and is currently under evaluation by "Oenobrands SAS, France". In this manuscript it is referred to by the abbreviation Hv.
- *Schizosaccharomyces pombe 938* belongs to the yeast collection of the Instituto de Fermentaciones Industriales (IFI, Spain). In this manuscript it is referred to by the abbreviation Sp.

2.5. Yeast Culture. The solid culture medium used was YPD-agar. It contains 1% yeast extract (Condalab, Madrid, Spain), 2% peptone (Condalab, Madrid, Spain), 2% pure anhydrous glucose (PanReac, Barcelona, Spain), and 1.7% agar (Condalab, Madrid, Spain). Incubation of the yeast seeded Petri dishes was carried out at 26 °C in an oven (J.P Selecta, Barcelona, Spain). Colony forming units (CFU/mL) were counted by preparing serial dilutions in sterile distilled water and plating 10^{-5} and 10^{-7} dilutions on YPD-agar plates. In all cases the cell count was around 8-log CFU/mL.

For biomass growth of the different yeasts, a YPD liquid culture was prepared. It also contains 1% yeast extract (Condalab, Madrid, Spain), 2% peptone (Condalab, Madrid, Spain), and 2% pure anhydrous glucose (PanReac, Barcelona, Spain). Two passages were performed prior to inoculation of the beer wort, the first in glass tubes with a volume of 5−10 mL of medium and the second in Erlenmeyer flask with 40% YPD medium. The amount of yeast inoculated at the different stages of the process corresponded to 2% of the final volume. The glass tubes with YPD medium were incubated at 26 °C for 24 h in a static incubator (J.P. Selecta, Barcelona, Spain), while the cultures in Erlenmeyer flasks, were incubated at 26 °C in an incubator with orbital shaking at 115 rpm (New Brunswick Innova 40/40R, Eppendorf, Barcelona, Spain) for 48 h.

2.6. Experimental Design. The following trials were designed and carried out in parallel (Figure 1). In experiment A, a main fermentation of 7 L of beer wort was carried out in a fermentation tank (Brew Bucket 13 L, Ss Brewtech, USA) being

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inoculated with a 2% pure culture of *S. cerevisiae*, while in experiment B, performed under the same conditions, the wort was fermented with a pure culture of *L. thermotolerans*. Each fermentation tank was equipped with a glycerol-filled muller valve (Panreac, Barcelona, Spain) and had a FTSs system (Ss Brewtech, USA) to control and maintain the temperature. The parameters monitored during the main fermentation were pH, concentration of ethanol, glycerol, and reducing sugars (glucose/fructose) and the process was stopped when pH and ethanol stabilized for two consecutive days.

After finishing these alcoholic fermentations and clarification at 4 °C for 5 days, the second fermentation, known as cellaring, conditioning, or bottle aging, was carried out. For this process, 245 mL of clarified beer wort was transferred to each 250 mL bottle, and each sample was inoculated with 2−3% pure culture of *S. cerevisiae*, *L. thermotolerans*, *H. vineae*, or *S. pombe*. In addition, 0.03% of anthocyanins from red grape skins was added as a natural coloring agent (E-163, powdered dye from red grapes obtained by extraction, then dehydrated by atomization (IC: EV 11.5−12.5). Secna, Valencia, Spain), to provide color, and 7 g/L of pure anhydrous glucose (Panreac, Barcelona, Madrid), to promote the start of fermentation. Incubation was carried out at 20 °C for 4 and 8 weeks for all samples in triplicate.

2.7. Instrumental Analysis. All beers were filtered using a 0.45 μM filter (Teknokroma, Barcelona, Spain) and stored at 4 °C until analytical assays were performed.

2.7.1. Enzyme Multianalyzer. A Y25 Biosystems enzyme multianalyzer (Biosystems, Barcelona, Spain) was used to determine the concentration of glucose/fructose and L-lactic acid during different fermentation times. The Food Quality-Enology enzyme kits for glucose/fructose and L-lactic acid (Biosystems, Barcelona, Spain) and the enzyme multianalyzer mentioned above were used for this purpose.[39]

2.7.2. High Performance Liquid Chromatography with Refractive Index Detector. HPLC 1200 chromatography equipment equipped with a refractive index detector (RID) (Agilent Technologies, Santa Clara, CA, USA) was used for the determination of glycerol and ethanol content. The temperature of the column and the RID detector were maintained at 35 °C during the entire chromatographic analysis, and the separation was performed in isocratic mode. Samples were placed in 1.5 mL Kimble 5.1 borosilicate chromatographic vials with a PTFE/silicone septum. In the case of glycerol, it was used with an Ascentis Expres 90 Å HILIC reverse phase column (15 cm × 4.6 mm; particle size 2.7 μm) (Supelco, Darmstadt, Germany). The eluent used was 99.8% pure acetonitrile for HPLC (Scharlau, Sentmenat, Spain) with deionized water (Milli-Q) in a 95:5 ratio. The flow rate of the system was 0.4 mL/min at a maximum pressure of 600 bar. Chromatographic peaks were integrated according to an external calibration performed from aqueous solutions with 99% pure glycerol (Panreac, Barcelona, Spain) of known concentrations: 1 g/L, 2.5 g/L, 5 g/L, 7.5 g/L, and 10 g/L, with an R^2 of 0.998. For ethanol, analyses were performed using a Phenosphere XDB C18 reverse phase column (4.6 mm × 150 mm; 5 μm particle size) (Phenomenex, Torrance, CA, USA). The solvent was a 50:50 v/v solution of deionized water (Milli-Q) and methanol (Panreac, Barcelona, Spain), injected at a flow rate of 0.8 mL/min and a maximum pressure of 600 bar. Calibration for the chromatographic peak integration was performed using known concentrations of 99.0% pure ethanol (Panreac, Barcelona, Spain): 5, 7.5, 10, 15, and 20% v/v, with R^2 in the range 0.984−0.998 since the calibration was repeated each time the samples were analyzed in the apparatus.[40]

2.7.3. High Performance Liquid Chromatography with Diode Array Detector. For the determination of added anthocyanins and derived pigments formed during bottle fermentation of the beers, HPLC 1200 chromatography equipment (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector (DAD) and a Kinetex C18 reverse phase column (4.6 mm × 100 mm; particle size 2.6 μm) (Phenomenex, Torrance, CA, USA) was used. The temperature of the column and DAD detector were maintained at 35 °C throughout the chromatographic analysis. The solvents used for sample elution were deionized water (Milli-Q)/formic acid (Panreac, Barcelona, Spain), 95:5 v/v (solvent A) and methanol 99.9% purity (Panreac, Barcelona, Spain)/formic acid, 95:5 v/v (solvent B). The gradient was as follows: 80% solvent A−20% solvent B from 0 to 6 min; 50% solvent A−50% solvent B from 6 to 11 min, and 80% solvent A−20% solvent B from 11 to 12 min. The elution flow rate was 0.4 mL/min at a maximum pressure of 600 bar. Detection was performed in the range 500−600 nm, and the quantification of anthocyanins was performed using external standards at 525 nm for the following compounds: delphinidin-3-glucoside (D3G), cyanidin-3-glucoside (C3G), petunidin-3-glucoside (Pt3G), peonidin-3-glucoside (P3G), malvidin-3-glucoside (M3G), malvidin-3-glucoside-acetylated (M3G-Ac), malvidin-3-glucoside-coumarilated (M3G-Cu), and vinylphenols.[41]

2.7.4. UV−Visible Spectrophotometry. The color parameters to monitor the added anthocyanins and derived pigments produced during bottle conditioning of the beers were determined using an Agilent 8453 UV−vis spectrophotometer (Agilent Technologies S.L., Madrid, Spain) and a 1 mm optical cuvette. The total polyphenol index (TPI) was determined from the absorbance at 280 nm, the color intensity as the sum of the absorbances at 420, 520, and 620 nm and the tonality as the ratio between the absorbance at 420 and 520 nm.[41]

2.7.5. Gas Chromatography with Flame Ionization Detector (GC-FID). Agilent Technologies 6850 gas chromatography equipment equipped with an integrated flame ionization detector (Hewlett-Packard, Palo Alto, CA, USA) and a DB-624 column (60 m × 0.250 mm, 1.40 μm) was used to determine the concentration of volatile compounds. The injector temperature was 250 °C, and the temperature detector was 300 °C, whereas, the column temperature was set at 40 °C for the first 5 min, then linearly increased by 10 °C per minute until the final temperature of 250 °C was reached and finally maintained for 5 min. Hydrogen produced from a generator (LNI Schmidlin SA, Geneva, Switzerland) was used as carrier gas. A flow rate of 2.2 mL/min was used, the split injection ratio was 1:10, and the limit of detection was 0.1 mg/L. The following external standards were used for calibration (Fluka, Sigma-Aldrich Corp, Buchs, Switzerland): acetaldehyde, methanol, 1-propanol, diacetyl, ethyl acetate, 2-butanol, isobutyl alcohol, 1-butanol, acetoin, 2-methyl-1 butanol, 3-methyl-1-butanol, isobutyl acetate, ethyl butyrate, ethyl lactate, 2,3-butanediol, 3-ethoxy-1-propanol, isoamyl acetate, hexanol, 2-phenylethanol, and 2-phenylethyl acetate. To the analyzed samples, 50 mg/L 4-methyl-2-pentanol (Fluka, Sigma-Aldrich Corp., Buchs, Switzerland) was added as internal standard. The samples were placed in 1.5 mL Kimble 5.1 borosilicate chromatographic vials with a PTFE/silicone septum. Automatic injection of 1 μL of sample into the GC-FID equipment was performed in triplicate for each beer sample.[42]

The different volatile compounds obtained were grouped into various categories in order to facilitate the discussion of the data,

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highlighting those components that enhance the sensory profile of the beer brewed.[23] The different categories considered are higher alcohols (1-propanol, 2-butanol, isobutyl alcohol, 1-butanol, 3-methyl-butanol, 2-methyl-butanol, and 2-phenylethyl alcohol[30,43]), esters (ethyl acetate, isobutyl acetate, ethyl butyrate, ethyl lactate, isoamyl acetate, 2-phenylethyl acetate[30,44]), and carboniyl compounds (diacetyl, acetoin[10,43]). Besides total volatiles have been considered to refer to the sum of all volatile compounds determined by GC-FIC and indicate the ability to produce secondary metabolites during fermentation (Table S3).

2.8. Sensory Analysis. The two sensory analyses were carried out according to ISO 6564:1985[45] and ISO 4121:2003[46] with a panel of trained tasters, who belong to the Department of Chemistry and Food Technology of the Universidad Politécnica de Madrid. A total of eight experimental beers were evaluated by nine panelists (five women and four men) for the first tasting (bottle conditioning after 4 weeks) and by eight panelists (four women and four men) for the second tasting (bottle storage after 8 weeks). The beers (25−30 mL/tasting glass) were served at 8 ± 2 °C in standard odorless tasting glasses. The panelists evaluated a total of 24 attributes (12 attributes per tasting) divided between visual, olfactory, and gustatory, as well as aftertaste and overall perception on a scale of intensity from low to high (score from 0 to 5).

2.9. Statistical Analysis. The results in this work were obtained from triplicate samples which allowed the mean and standard deviations of the samples to be calculated. The treatment of the data to study significant differences was carried out by analysis of variance (ANOVA), using the least significant difference (LSD) test. Statgraphics 18-X64 software (Graphics Software System, Rockville, MD, USA) was used for data processing. The significance level was set at $p < 0.05$. In addition, in order to study the similarities and differences between the results obtained from the instrumental and sensory analysis, a correlation test with Pearson's statistic was performed using the XLSTAT software (Addinsoft, Paris, France). This software made it possible to establish positive and negative correlations (+1/−1) between the different results observed.

3. RESULTS

3.1. Yield of Milled Malt. The Pilsen malt was milled and weighed to calculate the mean, standard deviation, and percentage of the process yield. The results in Table S1 show that the fine (>0.5 mm) and medium (<1 mm) grain fractions were less than 5%, while the coarse (>1 mm) fraction obtained was more than 90%. Moreover, the general yield of this process is 99.68%.

3.2. Beer Wort Yield: Temperature, pH, and Density. The different parameters determined (temperature, pH, and density) in the brewing of the beer wort are shown in Table S2. After completion of the mashing and boiling of the beer wort, the pH and density values obtained were 5.75 and 1066. These were close to the optimum range of 5.2−5.7 pH and approximately 1060 kg/m^3 density. According to the manual "Bier brouwen voor begginers" (Brouwland, Beverlo, Belgium), the alcoholic strength of the beer wort was estimated from the density values of 5.9−7.9% v/v ethanol at the end of mashing, 5.0−6.6% v/v ethanol before boiling, and 6.5−8.7% v/v ethanol after boiling.

3.3. Main Fermentation. The main fermentation of 7 L of beer wort was carried out in each fermentation tank. The inoculated yeast population was ∼log 10^8 CFU/mL for *S. cerevisiae* and *L. thermotolerans*, and the fermentation was carried out for 7 days at a constant temperature of 19−20 °C using the FTSs system (Brew Bucket, Ss BrewTech, USA). The parameters monitored daily were pH, consumption of reducing sugars (glucose/fructose), and metabolites of ethanol and glycerol. Figure 2 shows the evolution of pH over the 7 days of

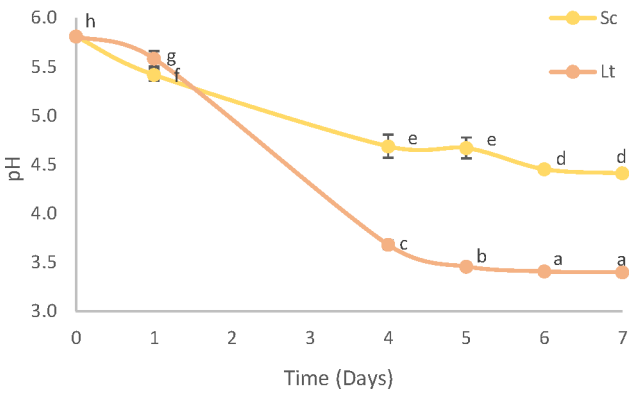

Figure 2. pH monitoring during fermentation by pH meter. Values represent the average ± standard deviation (n = 3) and significance level alpha = 0.05. The yellow line corresponds to *S. cerevisiae* (Sc) and the orange line to *L. thermotolerans* (Lt). In the ANOVA, the different letters indicate significant differences for the set of samples.

fermentation. The pH of beer fermented with *S. cerevisiae* decreased from 5.81 to 4.4. However, there was a marked drop in pH in *L. thermotolerans* to values of 3.4. Next, the consumption of reducing sugars is shown in Figure 3. The concentration of the

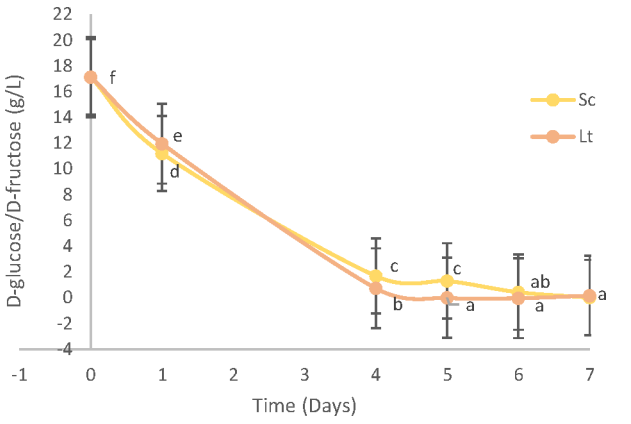

Figure 3. Evolution of reducing sugar content (g/L) during main fermentation using a Y25 enzymatic multianalyzer and a kit to quantify glucose/fructose. Values represent the average ± standard deviation (n = 3) and significance level alpha = 0.05. The yellow line corresponds to *S. cerevisiae* (Sc) and the orange line to *L. thermotolerans* (Lt). In the ANOVA the different letters indicate significant differences for the set of samples.

initial glucose/fructose mixture (17.13 g/L, not counting disaccharides and trisaccharides) decreased progressively to values close to zero from day 4−5 of fermentation for both yeasts.

The evolution of ethanol (% v/v) and glycerol (g/L) concentrations, the production of which developed in parallel for both yeasts, is plotted (Figure 4). The growth was abrupt from day 0 to 4, and then slowed down until day 7. The final amounts of ethanol were estimated at 5.57 and 5.45% v/v for *S. cerevisiae* and *L. thermotolerans*, respectively. As for glycerol

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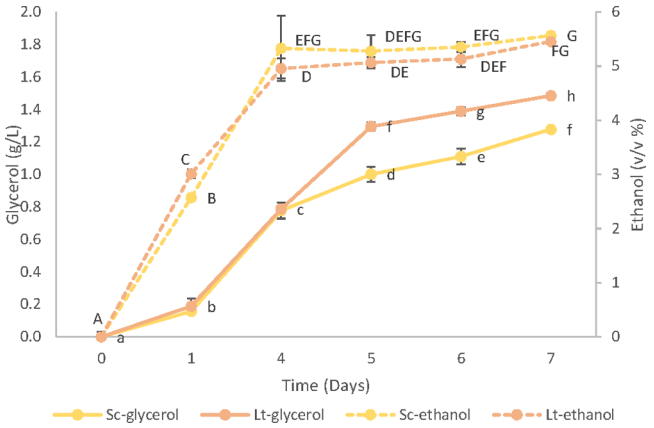

Figure 4. Evolution of glycerol (g/L) and ethanol (% v/v) during primary fermentation using HPLC-RID equipment. Values represent the average ± standard deviation (n = 3) and significance level alpha = 0.05. The solid line corresponds to glycerol and the dashed line to ethanol: *S. cerevisiae* (Sc) in yellow and *L. thermotolerans* (Lt) in orange. The different letters in the ANOVA indicate significant differences for the set of samples for each parameter analyzed.

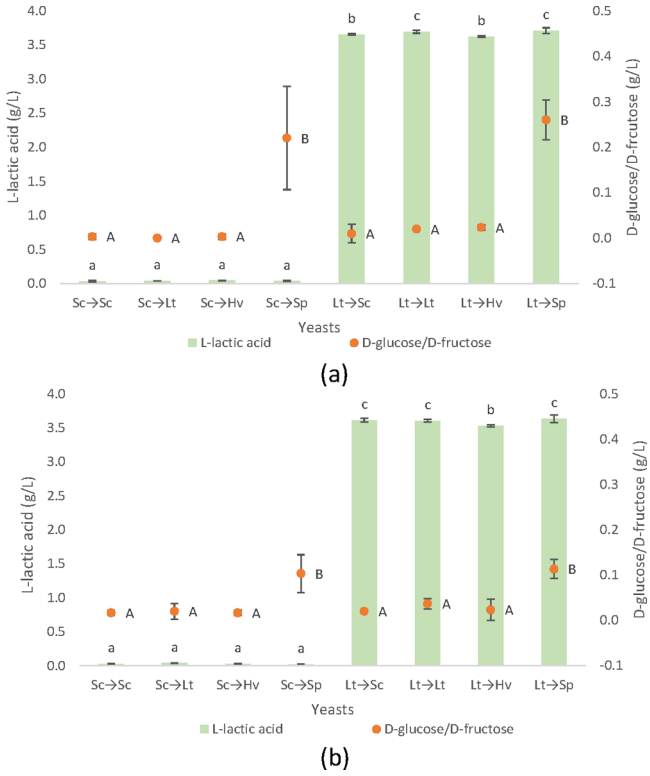

Figure 5. Determination of glucose/fructose and L-lactic acid by enzymatic multianalyzer: (A) 4 weeks of secondary fermentation; (B) 8 weeks of secondary fermentation. Values represent mean ± standard deviation (n = 3). Analyses of variance (ANOVA) were performed independently for each of the weeks. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

production, it was constant for both yeasts until day 4, thereafter the production of this metabolite slightly increased for *L. thermotolerans* compared to *S. cerevisiae*. The final glycerol concentration was 1.28 g/L and 1.48 g/L for *Saccharomyces* and *non-Saccharomyces* yeasts, respectively.

3.4. Evolution of Bottle Conditioning. *3.4.1. Reducing Sugars.* For the second fermentation, an extra 7 g/L of anhydrous glucose was added to encourage yeast implantation in the green beer. As it is shown in Figure 5a after 4 weeks of fermentation in the bottle, the concentration of reducing sugars in the samples with *S. cerevisiae*, *L. thermotolerans*, and *H. vineae* dropped below 0.1 g/L. However, in the case of beers inoculated with *S. pombe* (Sc→Sp; Lt→Sp), the glucose/fructose concentration remained around 0.2−0.26 g/L. After 8 weeks of bottle fermentation (Figure 5b) no noticeable changes in the concentration of reducing sugars were observed except for the glucose/fructose concentration in the samples with *S. pombe* which decreased by half.

3.4.2. L-Lactic Acid. In the case of lactic acid, after the main fermentation, the concentration of this metabolite remained close to zero in the tank containing *S. cerevisiae* (0.04 ± 0.01 g/L), while it increased to 3.98 ± 0.08 g/L for *L. thermotolerans* (Table 1). During secondary fermentation in the bottle, no changes in L-lactic acid concentrations were observed in the beers that had been inoculated with *S. cerevisiae* (experiment A) in the main fermentation, despite the fact that *L. thermotolerans* was also inoculated in the bottle fermentation. Only a subtle decrease in L-lactic acid concentration was perceived for all samples that were initially fermented with *L. thermotolerans* (experiment B). After 8 weeks of bottle fermentation, concentrations between 3.53 and 3.63 g/L of this organic acid were reached, the minimum value of which corresponds to Lt→Hv (Figure 5b).

3.4.3. pH/L-Lactic Acid. There is a relationship between pH and the concentration of L-lactic acid produced by the yeast. Figure 6 clearly shows the decrease of pH in the samples that have been fermented mainly with *L. thermotolerans* (experiment B).

3.4.4. Ethanol Content. The ethanol production, resulting from the alcoholic fermentation, was determined using HPLC-RID equipment. The main results are shown in Table 2 and Figure 7. In general, during secondary fermentation in the bottle, there was an increase in alcoholic strength ranging from 0.2 to 3 alcoholic strength. While after 4 weeks of bottle fermentation the sample with Lt→ Lt only increased the ethanol concentration to 5.68% v/v ethanol, the sample with Lt→Hv experienced a slight decrease in alcoholic strength. In addition, four of the beers reached between 6.57 and 6.74% v/v ethanol (Sc→ Sc; Sc→Lt; Sc→ Hv; Lt→Sc). Most relevant, the fermentation performed with *S. pombe* allowed reaching an alcoholic strength of 8.49 and 8.85% v/v ethanol for Sc→Sp and Lt→Sp, respectively.

3.4.5. Glycerol Content. The results obtained for glycerol production are shown in Table 2 and Figure 8. The glycerol concentration (g/L) increases after completing 4 weeks in

Table 1. Consumption of Reducing Sugars (Glucose/Fructose) and Production of L-Lactic Acid during Primary Fermentation[a]

	yeasts	glucose/fructose (g/L)	L-lactic acid (g/L)
wort beer		17.03 ± 0.64	0.03 ± 0.01
main fermentation	Sc (expt A)	0.01 ± 0.02[a]	0.04 ± 0.01[A]
	Lt (expt B)	0.01 ± 0.01[a]	3.98 ± 0.08[B]

[a]Values represent the mean ± standard deviation (n = 3). Analyses of variance (ANOVA) were performed independently for each of the fermentations. The different letters in the ANOVA indicate significant differences for the set of samples for each parameter analyzed. Yeasts: *S. cerevisiae* (Sc) and *L. thermotolerans* (Lt).

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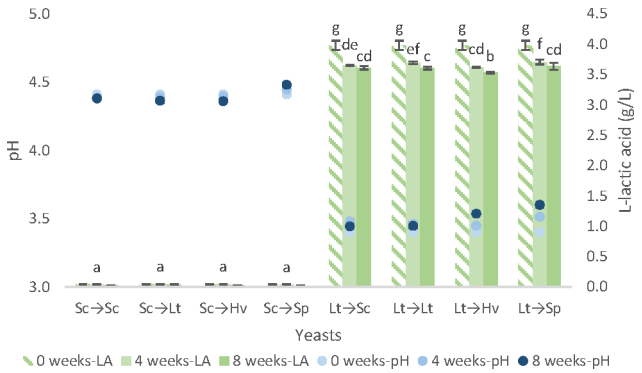

Figure 6. Relationship between pH and lactic acid accumulation throughout the fermentations carried out: end of main fermentation (0 weeks), secondary fermentation (4 and 8 weeks). Values represent the average ± standard deviation (n = 3). The different letters in the ANOVA indicate significant differences for the set of samples. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

Table 2. Determination of Ethanol Content (% v/v) and Glycerol Content by HPLC-RID[a]

yeasts	ethanol (% v/v)	glycerol (g/L)
	Main Fermentation	
Sc (expt A)	5.35 ± 0.29^{a}	1.28 ± 0.05^{A}
Lt (expt B)	5.45 ± 0.15^{a}	1.48 ± 0.02^{B}
	Secondary Fermentation (4 Weeks)	
Sc→Sc	6.74 ± 0.06^{b}	1.92 ± 0.05^{AB}
Sc→Lt	6.64 ± 0.01^{b}	1.88 ± 0.06^{A}
Sc→Hv	6.74 ± 0.16^{b}	1.85 ± 0.06^{A}
Sc→Sp	8.49 ± 0.22^{c}	2.06 ± 0.06^{BC}
Lt→Sc	6.57 ± 0.11^{b}	2.57 ± 0.05^{E}
Lt→Lt	5.68 ± 0.55^{a}	2.16 ± 0.10^{CD}
Lt→Hv	5.31 ± 0.20^{a}	2.25 ± 0.02^{D}
Lt→Sp	8.85 ± 0.18^{c}	2.71 ± 0.17^{E}
	Secondary Fermentation (8 Weeks)	
Sc→Sc	6.16 ± 0.42^{b}	1.93 ± 0.06^{BC}
Sc→Lt	6.66 ± 0.14^{b}	1.75 ± 0.11^{A}
Sc→Hv	6.39 ± 0.18^{b}	2.04 ± 0.11^{B}
Sc→Sp	8.36 ± 0.13^{c}	2.02 ± 0.04^{B}
Lt→Sc	6.16 ± 0.26^{b}	2.85 ± 0.11^{D}
Lt→Lt	5.20 ± 0.35^{a}	2.74 ± 0.07^{CD}
Lt→Hv	5.25 ± 0.76^{a}	2.68 ± 0.10^{C}
Lt→Sp	8.02 ± 0.16^{c}	2.86 ± 0.10^{D}

[a]Values represent the average ± standard deviation (n = 3). Analyses of variance (ANOVA) were performed independently for each of the fermentations. The different letters in the ANOVA indicate significant differences for the set of samples. Yeasts: *S. cerevisiae* (Sc), L. *thermotolerans* (Lt), *H. vineae* (Hv) and *S. pombe* (Sp).

secondary fermentation, being higher for beers whose main fermentation was carried out with *L. thermotolerans* (experiment B). However, between 4 and 8 weeks of bottle fermentation, the concentrations for the samples of experiment A (Sc→Sc, Sc→Lt, Sc→Hv, Sc→Sp) remained stable, but the concentrations of beers of experiment B (Lt→Sc, Lt→Lt, Lt→Hv, Lt→Sp) increased up to a maximum of 2.86 g/L.

3.4.6. Evolution of Anthocyanins from Red Grape Skins. The monitoring of anthocyanins compounds was carried out by HPLC-DAD and the main results are shown in Table 3. In the analysis of the anthocyanin mixture before the start of secondary fermentation in bottle (0 weeks), the following molecules were

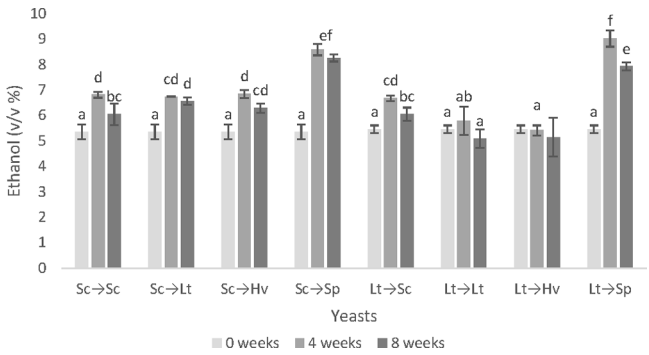

Figure 7. Evolution of ethanol concentration (% v/v) over time. Values represent the mean ± standard deviation (n = 3). Analyses of variance (ANOVA) were performed comparing all weeks with each other. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

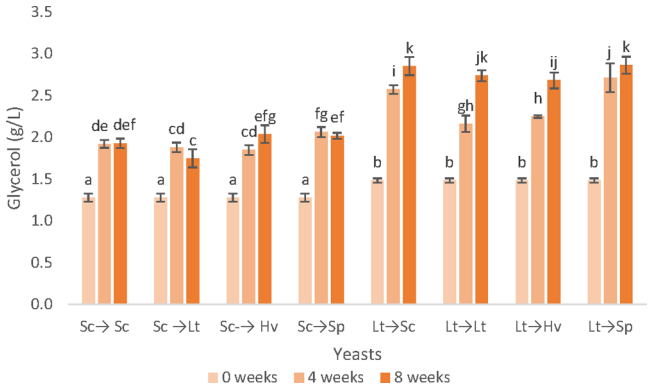

Figure 8. Evolution of glycerol concentration (g/L) over time. Values represent the mean ± standard deviation (n = 3). Analyses of variance (ANOVA) were performed comparing all weeks with each other. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

identified: delphidin-3-*O*-glycoside (D3G), cyanidin-3-*O*-glycoside (C3G), petunidin-3-*O*-glycoside (Pt3G), malvidin-3-*O*-glycoside (M3G), acetylated malvidin-3-*O*-glycoside (M3G-Ac), and coumarilated malvidin-3-*O*-glycoside (M3G-Cu). Of these, the acylated compounds M3G, Pt3G, and C3G were in the majority. After 4 weeks of bottle fermentation, a decrease of all the above-mentioned anthocyanins was observed, while vinylphenolic compounds in the order of 3 mg/L could be determined. Samples that had been fermented mainly with *L. thermotolerans* (experiment B), whose pH was lower, experienced a milder decrease. After 8 weeks of fermentation in bottle, the trend continued, that is, anthocyanins decreased, even to the point where the proportion of M3G-Cu disappeared, and the pyroanthocyanidin-vinylphenolic compounds remained in the same range as described (~3 mg/L).

3.4.7. Total Polyphenol Index, Color Intensity, and Color. In order to further study the evolution of anthocyanins during the second fermentation in the bottle, the different beers were analyzed by UV−vis spectrophotometry at 280 nm, 420 nm (yellow color), 520 nm (red color), and 620 nm (blue color). Spectral analysis of the anthocyanins added to beers brewed with *S. cerevisiae* (experiment A) and *L. thermotolerans* (experiment B) before the start of secondary fermentation revealed no significant differences between them, as there might be a hyperchromic effect due to the high acidity of *L. thermotolerans*.

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Table 3. Anthocyanin Composition before, during, and after Completion of Secondary Fermentation in Bottle[a]

secondary fermentation	yeasts	D3G	C3G	Pt3G	M3G	M3G-Ac	M3G-Cu	pyranoanthocyanvinylphenolics
0 weeks	Sc (expt A)	1.84 ± 0.01^{a}	5.36 ± 0.05^{a}	5.58 ± 0.05^{a}	9.20 ± 0.03^{a}	3.13 ± 0.02^{a}	1.76 ± 0.13a	
	Lt (expt B)	1.83 ± 0.01^{a}	4,97 ± 0.00^{b}	5.63 ± 0.00^{a}	9.25 ± 0.01^{a}	3.15 ± 0.04^{a}	1.71 ± 0.09a	
4 weeks	Sc→Sc	1.60 ± 0.02^{b}	2.92 ± 0.06^{c}	3.49 ± 0.04^{c}	5.22 ± 0.09^{c}	2.17 ± 0.02^{c}	1.51 ± 0.01^{bc}	3.02 ± 0.03^{d}
	Sc→Lt	1.48 ± 0.01^{a}	2.00 ± 0.02^{a}	2.20 ± 0.01^{a}	2.89 ± 0.01^{a}	1.69 + 0.00^{a}	1.54 ± 0.04^{d}	2.97 ± 0.01^{abc}
	Sc→Hv	1.49 ± 0.02^{a}	1.96 ± 0.04^{a}	2.20 ± 0.02^{a}	2.88 ± 0.01^{a}	1.70 ± 0.03^{a}	1.49 ± 0.03^{b}	2.95 ± 0.02^{ab}
	Sc→Sp	1.59 ± 0.01^{b}	2.79 ± 0.09^{b}	3.36 ± 0.01^{b}	4.91 ± 0.04^{b}	2.10 + 0.04^{b}	0.00 ± 0.00^{a}	3.00 ± 0.02^{cd}
	Lt→Sc	1.66 ± 0.0l^{d}	3.13 ± 0.09^{d}	4.21 ± 0.11^{e}	6.54 ± 0.19^{e}	2.45 ± 0.03^{e}	1.49 ± 0.00^{b}	2.94 ± 0.01^{a}
	Lt→Lt	1.70 ± 0.02^{d}	3.35 ± 0.07^{e}	4.73 ± 0.04^{f}	7.46 ± 0.09^{f}	2.68 ± 0.00^{f}	1.51 ± 0.01^{bc}	2.93 ± 0.00^{a}
	Lt→HV	1.63 ± 0.0l^{c}	2.91 ± 0.02^{c}	4.04 ± 0.02^{d}	6.21 ± 0.02^{d}	2.41 ± 0.02^{d}	1.48 ± 0.01^{b}	2.96 ± 0.04^{abc}
	Lt→Sp	1.66 ± 0.02^{d}	3.17 ± 0.04^{d}	4.29 ± 0.02^{e}	6.47 ± 0.03^{e}	2.44 ± 0.02^{de}	1.47 ± 0.00^{b}	3.00 ± 0.04^{bcd}
8 weeks	Sc→Sc	1.47 ± 0.0l^{a}	2.22 ± 0.06^{bc}	2.66 ± 0.07^{c}	3.45 ± 0.12^{c}	1.76 ± 0.01^{c}		3.07 ± 0.01^{cd}
	Sc→Lt	1.47 ± 0.00^{a}	2.06 ± 0.10^{a}	2.48 ± 0.02^{a}	3.14 ± 0.03^{a}	1.70 ± 0.01^{a}		3.10 ± 0.06^{de}
	Sc→Hv	1.47 ± 0.00^{a}	2.14 ± 0.06^{a}	2.73 ± 0.03^{d}	3.53 ± 0.06^{c}	1.76 ± 0.01^{c}		3.12 ± 0.04^{e}
	Sc→Sp	1.47 ± 0.00^{a}	2.23 ± 0.02^{c}	2.58 ± 0.04^{b}	3.31 ± 0.06^{b}	1.73 ± 0.01^{b}		3.04 ± 0.02^{c}
	Lt→Sc	1.48 ± 0.00^{b}	2.25 ± 0.01^{c}	3.34 ± 0.01^{f}	4.66 ± 0.01^{e}	2.00 ± 0.00^{e}		2.94 ± 0.01^{b}
	Lt→Lt	1.47 ± 0.00^{a}	2.06 ± 0.05^{a}	2.95 ± 0.01^{e}	4.04 ± 0.01^{d}	1.89 ± 0.01^{d}		1.48 ± 0.01^{a}
	Lt→Hv	1.47 ± 0.00^{a}	2.14 ± 0.03^{a}	3.34 ± 0.02^{f}	4.72 ± 0.01^{e}	2.02 ± 0.01^{d}		2.98 ± 0.03^{b}
	Lt→Sp	1.50 ± 0.00^{c}	2.39 ± 0.02^{d}	3.54 ± 0.01^{g}	4.95 ± 0.02^{f}	2.04 ± 0.00^{g}		2.96 ± 0.02^{b}

[a]Values represent the mean ± standard deviation (n = 3). Analysis of variance (ANOVA) was performed independently for each of the weeks. The different letters in the ANOVA indicate significant differences for the set of samples for each parameter analyzed. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp). Anthocyanins: delphidin-3-*O*-glycoside (D3G), cyanidin-3-*O*-glycoside (C3G), petunidin-3-*O*-glycoside (Pt3G), malvidin-3-*O*-glycoside (M3G), acetylated malvidin-3-*O*-glycoside (M3G-Ac), and coumarilated malvidin-3-*O*-glycoside (M3G-Cu).

The initial TPI was around 27, the intensity was ∼1.8, and the tonality was ∼1.5 for both yeasts (Figure 9). After completing 4

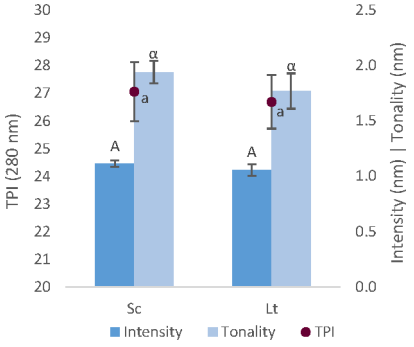

Figure 9. TPI, intensity, and tonality determined for samples before secondary fermentation in bottle (0 weeks). Values represent the average ± standard deviation (n = 3). In the ANOVA, the different letters indicate significant differences within each parameter. Yeasts: *S. cerevisiae* (Sc) and *L. thermotolerans* (Lt).

weeks of bottle conditioning, significant differences in the TPI content of the beers were observed, as they were in the range of 26−27 (Figure 10). After 8 weeks of secondary fermentation in the bottle, we can observe that in this case there were significant differences for all the parameters studied (Figure 11). TPI continued to decrease to values closer to 26, being a hypochromic effect probably due to oxidation and binding with other compounds, as did color intensity with values around ∼1, while color tonality increased to values close to or above 2, being a hypochromic effect. After 4 weeks of bottle conditioning, absorbance at 520 nm remained similar for all samples; whereas, after 8 weeks of secondary fermentation, absorbance values fell in the samples that had been inoculated in the main fermentation with *S. cerevisiae* (experiment A). In contrast, the absorbance at 520 nm maintained equal or higher values in the

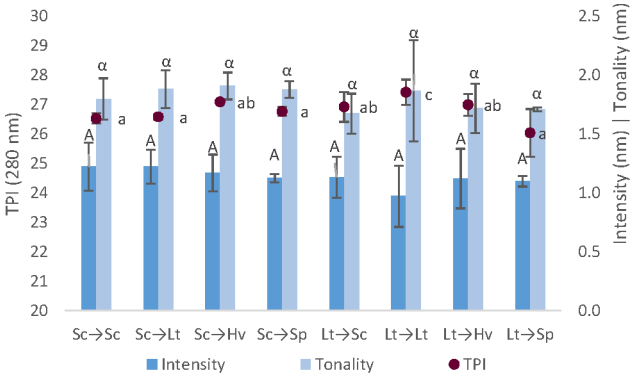

Figure 10. TPI, intensity, and tonality determined after secondary fermentation in bottle (4 weeks). Values represent the average ± standard deviation (n = 3). In the ANOVA, the different letters indicate significant differences within each parameter. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

case of *L. thermotolerans* for the main fermentation, a slight bathochromic effect (experiment B).

3.4.8. Volatile Compounds. The determination of the volatile compounds resulting from both main fermentation and bottle conditioning was carried out using GC-FID equipment. All the values were analyzed according to the detection threshold in Table S1. The results obtained after the main fermentation are shown in Table 4. The total number of compounds determined was higher in the fermentation with *L. thermotolerans* (480.61 mg/L) than for *S. cerevisiae* (441.77 mg/L). Notably, the concentration of acetaldehyde was more than four times lower in *L. thermotolerans* than in *S. cerevisiae* (82.38 mg/L), exceeding the detection threshold (2−20 mg/L). For alcohols such as methanol and hexanol, associated with alcohol/solvent and herbaceous descriptors, respectively, both yeasts show concentrations well above the established limits. The same was true for higher alcohols such as 1-propanol (descriptor

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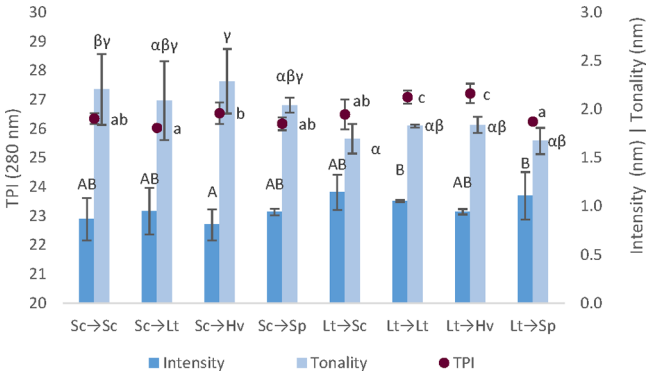

Figure 11. TPI, intensity, and tonality determined after secondary fermentation in bottle (8 weeks). Values represent the average ± standard deviation ($n = 3$). In the ANOVA, the different letters indicate significant differences within each parameter. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

Table 4. Volatile Compounds Determined (mg/L) by GC-FID, after Main Fermentation[a]

volatile compounds	Sc	Lt
acetaldehyde	82.38 ± 5.63[b]	17.33 ± 3.73[a]
methanol	13.33 ± 1.13[a]	17.09 ± 2.01[b]
1-propanol	25.05 ± 2.93[a]	10.83 ± 11.19[a]
diacetyl	1.95 ± 0.82[a]	4.32 ± 0.83[b]
ethyl acetate	16.92 ± 2.65[b]	10.46 ± 1.27[a]
2-butanol	1.71 ± 1.48[a]	0.00 ± 0.00[a]
isobutyl alcohol	13.55 ± 1.97[a]	43.76 ± 5.62[b]
1-butanol	0.00 ± 0.00[a]	0.00 ± 0.00[a]
acetoin	9.23 ± 1.84[a]	11.37 ± 1.60[a]
3-methyl-1-butanol	38.45 ± 1.05[b]	31.38 ± 1.35[a]
2-methyl-1-butanol	20.28 ± 0.74[a]	18.09 ± 1.79[a]
isobutyl acetate	4.39 ± 3.95[a]	5.98 ± 0.71[a]
ethyl butyrate	2.55 ± 4.41[a]	0.00 ± 0.00[a]
ethyl lactate	14.20 ± 3.83[a]	16.66 ± 2.10[a]
2−3-butanediol	144.12 ± 8.59[a]	205.67 ± 3.79[b]
isoamyl acetate	3.26 ± 0.77[a]	2.15 ± 1.88[a]
hexanol	8.33 ± 0.84[a]	9.49 ± 1.49a
2-phenylethyl alcohol	28.10 ± 2.37[a]	45.57 ± 6.60[b]
2-phenylethyl acetate	13.97 ± 1.38[a]	30.46 ± 1.67[b]
total volatile compounds	441.77 ± 3.78[a]	480.61 ± 17.46[a]

[a]Values represent the average ± standard deviation ($n = 3$). In the ANOVA the different letters for each row indicate significant differences between yeasts. Yeasts: *S. cerevisiae* (Sc) and *L. thermotolerans* (Lt).

alcohol, rancid), which is above the perception threshold for beer fermented with *S. cerevisiae*. While in the case of 2-phenylethyl alcohol (descriptor rose petal, bitter, perfume) significant differences were found between the yeasts, since the concentration analyzed was almost double in *L. thermotolerans* and exceeded the sensory threshold. Significant differences also appear for the compound isobutyl alcohol (descriptor alcohol, solvent) the concentration of which in non-*Saccharomyces* yeast was three times higher (43.76 mg/L) than in *Saccharomyces* yeast (13.55 mg/L). As for carbonyl compounds, the concentration of diacetyl (dairy descriptor, butter) was 10 times higher than the detection threshold established in both yeasts, while acetoin was found in a low proportion compared to the limit of perception. Finally, among the esters determined, the concentration of ethyl butyrate in *S. cerevisiae* (descriptor papaya) was 10 times higher than the established threshold, as was the case in both yeasts for the volatile compound 2-phenylethyl acetate (descriptor roses, honey, apple, sweet) with a concentration between 5 and 15 times higher.

After 4 weeks of bottle conditioning in which combinations have been carried out as sequential fermentation, the results are shown in Table 5. It was remarkable that for all samples the sum of volatile compounds was reduced between 40 and 120 mg/L. After this fermentation, the concentrations of acetaldehyde (apple and green leaves descriptor) were reduced, with all the samples presenting a similar range between 6 and 12 mg/L. With regard to alcohols, the concentration of methanol (descriptor alcohol and solvent) increased slightly for all the beers brewed, while the concentration of hexanol (descriptor herbaceous) decreased with all the yeasts and, particularly, those whose conditioning was carried out with *S. pombe*. In the case of higher alcohols, the amount of 1-propanol (descriptor alcohol) remained stable for all samples starting from the main fermentation with *S. cerevisiae* (experiment A) and doubled for those with *L. thermotolerans* (experiment B). Moreover, the concentration of 2-phenylethyl alcohol (descriptor rose petal, bitter, perfume) was close to 25 mg/L in all samples, staying within the established detection threshold (8−35 mg/L). Regarding isobutanol (descriptor alcohol, solvent) this volatile compound increased for all samples starting from the main fermentation with *S. cerevisiae* (experiment A), and decreases for those fermented with *L. thermotolerans* (experiment B), with the exception of the combination Lt→Sc, which presented a much higher concentration around 47 mg/L. In the carbonyl compounds, diacetyl (dairy descriptor, butter) was still 8−10 times above the detection threshold, but the beers showed concentrations without significant differences. Acetoin (descriptor butter) had slightly decreased its concentration with all yeasts except for Lt→Sc and Lt→ Hv. Finally, as for the quantified esters, ethyl butyrate (descriptor papaya) appeared again for two of the samples starting from the main fermentation with *L. thermotolerans* (experiment B), namely for Lt→Lt and Lt→Sp. While the values for isoamyl acetate (descriptor banana, sweet, fruit) remained within the established detection threshold, the concentration of 2-phenylethyl acetate (descriptor roses, honey, apple, sweet) considerably exceeds this threshold. The 2-phenylethyl acetate is particularly higher for samples conditioned with the yeast *H. vineae*.

The most relevant results of the last analysis of volatile compounds, after 8 weeks of bottle conditioning, are shown in Table 6. Once again, it can be observed that the total content of volatile compounds has decreased compared to that at the beginning. As for acetaldehyde (apple and green leaves descriptor), we can observe that it had decreased to values below 10 mg/L in all samples. As for alcohols, a decrease can also be noted, which in the case of methanol was below 20 mg/L for all yeasts, although it was still well above the sensory threshold of perception. On the other hand, the amount of hexanol (herbaceous descriptor) increased for all samples analyzed and remained above the sensory perception limits (Table S1). Higher alcohols such as 1-propanol remained in the same concentration range (25 mg/L), while 2-phenylethyl alcohol (descriptor rose petal, bitter, perfume) was reduced in all conditioned samples, reaching maximum values around 25 mg/L. Carbonyl compounds (diacetyl and acetoin) also decreased in all beers analyzed, and 2−3-butanediol again increased. Again, it seems that their decrease is in favor of the increase of 2−3-butanediol. Finally, among the esters, it should be noted that

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Table 5. Volatile compounds determined (mg/L) by GC-FID, after secondary fermentation in bottle (4 weeks) Values represent the average ± standard deviation (n = 3). In the ANOVA, the different letters for each row indicate significant differences between yeasts. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv) and *S. pombe* (Sp)

volatile compounds	Sc→Sc	Sc→Lt	Sc→Hv	Sc→Sp	Lt→Sc	Lt→Lt	Lt→Hv	Lt→Sp
acetaldehyde	8.59 ± 1.35^{ab}	8.63 ± 2.93^{ab}	9.01 ± 3.32^{ab}	12.02 ± 7.06^{b}	6.96 ± 2.29^{ab}	12.44 ± 1.92^{b}	5.47 ± 1.20^{ab}	8.41 ± 0.54^{ab}
methanol	19.80 ± 1.l0^{bcd}	18.16 ± 1.73^{bc}	17.87 ± 1.45^{e}	22.96 ± 1.96’	21.80 ± 2.14^{de}	20.95 ± 0.54^{abab}	18.91 ± 3.04$^{ab«}$	10.77 ± 0.93^{ab}
1-epropanol	24.67 ± 3.50^{de}	27.63 ± 3.28^{e}	23.73 ± 1.38^{bed}	26.44 ± 1.37^{de}	23.39 ± 2.85^{ab}	20.08 ± 2.73^{b}	12.10 ± 1.26^{ab}	15.55 ± 1.08^{ab}
diacetyl	2.33 ± 2.13^{ab}	2.14 ± 0.34^{ab}	2.65 ± 0.53^{ab}	1.87 ± 0.29^{ab}	2.82 ± 0.37^{ab}	3.20 ± 0.39^{ab}	3.31 ± 1.04^{ab}	2.31 ± 0.31^{ab}
ethyl acetate	2.82 ± 0.92^{ab}	2.18 ± 0.32^{ab}	4.71 ± 0.47^{bcd}	3.57 ± 0.0S^{abab}	4.04 ± 0.3S^{abab}	4.50 ± 1.28^{ab}	5.31 ± 2.66^{c}	2.39 ± 0.58^{ab}
2-butanol	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	2.31 ± 2.16^{b}	0.00 ± 0.00^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
isobutyl alcohol	20.57 ± 5.71^{ab}	14.25 ± 1.83^{a}	25.25 ± 4.86^{b}	18.54 ± 1.09^{a}	47.23 ± 0.84^{c}	20.15 ± 6.38^{ab}	16.12 ± 2.94^{ab}	18.81 ± 0.66^{ab}
1-butanol	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
acetoin	8.25 ± 1.43^{a}	7.46 ± 1.30^{a}	9.97 ± 0.62^{abc}	8.99 ± 1.09^{ab}	12.00 ± 2.47^{bc}	8.21 ± 0.83^{a}	13.03 ± 3.63^{c}	6.91 ± 0.24^{ab}
3-methyl-l-butanol	42.68 ± 2.51^{d}	41.88 ± 3.16^{cd}	42.29 ± 2.30^{ab}	47.70 ± 1.68^{e}	37.90 ± 0.92^{bc}	34.29 ± 3.36^{ab}	30.87 ± 4.60^{ab}	37.63 ± 1.26^{ab}
2-methyl-1-butanol	24.95 ± 2.58^{c}	22.29 ± 1.45^{bc}	22.59 ± 1.83^{ab}	25.88 ± 3.69^{c}	19.35 ± 0.43^{ab}	16.23 ± 1.79^{a}	19.11 ± 4.25^{ab}	17.92 ± 0.84^{a}
isobutyl acetate	6.04 ± 0.60^{ab}	2.45 ± 2.37^{de}	1.31 ± 2.26^{ab}	4.07 ± 0.28^{cd}	3.71 ± 0.47^{c}	3.92 ± 0.85^{cd}	6.98 ± 0.83^{e}	0.00 ± 0.00^{a}
ethyl butyrate	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.28 ± 1.1 l^{b}	0.00 ± 0.00^{a}	1.54 ± 0.39^{b}	0.00 ± 0.00^{a}	1.43 ± 0.20^{b}
ethyl lactate	13.81 ± 0.11^{cd}	8.19 ± 1.05^{a}	12.50 ± 0.97^{bc}	10.45 ± 1.10^{ab}	25.21 ± 1.87^{g}	15.58 ± 1.10^{de}	17.55 ± 1.80^{e}	20.95 ± 2.79^{f}
2−3-butanediol	148.56 ± 11.64^{a}	161.03 ± 9.21^{ab}	152.34 ± 10.04^{a}	173.75 ± 9.14^{b}	156.24 ± 6.49^{ab}	226.55 ± 6.58^{c}	231.32 ± 20.40^{c}	172.92 ± 7.37^{b}
isoamvl acetate	3.34 ± 0.13^{e}	2.82 ± 0.21^{d}	3.67 ± 0.40^{e}	3.67 ± 0.37^{e}	2.71 ± 0.18^{cd}	2.36 ± 0.19a^{b}	2.18 ± 0.32^{a}	2.43 ± 0.21^{ab}
hexanol	6.22 ± 1.10^{ab}	7.87 ± 0.87^{de}	7.27 ± 0.72^{cd}	4.90 ± 0.16^{a}	7.19 ± 0.13^{bcd}	8.88 ± 0.72^{e}	6.17 ± 0.28^{b}	4.74 ± 0.17^{a}
2-phenylethylalcohol	30.07 ± 3.09^{cd}	23.94 ± 1.19a	28.35 ± 1.96^{bc}	32.36 ± 0.80^{d}	27.82 ± 2.00^{bc}	26.11 ± 2.58^{ab}	27.21 ± 2.37^{abc}	26.55 ± 1.51^{abc}
2-phenylethyl acetate	10.22 ± 1.32^{ab}	8.36 ± 0.85^{a}	11.14 ± 0.45^{ab}	7.70 ± 0.16^{a}	14.14 ± 0.37^{bc}	18.27 ± 3.06^{c}	25.23 ± 6.47^{d}	8.84 ± 1.38^{a}
total volatile compounds	372.93 ± 28.46^{ab}	359.28 ± 5.67^{a}	376.07 ± 11.56^{ab}	406.16 ± 7.79^{bc}	412.51 ± 7.69^{cd}	443.26 ± 22.66^{d}	440.86 ± 41.80^{cd}	358.56 ± 4.54^{a}

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Table 6. Volatile Compounds Determined (mg/L) by GC-FID, after Secondary Fermentation in Bottle (8 Weeks)[a]

volatile compounds	Sc→Sc	Sc→Lt	Sc→Hv	Sc−Sp	Lt→Sc	Lt→Lt	Lt→Hv	Lt→Sp
acetaldehyde	9.29 ± 2.64^{ab}	6.36 ± 1.82^{a}	8.07 ± 0.86^{ab}	8.16 ± 0.13^{ab}	6.14 ± 0.31^{a}	6.40 ± 1.08^{a}	6.21 ± 0.61^{a}	7.63 ± 0.29^{ab}
methanol	14.18 ± 0.47^{b}	15.92 ± 2.08^{bc}	14.21 ± 0.49^{b}	14.31 ± 1.45^{b}	14.82 ± 0.30^{bc}	16.52 ± 1.25^{c}	15.63 ± 1.98^{bc}	11.46 ± 0.39^{a}
1-propanol	24.85 ± 1.03^{b}	25.32 ± 1.58^{c}	24.57 ± 1.39^{c}	24.02 ± 1.13^{c}	18.46 ± 1.52^{a}	21.21 ± 1.63^{b}	21.18 ± 2.61^{b}	17.04 ± 1.01^{a}
diacetyl	1.98 ± 0.17^{a}	1.72 ± 0.04^{a}	2.11 ± 0.33^{a}	1.79 ± 0.16^{a}	2.20 ± 0.43^{ab}	2.74 ± 0.44^{bc}	2.76 ± 0.47^{c}	1.97 ± 0.20^{c}
ethyl acetate	3.82 ± 0.36^{bc}	3.76 ± 0.88^{bc}	4.73 ± 0.57^{cd}	3.88 ± 0.39^{bc}	3.81 ± 0.36b^{c}	5.05 ± 0.33^{d}	4.72 ± 0.90^{cd}	2.16 ± 0.20^{a}
2-butanol	2.14 ± 1.86^{b}	2.64 ± 0.06^{b}	2.85 ± 0.11^{b}	2.74 ± 0.09^{b}	2.73 ± 0.01^{b}	2.83 ± 0.12^{b}	2.96 ± 0.06^{b}	0.00 ± 0.00^{a}
isobutyl alcohol	22.33 ± 3.01^{a}	19.00 ± 5.80^{a}	14.42 ± 1.75^{a}	16.62 ± 1.62^{a}	17.93 ± 9.03^{a}	47.24 ± 1.59^{c}	34.37 ± 6.37^{b}	16.66 ± 1.73^{a}
1-butanol	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
acetoin	8.13 ± 1.74^{ab}	8.21 ± 1.53^{ab}	9.58 ± 0.34^{bc}	8.35 ± 0.58^{ab}	7.05 ± 1.61^{a}	9.89 ± 1.78^{bc}	10.95 ± 1.62^{c}	7.20 ± 0.91^{a}
3-methyl-1- butanol	38.25 ± 1.16^{bcd}	39.58 ± 1.45^{cd}	40.46 ± 1.82^{d}	44.01 ± 1.73^{e}	37.20 ± 1.99^{bc}	31.11 ± 1.53^{a}	31.29 ± 2.91^{a}	35.60 ± 1.67^{b}
2-methyl-l-butanol	22.41 ± 0.99^{c}	20.98 ± 2.39^{b}	21.48 ± 2.49^{bc}	24.33 ± 1.28^{bc}	15.52 ± 0.51^{a}	17.01 ± 2.60^{a}	16.38 ± 0.61^{a}	15.93 ± 1.10^{b}
isobutyl acetate	0.00 ± 0.00^{a}	4.71 ± 3.02^{b}	3.27 ± 0.36^{b}	5.19 ± 2.06^{b}	4.49 ± 0.44^{b}	5.36 ± 0.62^{b}	4.72 ± 0.81^{b}	4.23 ± 1.96^{b}
ethyl butyrate	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.79 ± 0.30^{b}	1.70 ± 0.18^{b}	1.51 ± 0.41^{b}	1.60 ± 0.3l^{b}	1.47 ± 0.23^{b}	1.55 ± 0.22^{b}
ethyl lactate	17.98 ± 2.23^{b}	9.50 ± 0.90^{a}	9.28 ± 0.75^{a}	19.09 ± 3.42^{b}	26.00 ± 0.52^{c}	22.50 ± 1.99^{bc}	21.83 ± 6.80^{bc}	25.86 ± 3.49^{c}
2−3-butanediol	158.97 ± 3.99^{cd}	157.26 ± 14.70^{cd}	158.66 ± 2.88c^{d}	163.23 ± 4.06^{d}	138.50 ± 4.08^{ab}	148.52 ± 10.16^{bc}	131.97 ± 2.56^{a}	160.65 ± 3.64^{cb}
isoamyl acetate	2.20 ± 0.09^{ab}	2.35 ± 0.13^{b}	2.35 ± 0.01^{b}	2.77 ± 0.21^{c}	2.10 ± 0.24^{ab}	2.27 ± 0.25^{b}	1.91 ± 0.10^{a}	2.28 ± 0.36^{b}
hexanol	7.87 ± 0.90^{d}	7.63 ± 1.25^{d}	7.23 ± 0.49^{cd}	5.04 ± 0.35^{a}	6.93 ± 0.23^{cd}	6.16 ± 0.51^{bc}	6.26 ± 0.43^{bc}	5.17 ± 0.32^{ab}
2-phenylethylalcohol	21.77 ± 1.40$^{a\ b}$	25.40 ± 1.14^{c}	23.35 ± 1.02^{abc}	24.92 ± 0.97^{c}	24.89 ± 0.95^{c}	23.90 ± 0.63^{bc}	21.17 ± 1.82^{a}	24.81 ± 1.77^{c}
2-phenylethyl acetate	8.58 ± 0.81^{bc}	9.59 ± 0.67^{bc}	9.88 ± 0.71^{c}	6.16 ± 0.23^{a}	12.11 ± 0.59^{d}	15.94 ± 1.42^{e}	16.23 ± 2.56^{e}	7.82 ± 0.19^{ab}
total volatile compounds	364.75 ± 4.04^{bcd}	359.93 ± 16.65^{abc}	358.27 ± 5.53^{abc}	376.34 ± 14.14^{cd}	342.40 ± 15.70^{a}	386.24 ± 14.81^{d}	352.01 ± 16.20^{ab}	348.02 ± 8.40^{ab}

[a]Values represent the average ± standard deviation (n = 3). In the ANOVA, the different letters for each row indicate significant differences between yeasts. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

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unfortunately the concentration of 2-phenylethyl acetate (descriptor roses, honey, apple, sweet) was reduced for all the samples analyzed, although the highest values are associated with the sequential fermentations of experiment B and, in particular, with Lt→Sc, Lt→Lt, and Lt→Hv. Finally, it should be noted that the ethyl lactate (descriptor cheese, fruity) concentration is higher with respect to the 4-week bottle conditioning and, in particular, in all beers that were inoculated in the main fermentation with *L. thermotolerans* (>20 mg/L).

The evolution of the higher alcohols (Figure 12a) seems to depend on the yeast used in the main fermentation. Beers

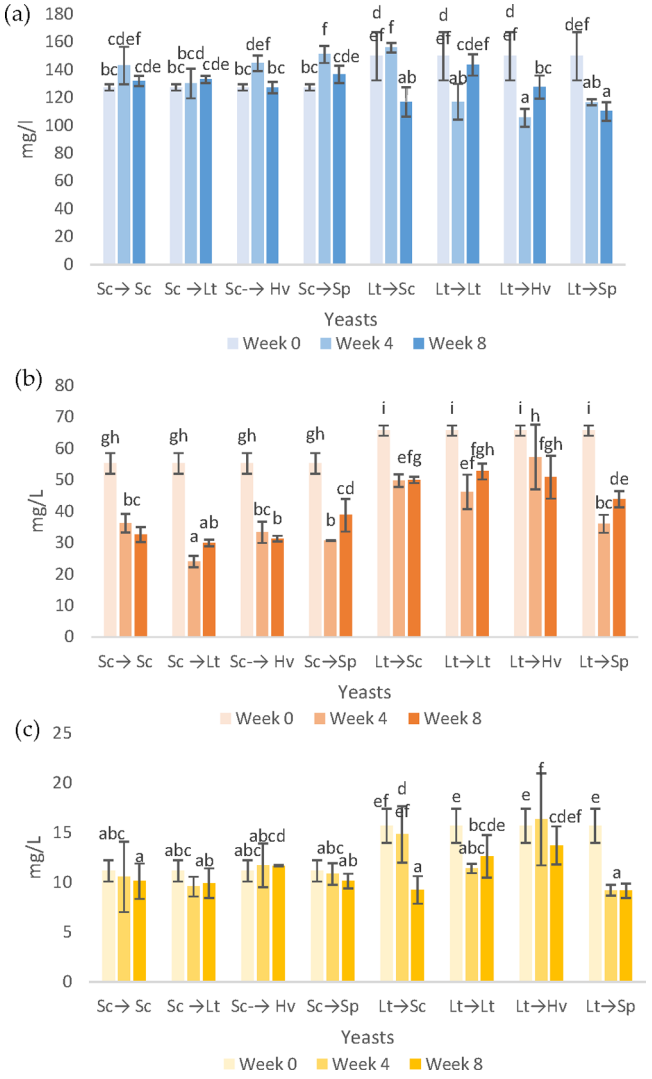

Figure 12. Evolution of the total concentration of volatile compounds during bottle conditioning: (A) total higher alcohols; (B) total esters; (C) total carbonyl compounds. Values represent the average ± standard deviation ($n = 3$). In the ANOVA, the different letters indicate significant differences for the set of samples. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

brewed with *S. cerevisiae* (experiment A), showed similar concentrations of these compounds before and after 8 weeks of conditioning. On the contrary, those brewed with *L. thermotolerans* in the main fermentation (experiment B) experienced a significant decrease in higher alcohols during bottle fermentation. As for the total esters (Figure 12b), it can be observed that they follow a decreasing trend during bottle conditioning. However, again, significant differences could be observed with respect to the yeasts used in the main fermentation. The concentration of esters produced in the main fermentation with *L. thermotolerans* (experiment B) was higher from the beginning to the end of the bottle conditioning than in the case of the samples with *S. cerevisiae* (experiment A). Finally, the production of carbonyl compounds (Figure 12c). In the case of experiment B (with *L. thermotolerans)*, a decrease of diacetyl and acetoin is observed during bottle conditioning.

3.5. Sensorial Profile. *3.5.1. First Sensory Evaluation: Acidity, Color, and Body.* The sensory evaluation of the brewed beers was carried out twice, after 4 and 8 weeks of bottle conditioning. In the first sensory evaluation (Table 7) by means of a spider web diagram, the parameters that show significant differences in the different yeasts are represented in a spider web diagram; specifically, 15 parameters were represented (Figure 13). The attribute "acidity" stands out as receiving the highest score in beers that have been inoculated with *L. thermotolerans* in the main fermentation (experiment B), and the combination Lt→Sc received the highest score. In relation to this attribute, the "beer color" also received the highest score for the conditioning made from the *L. thermotolerans* beer. The attributes "body", "effervescence" and "aromatic quality" also stood out, with intermediate scores for all beers evaluated. As for the attribute "astringency", the set of beers that were inoculated with *S. cerevisiae* in the main fermentation (experiment A) are highlighted. Finally, the Sc→Lt and Lt→Lt combinations received the highest scores for the attribute "banana".

3.5.2. Second Sensory Evaluation: Acidity, Color, Aromatic Quality, And Overall Perception. The results of the second tasting are shown in Table 8, where a total of 12 attributes showed significant differences (Figure 14). In this evaluation, the high scores for the attributes "acidity" and "beer color" in the combinations belonging to the main fermentation with *L. thermotolerans* (experiment B) were confirmed. The high aromatic quality was also confirmed for all evaluated beers, with the exception of Sc→Sp. Finally, the Sc→Hv and Lt →Hv combinations received the highest scores for the "overall perception" parameter, which also report high scores for attributes such as visual effervescence and aromatic quality.

3.6. Pearson Correlation. The correlation between the instrumental parameters analyzed (pH, ethanol, glycerol, L-lactic acid, volatile compounds) and the sensory parameters (attributes) evaluated (attributes) were studied. As two sensory tests were carried out, after 4 weeks and 8 weeks of bottle conditioning, two correlation tests were performed and are shown in the Supporting Information (Table S4 and Table S5).

For the first sensory test (4 weeks bottle conditioning) the statistically significant positive correlations were as follows: beer color with L-lactic acid, ethyl lactate, and esters; turbidity with ethyl acetate and total volatile compounds; yeast with methanol; floral aroma with hexanol; hop aroma with glycerol and ethyl lactate; cereal aroma with pH and 2-methyl-1-butanol; bitterness with ethanol; saltiness with glycerol and L-lactic acid; and astringency with glycerol. The negative correlations were beer color with pH; aromatic quality with isobutyl acetate; cereal aroma with L-lactic acid; sweetness with acetaldehyde; bitterness with diacetyl; bitterness and 2-phenyl-ethyl acetate; bitterness and esters; saltiness and 2-methyl-1-butanol.

For the second sensory test (8 weeks), the correlations with positive statistical significance were beer color with glycerol; L-lactic acid and total esters; visual effervescence with acetoin and total volatile compounds; foam consistency with methanol;

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Table 7. Sensory Analysis Results after 4 Weeks of Bottle Conditioning[a]

parameters	Sc→ Sc	Sc→ Lt	Sc→Hv	Sc →Sp	Lt→Sc	Lt →Lt	Lt →Hv	Lt →Sp
beer color	3.00 ± 0.50^{c}	1.44 ± 0.73^{a}	2.11 ± 0.78^{b}	3.00 ± 0.87^{c}	1.11 ± 0.60^{d}	4.11 ± 0.60^{d}	4.00 ± 0.50^{d}	4.00 ± 0.50^{d}
turbidity	2.33 ± 0.50^{a}	2.11 ± 0.60^{a}	2.44 ± 0.53^{a}	2.33 ± 0.50^{a}	2.33 ± 0.50^{a}	2.56 ± 0.73^{a}	2.50 ± 1.01^{a}	2.20 ± 0.67^{a}
visual effervescence	1.78 ± 0.67^{a}	3.22 ± 0.67^{c}	3.67 ± 0.71^{c}	3.22 ± 0.97^{c}	2.22 ± 0.97^{a}	3.78 ± 0.97^{c}	3.11 ± 0.60^{bc}	2.40 ± 0.73^{ab}
foam consistency	1.44 ± 0.53^{a}	2.56 ± 1.01^{bc}	2.56 ± 0.53^{bc}	2.56 ± 1.24^{bc}	178 ± 0.97^{ab}	2.78 ± 1.09^{c}	2.78 ± 0.67^{c}	1.80 ± 0.67^{ab}
foam persistence	1.56 ± 0.53^{a}	2.67 ± 1.12^{bcd}	2.78 ± 1.20^{bcd}	3.00 ± 1.50^{a}	2.00 ± 0.87^{ab}	3.56 ± 1.13^{d}	2.89 ± 0.60^{bcd}	2.20 ± 0.83^{abc}
foam color	1.33 ± 0.50^{a}	1.44 ± 0.73^{a}	1.22 ± 0.44^{a}	1.44 ± 0.53^{a}	1.33 ± 0.50^{a}	1.67 ± 0.87^{a}	1.33 ± 0.50^{a}	1.30 ± 0.50^{a}
aromatic intensity	3.67 ± 1.00^{ab}	1.44 ± 1.13^{a}	3.33 ± 0.71^{a}	3.11 ± 0.78^{a}	3.22 ± 0.83^{a}	3.22 ± 0.97^{a}	3.44 ± 0.73^{a}	3.20 ± 0.83^{a}
aromatic quality	2.67 ± 0.71^{a}	3.11 ± 0.60^{ab}	3.44 ± 1.01^{b}	2.67 ± 1.71^{a}	2.78 ± 1.39^{ab}	2.67 ± 1.12^{ab}	2.33 ± 1.12^{a}	3.00 ± 0.87^{ab}
malt	2.56 ± 1.24^{a}	2.22 ± 0.97^{a}	1.78 ± 0.67^{a}	1.89 ± 0.78^{a}	2.44 ± 0.73^{a}	2.00 ± 0.87^{a}	1.89 ± 0.78^{a}	1.90 ± 1.05^{a}
yeast	1.78 ± 0.83^{ab}	1.89 ± 0.93^{ab}	1.78 ± 0.83^{a}	2.00 ± 0.71^{a}	1.78 ± 0.83^{ab}	1.67 ± 0.71^{ab}	1.89 ± 0.93^{ab}	1.20 ± 0.44^{a}
banana	1.56 ± 0.88^{a}	2.33 ± 0.87^{ab}	2.00 ± 1.32^{a}	1.89 ± 0.78^{a}	1.78 ± 0.83^{ab}	2.56 ± 1.13^{ab}	1.78 ± 0.20^{ab}	1.80 ± 0.83^{ab}
floral	1.67 ± 0.87^{ab}	1.89 ± 0.60^{ab}	2.11 ± 0.78^{a}	1.33 ± 0.50^{a}	1.78 ± 0.44^{ab}	2.11 ± 0.78^{b}	1.67 ± 0.50^{a}	1.70 ± 0.71^{ab}
fruity hoppy	2.44 ± 0.73^{a}	2.56 ± 1.01^{a}	2.44 ± 1.42^{a}	1.78 ± 1.39^{a}	2.11 ± 1.05^{a}	2.11 ± 1.05^{a}	2.11 ± 0.60^{a}	2.60 ± 1.32^{a}
hoppy	2.33 ± 0.87^{a}	2.11 ± 0.60^{a}	2.11 ± 0.78^{a}	2.33 ± 0.50^{a}	2.56 ± 0.88^{a}	2.33 ± 0.50^{a}	2.44 ± 0.73^{a}	2.60 ± 0.53^{a}
body	2.33 ± 0.50^{a}	2.89 ± 0.60^{abc}	2.67 ± 0.50^{a}	3.33 ± 0.87^{a}	2.44 ± 0.53^{ab}	3.00 ± 0.50^{bc}	3.00 ± 0.71^{bc}	3.00 ± 0.71^{bc}
cereal	2.78 ± 0.44^{d}	2.33 ± 0.50^{cd}	2.33 ± 0.71^{a}	2.22 ± 0.67^{a}	1.67 ± 0.50^{ab}	1.78 ± 0.67^{abc}	1.56 ± 0.53^{a}	1.70 ± 0.71^{ab}
sweetness	2.22 ± 0.44^{a}	2.33 ± 1.12^{a}	2.22 ± 1.09^{a}	1.89 ± 0.60^{a}	2.11 ± 1.17^{a}	1.89 ± 0.93^{a}	2.44 ± 1.13^{a}	2.20 ± 0.97^{a}
acidity	1.89 ± 0.78^{a}	2.22 ± 1.20^{ab}	2.89 ± 1.36^{abc}	2.56 ± 1.24^{abc}	4.33 ± 0.87^{d}	3.44 ± 1.01^{cd}	3.33 ± 1.22^{cd}	3.00 ± 1.22^{bc}
bitterness	1.78 ± 0.67^{ab}	2.11 ± 0.78^{a}	2.11 ± 0.93^{a}	2.67 ± 1.12^{c}	1.22 ± 0.44^{a}	1.33 ± 0.50^{a}	1.22 ± 0.44^{a}	2.30 ± 1.12^{bc}
salty	1.33 ± 0.50^{a}	1.44 ± 0.73^{a}	1.33 ± 0.71^{a}	1.56 ± 0.53^{a}	1.89 ± 0.78^{a}	1.89 ± 1.05^{a}	1.78 ± 0.67^{a}	1.80 ± 0.83^{a}
astringency	1.44 ± 0.53^{abc}	1.44 ± 0.73^{abc}	1.67 ± 0.50^{abc}	2.00 ± 0.71^{c}	1.33 ± 0.71^{ab}	1.44 ± 1.01^{abc}	1.11 ± 0.33^{a}	1.80 ± 0.67^{bc}
effervescence	1.89 ± 0.78^{a}	3.00 ± 1.12^{b}	3.00 ± 1.12^{a}	2.78 ± 0.97^{ab}	3.11 ± 1.27^{b}	3.11 ± 1.05^{b}	3.22 ± 0.97^{b}	3.10 ± 1.27^{b}
aftertaste	2.56 ± 0.53^{a}	3.11 ± 0.60^{a}	2.67 ± 0.71^{a}	2.89 ± 0.83^{a}	2.67 ± 0.71^{a}	2.78 ± 10.83^{a}	2.78 ± 0.67^{a}	2.60 ± 0.88^{a}
overall perception	2.56 ± 0.53^{b}	3.22 ± 0.44^{b}	2.67 ± 0.87^{a}	2.78 ± 0.33^{ab}	3.00 ± 0.71^{ab}	2.89 ± 0.33^{ab}	3.22 ± 1.09^{b}	2.90 ± 0.60^{ab}

[a]Values represent the mean ± standard deviation (n = 9). In the ANOVA, the different letters for each line indicate significant differences between yeasts. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

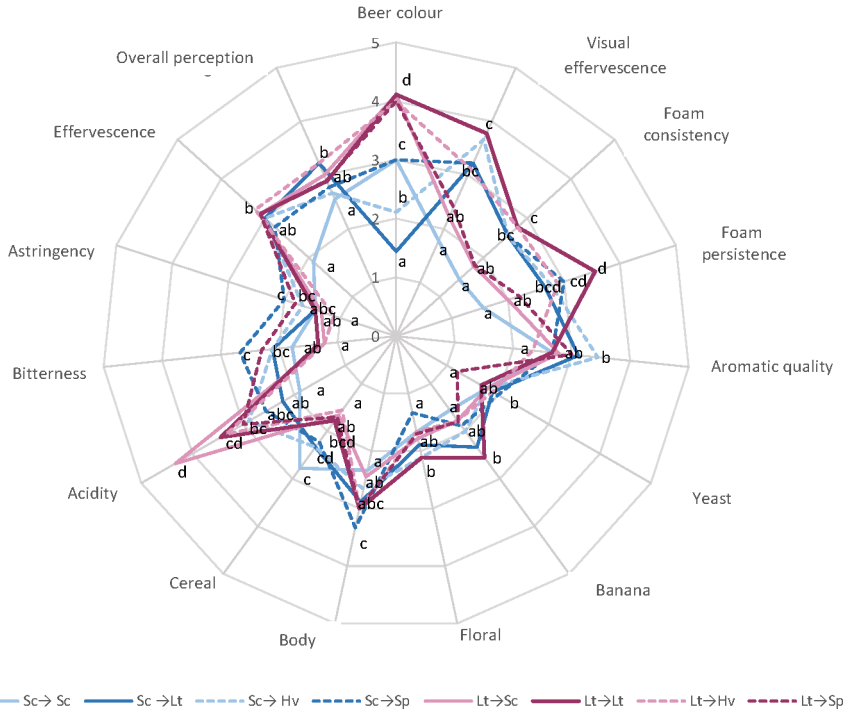

Figure 13. Spider web plot for sensory analysis after 4 weeks of bottle conditioning. Values represent the average ± standard deviation (n = 9). In the ANOVA the different letters for each parameter indicate significant differences between yeasts. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

foam persistence with acetoin and total volatile compounds; cereal aroma with 3-methyl-1-butanol; acidity with glycerol, lactic acid, and total esters; bitterness with pH, 3-methyl-1-butanol, 2-methyl-1-butanol, and isoamyl acetate; astringency

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Table 8. Sensory Analysis Results after 8 Weeks of Bottle Conditioning[a]

parameters	Sc→Sc	Sc →Lt	Sc→Hv	Sc→Sp	Lt→Sc	Lt→Lt	Lt→Hv	Lt→Sp
beer color	3.25 ± 0.46^{bc}	3.00 ± 0.53^{a}	3.25 ± 3.25^{ab}	2.88 ± 0.83^{a}	4.00 ± 0.00^{c}	3.63 ± 0.74^{bc}	4.00 ± 0.00^{c}	3.75 ± 0.71^{bc}
turbidity	2.50 ± 0.76^{ab}	2.13 ± 0.35^{ab}	2.75 ± 2.75^{b}	2.25 ± 0.46^{ab}	1.88 ± 0.35^{a}	2.50 ± 1.07^{ab}	2.00 ± 0.76^{a}	1.88 ± 0.64^{a}
visual effervescence	2.25 ± 1.28^{abcd}	1.75 ± 0.46^{ab}	3.00 ± 3.00^{bc}	2.00 ± 0.76^{abc}	1.50 ± 0.76^{a}	2.63 ± 0.92^{bcd}	3.25 ± 1.04^{d}	2.25 ± 1.28^{abcd}
foam consistency	2.00 ± 0.93^{a}	2.25 ± 1.04^{a}	2.00 ± 2.00^{a}	1.75 ± 0.71^{a}	1.88 ± 0.64^{a}	2.25 ± 0.46^{a}	2.25 ± 0.71^{a}	1.75 ± 0.71^{a}
foam persistence	2.13 ± 0.99^{abc}	2.25 ± 1.16^{abc}	2.38 ± 2.38^{abc}	1.88 ± 0.64^{ab}	1.50 ± 0.76^{a}	2.75 ± 0.89^{bc}	2.88 ± 0.99^{c}	2.00 ± 0.93^{abc}
foam color	1.13 ± 0.35^{a}	1.13 ± 0.35^{a}	1.25 ± 1.25^{a}	1.25 ± 0.46^{a}	1.25 ± 0.46^{a}	1.50 ± 0.76^{a}	1.25 ± 0.46^{a}	1.25 ± 0.46^{a}
aromatic intensity	3.75 ± 0.46^{a}	3.13 ± 0.64^{a}	3.25 ± 3.25^{a}	3.25 ± 0.71^{a}	3.50 ± 0.93^{a}	3.25 ± 0.71^{a}	3.38 ± 0.92^{a}	3.38 ± 0.92^{a}
aromatic quality	3.38 ± 0.74^{ab}	4.00 ± 0.76^{b}	3.25 ± 3.25^{ab}	2.63 ± 0.92^{a}	3.75 ± 1.04^{b}	3.50 ± 0.76^{b}	3.63 ± 0.74^{b}	3.63 ± 0.74^{b}
malt	2.63 ± 0.74^{a}	2.88 ± 0.99^{a}	2.38 ± 2.38^{a}	2.50 ± 1.20^{a}	2.13 ± 0.64^{a}	2.38 ± 0.52^{a}	2.75 ± 0.46^{a}	2.13 ± 0.64^{a}
yeast	2.25 ± 1.04^{a}	2.13 ± 0.99^{a}	2.38 ± 2.38^{a}	1.88 ± 0.99^{a}	1.63 ± 0.92^{a}	1.88 ± 1.13^{a}	2.13 ± 0.99^{a}	1.75 ± 0.89^{a}
banana	2.50 ± 0.93^{ab}	2.50 ± 0.76^{ab}	3.13 ± 3.13^{b}	2.63 ± 1.19^{ab}	2.63 ± 1.30^{ab}	2.13 ± 0.35^{a}	3.00 ± 1.07^{ab}	2.38 ± 0.92^{ab}
floral	1.88 ± 0.64^{a}	1.63 ± 0.74^{a}	2.00 ± 2.00^{a}	2.38 ± 0.92^{a}	2.38 ± 0.92^{a}	2.00 ± 0.76^{a}	2.13 ± 0.83^{a}	2.00 ± 0.76^{a}
fruity hoppy	2.13 ± 0.99^{a}	2.25 ± 0.46^{ab}	3.00 ± 3.00^{b}	2.38 ± 1.19^{ab}	2.25 ± 1.04^{ab}	2.13 ± 0.64^{a}	2.75 ± 0.71^{ab}	3.00 ± 0.76^{b}
hoppy	2.25 ± 0.71^{a}	2.88 ± 0.64^{a}	2.75 ± 2.75^{a}	2.38 ± 1.06^{a}	2.25 ± 0.71^{a}	2.38 ± 0.92^{ab}	2.13 ± 0.64^{a}	2.63 ± 0.92^{a}
body	2.38 ± 1.06^{ab}	2.38 ± 1.06^{ab}	2.50 ± 2.50^{ab}	2.63 ± 0.92^{ab}	1.88 ± 0.64^{a}	2.75 ± 0.71^{b}	2.38 ± 0.92^{ab}	2.75 ± 0.71^{b}
cereal	2.63 ± 0.92^{a}	2.88 ± 1.25^{a}	2.50 ± 2.50^{a}	2.75 ± 0.71^{a}	2.63 ± 0.52^{a}	2.25 ± 0.46^{a}	2.38 ± 0.74^{a}	2.50 ± 0.76^{a}
sweetness	1.63 ± 0.74^{a}	1.38 ± 0.52^{a}	2.13 ± 2.13^{a}	1.50 ± 0.76^{a}	2.13 ± 1.13^{a}	1.63 ± 0.92^{a}	1.88 ± 0.83^{a}	1.63 ± 0.52^{a}
acidity	2.38 ± 1.30^{a}	2.38 ± 1.19^{a}	2.75 ± 2.75^{ab}	2.50 ± 0.93^{a}	4.00 ± 1.41^{c}	4.25 ± 0.71^{c}	3.75 ± 1.28^{bc}	4.13 ± 0.83^{c}
bitterness	2.63 ± 1.06^{bc}	3.38 ± 1.06^{cd}	2.50 ± 2.50^{abc}	3.63 ± 1.06^{d}	1.75 ± 0.71^{ab}	1.88 ± 0.83^{ab}	1.63 ± 0.52^{a}	2.38 ± 0.74^{ab}
salty	2.50 ± 0.76^{a}	2.00 ± 0.53^{a}	1.88 ± 1.88^{a}	2.50 ± 1.07^{a}	2.00 ± 1.07^{a}	2.13 ± 0.99^{a}	2.13 ± 0.83^{a}	2.25 ± 1.04^{a}
astringency	1.63 ± 0.52^{a}	1.63 ± 0.52^{a}	1.38 ± 1.38^{a}	1.88 ± 0.83^{a}	1.75 ± 0.71^{a}	1.50 ± 0.76^{a}	1.38 ± 0.52^{a}	1.88 ± 0.99^{a}
effervescence	2.50 ± 1.31^{a}	2.88 ± 1.13^{abc}	3.25 ± 3.25^{abc}	2.75 ± 1.04^{ab}	2.75 ± 1.04^{ab}	3.75 ± 0.89^{bc}	3.88 ± 0.99^{c}	3.63 ± 0.92^{bc}
aftertaste	2.38 ± 0.92^{a}	2.63 ± 0.92^{a}	3.00 ± 3.00^{a}	3.00 ± 0.53^{a}	2.75 ± 0.71^{a}	2.50 ± 0.76^{a}	2.75 ± 0.46^{a}	2.63 ± 0.92^{a}
overall perception	2.88 ± 0.99^{ab}	3.13 ± 1.13^{ab}	3.63 ± 3.63^{a}	3.00 ± 0.76^{ab}	2.63 ± 1.06^{a}	2.75 ± 0.89^{ab}	3.50 ± 0.93^{ab}	2.88 ± 0.64^{ab}

[a]Values represent the mean ± standard deviation (n = 9). In the ANOVA, the different letters for each line indicate significant differences between yeasts. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

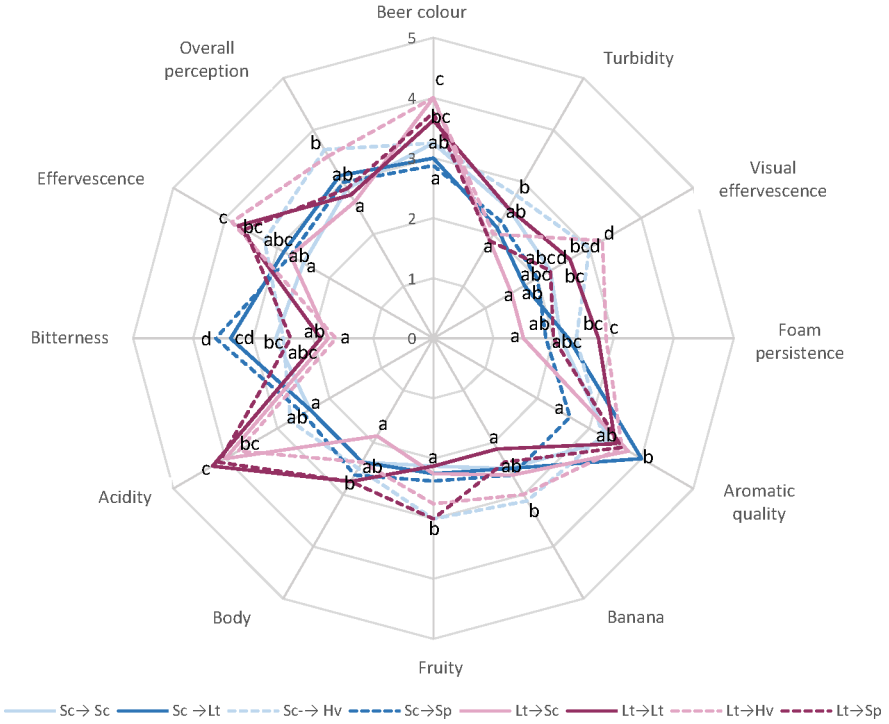

Figure 14. Spider web plot for sensory analysis after 8 weeks of bottle conditioning. Values represent the average ± standard deviation (n = 8). In the ANOVA the different letters for each parameter indicate significant differences between yeasts. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv), and *S. pombe* (Sp).

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with ethanol. Negatively the correlations found were beer color with pH, 1-propanol, 2-methyl-1-butanol, and 2−3 butanediol; foam consistency with ethanol; yeast aroma with ethyl lactate; cereal aroma with diacetyl; acidity with pH, 1-propanol, and 2-methyl-butanol; bitterness with glycerol, L-lactic acid, and diacetyl; and finally, astringency with acetoin and total volatile compounds.

4. DISCUSSION

The production of craft beers from different yeasts of *Saccharomyces* and non-*Saccharomyces* genera allows the sensory profile of the beers to be modified, thanks to the generation of different metabolites and the different fermentative capacities of the microorganisms.[8−11] In the present work, the following yeasts have been used for the production of craft beers with differentiated characteristics: *S. cerevisiae*, *L. thermotolerans*, *H. vineae*, and *S. pombe*.

In general, in the monitoring of the main fermentation of beer wort using *S. cerevisiae* (experiment A) and *L. thermotolerans* (experiment B), we observed a relationship between different parameters analyzed. The decrease in the concentration of reducing sugars to values close to 0 g/L coincided with the stabilization of ethanol production and pH through the production of organic acids, as well as the generation of glycerol, which slows down their growth from that moment onward in both yeasts. It should be noted that for each alcoholic strength generated, ∼17g/L sugars must be consumed;[47,48] therefore, to reach the ethanol concentrations determined, ∼94 g/L had to be consumed from a mixture of polysaccharides such as glucose, fructose, sucrose, galactose, maltose, maltotriose, or trehalose present in the beer wort. In fact, the assimilation of sugars ranges from the simplest (glucose and fructose) to the most complex (sucrose, maltose, and galactose).[13,16,49] In addition, beer brewing with *L. thermotelorans* was noted for the generation of L-lactic acid from fermentable sugars, as a product of lactate dehydrogenase enzyme activity on pyruvate.[50] As reported by Domizio et al.,[20] this yeast has an acidifying metabolism by which it is able to produce significant amounts of L-lactic acid in beer, and in the present study, the amount of L-lactic acid achieved is within the range of concentrations (0.26−10.54 g/L) that has been determined by different strains of *L. thermotolerans* for winemaking.[51] As can be seen, the production of this organic acid leads to a considerable decrease in pH. Moreover, ethanol is not the only metabolite produced in alcoholic fermentation, since glycerol is generated in parallel, in order to alleviate the osmotic stress caused by the high concentration of sugars in the must,[10,52] and its production is modulated as a consequence of temperature[19] and oxygenation level.[53] The increase in glycerol production from day 4 for *L. thermotolerans* compared to *S. cerevisiae* is consistent with previous studies[15,20,51] as *L. thermotolerans* is a species that produces higher concentrations of glycerol during alcoholic fermentation.[16,20]

Although the clarification of the beer is essential to remove the maximum amount of yeast, its cold clarification, without filtration equipment, caused some residual yeast from the main fermentation to remain in the bottle conditioning. This fact must be taken into account in the analyses carried out after the second fermentation, storage, or bottle conditioning.

The secondary fermentation was carried out by inoculating *S. cerevisiae*, *L. thermotolerans*, *H. vineae*, or *S. pombe* in the bottle. This process will form the foam, develop carbonation and yeast sedimentation, and promote aromatic maturation and colloidal stabilization.[10,12] The use of different yeasts made it possible to modify the sensory profile of the beers, finding significant differences in both instrumental and sensory analyses. In addition, it was interesting to evaluate the added anthocyanins, which potentially evolved into more stable forms according to the metabolism of each yeast.[33]

The difference in the concentration of reducing sugars present during bottle fermentation can be explained by the fact that *S. pombe* is a yeast with a slower metabolism due to nutritional requirements,[27] although it has a high fermentative power.[25,26] As for the differences in L-lactic acid concentration after bottle conditioning in all samples whose main fermentation was performed with *L. thermotolerans* (experiment B), these could be due to the transformation of L-lactic acid together with ethanol into the volatile compound ethyl lactate.[16] As explained above, lower pH in beers has been shown to be related to the generation of high amounts of L-lactic acid by *L. thermotolerans*.[20,51] The production of sour beers commonly known as sour-style beers has been associated with the use of lactic acid bacteria (LAB) by kettle souring or mixed-culture fermentation.[54] However, Osburn et al. proposed the use of non-*Saccharomyces* heterolactic yeasts. The application of this type of yeast in the main fermentation of beer, in the absence of LAB, is known as primary souring.[18] In the present experimental design, acidification of beers was not achieved when *L. thermotolerans* was inoculated exclusively for bottle conditioning (Sc→Lt). Therefore, *L. thermotolerans* proves to be a biotechnological tool for the realization of primary souring during primary fermentation. In terms of alcohol content, *S. pombe* stands out because it is a yeast characterized by a high fermentative power (10−14% v/v ethanol),[25] which leads to high alcohol content as observed in this work. The increase in alcoholic strength in the Sc→Hv combination is not due to the second yeast used, since it is unable to assimilate saccharides other than glucose and fructose,[22] being interesting for production of NABLAB.[4] These increases could be explained by two approaches: (i) due to the activity of residual *S. cerevisiae* from the main fermentation that have remained in the green beer after the clarification process, being able to metabolize sugars such as sucrose, galactose, or maltose;[13] (ii) due to the transformation of acetaldehyde into ethanol as part of the yeast metabolism.[55] By extension, the increase in alcoholic strength in the Sc→ Lt combination could be explained in the same way, as the contribution to ethanol concentration by *L. thermotolerans* is minimal. Furthermore, it has been shown that *L. thermotolerans* continues to produce L-lactic acid from sugars, and this is detrimental to the alcoholic strength, reaching 0.3−0.7% v/v less ethanol.[39] As mentioned above, glycerol production is directly related to alcoholic fermentation as this metabolite is generated in response to cellular stress. According to the results, it was expected to find an increase in the production of this metabolite during secondary fermentation with increasing alcoholic strength,[20] as is the case in beers conditioned with *S. pombe*.

As for anthocyanins added prior to bottle conditioning, the formation of vinyl−phenolic pyranoanthocyanin compounds, which are stable pigments with a double ring, can be formed in two ways: chemically or enzymatically. In the case of the chemical reaction, condensation occurs between hydroxycinnamic acids and grape anthocyanins and their concentration increases over time.[36,56] While biological action involves the enzyme hydroxycinnamate decarboxylase (HCDC), which transforms hydroxycinnamic acids into vinifenols[37] and these undergo a condensation reaction with the grape anthocyanins to form these stable pigments.[38] The enzymatic strategy by which

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positive HCDC activity in *S. cerevisiae* and *L. thermotolerans* is responsible for the production of vinylphenolic−pyranoanthocyanins is gaining momentum.[57] The formation of these compounds in all beers tested could again be explained by the residual presence of both yeasts coming from the main fermentation and remaining in the secondary fermentation in the bottle. However, further studies would be desirable to prove this thesis. Also, it has been observed that pH is an important parameter affecting anthocyanins and, consequently, the color of the beverage. At acidic pH this molecule shows an equilibrium between the different chemical forms which are shifted in favor of the flavinium cation which is red in color; that is, it absorbs more at wavelengths of 520 nm.[58] The increase in absorbance at 520 nm for beers for which the main fermentation was carried out with *L. thermotolerans* (experiment B) translates into a hyperchromic effect, since the intensity of absorption at this wavelength increases.[33,35] A priori, a clear relationship can be established between pH and color, which means that anthocyanins are more protected, and consequently, absorption is greater the lower is the pH of the sample, as has been observed in wine.[39]

4.1. Volatile Compounds. The balance of secondary metabolite production is biased toward non-*Saccharomyces* yeasts in contrast to the production of biomass and ethanol by *Saccharomyces* spp.[10,59] However, some metabolic products can act as undesirable volatiles when they exceed certain thresholds of perception,[60] such as methanol or diacetyl. Among the different categories in which metabolites are grouped are higher alcohols, esters, and carbonyl compounds.

From the main fermentation, acetaldehyde was highlighted in those beers that were made with *S. cerevisiae*. This is a compound associated with the apple and green leaf descriptor and is a direct product of alcoholic fermentation under anaerobic conditions (transformation of sugars into pyruvic acid, decarboxylation into acetaldehyde, and reduction into ethanol).[43] Also noteworthy is the concentration of diacetyl in both yeasts, which is much higher than the established perception threshold and could give a buttery taste, linked to rancidity notes in the mouth.[61]

After 4 weeks of bottle conditioning, acetaldehyde concentrations were reduced, which would explain the increase in alcoholic strength compared to the main fermentation.[30,43] A reduction in the concentration of diacetyl and acetoin also was observed, which is reflected in the increase of 2−3-butanediol, which is the last product of the same biosynthetic pathway.[30,43] This is in line with the reduction of diacetyl as one of the objectives of bottle aging.[10] Ethyl butyrate appears de novo for two of the samples starting from the main fermentation with *L. thermotolerans* (experiment B), namely for Lt→Lt and Lt→Sp.[62,63] As reported in previous studies it is an aromatic ester associated with the descriptor pineapple. Moreover, 2-phenylethyl acetate is particularly superior for samples conditioned with the yeast *H. vineae*, with respect to the other yeasts, as demonstrated in previous studies in beer.[18,22]

After 8 weeks of bottle conditioning, the decrease of acetaldehyde continued in favor of an increase of ethanol from acetaldehyde,[30,43] while the persistent concentration of methanol could be responsible for solvent aromas. Again, the decrease of carbonyl compounds (diacetyl and acetoin) in all beers analyzed could be justified in favor of the increase of 2,3-butanediol.[30,43] The biosynthetic pathway in which diacetyl and acetoin are produced concludes with the dehydrogenation of the second molecule into 2−3-butanediol.[55] It could be expected that the characteristics of the green beer produced with *L. thermotolerans* (experiment B) would favor the dehydrogenation of more or less acetoin to 2−3-butanediol, but further in-depth studies would be necessary to reach a conclusion.

Among the esters, it should be noted that unfortunately, the concentration of 2-phenylethyl acetate (descriptor roses, honey, apple, sweet) was reduced for all the samples analyzed, although the highest values are associated with the sequential fermentations of experiment B (*L. thermotolerans*) and, in particular, with Lt→Sc, Lt→Lt, and Lt→Hv.[23] Finally, the generation of ethyl lactate (descriptor cheese, fruit) could be due to the reaction of L-lactic acid with ethanol, which can be clearly justified for all samples starting from a fermentation with *L. thermotolerans* (experiment B).[21] In fact, this metabolite in high concentrations is synonymous with sour style beers.[64] Finally, it is noteworthy that the concentration of esters when using *L. thermotolerans* and *S. pombe* for bottle conditioning is higher after 8 weeks than after 4 weeks.[12,18] It is essential to bear in mind that the production of volatile esters is tremendously complex and difficult to modulate, because numerous factors such as the availability of nutrients and the yeast metabolism itself are key to the generation of these compounds that will confer fruity aromas.[44,65]

4.2. Sensory Analysis: Acidity, Beer Color, and pH. After the sensory analyses, a clear relationship can be established between the attributes "acidity" and "beer color", the highest scores of which were found in the beers whose main fermentation was carried out with *L. thermotolerans*. This conclusion is in agreement with the instrumental analyses presented above, since *L. thermotolerans* by its acidifying metabolism generates a high production of lactic acid, which consequently lowers the pH and favors the higher absorption at 520 nm (red color).[58] Furthermore, in the first sensory evaluation, the banana attribute could be related to the concentration of esters such as isoamyl acetate and isobutyl acetate, and even higher alcohols such as 2- and 3-methyl-1-butanol.[66−70] Whereas, in the second sensory evaluation, the aromatic quality can be justified because it corresponds to the high concentration of higher alcohols present in most yeasts, as observed in instrumental analyses. Besides, the application of *H. vineae* for bottle conditioning is shown to be beneficial with respect to the attributes "overall perception", "visual effervescence" and "aromatic quality". In particular the last attribute could be related to the high production of 2-phenylethyl acetate.[22,23]

4.3. Correlations between Parameters and Attributes. After 4 weeks of bottle conditioning, there is a positive correlation between beer color and L-lactic acid, which is behind the acidification of the beer. Linked to this is the negative correlation between pH and beer color, for the same reasons. Thus, samples with lower pH received higher color scores, that is, redder colors. Another notable negative correlation was between sweetness and acetaldehyde, as acetaldehyde is a volatile compound associated with apple and leafy greens that is characterized by its acidity and can mask sweetness in high concentrations. After 8 weeks of bottle conditioning, some results of the previous matrix were repeated, such as the positive correlation between color and L-lactic acid, together with the negative correlation between pH and beer color. Furthermore, a positive correlation appears between acidity and L-lactic acid related to the fermentative metabolism of *L. thermotolerans*.[18,19] With regard to the negative correlations, the one between bitterness and glycerol stands out, since the higher is the concentration of glycerol, the less bitterness the beer evaluated

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has, because this polyol contributes to the softening of the sensory profile of the beer, providing smoothness and a slight sweetness.[5] However, not all correlations established with significant differences can be explained by the analyses performed. Further instrumental and sensory analyses are needed to clarify these relationships between parameters and attributes.

5. CONCLUSIONS

It has been demonstrated how the use of non-*Saccharomyces* yeasts is a useful biotool for modulating the sensory profile of beers with respect to different parameters (pH, glycerol concentration, alcohol content, and even secondary metabolites) from the same beer wort (Pilsen malt and *Nugget* pellet hops). In particular, the use of *L. thermotolerans* makes it possible to obtain high concentrations of L-lactic acid and, consequently, of the secondary metabolite ethyl lactate. Therefore, the use of this yeast in the early stages of the process is postulated as an interesting alternative for the acidification of beer without using lactic acid bacteria (BAL), in order to formulate sour beers as suggested by previous studies. Moreover, in the case of *H. vineae*, the production of 2-phenylethyl acetate stands out, which has a positive impact on the aromatic quality, as well as its inability to increase the alcohol content, so it could be postulated as a key yeast for the production of NABLAB beers. On the other hand, *S. pombe* stands out for reaching the highest ethanol concentrations in the present experiment due to its high fermentative power. As for the color, aging caused it to lose some color except for the one fermented with *L. thermotolerans*. Therefore, although the potential of biotechnology in craft beer brewing has been demonstrated, more experiments with this type of matrix and these yeasts are needed for a deeper understanding of their behavior and desired organoleptic characteristics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c01035.

Characterization of the milled malt; parameters during the wort brewing process; perception thresholds; correlation matrix between instrumental and sensory analysis (4 and 8 weeks) (PDF)

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R.P.-P., experimental work; R.P.-P., C.V., M.J.C., and A.M., literature review, writing, and editing; A.M., C.V., and R.P.-P., image design; M.J.C., critical reading; A.M. and C.V., conceptualization and experimental design. All authors have read and agreed to the published version of the manuscript.

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Notes

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