CHAPTER 10. APPENDIX

10.1. Article 6.

Cabernet Sauvignon Red Must Processing by UHPH to Produce Wine Without SO_2: the Colloidal Structure, Microbial and Oxidation Control, Colour Protection and Sensory Quality of the Wine

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Abstract:

A cryo-macerated must of *V. vinifera* L. cabernet sauvignon was processed by ultra-high pressure homogenisation (UHPH) sterilisation without the use of SO_2. The UHPH treatment of the must was carried out continuously at a pressure of 300 MPa and reaching a maximum temperature of 77 °C for less than 0.2s. The colloidal structure of the UHPH must was evaluated by atomic force microscopy (AFM) measuring an average particle size of 457nm. The initial microbial load was 4-log CFU/mL (yeast), 3-log CFU/mL (bacteria). No yeast and non-sporulating bacteria were detected in 1mL and 10mL of the UHPH-treated must, respectively. Furthermore, no fermentative activity was detected in the non-inoculated UHPH-treated musts for more than 50 days. A strong inactivation of the oxidative enzymes was observed, with lower oxidation (≈x3) than controls. The antioxidant activity of the UHPH-treated must was much higher (106%) than that of the control must. UHPH had a protective effect in total anthocyanins, and especially in acylated anthocyanins (+9.3%); furthermore, the fermentation produces fewer higher alcohol (-44,3%) and more 2-phenylethyl acetate (+63%).

Keywords: Ultra-High Pressure Homogenisation (UHPH) sterilisation, microbial control, cabernet sauvignon, wine colour, SO_2, oxidation

1. Introduction

Elimination of SO_2 is currently a topic of great interest in oenology, and the use of ultra-high-pressure homogenisation (UHPH) to eliminate oxidative enzymes and wild microorganisms is a powerful tool to reach this objective. UHPH is the continuous pressurisation at higher than 200 MPa of a fluid and the instantaneous release at atmospheric pressure (or low pressure) across a specially designed valve. UHPH has an ultrashort in-valve time (<0.2 seconds) which helps to protect the nutritional and sensory quality, even when high temperatures can be reached in the valve. Several recent reviews have included the most important features and applications of UHPH processing (Zamora & Guamis, 2015; Patrignani & Lanciotti, 2016; Comuzzo & Calligaris, 2019; Morata & Guamis, 2020). UHPH is more efficient than high-pressure

homogenisation (HPH) or microfluidisation concerning the elimination of microorganisms and enzyme inactivation, which normally needs multi-passes for efficient microbial control (Szczepańska et al., 2021). Additionally, HPH or microfluidisation are used to improve colloidal stability (Leite et al., 2016; Oliete et al., 2019); however, UHPH produces nanofragmentation, with lower than average size particles stabilising better colloidal structure (Morata & Guamis, 2020). This is because the higher pressure (300 MPa) used in UHPH increases collision effects in valves compared with HPH or microfluidisation (<200 MPa).

The antimicrobial effect is produced by the intense impact and extreme shear efforts in colloidal structures and microorganisms during the in-valve depressurisation, which produces the nanofragmentation of cells and particles (Bañuelos et al., 2020; Morata & Guamis, 2020). Concerning the antimicrobial effect, it is highly effective, destroying yeast and bacteria in the grape musts (Loira et al., 2018) and, depending on the in-valve temperature, sporulated bacteria (Bañuelos et al., 2020). Compared with discontinuous high hydrostatic pressure (HHP), it shows a more intense antimicrobial effect in grape must; even in intense HHP treatments at 550 MPa-10min, residual bacterial cells are detected (Morata et al., 2015). The intense microbial inactivation by HHP and UHPH technologies facilitates the use of new wine biotechnologies, such as non-*Saccharomyces* yeasts and yeast-bacteria co-inoculations, providing better implantation of fewer competitive starters (Bañuelos et al., 2016).

As with HHP, the continuous UHPH produces a gentle effect on fruit juices, with a low impact in colour (Patrignani et al., 2019), and delicate aroma compounds such as terpenes (Bañuelos et al., 2020), protecting, therefore, the sensory quality. A short processing time produces a low number or an absence of thermal markers. No HMF has been detected in musts processed by UHPH, and neither has 5-methyl furfural (Suárez-Jacobo et al., 2012; Bañuelos et al., 2020).

Discontinuous HHP technologies used in batch processes in grapes can control yeasts and moulds, but can also increase the extraction of phenols (Corrales et al., 2009; Morata et al., 2015). Even when continuous UHPH must be applied in liquids, the intense nanofragmentation of colloidal particles from fruit cells has proven to have positive effects on the extraction of phenolic compounds, thereby increasing their availability

and the antioxidant capacity of juices (Suárez-Jacobo et al., 2012; Loira et al., 2018; Patrignani et al., 2019; Bañuelos et al., 2020).

HHP has a limited effect on the inactivation of oxidative enzymes such as polyphenol oxidases (PPOs) and, frequently, its effectiveness depends on the use of simultaneous thermal treatments (Buckow et al., 2009; Sulaiman et al., 2015), even though higher browning effects can be observed when used with high pressure levels (Martínez-Hernández et al., 2019). Conversely, UHPH has proven to cause intense inactivation of PPOs, with the consequent improvement of the antioxidant capacity in fruit juices (Velázquez-Estrada et al., 2013; Loira et al., 2018; Patrignani et al., 2019; Bañuelos et al., 2020).

UHPH technology is available at an industrial scale reaching 50.000 L/h based on modular systems. UHPH pumps that can work at 300 MPa are available to process at 10.000 L/h (Ypsicon, 2018). The technology was approved by the International Organization of Vine and Wine (OIV) as an authorised practice in oenology in 2020 (OENO-MICRO 16–594B, OIV, 2020) and has been approved by the EU (European Commission, 2020).

This work aims to evaluate the effectiveness of UHPH in the production of red wine in controlling wild microorganisms and the oxidative processes, as well as analyse the repercussions in the colloidal structure, fermentation, colour, aroma, and sensory profile of the wine. The effect of UHPH on colour, stability of anthocyanin pigments, inhibition of polyphenol oxidases (PPO) monitoring colour hue, and colloidal average size using AFM has been studied and assessed.

2.Materials and Methods

2.1. Must preparation

The must used in this study was obtained from destemmed *Vitis vinifera* L. cabernet sauvignon grapes collected in the *Costers del Segre* region (Lérida, Spain), which were crushed and added to 3 g/HL of pectolytic enzymes (Rapidase™, Oenobrands, Montpellier, France) and then left to macerate at 0 °C for 15 days. This process is

necessary to reach enough tannins and anthocyanins in order to produce red wines in the absence of skin contact. UHPH technology can only be applied to liquids with colloidal size below 0.5 mm. After this maceration of the grapes, the must was separated using a pneumatic press, then settled at 8 °C and kept in an inert CO_2 atmosphere until the start of the experiment. Half of this clean must was used as a control treatment and the other half was treated by UHPH. The must processing by UHPH was carried out in a continuous operating mode using equipment including an improved tungsten carbide valve patented by the company Ypsicon Advanced Technologies (Barcelona, Spain; patent number: EP2409583B1). The working flow rate was 60 L/h at 300 ± 3 MPa, with an inlet temperature of 4 °C, an in-valve temperature of 78 ± 2 °C for less than 0.2s (measured by a sensor placed immediately downstream of the valve), and an outlet temperature of 15 °C (Figure 1). At the outlet of the valve, the UHPH-treated must was cooled in a heat exchanger through which water was circulating at 3 °C. The total volume of must processed by UHPH was 100 L.

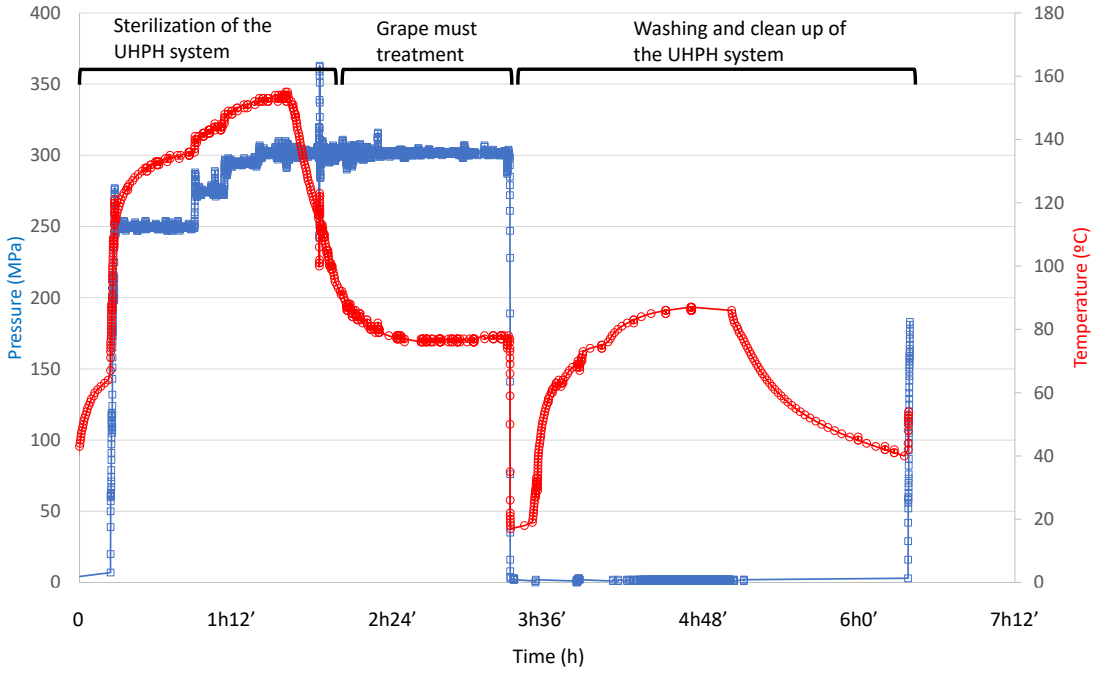

Figure 1. Pressure (MPa): blue dots; in-valve temperature (°C): red dots.

2.2. Optical and atomic force microscopy (AFM)

Samples of control and UHPH musts were observed and photographed using an optical microscope at 630x (63x objective) using a trinocular Leitz Diaplan microscope and a Jenoptik Gryphax digital camera. For AFM measurements, samples were prepared by freezing 10 µL drops of the control and UHPH-processed musts on a coverslip in a CO_2 freezer at 80 °C.

The samples were then lyophilised. Topographic measurements of the cells were carried out using a Nano-Observer AFM (Concept Scientific Instruments, Les ULIS, France) operating in resonant mode. A 1 N/m rectangular silicon cantilever (model Fort, AppNano, Mountain View, CA, USA) with an 8 nm nominal tip radius was selected. Typical setpoint amplitudes of 4–5 volts were used during the measurements, with high values of feedback of proportional and integral gains (P and I) to compensate for the high topographic variations (1–4 microns).

2.3. Microbial counts

Microbiological counts were performed on both control and UHPH-treated must to verify the antimicrobial effectiveness of the non-thermal processing technique and assess the degree of implantation of the inoculated starters. Vegetative forms were assessed by growth on plates with selective media. In the case of total aerobic bacteria and lactic acid bacteria, 1 mL of serial decimal dilutions in saline peptone (0.85% NaCl and 0.1% peptone) were pour-plated into PCA supplemented with nystatin (50 mg/L) and into MRS agar (Pronadisa, Barcelona, Spain) and MLO agar (Pronadisa, Barcelona, Spain) supplemented with nystatin (50 mg/L), respectively. The inoculated plates were incubated for six days at 30 °C until colony growth, maintaining anaerobic conditions for lactic acid bacteria by placing the plates in a jar with CO_2 atmosphere. For yeast counts, 100 µL or 1 mL were spread-plated into glucose chloramphenicol agar (GCA; Pronadisa, Barcelona, Spain) for total yeast count and into synthetic lysine agar (Oxoid, Hampshire, UK) for non-*Saccharomyces* counts. These plates were incubated aerobically at 25 °C for four and six days, respectively. Aerobic bacterial endospores were analysed from 10 mL of must pasteurised at 80 °C for 30 min (to remove vegetative forms), which was cooled

down and filtered through 0.45 µm membrane filters (Millipore) and surface incubated on PCA plates for 6 days at 30 °C.

2.4. Fermentations

Fermentations were carried out at 20 °C and assayed in triplicate in 1 L flasks filled with must at 90% capacity, because the UHPH system produces two 2 L bags in conditions of full sterility. The fermentation temperature was set at 20 °C because it is suitable for young red wines and avoids excessive aroma losses during fermentation. All fermentation flasks were inoculated with 20 mL of *Saccharomyces cerevisiae* 7VA starter (enotecUPM, Madrid, Spain) pre-cultured for 24h in YPD broth (Conda, Madrid, Spain), containing 5×10^7 CFU/mL (checked by plating).

A parallel assay was performed in triplicate in 100 mL vials to further confirm the antimicrobial power of the UHPH technique. These vials were filled with 60 mL of must, sealed with Müller valves and left to ferment at 20 °C with the native population (without microbial inoculation). The weight loss in the vials was recorded daily to monitor fermentation.

2.5. Oenological parameters by infrared spectroscopy

Oenological parameters were evaluated by Fourier Transform Infrared Spectroscopy (FTIR) with OenoFoss™ equipment (FOSS, Barcelona, Spain).

2.6. Determination of Polyphenol Oxidase (PPO) enzymatic activity

PPO activity was evaluated spectrophotometrically (Agilent Technologies™ 8453, Palo Alto, CA, USA) by measuring the ratio of absorbances 420/520 nm at a path length of 1 mm and with a high aeration surface of 1 cm^2/mL at room temperature. The ratio between absorbance at 420 nm (yellow) and 520 nm (red) is an estimation of the browning processes, which, over a short period of time and exposure to air, is due to enzymatic oxidation.

2.7. Antioxidant capacity (ABTS method)

The transformation in the colourless form of the cationic $ABTS^{\bullet+}$ [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical cation] by phenols and other compounds was used to evaluate the antioxidant activity of musts (Re et al., 1999). Samples of 50 µL of diluted wines were analysed at four concentrations in duplicate.

2.8. Colour parameters and total phenol content

The colour intensity and hue were analysed according to the Glories method (1984), spectrophotometrically with a device 8453 Agilent Technologies™ (Palo Alto, CA, USA). Total phenols were also analysed in the same spectrophotometer at 280 nm in a 1 mm quartz cuvette (Ribéreau-Gayon et al., 1980).

2.9. Anthocyanins by LC-DAD

Must and wine anthocyanins and pyranoanthocyanins were analysed using an LC-DAD chromatograph system (Agilent Technologies 1260, Palo Alto, CA) according to Bañuelos et al. (2016). Concentrations were calculated with a calibration curve of malvidin-3-O-glucoside (r^2 = 0.9999, LOD = 0.1 mg/L) and an injection volume of 50 µL.

2.10. Analysis of volatile fermentative compounds by gas chromatography with flame ionisation detection (GC-FID)

Fermentative aroma compounds were analysed by GC-FID using an Agilent Technologies 6850 GC (Network GC System) in a DB-624 column, according to Bañuelos et al. (2020). One µL with 10% internal standard (4-methyl-2-pentanol at 500 mg/L) and prefiltered at 0.45 µm was injected. External standards calibration (r2 > 0.999) was performed using compounds from Fluka (Buchs SG, Switzerland, GC quality > 98%).

2.11. Sensory evaluation

The sensory quality of the wines was evaluated by means of a blind attribute difference test, following a procedure similar to that described by Loira et al. (2018). The tasting panel consisted of nine expert tasters from the Department of Food Chemistry and Technology of the Universidad Politécnica de Madrid (age range: 30 to 60 years old, four women and five men). The wines were served randomly at a temperature of 20 ± 2 °C in two standard tasting glasses. After reaching a general consensus on the description of the attributes to be evaluated, the panellists used a scale from 0 to 5 to rate the intensity of the attributes, the lowest rating being considered as a "not perceived attribute" and the highest rating as a "strongly perceived attribute". Finally, the global perception of the wines was also evaluated, taking into account visual, olfactory, and taste characteristics. Significant differences between samples were determined by ANOVA analysis ($p < 0.05$).

2.12. Statistical analysis

Statistical analyses were performed with PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). The significance level was 5%.

3.Results and Discussion

3.1. Colloidal and molecular structures produced by UHPH

Must processed by UHPH is affected by ultra-high impact forces, and shear efforts strongly affect the nature of the molecular structure of the colloidal particles. The effect is a nanofragmentation of the suspended particles in the must, producing higher colloidal stability. It was observed that, after centrifugation, the colloidal particles appeared intensively dyed in the control (Figure 2A, left) but only slightly in the must processed by UHPH (Figure 2A, right), probably because the smaller average size reduces the adsorbent capacity of the anthocyanins. This could have an impact on colour stability. The optical microscopy of the control must without UHPH treatment showed a colloidal structure with large fragments (several micrometres) dyed by anthocyanins (Figure 2B). Fibres of vegetal structures could be observed, as could other cell wall

fragments from the vegetal cells of the grapes. The optical image of the UHPH must showed smaller particles in the submicron range and, again, less colour (Figure 2C). Additionally, more tartrate crystals could be observed, probably because of their formation in the intense depressurisation after the UHPH valve. Using high-resolution AFM, we characterised and measured the size of the fragments of a dry drop of must by topographic scanning in resonant mode. A flat surface could be observed in the control, with many polyhedric fragments spread across the entire surface (Figure 2D). A thinner mass with fewer and smaller polyhedric structures could be observed in the UHPH-processed must (Figure 2D).

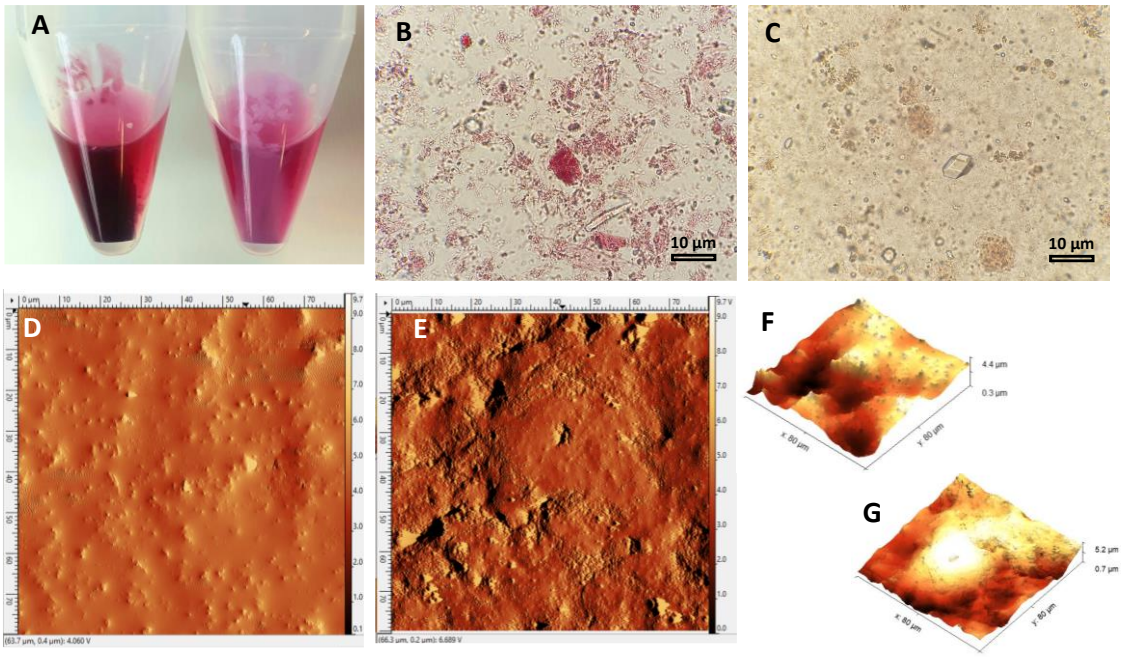

Figure 2. External appearance of the control and UHPH-processed must after centrifugation **(A)**, optical microscopy (600x) of a centrifuged drop of cabernet sauvignon must **(B)**, optical microscopy (600x) of the UHPH centrifuged must **(C)**, AFM scanning in resonant mode of a drop of dried control must **(D)**, and the UHPH dried must **(E)**. Topographic renders of the 3D external topography in the control **(F)** and UHPH dried drops **(G)**

In a previous study, the size of the colloids in the UHPH must was estimated by laser diffraction (Bañuelos et al., 2020), and it was observed that most particles were in the range of 100–400 nm. In the current study, the size of the fragments was measured by AFM microscopy, obtaining an average size of 1,342 ± 464 nm in the control must (range 824–3,180 nm Figure 3A) and 457 ± 140 nm in the UHPH must (range 235–744 Figure 3B), which are relatively close to our previous results, and showing fragments in the

UHPH product higher than 200 nm without nano-safety impact (which must be considered when the size is <100 nm).

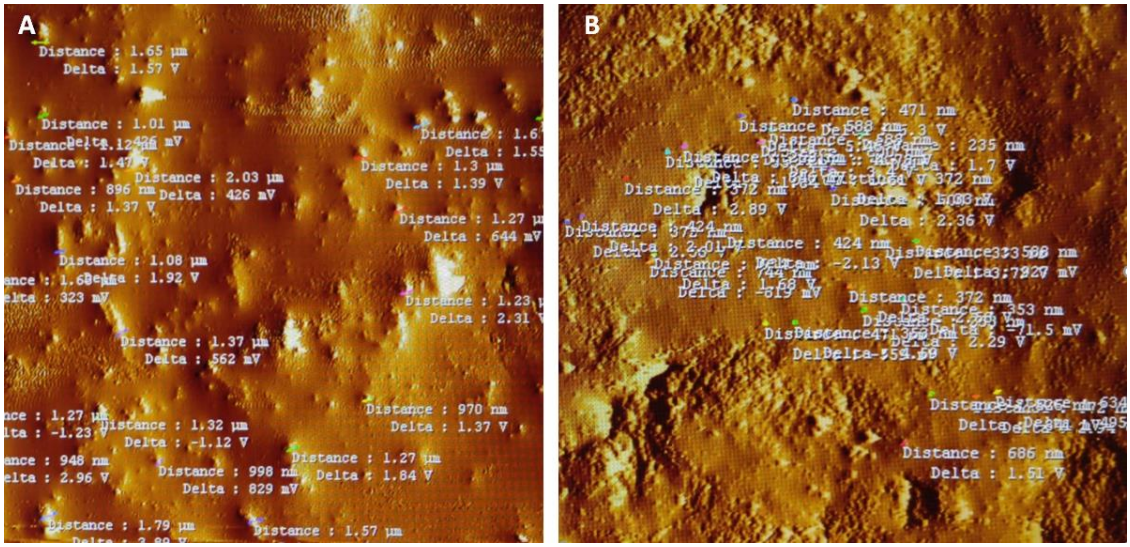

Figure 3. Measurement of colloidal fragments by AFM in a drop of dried control must **(A)** and in the UHPH dried must **(B)**

3.2. Microbial loads

In the control must, *Saccharomyces cerevisiae* counts were $8x10^3$ CFU/mL and non-*Saccharomyces* were $1.2x10^4$ CFU/mL; therefore, they were globally approximately 4-log CFU/mL, which is the typical value in healthy grapes (Fleet, 2003) (Figure 4A). In the UHPH-processed musts, neither *Saccharomyces* nor non-*Saccharomyces* yeasts were detected in 1 mL. This highly antimicrobial effectivity of UHPH follows previous works regarding grape musts (Loira et al., 2018; Bevilacqua et al., 2018; Bañuelos et al., 2020) and other juices (Bevilacqua et al., 2012; Calligaris et al., 2012; Patrignani et al., 2019 and 2020). Initial bacterial counts were $1.7x10^3$ CFU/mL in the control must and undetected in the UHPH. Sporulated bacteria in 10 mL were four viable cells, undetected in UHPH musts. This result supports the high efficiency of UHPH controlling bacteria in grape juices compared with discontinuous pressurisation techniques, such as HHP, which is unable to eliminate them completely, even at pressures higher than 500 MPa/10 min (Morata et al., 2015). Also, UHPH is much more efficient than HPH, which normally needs multi-passes to reach inactivation lower than 2-log at 200 MPa (Szczepańska et al., 2021). *Saccharomyces* yeast starters were inoculated in both the

control and UHPH musts at 5×10^7CFU/mL, and the population of *Saccharomyces* and non-*Saccharomyces* yeasts was monitored every two days. It could be observed that non-*Saccharomyces* were undetected in the UHPH fermenters during the entire fermentation; however, they remained above or close to 4-log in the control fermentations (Figure 4A) until day 7, when the ethanol was higher than 9 % v/v (see Figure 5A). The *Saccharomyces* populations were a little lower in the UHPH than in the control fermentations (but significant, $p < 0.05$) because the inoculated strain was the only one present.

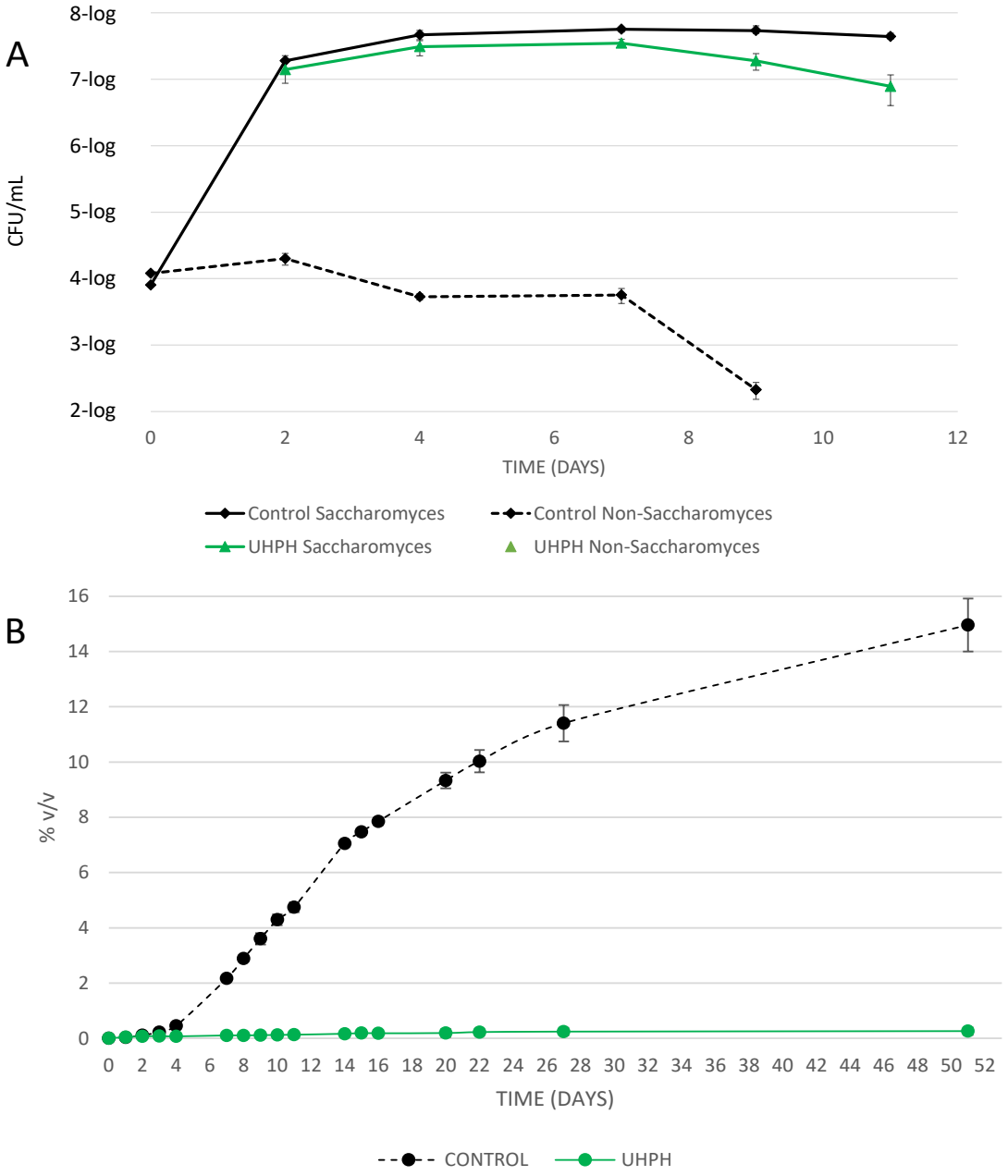

Figure 4. (A) *Saccharomyces* yeast counts for the control (continuous black line) and UHPH treatments (continuous green line) during fermentation in 1 L flasks. Non-*Saccharomyces* counts for control (dashed

black line) and UHPH treatments (undetected). **(B)** Evolution of fermentation in non-inoculated control and UHPH-processed musts by ethanol content calculated from the CO_2 losses in the 100 mL fermenters. All the values are means and standard deviations of three independent fermentations.

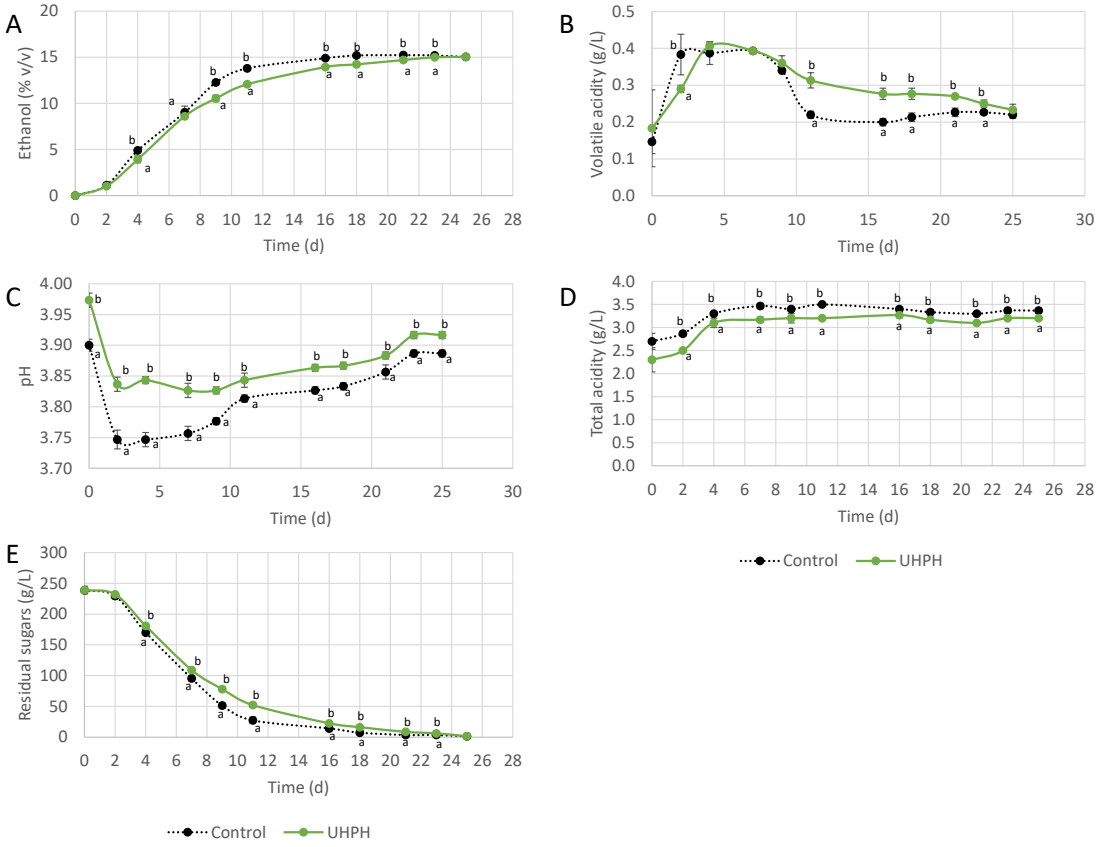

Figure 5. Evolution during fermentation of ethanol in % v/v **(A)**, volatile acidity expressed in g/L of acetic acid **(B)**, pH **(C)**, total acidity expressed in g/L of tartaric acid **(D)**, and residual sugars **(E)**. Values are means and standard deviations of three independent fermentations.

Additionally, triplicate samples of the UHPH and control musts were left to evolve without inoculation, and the fermentation was completed by the wild yeast population in the control musts in 50 days at 20 °C (Figure 4B). The control wines reached 15 % v/v in ethanol. The long duration was due to the high initial sugar content and, probably, due to the unsuitable fermentative ability of the wild yeast population. Fermentation was undetected in the uninoculated UHPH controls for longer than 50 days (Figure 4B). The absence of fermentative activity in the long-term in the UHPH musts ensured the total elimination of yeasts, especially the damaged non-culturable cells that are sometimes observed in treatments by discontinuous HHP (Lado & Yousef, 2002).

Full elimination of fermentative yeasts allows the use of new fermentation biotechnologies, as the use of non-*Saccharomyces* yeasts and the yeast-bacteria allow

co-inoculation to perform simultaneous alcoholic and malolactic fermentation (Bañuelos et al., 2016, Morata et al., 2017, Vaquero et al., 2021). In the UHPH must, there was full implantation of the inoculated yeast starter without any impact on the wild yeast or bacteria, which were completely eliminated. Therefore, UHPH helps to reduce the use of chemical additives as sulphites (Morata et al., 2017; Morata & Guamis., 2020; Christofi et al., 2020) and can help to delay fermentation processes at the winery, scheduling them at appropriate dates, favouring a better distribution of work and allowing non-stational wine production.

3.3. Oenological parameters in grape must. Effect of UHPH processing on must composition

The general analysis of the standard oenological parameters was done by means of FTIR spectroscopy (Figure 5). Fermentations proceed at a regular rate, reaching the highest potential alcoholic degree (15 % v/v) in 25 days (Figure 5A). The UHPH fermentations were slower than the controls (statistically significant, $p < 0.05$), but it must be considered that they were carried out in small volumes of 0.9 L and at 20 °C. Volatile acidity was controlled throughout all the fermentations at values close to or below 0.4 g/L, finishing in both the controls and UHPH at less than 0.25 g/L, without significant differences ($p>0.05$), which is an optimal value for wine fermentation (Figure 5B).

The pH was slightly higher in the UHPH musts, which is normal because of the higher extraction of salts and cations by the fragmentation of solid colloids (Figure 5C). This has also been previously observed (Loira et al., 2018). However, the final values after fermentation did not have an oenological impact: pH 3.92 in the UHPH wines and 3.89 in the controls, although they were significant ($p<0.05$). As a result of the pH values, acidity was somewhat higher in the control (3.37 g/L) than in the UHPH wines (3.20 g/L) but, again, without oenological impact (Figure 5D). Finally, both wines finished dry, with residual sugars of <1.6 g/L (Figure 5E).

3.4. Antioxidant power and control of oxidative enzymes by UHPH

Oxidative damage by polyphenol oxidases (PPO) affects wine quality, producing detrimental colour and aroma quality (Hendrickx et al., 1998). The oxidation effect was monitored by the evolution of the hue (Figure 6). The air exposure was significantly high (1 cm^2/mL in cuvettes at room temperature) and produced an evolution to red-brown colour in the controls, while better colour stability was observed in the UHPH musts (Figure 6). The control musts had a higher hue than the UHPH musts ($p < 0.05$). Using polynomic regression and future extrapolation, a high correlation could be observed (R^2 >0.99) with the measured values. After six days of exposure to air, the hue in the controls had noticeably increased (≈x6), and in the UHPH musts, it remained at lower values (≈x2). Therefore, the UHPH musts were much more stable, even with high aeration, at room temperature, and without SO_2, which is in accordance with the findings reported in previous works (Suárez-Jacobo et al., 2012; Loira et al., 2018; Bañuelos et al., 2020). The powerful inactivation of PPO enzymes (≈90%, Loira et al., 2018) is an advantage of UHPH compared with discontinuous HHP, in which unclear enzyme inactivation is observed (Buckow et al., 2009), or HPH with inactivation not higher than 20% (Szczepańska et al., 2021). The antimicrobial, but also antioxidative, effect of UHPH opens the clear potential for using this technique to produce wines with low levels of SO_2, or without it.

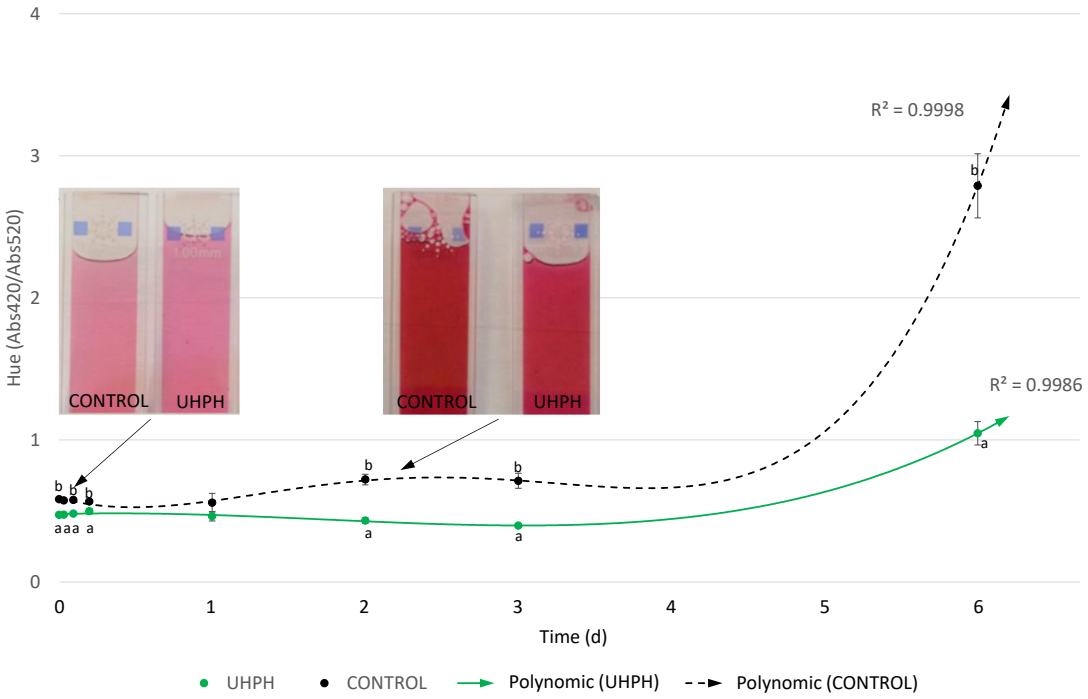

Figure 6. Evolution of the colour hue measured by the ratio of absorbances at 420nm (yellow) and 520nm (red). Oxidation was promoted by leaving the musts at room temperature in cuvettes with a surface exposure to air of 1 cm^2/mL. Values are means with standard deviations (SDs) of triplicate samples. Values were approximated and extrapolated by polynomic regressions with a high r^2 value (>0.99). The real colour in 1 mm cuvettes is also shown, at 45 min and 48 h.

Even when the initial antioxidant activity of the red musts was much higher compared to white musts processed in previous works (Loira et al., 2018; Bañuelos et al., 2020), due to the elevated content of polyphenols, the continuous treatment by UHPH at 300MPa produced a 6.5% higher antioxidant activity (Trolox equivalents) in comparison with the untreated red must (control). The observed value in the UHPH-processed must was 7242 µmol/L compared to 6797 µmol/L in the unprocessed must (with significant differences p<0.05), which is in accordance with previous research (Velázquez-Estrada et al., 2013; Loira et al.; 2018, Patrignani et al., 2019; Bañuelos et al., 2020). This increased antioxidant activity is related to the better preservation of the flavonoids and other polyphenols by PPO inactivation.

3.5. Polyphenol content and colour intensity

The UHPH-treated and control musts showed similar initial contents of polyphenols, which are better stabilised in UHPH fermentations and remain at higher values, with a

more stable trend at the end of fermentation (Figure 7A). The content of total polyphenols in the controls was significantly lower at the end of fermentation, probably because of the effect of the PPOs in the absence of SO_2. Usually, polyphenols show a typically increasing trend during red wine fermentation due to their better solubility in less polar solutions caused by the production of ethanol by yeasts (Morata et al., 2019). Conversely, anthocyanins and, therefore, colour show a decreasing trend because of their insolubilisation in higher ethanol contents. Previously, increased extraction of polyphenols by UHPH in kiwi juice has been observed (Patrignani et al., 2019). However, a reduction in phenolic compounds in mulberry juice treated by UHPH, but in different working conditions, in a multi-pass mode at 200 MPa, has also been found (Yu et al., 2014). Our results highlight the gentle but effective processing of grape juice in a single pass at 300 MPa.

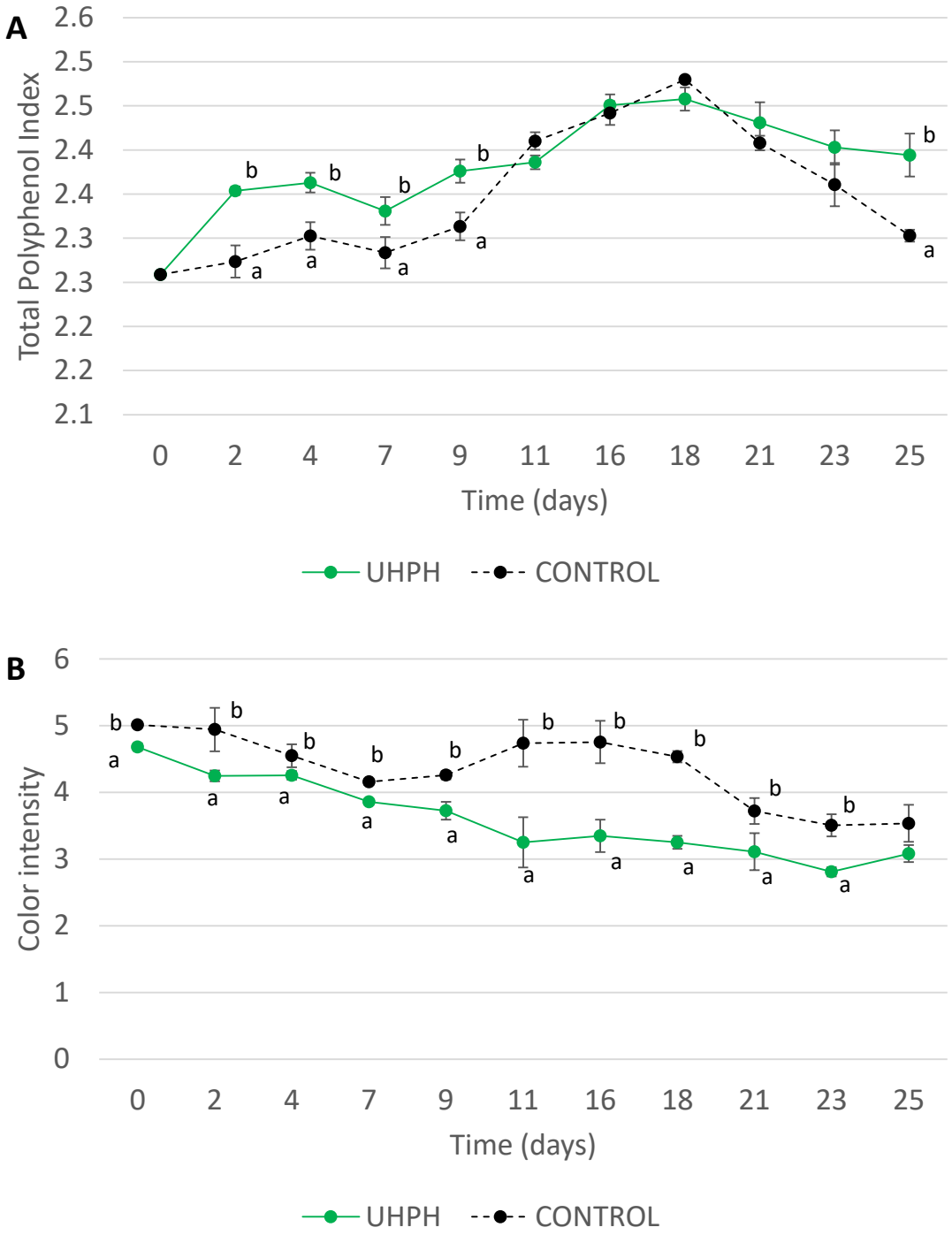

Figure 7. Evolution of polyphenols (TPI) measured at 280 nm during fermentation **(A)**, and changes in colour intensity during fermentation **(B)**. Values are means and standard deviations of three independent fermentations. Values with the same letter in the same row are not significantly different ($p < 0.05$).

Regarding colour intensity, as can be seen in Figure 7B, because of the effect of UHPH, the colour was slightly weaker in the UHPH musts. However, there was a suitable evolution during fermentation and, in the end, the results of both fermentations were without significant differences ($p<0.05$). Therefore, UHPH can be considered to be a

protective technique in both total polyphenols as well as colour intensity (Figure 7B) and appearance (Figure 6).

3.6. Effect of UHPH on anthocyanin content

The grape anthocyanin contents (monoglucosides and acylated derivatives), together with the pyranoanthocyanins formed during fermentation, were analysed using LC-DAD (Table 1). A slightly higher concentration of anthocyanins can be observed in the UHPH musts, especially due to the selective protection of the UHPH in the acylated derivatives (Table 1), which show a 9.3% increment. This difference can be clearly observed in the peak areas for these derivatives in the LC-DAD chromatograms (Figure 8). Acylated anthocyanins are especially interesting in wine colouration because they absorb at higher wavelengths compared to non-acylated ones (Mazza and Francis, 1995), producing bluish-red hues that are highly valued by consumers. The formation of stable pyranoanthocyanins during fermentation (Morata et al., 2019) was similar in the control and UHPH musts (Table 1). The similar contents indicate the protective effect on delicate pigments of this non-thermal technology, allowing the elimination of microorganisms and oxidative enzymes with a gentle effect on colour. This protective effect has been reported in other fruit pigments (Patrignani et al., 2019). The use of a single-pass 300 MPa UHPH process is more protective of colour than a multi-pass mode at 200 MPa (Yu et al., 2014).

Table 1. Anthocyanin contents (mg/L) in the control and UHPH-processed musts at the beginning (Day 0) and end of fermentation (Day 25). Values are means and standard deviations of three independent fermentations. Values with the same letter in the same row are not significantly different ($p < 0.05$).

Anthocyanins	Day 0		Day 25	
	Control	UHPH	Control	UHPH
D3G	11.7±0.3b	16.8±0.0a	0.5±0.0b	0.7±0.0a
C3G	2.3±0.0b	2.7±0.0a	0.1±0.0a	0.1±0.0a
Pt3G	11.2±0.1b	13.7±0.0a	1.0±0.3a	1.0±0.3a
Pn3G	12.1±0.0a	11.7±0.2b	3.8±0.3a	3.1±0.5a
M3G	255.3±0.3a	248.3±0.7b	39.6±0.4a	35.5±1.6b
monomeric non-acylated	**292.6±0.6a**	**293.3±0.6a**	**45.0±0.7a**	**40.3±2.4b**
Vit B	0.0±0.0	0.0±0.0	0.6±0.0a	0.5±0.0a
unknown (λ 495nm)	0.0±0.0	0.0±0.0	1.0±0.0a	0.8±0.1b
unknown (λ 496 nm)	0.0±0.0	0.0±0.0	0.2±0.0a	0.1±0.0b
Vitisin type	**0.0±0.0**	**0.0±0.0**	**1.8±0.0a**	**1.5±0.1b**
D3G-Ac	1.7±0.0a	1.1±0.0b	0.0±0.0b	0.1±0.0a
C3G-Ac	0.2 ±0.0b	0.3±0.0a	0.0±0.0	0.0±0.0
Pt3G-Ac	1.8±0.0b	2.3±0.0a	0.2±0.0b	0.3±0.0a
M3G-Ac	65.6±0.1b	71.1±0.3a	10.6±0.2a	11.2±0.4a
M3G-Cm cis	3.5±0.1a	3.5±0.0a	0.3±0.0a	0.3±0.0a
M3G-Cm trans	20.0±0.2b	23.1±0.1a	2.6±0.0b	3.3±0.1a
monomeric acylated	**92.8±0.3b**	**101.4±0.4a**	**13.8±0.2b**	**15.2±0.5a**
M3G-VPh	0.0±0.0	0.0±0.0	0.5±0.0b	0.6±0.0a
M3G-VG	0.0±0.0	0.0±0.0	0.1±0.0b	0.1±0.0a
unknown (λ 508 nm)	0.0±0.0	0.0±0.0	0.1±0.0b	0.2±0.0a
unknown (λ 512 nm)	0.0±0.0	0.0±0.0	0.1±0.0b	0.1±0.0a
Vinylphenol adducts	**0.0±0.0**	**0.0±0.0**	**0.7±0.0b**	**1.0±0.0a**
Pyranoanthocyanins	**0.0±0.0**	**0.0±0.0**	**2.5±0.0a**	**2.5±0.1a**
Total	**385.5±0.9b**	**394.6±1.0a**	**61.3±0.6a**	**58.0±3.0a**

D3G (delphinidin-3-O-G), C3G (cyanidin-3-O-G), Pt3G (petunidin-3-O-G), Pn3G (peonidin-3-O-G), M3G (malvidin-3-O-G), Vit B (M3G-acetaldehyde adduct), -Ac (-(6″-acetylglucoside)-), -Cm (-(6″-p-coumaroylglucoside)), -VPh (-4-vinylphenol adduct) and VG (-4-vinylguaiacol adduct)

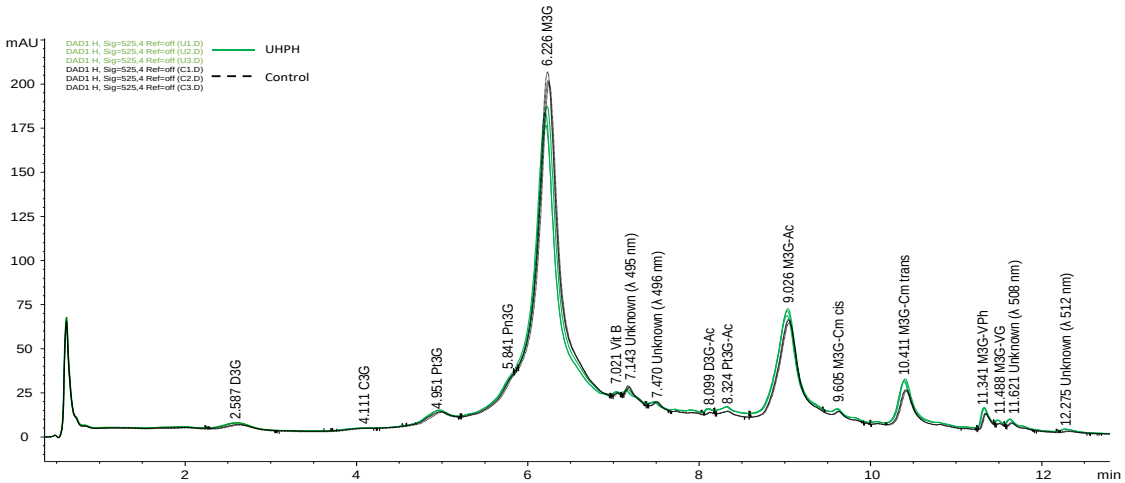

Figure 8. Anthocyanin LC-DAD chromatograms extracted at 525 nm at the end of fermentation in triplicate. Black lines: controls; green lines: UHPH.

The evolution of total anthocyanins and pyranoanthocyanins was monitored along with fermentation by LC-DAD, and similar trends in the insolubilisation of grape anthocyanins from controls and UHPH musts were observed (Figure 9). Additionally, the formation of pyranoanthocyanins (vinylphenolic and vitisins) during fermentation by yeast showed a similar tendency in the controls and the UHPH.

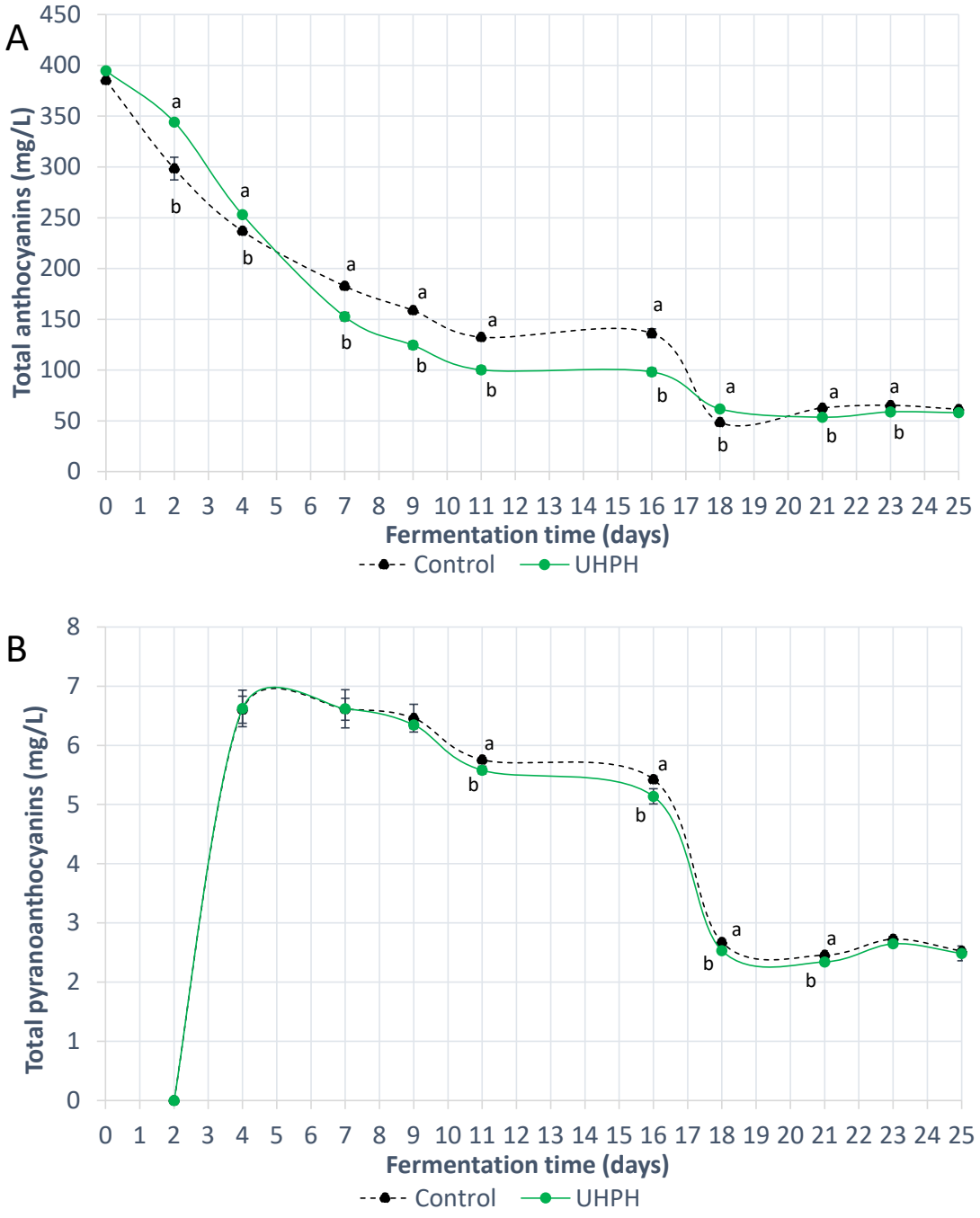

Figure 9. Evolution of total anthocyanin contents (mg/L) during fermentation **(A)**, and formation of pyranoanthocyanins **(B)**. Values are means and standard deviations of three independent fermentations. Values with the same letter in the same row are not significantly different ($p < 0.05$).

3.7. Effect of UHPH on fermentative aroma

The fermentative volatiles had a normal profile in the wines (Table 2). However, it should be noted that the controls had a much higher content of higher alcohols than the UHPH wines (+78%). Higher alcohols are responsible for the winey flat smell of low-quality wines when they are present at concentrations higher than 350-400 mg/L. In the fermentation, both trial samples were under these values, but the control was closer to the threshold, which can shade and hide other floral and fruity aromas (de-la-Fuente-Blanco et al., 2016). Additionally, at moderate concentrations, they have a low impact on the sensory profile of wines (Ferreira et al., 2002). Concerning acetaldehyde, both samples showed a concentration found in the normal range of wines (Liu & Pilone, 2000). Ethyl acetate is responsible for fruity scents at low concentrations, but solvent odour at high amounts. The sensory threshold was around 12.3 mg/L (Culleré et al., 2019). So, in the case of the UHPH, the odour activity value (OAV) was 2.2, while it was 3.2 in the controls, thus, probably expressing more solvent-glue aroma in the last ones.

Table 2. Fermentative volatiles (mg/L) in the control and UHPH-processed musts at the end of fermentation (Day 25). Values are means and standard deviations of three independent fermentations. Values with the same letter in the same row are not significantly different ($p < 0.05$).

	Control	**UHPH**
acetaldehyde	19.09±0.28a	16.02±2.88a
methanol	144.31±6.15a	105.04±17.47b
1-propanol	17.37±0.90a	15.00±3.82a
diacetyl	1.80±0.11a	1.16±1.03a
ethyl acetate	42.10±1.52a	26.82±5.20b
2-butanol	0.00±0.96a	0.00±2.01a
isobutanol	22.28±0.00a	15.94±0.00b
1-butanol	0.00±0.00a	2.76±2.39a
acetoin	5.88±0.64a	8.33±2.26a
2-methyl-1-butanol	48.67±7.53a	30.32±7.52b
3-methyl-1-butanol	183.15±10.77a	92.97±25.83b
isobutyl acetate	1.31±1.13a	0.50±0.87a
ethyl butyrate	1.49±0.19a	0.80±0.69a
ethyl lactate	15.99±5.62a	33.50±28.03a
2,3-butanediol	683.20±166.22a	684.02±93.03a
isoamyl acetate	3.07±0.09a	3.00±0.69a
hexanol	0.00±0.00a	1.14±1.98a
2-phenylethanol	73.53±8.32a	34.13±8.51b
2-phenylethyl acetate	5.29±0.04b	8.66±1.56a
Acetate esters	9.67±3.60a	12.16±27.52a
Ethyl esters	59.58±1.17a	61.12±1.65a
Total esters	69.25±4.62a	73.28±29.01a
Higher alcohols	344.99±25.84a	192.26±47.05b
Total volatiles	1,268.51±179.21a	1,080.12±148.61a

Acetate esters have a powerful impact on the sensory profile because they have fruity or floral descriptors and low sensory thresholds. In this research, they did not show significant differences at the global level. However, 2-phenylethyl acetate showed a significantly higher concentration in the UHPH wines compared to the controls (x1.6), both at higher sensory threshold values. 2-phenylethyl acetate is a key compound in wine fermentative aroma because of its floral impact with a rose petal descriptor (Morata et al., 2020). The high level of this ester in the UHPH wines could have been produced by the release of higher contents of the amino acid precursor from the grape cell fragments due to the intense impact forces. Higher contents of yeast-assimilable nitrogen were observed in the UHPH-processed musts (Loira et al., 2018).

3.8. Sensory evaluation

The sensory attribute evaluation of the wines exhibited similar profiles, but significant differences were found between the control and UHPH wines, corresponding to the attributes' global perception and aromatic quality (Figure 10). The better aromatic quality and the probable consequence of a better global perception can be correlated with the lower concentrations of higher alcohols and the higher content of 2-phenylethyl acetate, which is representative of the floral esters with a rose petal descriptor. This molecule was above its sensory threshold in both cases (0.25 mg/L, Carrau et al., 2008), but at 63% higher in the UHPH than in the control wines. Both parameters were better valued in the UHPH wines. Concerning colour intensity and hue, the controls had the worst average values, but without statistical significance.

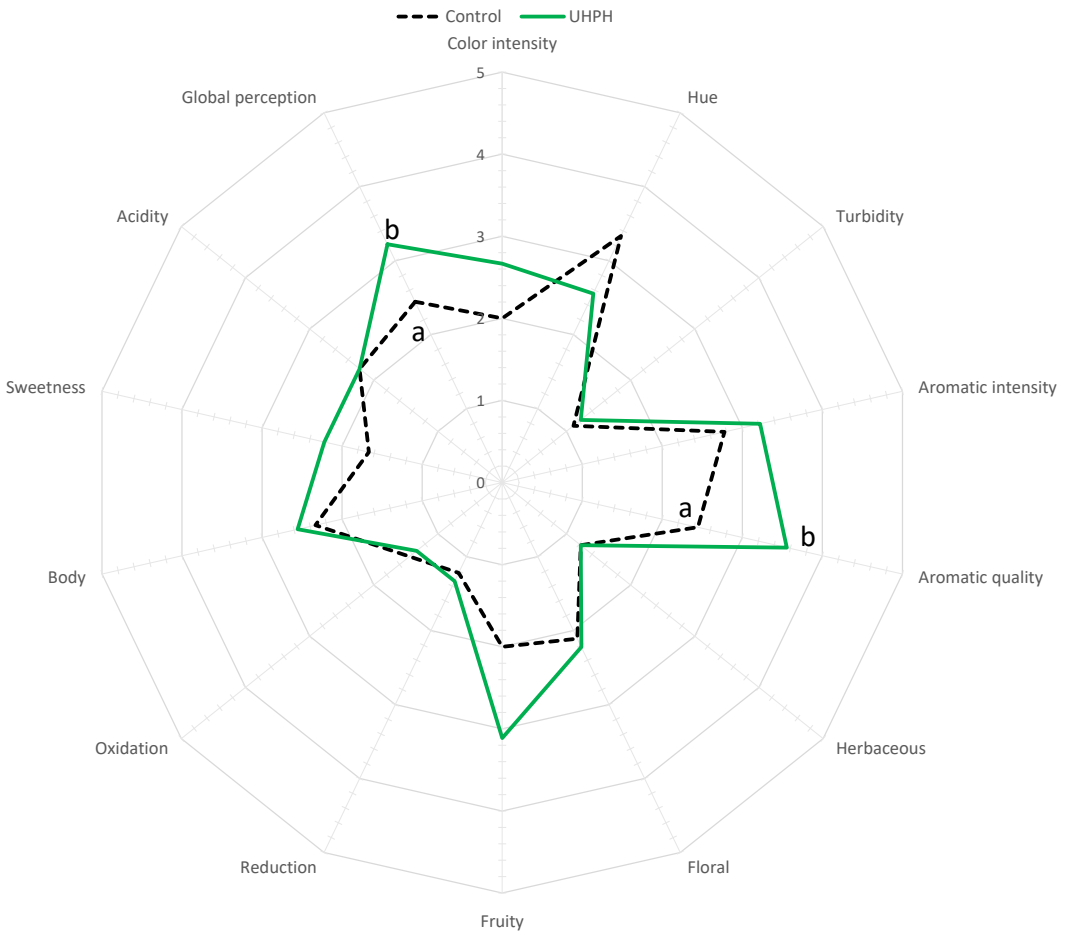

Figure 10. Sensory evaluation of wines: UHPH: green continuous line; control: black dashed line. Values are means and standard deviations of nine tasters. Values with the same letter in the same attribute are not significantly different ($p < 0.05$).

4. Conclusion

UHPH sterilisation eliminates wild microorganisms, avoiding spontaneous fermentation and favouring the implantation of yeast starters. The use of uncompetitive non-*Saccharomyces* yeasts can be strongly promoted, as can the use of yeast–bacteria co-inoculations. Additionally, the oxidative processes mediated by PPOs are inactivated following the denaturation of the enzyme structure by the impact and shearing forces during UHPH treatments. Colour is preserved, and oxidation is delayed because of the high air exposure in the absence of SO_2. The results confirm that UHPH is a powerful tool in the production of healthier and more stable red wines without sulphites or other chemical additives. The intense forces produced by UHPH produce a different colloidal structure, with a lower average particle size, which has an impact on wine stability.

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Data Availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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

10.2. Article 7.

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 thanks to the demand for new beverages and the consumer's willingness to pay higher prices. This article explore the sensorial changes produced in craft beers by using different *Saccharomyces* and non-*Saccharomyces* yeasts with several instrumental and sensory analyses performed. After 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 *S. cerevisiae.* Following, the bottle conditioning was carried out with 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.

Keywords: *Saccharomyces cerevisiae*; *Lachancea thermotolerans*; *Hanseniaspora vineae; Schizosaccharomyces pombe***;** volatile compounds; primary souring.

1. Introduction

Beer is an alcoholic beverage fermented from four basic ingredients: water, malt (usually barley), hops and yeast (Briggs et al., 2004), plus other ingredients specific to each brewmaster and geographical area (Baiano, 2021). 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 (EU Report, 2020). This diversity creates an additional value for consumers who demand the existence of new beers such as radlers and non-alcohol and low alcohol beers (NABLAB) (Bellut & Arendt, 2019). In fact, consumption of craft beers has increased due to consumers' willingness to pay higher prices for a high-value product (EU Report, 2020). 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 of generating desirable metabolites in beers, and

with diverse fermentative capabilities, which can facilitate the production of beers with non or low alcohol content (Buiatti, 2009; Callejo MJ, Tesfaye W, González MC, 2019).

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 (Basso et al., 2016). 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, favoured by the Crabtree effect and, finally, their tolerance to environmental stress caused by ethanol (cell-toxic compound) or other metabolites (Ernandes et al., 1993; Steensels & Verstrepen, 2014). 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 (Lodolo et al., 2008; Pires et al., 2014). 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) (Callejo et al., 2017). Its fermentative power is between 12-18 % 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 (Martini, 1991; Suárez-Lepe & Morata, 2012).

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 (Morata et al., 2018). It is characterised by its ability to ferment sugars such as glucose, fructose and galactose and is also variably able to metabolise maltose (Comitini et al., 2011). 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 (Banilas et al., 2016). It is positioned as a yeast suitable to produce beers in a single fermentation step and without the use of lactic acid

bacteria (LAB) (Osburn et al., 2018). 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 (Gobbi et al., 2013). It produces controlled levels of acetaldehyde and higher alcohols, while it is characterised by high production of both glycerol, giving it osmophilic characteristics, and aromatic esters such as 2-phenylethyl acetate and ethyl lactate (Domizio et al., 2016; Gobbi et al., 2013).

H. vineae is an apiculate yeast (Ribereau-Gayon, P., Dubourdieu, D., Doneche, B., and Lonvad, 2003). It is characterised 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 (Larroque et al., 2021), 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 two weeks (Osburn et al., 2018). It is noted for its positive aromatic contribution through the production of fruity and floral volatile compounds (Michel et al., 2016) such as 2-phenylethyl acetate and benzyl acetate (Martin et al., 2016).

S. pombe is a rod-shaped yeast with dimensions of 3-4 µm in diameter and 7-20 µm in length (Martini, 1991). It has a high fermentative power reaching up to 10-13 % v/v under anaerobic conditions (Rankine, 1968; Suárez-Lepe et al., 2012). However, its growth rate is low due to its high vitamin requirement (Benito et al., 2012). As a carbon source it is able to use glucose, fructose, sucrose, maltose and even raffinose and glycerol (Petersen & Russell, 2016). The generation of higher concentrations of pyruvate as an intermediate product highlights its oenological interest in red wine, as it favours the formation of vitisin A, by condensation of pyruvate and anthocyanins (Morata et al., 2003). Finally, it is worth mentioning its favourable impact in terms of food safety. On the one hand, because it has low assimilable nitrogen requirements compared to *S. cerevisiae,* which minimises the formation of biogenic amines (Benito et al., 2012) and, on the other hand, because of the reduction of urea content and, consequently, of ethylcarbamate through its urease activity (Suárez-Lepe et al., 2012).

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 flavour of beer (higher alcohols, organic acids, esters, aldehydes, ketones and sulphur compounds) (Buiatti, 2009). 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) (Humia et al., 2019),(Mozzon et al., 2020). Yeasts use sugars, nitrogen compounds and sulphur compounds for the synthesis of components for their growth, i.e. amino acids, proteins, lipids or nucleic acids among others. Aromatic compounds are a catabolic product of metabolising the must, among which we can find aliphatic and aromatic alcohols, esters, aldehydes, organic acids, carbonyl compounds and terpenic substances. Non-*Saccharomyces* yeasts are characterised by a shift in metabolism towards the production of secondary metabolites as opposed to the biomass and ethanol production of the classical *Saccharomyces* spp. (Lodolo et al., 2008).

At last, anthocyanins have been added previous second fermentation in bottle to change the colour of beer. Anthocyanins are phenolic compounds, belonging to the flavonoid type, which have the following rings: benzopyrillium, flavilium cation (B) and pyrillium cation. The colour 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 (Mazza & Francis, 1995; Morata et al., 2019). In the wine fermentation process, anthocyanins are transformed into derived pigments, called pyranoanthocyanins, which are more stable with respect to colour, pH variations or SO_2 bleaching, as they increase the resonant forms due to the double pyrilium ring (Bakker & Timberlake, 1997; Morata et al., 2016). The formation of pyranoanthocyaninins is a consequence of 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 (Schwarz et al., 2003). Whereas the second strategy occurs through the intervention of the enzyme hydroxycinnamate decarboxylase (HCDC) for the transformation of hydroxycinnamic acids into vinylphenol adducts (Oelofse et al., 2008), which are highly reactive and will condense with the anthocyanins to generate vinylphenolic-pyranoanthocyanins (Cameira-dos-Santos et al., 1996).

The general objective of this project is to modulate the sensory profile of craft beers thanks to biotechnology, i.e. 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 colouring 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). 5500 g were 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 were used to characterise 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) favours 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 non-reducing 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 favour 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. 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, 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 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 stabilised 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 ageing, was carried out. For this process, 245 ml of clarified beer wort was transferred to each 250 ml bottle, each sample was inoculated with 2-3% pure culture of *S. cerevisiae*, *L. thermotolerans*, *H. vineae* or *S. pombe*. In addition, 0.03% anthocyanins from red grape skins were added as a natural colouring 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 colour, 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.

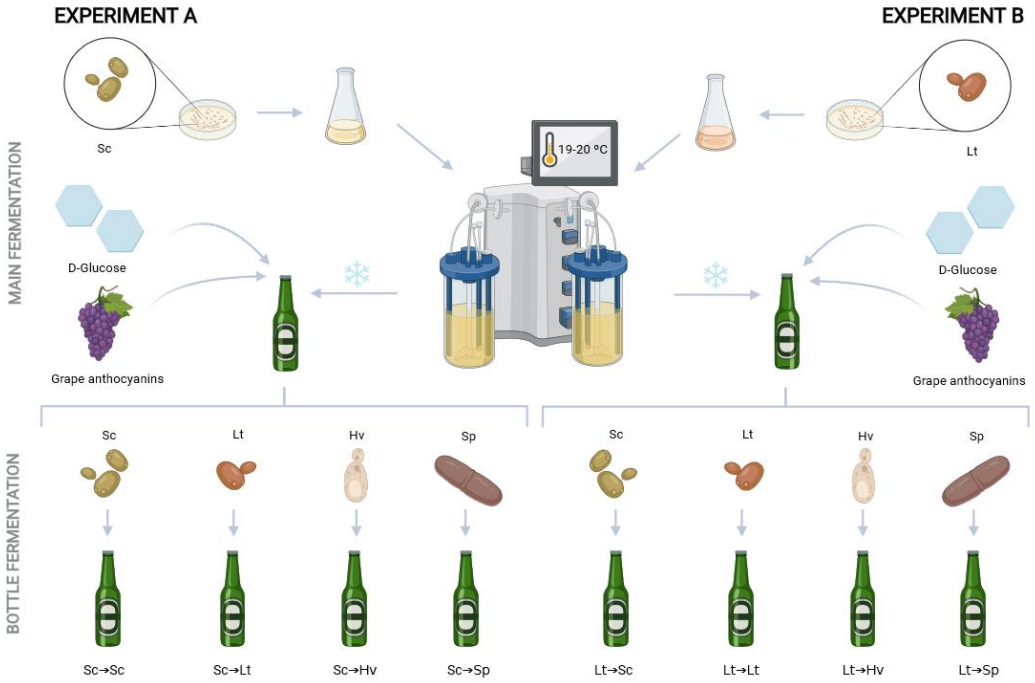

Figure 1. Experimental design performing to craft beers brewery with different *Saccharomyces* and non-*Saccharomyces yeasts: S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv) y *S. pombe* (Sp).

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 multi-analyser

A Y25 Biosystems enzyme multi-analyser (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 multi-analyser mentioned above were used for this purpose (Vaquero et al., 2020).

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 whole 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 x 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 deionised water (milliQ) 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 0.998 µl. 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 deionised water (milliQ) and methanol (Panreac, Barcelona, Spain), injected at a flow rate of 0.8 ml/min and a maximum pressure of 600 bar. Calibration for 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 analysed in the apparatus (Escott, 2018).

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 x 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 deionised water (milliQ) / 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 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 (Kulkarni et al., 2015).

2.7.4. UV-Visible Spectrophotometry

The colour 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 colour intensity as the sum of the absorbances at 420 nm, 520 nm and 620 nm and the tonality as the ratio between the absorbance at 420 nm and 520 nm (Kulkarni et al., 2015).

2.7.5. Gas chromatography with flame ionisation detector (GC-FID)

Agilent Technologies 6850 gas chromatography equipment equipped with an integrated flame ionisation detector (Hewlett-Packard, Palo Alto, CA, USA) and a DB-624 column (60 m x 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 at 300 °C. Whereas, the column temperature was set at 40 °C for the first five minutes, then linearly increased by 10 °C per minute until the final temperature of 250 °C was reached and finally maintained for 5 minutes. 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): acetalcehyde, methanol, 1-propanol, diacetyl, ethyl acetate, 2-butanol, isobutanol, 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 analysed 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 (Abalos et al., 2011).

The different volatile compounds obtained were grouped into various categories in order to facilitate the discussion of the data, highlighting those components that enhance the sensory profile of the beer brewed (Michel et al., 2016). The different categories considered are: higher alcohols (1-propanol, 2-butanol, isobutanol, 1-butanol, 3-methyl-butanol, 2-methyl-butanol and 2-phenylethyl alcohol (Humia et al., 2019; M. Kobayashi et al., 2008)), esters (ethyl acetate, isobutyl acetate, ethyl butyrate, ethyl lactate, isoamyl acetate, 2-phenylethyl acetate (Humia et al., 2019; Verstrepen et al., 2003)) and carboniyl compounds (diacetyl, acetoin (M. Kobayashi et al., 2008; Lodolo et al., 2008)). Besides total volatiles have been considered to refer to the sum of all volatile compounds determined by GC- FIC and indicates 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 (ISO, 1985) and ISO 4121:2003 (ISO, 2003) with a panel of trained tasters, who belonged to the Department of Chemistry and Food Technology of the Universidad Politécnica de Madrid. A total of eight experimental beers were evaluated by 9 panellists (5 women and 4 men) for the first tasting (bottle conditioning after 4 weeks) and by 8 panellists (4 women and 4 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 odourless tasting glasses. The panellists 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

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 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 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.

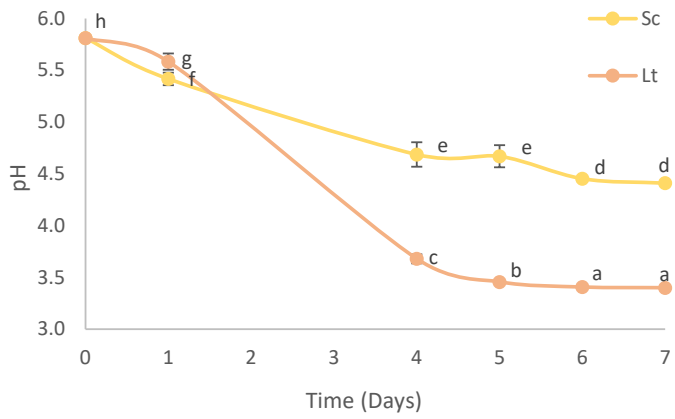

Figure 2. pH monitoring during fermentation by pH meter. Values represent the average ± standard deviation (n= 3) and significance level alpha=0.05. The yellow line corresponds to *S. cerevisiae* (Sc) and the orange line to *L. thermotolerans* (Lt). In the ANOVA, the different letters indicate significant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

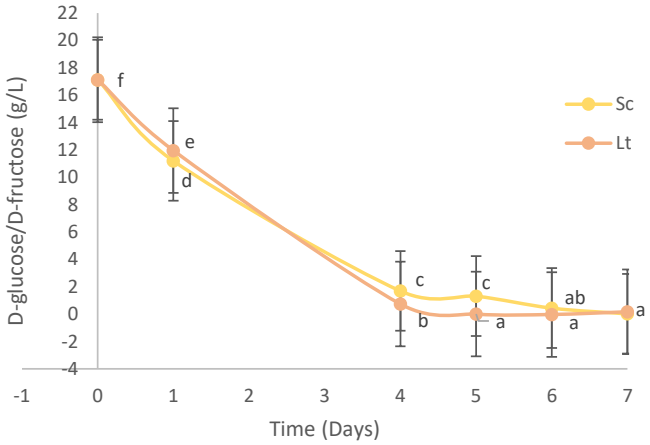

Figure 3. Evolution of reducing sugar content (g/L) during main fermentation using a Y25 enzymatic multianalyser and a kit to quantify glucose/fructose. Values represent the average ± standard deviation (n= 3) and significance level alpha=0.05. The yellow line corresponds to *S. cerevisiae* (Sc) and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

The evolution of ethanol (% v/v) and glycerol (g/L) concentrations (Figure 4), whose production developed in parallel for both yeasts, is plotted below. 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 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.

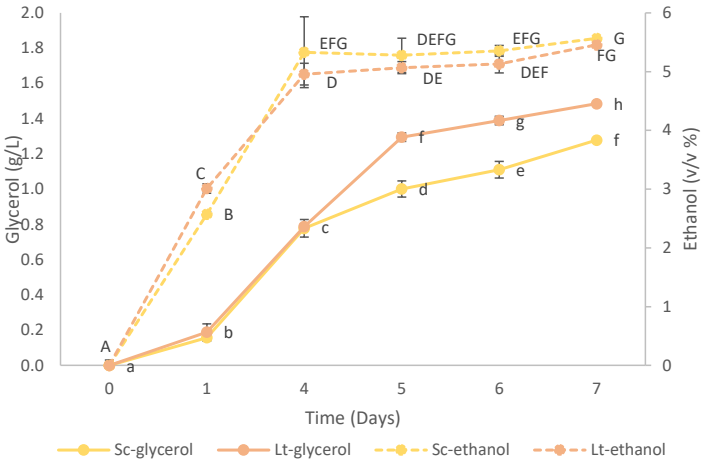

Figure 4. Evolution of glycerol (g/L) and ethanol (% v/v) during primary fermentation using HPLC- RID equipment. Values represent the average ± standard deviation (n= 3) and significance level alpha=0.05. The solid line corresponds to glycerol and the dashed line to ethanol: *S. cerevisiae* (Sc) in yellow and *L.*

thermotolerans (Lt) in orange. The different letters in the ANOVA indicate significant differences for the set of samples for each parameter analysed.

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 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).

Table 1. Consumption of reducing sugars (glucose/fructose) and production of L- lactic acid during primary fermentation. Values represent the mean ± standard deviation (n=3). Analyses of variance (ANOVA) were performed independently for each of the fermentations. Yeasts: *S. cerevisiae* (Sc) and *L. thermotolerans* (Lt).

	Yeasts	Glucose/ fructose (g/L)	L-lactic acid (g/L
Wort beer	-	17.03 ± 0.64	0.03 ± 0.01
Main fermentation	Sc (Experiment A)	0.01 ± 0.02^{a}	0.04 ± 0.01^{A}
	Lt (Experiment B)	0.01 ± 0.01^{a}	3.98 ± 0.08^{B}

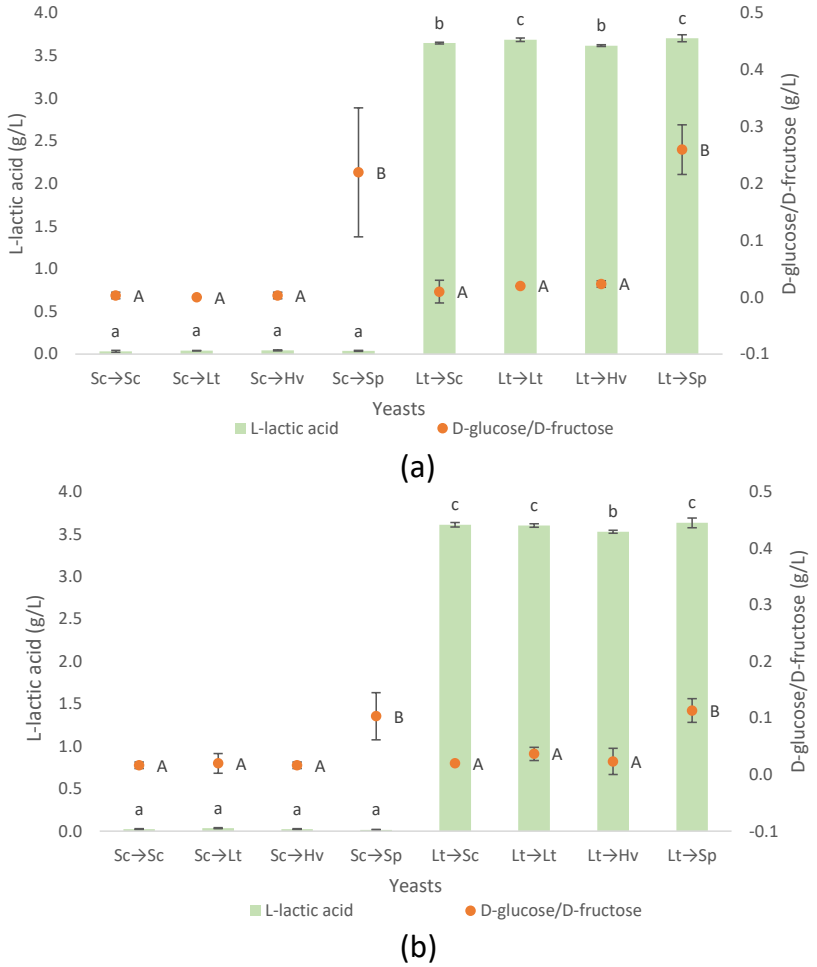

Figure 5. Determination of glucose/fructose and L-lactic acid by enzymatic multi-analyser. (A) 4 weeks of secondary fermentation; (B) 8 weeks of secondary fermentation. Values represent mean ± standard deviation (n=3). Analyses of variance (ANOVA) were performed independently for each of the weeks. Yeasts*: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

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 how the decrease of pH in the samples that have been fermented mainly with *L. thermotolerans* (experiment B).

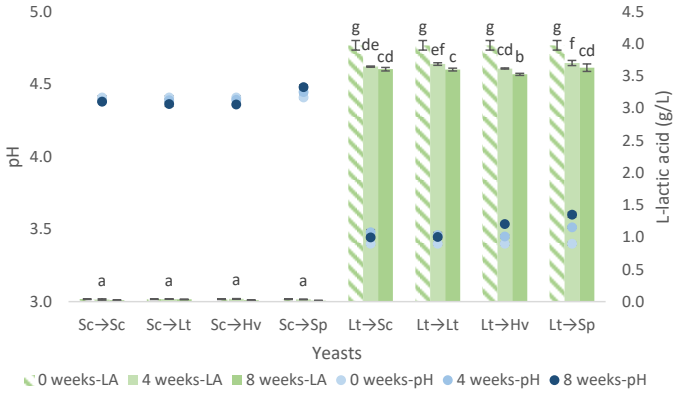

Figure 6. Relationship between pH and lactic acid accumulation throughout the fermentations carried out: end of main fermentation (0 weeks), secondary fermentation (4 and 8 weeks). Values represent the average ± standard deviation (n=3). The different letters in the ANOVA indicate significant differences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

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 0.2-3 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 6.57-6.74 % 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.

Table 2. Determination of ethanol content (% v/v) and glycerol content by HPLC-RID. Values represent the average ± standard deviation (n=3). Analyses of variance (ANOVA) were performed independently for each of the fermentations. Yeasts: *S. cerevisiae* (Sc), L*. thermotolerans* (Lt), *H. vineae* (Hv) and *S. pombe* (Sp).

	Yeasts	Ethanol (% v/v)	Glycerol (g/L)
Main fermentation	Sc (experiment A)	5.35 ± 0.29^{a}	1.28 ± 0.05^{A}
	Lt (experiment 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}

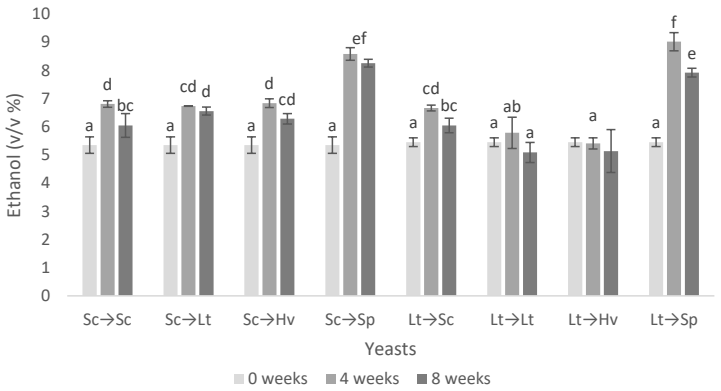

Figure 7. Evolution of ethanol concentration (% v/v) over time. Values represent the mean ± standard deviation (n=3). Analyses of variance (ANOVA) were performed comparing all weeks with each other. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv) and *S. pombe* (Sp).

3.4.5. Glycerol content

The results obtained for glycerol production are showed in Table 2 and Figure 8. The glycerol concentration (g/L) increases after completing 4 weeks in 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 beers of experiment B (Lt→Sc, Lt→Lt, Lt→Hv, Lt→Sp) increased up to a maximum of 2.86 g/L.

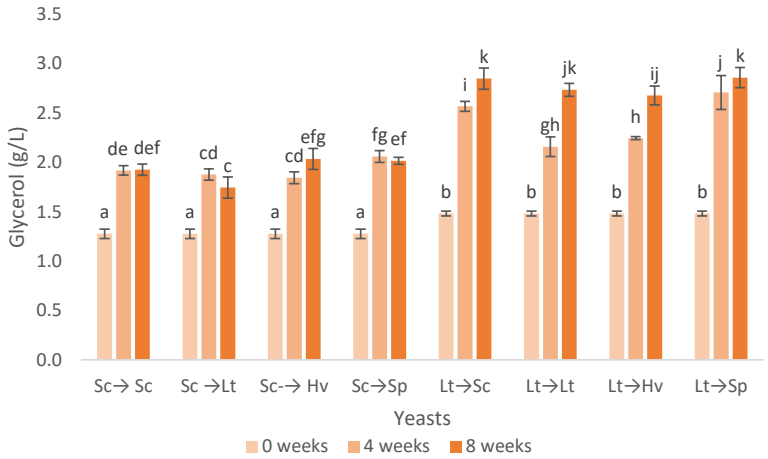

Figure 8. Evolution of glycerol concentration (g/L) over time. Values represent the mean ± standard deviation (n=3). Analyses of variance (ANOVA) were performed comparing all weeks with each other. Yeasts*: S. cerevisiae* (Sc), *L. thermotolerans* (Lt), *H. vineae* (Hv) and *S. pombe* (Sp).

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 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, i.e. 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).

Table 3. Anthocyanin composition before, during and after completion of secondary fermentation in bottle. Values represent the mean ± standard deviation (n=3). Analysis of variance (ANOVA) was performed independently for each of the weeks. 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).

Secondary fermentation	Yeasts	D3G	C3G	Pt3G	M3G	M3G-Ac	M3G-Cu	Pyranoanthocyan-vinylphenolics
0 weeks	Sc (experiment 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 (experimentb B)	1.83 ± 0.01^{a}	4.97 ± 0.00^{b}	5.63 ± 0.00^{a}	9.25 ± 0.01^{b}	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 ± 0.01^{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 ± 0.01^{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 ± 0.01^{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 ± 0.6^{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^{f}	-	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}

3.4.7. Total polyphenol index, colour intensity and colour

In order to further study the evolution of anthocyanins during the second fermentation in the bottle, the different beers were analysed by UV-Vis spectrophotometry at 280 nm, 420 nm (yellow colour), 520 nm (red colour) and 620 nm (blue colour). 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.* 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 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 colour intensity with values around ~1, while colour 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 case of *L. thermotolerans* for the main fermentation, a slight bathochromic effect (experiment B).

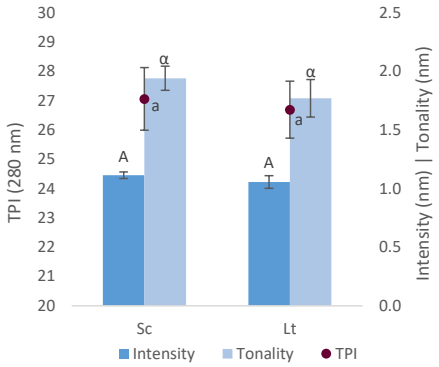

Figure 9. TPI, intensity and tonality determined for samples before secondary fermentation in bottle (0 weeks). Values represent the average ± standard deviation (n= 3). In the ANOVA, the different letters indicate significant differences within each parameter. Yeasts: *S. cerevisiae* (Sc) and *L. thermotolerans*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

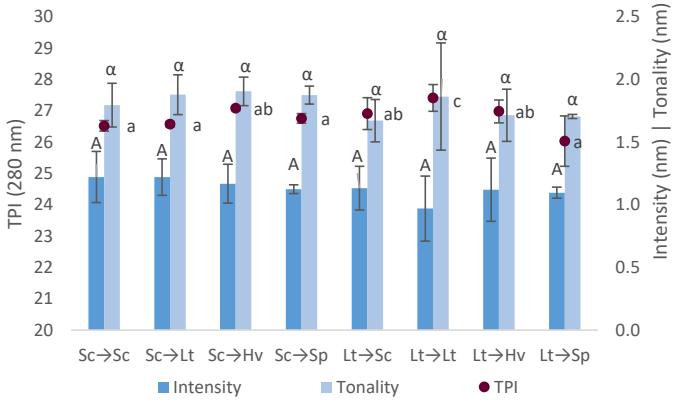

Figure 10. TPI, intensity and tonality determined after secondary fermentation in bottle (4 weeks). Values represent the average ± standard deviation (n= 3). In the ANOVA, the different letters indicate significant differences within each parameter. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans (*Lt),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

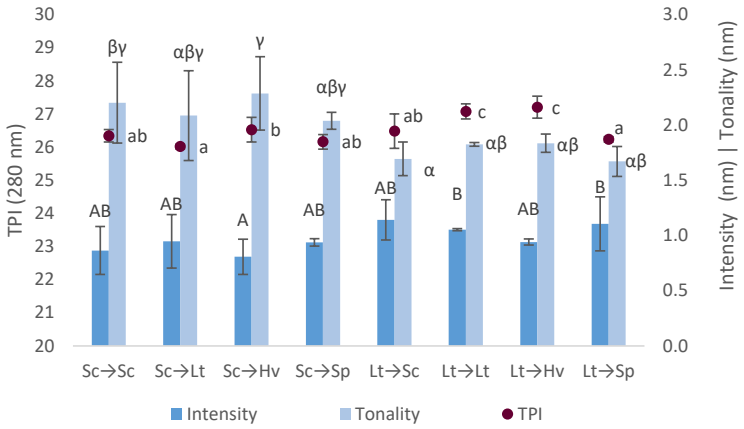

Figure 11. TPI, intensity and tonality determined after secondary fermentation in bottle (8 weeks). Values represent the average ± standard deviation (n= 3). In the ANOVA, the different letters indicate significant differences within each parameter. Yeasts: *S. cerevisiae* (Sc*), L. thermotolerans* (Lt),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

3.4.7. 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 analysed 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 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 analysed was almost double in *L. thermotolerans* and exceeded the sensory threshold. Significant differences also appear for the compound isobutanol (descriptor alcohol, solvent) whose concentration 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.

Table 4. Volatile compounds determined (mg/L) by GC-FID, after main fermentation 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).

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]
Isobutanol	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]
;obutyl 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]
;oamyl 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]

After 4 weeks of bottle conditioning in which the following 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 has been reduced between 40-120 mg/L. After this fermentation, the concentrations of acetaldehyde (apple and green leaves descriptor) have been reduced, with all the samples presenting a similar range between 6-12 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*.

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^{a}	8.41 ± 0.54^{ab}
Methanol	19.80 ± 1.10^{bcd}	18.16 ± 1.73^{bc}	17.87 ± 1.45^{b}	22.96 ± 1.96^{e}	21.80 ± 2.14^{de}	20.95 ± 0.54^{cde}	18.91 ± 3.04^{bcd}	10.77 ± 0.93^{a}
1-propanol	24.67 ± 3.50^{de}	27.63 ± 3.28^{e}	23.73 ± 1.38^{bcd}	26.44 ± 1.37^{de}	23.39 ± 2.85^{bc}	20.08 ± 2.73^{b}	12.10 ± 1.26^{a}	15.55 ± 1.08^{a}
Diacetyl	2.33 ± 2.13^{a}	2.14 ± 0.34^{a}	2.65 ± 0.53^{a}	1.87 ± 0.29^{a}	2.82 ± 0.37^{a}	3.20 ± 0.39^{a}	3.31 ± 1.04^{a}	2.31 ± 0.31^{a}
Ethyl acetate	2.82 ± 0.92^{ab}	2.18 ± 0.32^{a}	4.71 ± 0.47^{bcd}	3.57 ± 0.05^{abc}	4.04 ± 0.35^{abc}	4.50 ± 1.28^{bc}	5.31 ± 2.66^{c}	2.39 ± 0.58^{a}
2-butanol	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	2.31 ± 2.16^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Isobutanol	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^{a}	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^{a}
3-methyl-1-butanol	42.68 ± 2.51^{d}	41.88 ± 3.16^{cd}	42.29 ± 2.30^{cd}	47.70 ± 1.68^{e}	37.90 ± 0.92^{bc}	34.29 ± 3.36^{ab}	30.87 ± 4.60^{a}	37.63 ± 1.26^{bc}
2-methyl-1-butanol	24.95 ± 2.58^{c}	22.29 ± 1.45^{bc}	22.59 ± 1.83^{bc}	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^{de}	2.45 ± 2.37^{bc}	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 ± 1.11^{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}
Isoamyl 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^{abc}
Hexanol	6.22 ± 1.10^{bc}	7.87 ± 0.87^{de}	7.27 ± 0.72cd	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-phenylethyl alcohol	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}

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 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 analysed 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 analysed, and 2-3- butanediol again increased. Again, it seems that their decrease is in favour of the increase of 2-3 butanediol. Finally, 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 analysed, 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).

Table 6. Volatile compounds determined (mg/L) by GC-FID, after secondary fermentation in bottle (8 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	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^{c}	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 ± 0.36^{bc}	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}
Isobutanol	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-1-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^{a}
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 ± 0.31^{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 ± 2.88^{cd}	163.23 ± 4.06^{d}	138.50 ± 4.08^{ab}	148.52 ± 10.16^{bc}	131.97 ± 2.56^{a}	160.65 ± 3.64^{cd}
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-phenylethyl alcohol	21.77 ± 1.40^{ab}	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}

The evolution of the higher alcohols (Figure 12a) seems to depend on the yeast used in the main fermentation. Beers 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 12 b), 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 12 c). In the case of experiment B (with *L. thermotolerans),* a decrease of diacetyl and acetoin is observed during bottle conditioning.

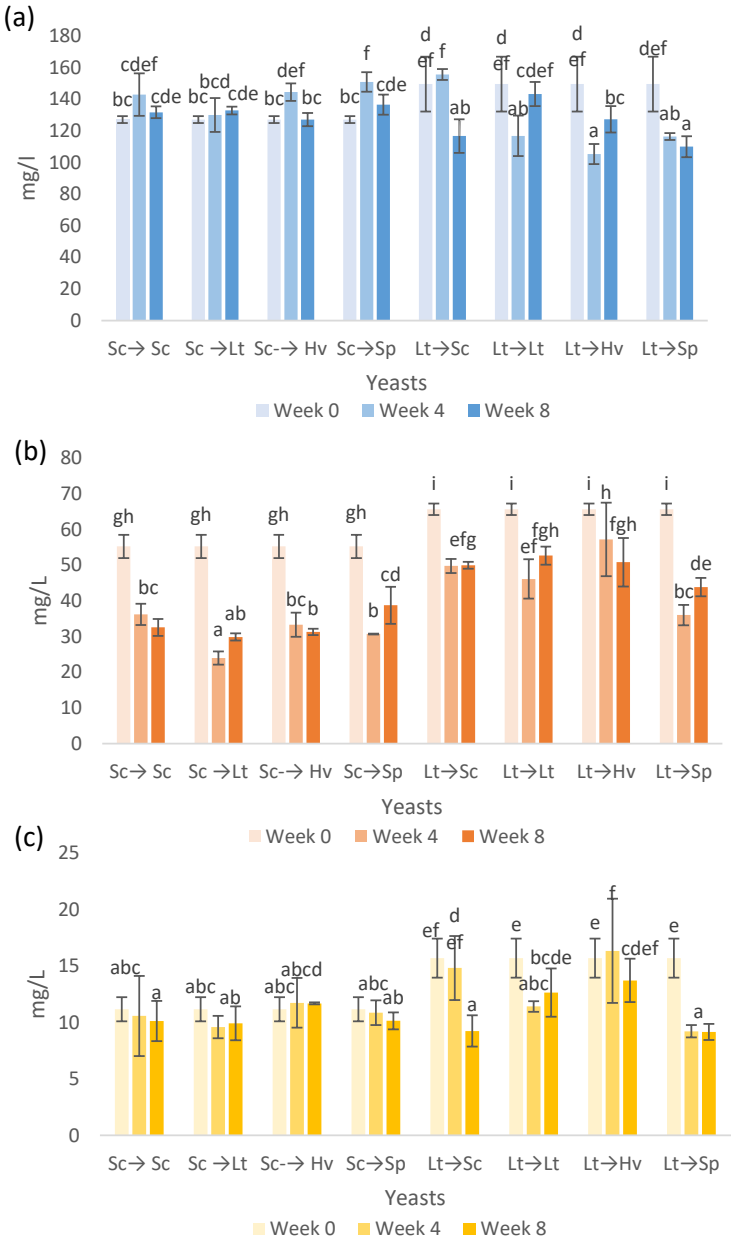

Figure 12. Evolution of the total concentration of volatile compounds during bottle conditioning: A) total higher alcohols; B) total esters; C) total carbonyl compounds. Values represent the average ± standard deviation (n= 3). In the ANOVA, the different letters indicate significant differences for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

3.5. Sensorial profile

3.5.1. First sensory evaluation: Acidity, colour 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 colour" also received the highest score for the conditioning made from the *L. thermotolerans* beer. The attributes 'body', 'effervescence' and 'aromatic quality' also standed 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) highligthed. Finally, the Sc→Lt and Lt→Lt combinations received the highest scores for the attribute "banana".

Table 7. Sensory analysis results after 4 weeks of bottle conditioning. 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).

Parameters	Sc→ Sc	Sc →Lt	Sc→ Hv	Sc→Sp	Lt→Sc	Lt→Lt	Lt→Hv	Lt→Sp
Beer colour	3.00 ± 0.50^{c}	1.44 ± 0.73^{a}	2.11 ± 0.78^{b}	3.00 ± 0.87^{c}	4.11 ± 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}	2.56 ± 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 consistecy	1.44 ± 0.53^{a}	2.56 ± 1.01^{bc}	2.56 ± 0.53^{bc}	2.56 ± 1.24^{bc}	1.78 ± 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^{cd}	2.00 ± 0.87^{ab}	3.56 ± 1.13^{d}	2.89 ± 0.60^{bcd}	2.20 ± 0.83^{abc}
Foam colour	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^{a}	3.44 ± 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^{ab}	3.11 ± 0.60^{ab}	3.44 ± 1.01^{b}	2.67 ± 1.12^{ab}	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^{ab}	2.00 ± 0.71^{b}	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^{ab}	1.89 ± 0.78^{ab}	1.78 ± 0.83^{ab}	2.56 ± 1.13^{b}	1.78 ± 1.20^{ab}	1.80 ± 0.83^{ab}
Floral	1.67 ± 0.87^{ab}	1.89 ± 0.60^{ab}	2.11 ± 0.78^{b}	1.33 ± 0.50^{a}	1.78 ± 0.44^{ab}	2.11 ± 0.78^{b}	1.67 ± 0.50^{ab}	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^{ab}	3.33 ± 0.87^{c}	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^{cd}	2.22 ± 0.67^{bcd}	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^{bc}	2.11 ± 0.93^{bc}	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^{b}	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 ± 0.60^{a}	2.67 ± 0.71^{a}	2.78 ± 0.83^{a}	2.78 ± 0.67^{a}	2.60 ± 0.88^{a}
Overall perception	2.56 ± 0.53^{a}	3.22 ± 0.44^{b}	2.67 ± 0.87^{ab}	2.78 ± 0.44^{ab}	3.00 ± 0.71^{ab}	2.89 ± 0.33^{ab}	3.22 ± 1.09^{b}	2.90 ± 0.60^{ab}

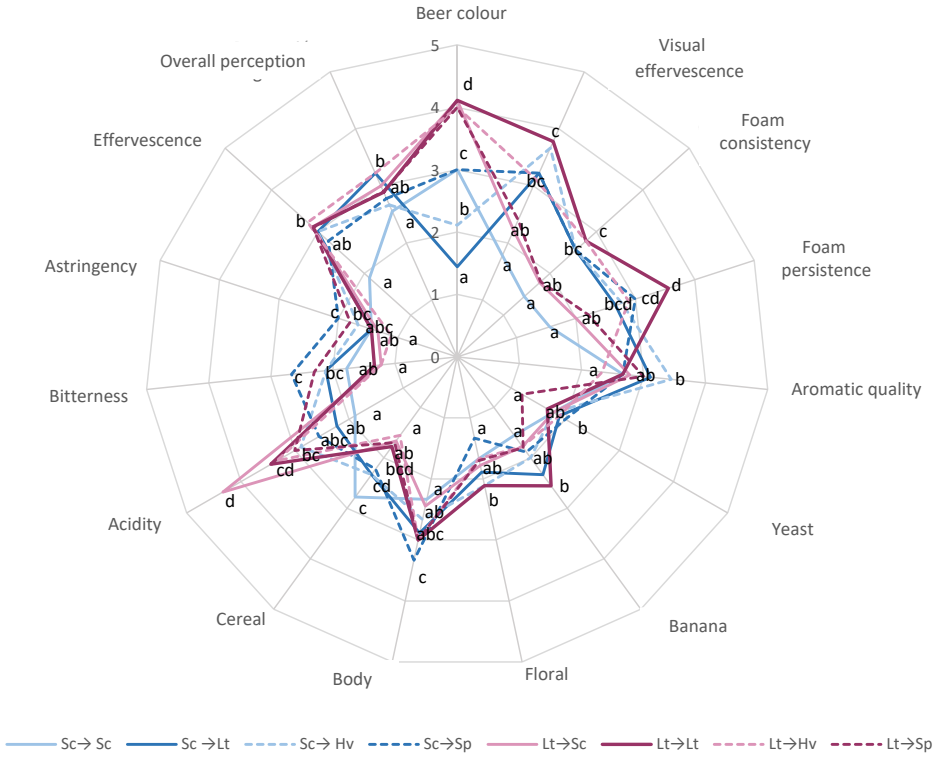

Figure 13. Spider web plot for sensory analysis after 4 weeks of bottle conditioning. Values represent the average ± standard deviation (n=9). In the ANOVA the different letters for each parameter indicate significant differences between yeasts. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt),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

3.5.2. Second sensory evaluation: acidity, colour, 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 colour" 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.

Table 8. Sensory analysis results after 8 weeks of bottle conditioning. 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).

Parameters	Sc→ Sc	Sc →Lt	Sc→ Hv	Sc→Sp	Lt→Sc	Lt→Lt	Lt→Hv	Lt→Sp
Beer colour	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 consistecy	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 colour	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^{a}	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}

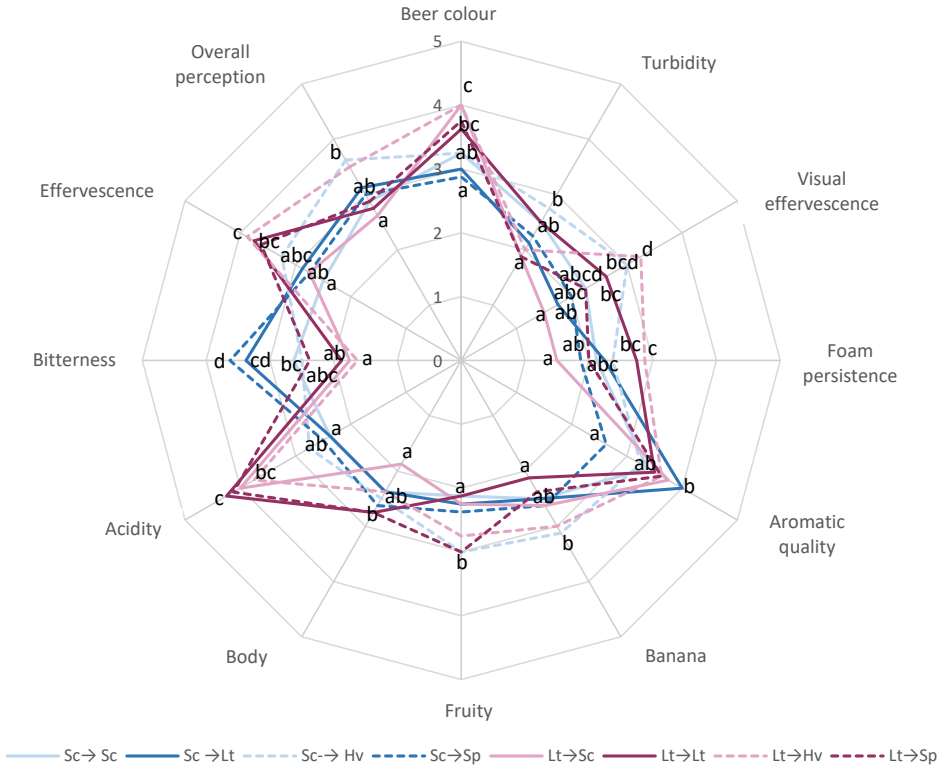

Figure 14. Spider web plot for sensory analysis after 8 weeks of bottle conditioning. Values represent the average ± standard deviation (n=8). In the ANOVA the different letters for each parameter indicate significant differences between yeasts. Yeasts: *S. cerevisiae* (Sc), *L. thermotolerans* (Lt),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

3.6. Pearson correlation

The correlation between the instrumental parameters analysed (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 supplementary material (Table S4 and Table S5).

For the first sensory test (4 weeks bottle conditioning) the statistically significant positive correlations were as follows: beer colour 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 colour 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 colour with glycerol, L-lactic acid and total esters, visual effervescence with acetoin and total volatile compounds, foam consistency with methanol, 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 with ethanol. Negatively the correlations found were: beer colour 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 (Ernandes et al., 1993; Lodolo et al., 2008; Pires et al., 2014; Steensels & Verstrepen, 2014). In the present work, the following yeasts have been used for the production of craft beers with differentiated characteristics by *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 analysed. The decrease in the concentration of reducing sugars to values close to 0 g/L coincided with the stabilisation 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 onwards in both yeasts. It should be noted that for each alcoholic strength generated, ~17g/L sugars must be consumed (Hidalgo et al., 2010; Noble, J;Ortiz-Julien, A; Silvano, A; Heras, JM; Théodore, n.d.), 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) (Capece et al., 2018; Comitini et al., 2011; Suárez-Lepe & Morata, 2012). 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 (Sauer et al., 2010). As reported by Domizio et al. (2016) (Domizio et al., 2016), 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 (Binati et al., 2019). 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 (Lodolo et al., 2008; Tilloy et al., 2014), and its production is modulated as a consequence of temperature (Gobbi et al., 2013) and oxygenation level (Shekhawat et al., 2018). The increase in glycerol production from day 4 for *L. thermotolerans* compared to *S. cerevisiae* is consistent with previous studies (Binati et al., 2019; Domizio et al., 2016; Morata et al., 2018) as *L. thermotolerans* is a species that produces higher concentrations of glycerol during alcoholic fermentation (Comitini et al., 2011; Domizio et al., 2016).

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, as well as promote aromatic maturation and colloidal stabilisation (Callejo et al., 2017; Lodolo et al., 2008). 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 (Morata et al., 2019).

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 (Benito et al., 2012), although it has a high fermentative power (Rankine, 1968; Suárez-Lepe et al., 2012). 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 (Comitini et al., 2011). 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* (Binati et al., 2019; Domizio et al., 2016). 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 (Tonsmeire, 2014). However, Osburn et al. (2018) 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 (Osburn et al., 2018). 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 realisation of primary souring during primary fermentation. In terms of alcohol content, *S. pombe* stands out because it is a yeast characterised by a high fermentative power (10-14 % v/v ethanol) (Suárez-Lepe et al., 2012), 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 (Larroque et al., 2021), being interesting for production of NABLAB (Bellut & Arendt, 2019). 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 metabolise sugars such as sucrose, galactose or maltose (Suárez-Lepe & Morata, 2012); ii) due to the transformation of acetaldehyde into ethanol as part of the yeast metabolism (K. Kobayashi et al., 2005). 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 (Vaquero et al., 2020). 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 (Domizio et al., 2016), 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 (Morata et al., 2007; Schwarz et al., 2003). While biological action involves the enzyme hydroxycinnamate decarboxylase (HCDC), which transforms hydroxycinnamic acids into vinifenols (Oelofse et al., 2008) and these undergo a condensation reaction with the grape anthocyanins to form these stable pigments (Cameira-dos-Santos et al., 1996). The enzymatic strategy by which positive HCDC activity in *S. cerevisiae* and *L. thermotolerans* is responsible for the production of vinylphenolic-pyranoanthocyanins is gaining momentum (Vaquero et al., 2021). 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 colour of the beverage. At acidic pH this molecule shows an equilibrium between the different chemical forms which are shifted in favour of the flavinium cation which is red in colour, i.e. it absorbs more at wavelengths of 520 nm (Brouillard et al., 1978). The increase in absorbance at 520 nm for beers whose main fermentation was carried out with *L. thermotolerans* (experiment B), translates into a hyperchromic effect, since the intensity of absorption at this wavelength increases (Morata et al., 2016, 2019). A priori, a clear relationship can be established between pH and colour, which means that anthocyanins are more protected

and, consequently, absorption is greater the lower the pH of the sample, as has been observed in wine (Vaquero et al., 2020).

4.1. Volatile compounds

The balance of secondary metabolite production is biased towards non-*Saccharomyces* yeasts in contrast to the production of biomass and ethanol by *Saccharomyces* spp. (Hammond, 1993; Lodolo et al., 2008). However, some metabolic products can act as undesirable volatiles when they exceed certain thresholds of perception (Toh et al., 2020), 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) (M. Kobayashi et al., 2008). 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 (Rosca et al., 2016).

After 4 weeks of bottle conditioning, acetaldehyde concentrations have been reduced, which would explain the increase in alcoholic strength compared to the main fermentation (Humia et al., 2019; M. Kobayashi et al., 2008). A reduction in the concentration of diacetyl and acetoin has also been observed, which is reflected in the increase of 2-3 butanediol, which is the last product of the same biosynthetic pathway (Humia et al., 2019; M. Kobayashi et al., 2008). This is in line with the reduction of diacetyl as one of the objectives of bottle ageing (Lodolo et al., 2008). 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 (Petitgonnet et al., 2019; Saberi et al., 2012). 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 (Larroque et al., 2021; Osburn et al., 2018).

After 8 weeks of bottle conditioning, the decrease of acetaldehyde continued in favour of an increase of ethanol from acetaldehyde (Humia et al., 2019; M. Kobayashi et al., 2008). While the persistent concentration of methanol could be responsible for solvent aromas. Again, the decrease of carbonyl compounds (diacetyl and acetoin) in all beers analysed could be justified in favour of the increase of 2,3-butanediol (Humia et al., 2019; M. Kobayashi et al., 2008). The biosynthetic pathway in which diacetyl and acetoin are produced concludes with the dehydrogenation of the second molecule into 2-3 butanediol (K. Kobayashi et al., 2005). It could be expected that the characteristics of the green beer produced with *L. thermotolerans* (experiment B) would favour 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 analysed, 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 (Michel et al., 2016). 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) (Ribereau-Gayon, P., Dubourdieu, D., Doneche, B., and Lonvad, 2003). In fact, this metabolite in high concentrations is synonymous with sour style beers (Thompson Witrick et al., 2017). 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 (Callejo et al., 2017; Osburn et al., 2018). 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 (Rijswijck et al., 2017; Verstrepen et al., 2003).

4.2. Sensory analysis: acidity, beer colour and pH

After the sensory analyses, a clear relationship can be established between the attributes "acidity" and "beer colour", whose highest scores 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 favours the higher absorption at 520 nm (red colour) (Brouillard et al., 1978). 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 (Baxter & Hughes, 2001; Kassemeyer & Berkelmann-Löhnertz, 2009; Meilgaard, 1975; Moll et al., 1991; Tan & Siebert, 2004). 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 (Larroque et al., 2021; Michel et al., 2016).

4.3. Correlations between parameters and attributes

After 4 weeks of bottle conditioning, there is a positive correlation between beer colour and L-lactic acid, which is behind the acidification of the beer. Linked to this is the negative correlation between pH and beer colour, for the same reasons. Thus, samples with lower pH received higher colour scores, i.e. redder colours. Another notable negative correlation was between sweetness and acetaldehyde, as acetaldehyde is a volatile compound associated with apple and leafy greens that is characterised 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 colour and L-lactic acid, together with the negative correlation between pH and beer colour. Furthermore, a positive correlation appears between acidity and L-lactic acid related to the fermentative metabolism of *L. thermotolerans* (Gobbi et al., 2013; Osburn et al., 2018). With regard to the negative correlations, the one between bitterness and glycerol stands out, since the higher the concentration of glycerol, the less bitterness the beer evaluated has, because this polyol contributes to

the softening of the sensory profile of the beer, providing smoothness and a slight sweetness (Buiatti, 2009). 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 colour, ageing caused it to lose some colour 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 behaviour and desired organoleptic characteristics.

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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)

10.3. Article 8.

Effect of acidification biotechnologies on the production of volatile compounds, lactic acid and colour in the red wines after the use of pulsed light pretreatment in grapes

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Abstract

The treatment of grapes with pulsed light is an emerging technique to reduce the populations of native microorganisms and thus implement fermentative biotechnologies in a more controlled manner with selected yeasts and bacteria. These biotechnologies can modify the profile of wines as the microbial populations thrive in different ways and produce different fermentative metabolites. The first objective of this research was to assess the use of pulsed light as a technology to reduce microbial population in grapes, and a second objective was to assess the viability of performing biological acidification in red wines with the use of *Lachancea thermotolerans* after the use of pulsed light. The treatment of grapes with pulsed light reduced the yeast and bacteria population 1.2 $\log_{10}$ CFU/mL and allowed the use of fermentative biotechnologies. Biological acidification with *Lachancea thermotolerans* produced more lactic acid than malolactic fermentation with *Oenococcus oeni*. Up to 4 g/L of lactic acid were reported in co-inoculations *of Lachancea thermotolerans* with *Saccharomyces cerevisiae*. On the other hand, the ester concentration, which provides floral and fruity aromas, was higher in wines that underwent malolactic fermentation. In this way, pulsed light treatment has produced wines with less aromatic volatiles and yet better perception of fruity and floral aromas as the lactic acid production was higher. Regarding colour, the wines were brighter and more intense due to the concentration of lactic acid although anthocyanins have decreased 9% and 18% in co-inoculation of *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in wines with treated and untreated grapes, respectively. A deeper evaluation during large-scale winemaking is advised to assess pulsed light to reduce native microbiota before the implementation of acidification biotechnologies.

Keywords: non-thermal technology, non-*Saccharomyces*, red wine, co-inoculation, acidification, freshness

Abbreviations

ADY	Active dry yeast
AF	Alcoholic fermentation
CI	Colour intensity
FTIR	Fourier-transform Infrared spectroscopy
HHP	High hydrostatic pressure
LAB	Lactic acid bacteria
LSD	Least significant difference
MLF	Malolactic fermentation
PCA	Principal component analysis
PEF	Pulsed electric fields
PL	Pulsed light
PMMA	Polymethyl methacrylate
TPI	Total polyphenol index
UHPH	Ultra-high pressure homogenisation
UV	Ultraviolet
YEPD	Yeast extract, peptone, D(+)glucose

Chemical compounds: Delphinidin-3-O-glucoside (PubChem CID: 443650), Cyanidin-3-O-glucoside (PubChem CID: 441667), Petunidin-3-O-glucoside (PubChem CID: 443651), Peonidin-3-O-glucoside (PubChem CID: 443654), Malvidin-3-O-glucoside (PubChem CID: 443652), Malvidin-3-O-glucoside-acetaldehyde adduct (vitisin B, Vit B) (PubChem CID: 16138152), Delphinidin-3-O-(6”-acetylglucoside) (PubChem CID: 15385440), Cyanidin-3-O-(6”-acetylglucoside) (PubChem CID: 15714477), Petunidin-3-O-(6”-acetylglucoside) (PubChem CID: 44256961), Malvidin-3-O-(6”-acetylglucoside) (PubChem CID: 74977116), Malvidin-3-O-(6”-p-coumaroylglucoside) (PubChem CID: 71308234), Malvidin-3-O-glucoside-4-vinylphenol (PubChem CID: 44257035), Malvidin-3-O-glucoside-4-vinylguaiacol (PubChem CID: 44257037), 2-Phenylethyl acetate (PubChem CID: 7654), 2-Phenylethanol (PubChem CID: 6054), Ethyl acetate (PubChem CID: 8857), Isobutyl acetate (PubChem CID: 8038), Ethyl butyrate (PubChem CID: 7762), Isoamyl acetate (PubChem CID: 31276), Acetaldehyde (PubChem CID: 177), Methanol (PubChem CID: 887), 1-Propanol (PubChem CID: 1031), Diacetyl (PubChem CID: 650), 1-Butanol

(PubChem CID: 263), 2-Butanol (PubChem CID: 6568), Isobutanol (PubChem CID: 6560), Acetoin (PubChem CID: 179), 2-Methyl-1-butanol (PubChem CID: 8723), 3-Methyl-1-butanol (PubChem CID: 31260), Ethyl lactate (PubChem CID: 7344), 2,3-Butanediol (PubChem CID: 262), 1-Hexanol (PubChem CID: 8103)

1. Introduction

The fermentation biotechnology involved in winemaking is currently aiming towards producing wines with a more complex profile as they seek starter cultures to perform optimally in that specific way. Contrary to what happens in spontaneous fermentation, fermentative biotechnologies can control the quality and reliability of wine fermentations. These technological approaches may include starters for pure culture fermentation to stress the metabolic features of a single strain, a practice that is widespread with the use of active dry yeast (ADY) of the species *Saccharomyces* spp. (Parapouli et al., 2020), and the increasing use of ADY of non-*Saccharomyces* yeasts. Another approach is the simultaneous fermentation or co-inoculation of two or more yeast strains that can thrive in synergy to improve the complexity of wines in parallel (Comitini et al., 2011). A third approach is the sequential fermentation which allows strains of first stages to express metabolically before another strain of higher fermentative power consumes residual sugars and carries on the fermentation (Del Fresno et al., 2017).

One limitation on the successful implantation of these technologies during winemaking is the initial population of native microorganisms that avoid a correct growth of selected strains, or that prevents the expression of characteristic metabolic features. This is the case for some strains of the yeast species *Lachancea thermotolerans*. The production of lactic acid by this species, which helps improve the freshness in wines with a pleasant acidity (Vilela, 2018), is inhibited in mixed fermentations with the presence of the apiculate species *Hanseniaspora vineae* (Vaquero et al., 2021). On the other hand, *Hanseniaspora* spp. strains, as well as other non-*Saccharomyces* yeast species such as *Metschnikowia pulcherrima* and *Torulsapora delbrueckii*, are well known for their high enzymatic activity towards the production of aromatic esters (Jolly et al., 2017). This enzymatic activity, which contributes to increasing the complexity of the aromatic

fraction, is greatly desired. Nonetheless, the competition of species during fermentation may endanger the performance of the selected culture yeasts. Therefore, and with the aim to reduce the initial microbial counts of native yeast and bacteria, the use of non-thermal emerging technologies is being tested to ensure the implantation of selected strains. Among the non-thermal technologies already tested for microbial reductions in grapes, the following are highlighted: high hydrostatic pressure (HHP) that allows the reduction of the use of SO_2 as an antiseptic additive for the preservation of wines (Christofi et al., 2020); ultra-high-pressure homogenisation (UHPH) able to eliminate viable cells of native microbiota (Morata et al., 2019) and also to prevent the proliferation of spoilage yeasts such as the species *Brettanomyces* spp. (Pinto et al., 2020); pulsed electric fields (PEF) which affect the cell wall structures and may increase the extraction of polyphenolic compounds on the one hand (Puértolas, López, Saldaña, et al., 2010), while also reducing the population of spoilage microorganisms on the other hand (Puértolas, López, Condón, et al., 2010). Another non-thermal technology rarely used in winemaking is the pulsed light (PL), which has been largely used in other food matrices, such as fresh fruits and vegetables, dairy products and meat (Leng et al., 2020; Tao et al., 2019; Valdivia-Nájar et al., 2018) to improve the safety and shelf life of food products. The use of PL for winemaking purposes is proposed as a treatment for grapes after selection and before crushing (Escott et al., 2017).

This article summarises the findings after carrying out three fermentative biotechnologies for the biologic acidification of red wines produced with untreated grapes and grapes treated with PL.

2. Materials and Methods

2.1 Yeast strains and growing media

The yeast strains used in this experimental set-up were all isolated in the Food Technology Laboratory at the School of Agronomic, Food and Biosystems Engineering (Universidad Politécnica de Madrid). The species used were two non-*Saccharomyces* yeast strains *(Lachancea thermotolerans* (Lt) strain L3.1, and *Hanseniaspora opuntiae* (Op) strain A56, and the species *Saccharomyces cerevisiae* strain 7VA. The lactic acid

bacteria (LAB) used for malolactic fermentation was a strain of the species *Oenoccocus oeni* Enoferm Alpha™ (Lallemand Bio, Madrid, Spain).

Since the initial microbial counts found on the grapes were below 1 log_{10} CFU/mL, yeasts were sprayed over the grapes to increase the initial population (Table 1). This pretreatment would simulate the yeast population typically found in the pruina of harvested grapes (Barata et al., 2012). The treatment with pulsed light described in section 2.2 was performed after this conditioning.

Table 1. Description of the addition of *H. opuntiae* and *S. cerevisiae* over the grapes to increase the initial population of yeasts before the PL treatment.

Strain ID.	Yeast species	Inoculum volume* (mL)	Inoculum population [1] (CFU/mL)	Population in must[2] (CFU/mL)
A56	*Hanseniaspora opuntiae*	10	8.4 log_{10}	7 log_{10}
7VA	*Saccharomyces cerevisiae*	5	8.2 log_{10}	5.8 log_{10}

*The volume used for the fermentation trials with the population described in the colum "Inoculum population"; [1]Measured in 1mL of the inoculum; [2]Measured in 1mL of must after the inoculation of A56 and 7VA.

For the fermentation trails, strains from the species *Saccharomyces cerevisiae* 7VA and *Lachancea thermotolerans* L3.1 were used. These two yeast strains were grown for 24 h in liquid YEPD media at constant 24 °C to reach a population of 8.3 log_{10} CFU/mL and 8.4 log_{10} CFU/mL respectively. The liquid medium was prepared by mixing 1% yeast extract (Laboratorios Conda; Madrid, Spain), 2% bacteriological peptone (Laboratorios Conda; Madrid, Spain), and 2% D(+)-glucose anhydrous (Panreac Química; Barcelona, Spain). The growing medium was autoclaved for 15 min at 120 °C. The LAB were rehydrated in water free of chlorine at 20 °C for 15 min prior to inoculation, in accordance with the instructions for use given by the manufacturer. The population achieved for LAB was 9.9 log_{10} CFU/mL.

2.2 Pulsed light treatment

The PL treatment was applied to one set of non-immobilised grapes to reduce the blind spots created by shadows as a consequence of the geometry of the berries. It is also a way to emulate a continuous PL treatment for future investigations in this regard. The

treatment consisted of a series of 120 pulses applied on destemmed berries placed on a two-layer arrangement in plastic trays. Every 40 pulses, the tray was removed from the chamber to reorganise the berries randomly without touching them directly. This step simulates the rotation that the berries are subjected to on a conveyor. The trays have the following dimensions: 13 cm × 20 cm × 5 cm. The PL apparatus (Claranor, Avignon, France) consists of a treatment chamber with a double xenon lamp with a flash width of 0.2–2 milliseconds. The distance between the berries and the lamps was adjusted to 7 cm. The applied pulses reached 1 MW per flash, which translates to a maximum fluence of 30 J/cm^2. After the treatment, the trays were placed inside a laminar flow hood, Aeolus V (Telstar, Madrid, Spain), to carry on the microbial counts in an aseptic environment.

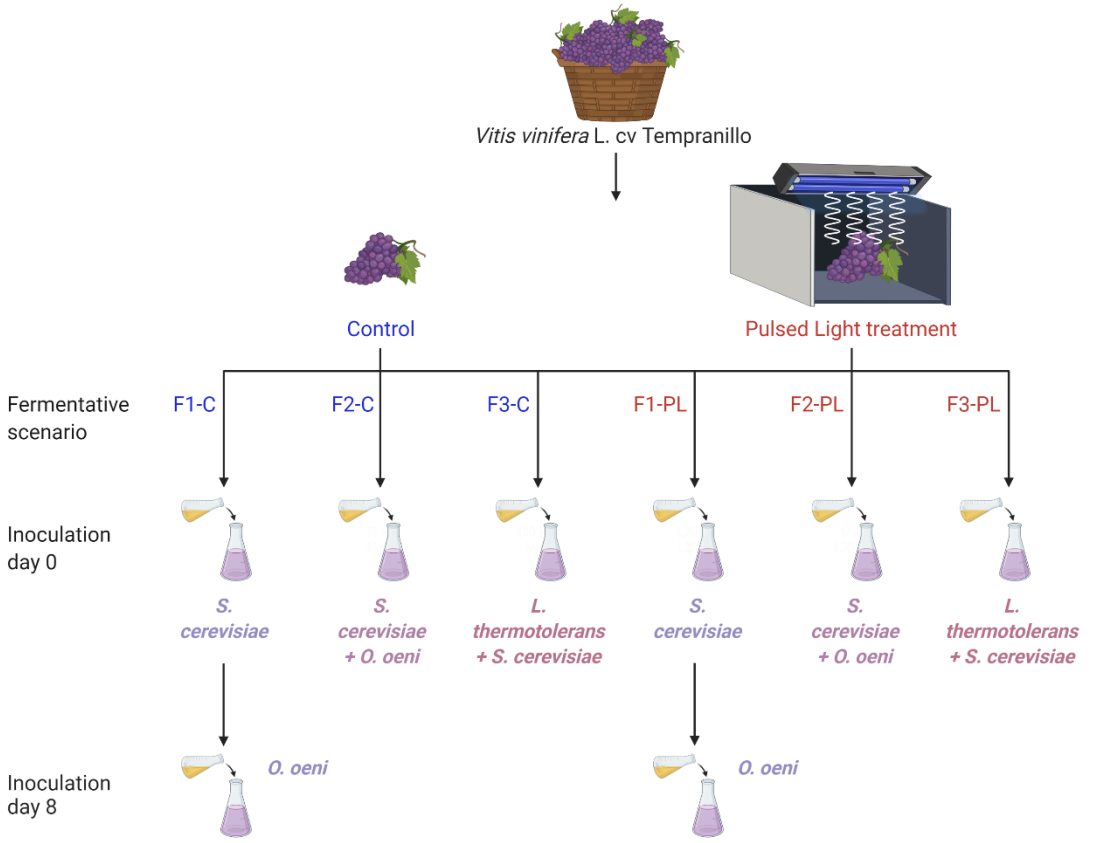
2.3 Must fermentation

Two sets of *Vitis vinifera* L. cv. Tempranillo grapes were crushed under sterile conditions inside a laminar flow hood. The first set corresponded to the untreated grapes, while the second corresponded to the PL-treated grapes. Both musts were left for 1 h with the skins, which were then removed from the juice. This maceration time intended to increase the concentration of phenolics and anthocyanins, but to limit the proliferation of microbial counts in the same time. The juice of each of the two musts was then divided into 9 flasks, 18 in total. The fermenters were 250 mL volume brown glass flasks filled with 220 mL to leave a headspace of 30 mL on top. The musts had a specific gravity of 1105 and pH values of 4.21 and 4.23 for untreated and treated grapes, respectively. The potential alcohol by volume expected in these musts was approximately 14.5% v/v. Microbiological analysis showed an initial population of 7.1 $\log_{10}$ CFU/mL and 5.8 $\log_{10}$ CFU/mL for inoculated *Saccharomyces* and non-*Saccharomyces* yeasts for the control must and the survival population after the PL treatment, respectively (See Table 1).

The experiment comprised three fermentative scenarios with treated grapes and the same three scenarios with untreated grapes. The fermentative scenarios were F1) alcoholic fermentation (AF) with *S. cerevisiae* and sequential malolactic fermentation (MLF) with *O. oeni*; F2) simultaneous AF and MLF with *S. cerevisiae* co-inoculated with

O. oeni; and F3) co-inoculation of *S. cerevisiae* and *L. thermotolerans* with no MLF (See Figure 1). The fermenters were closed with the use of Müller valves and they were placed at steady 20 °C right after the initial weight was recorded. Each fermentation scenario was carried out in triplicate. In scenario F1, the inoculum of *O. oeni* was added to the fermenters after the eighth day. A similar volume of sterile YEPD media was added to all the fermenters of scenarios F2 and F3 to keep the volume constant in all three fermentative scenarios. The evolution of the fermentations was followed up until no changes in weight were observed. The fermentation lasted 24 days.

Figure 1. Experimental setup for the fermentation of must from PL treatment on destemmed grapes.



2.4 Oenological parameters

A Fourier transform infrared spectroscopy (FTIR) equipment, OenoFoss™ (FOSS Iberia, Barcelona, Spain), was used to characterise glucose, fructose, gluconic acid, and nitrogen compounds in musts. One millilitre was needed for the analysis. The ethanol, glucose, fructose, and volatile acidity were determined in the finished wines. Malic acid and lactic acid were determined with an enzymatic analyser Y25 (Biosystems, Barcelona, Spain). Ethanol is expressed as % v/v, sugars and nitrogen compounds are expressed as

g/L, and all organic acids are expressed as tartaric acid equivalents in g/L. The pH was determined in musts and finished wines using a GLP 21 Crison Instruments (Hach Lange Spain, S.L.U., Madrid, Spain). All samples were previously stirred with a vortex mixer to release the trapped CO_2 to avoid any errors in the measurements.

2.5 Major volatile compounds analysis

The volatile compounds were determined with the use of a gas chromatograph with a flame ionisation detector (GC-FID). The chromatograph is an Agilent Technologies™ 6850 (Palo Alto, CA, USA) with a column DB-624 (60 m x 250 µm x 1.4 µm). The injector's temperature was set at 250 °C and the detector's temperature was 300 °C. Finally, the temperature went from 40 °C for 5 min to 250 °C with a gradient of 10 °C/min and was maintained for 5 min. Hydrogen was used as the carrying gas with a flow of 2.2 L/min and the split ratio set at 1:10. The identification and the quantification of volatile organic compounds were performed with calibration curves for each of the following compounds (R^2): 2-phenylethyl acetate (0.99460), 2-phenylethanol (0.99933), ethyl acetate (0.99980), isobutyl acetate (0.99987), ethyl butyrate (0.99987), isoamyl acetate (PubChem CID: 31276), acetaldehyde (0.99973), methanol (0.99917), 1-propanol (0.99938), diacetyl (0.99977), 1-butanol (0.99932), 2-butanol (0.9954), isobutanol (0.99941), acetoin (0.99949), 2-methyl-1-butanol (0.99925), 3-methyl-1-butanol (0.99958), ethyl lactate (0.99916), 2,3-butanediol (0.99109), 1-hexanol (0.99935). Additionally, 100 µL of 4-methyl-2-pentanol (500 mg/L) were used as an internal standard in accordance with a procedure previously described (Abalos et al., 2011).

2.6 Anthocyanins characterisation

The anthocyanins were determined with a liquid chromatograph Agilent Technologies™ 1100 (Palo Alto, CA, USA) with a column RP Kinetex C18 (100 × 4.6 mm; 2.6 µm) (Phenomenex, Torrance, CA, USA) and a diode array detector. This chromatographic technique was used to identify and characterise the following pigments: anthocyanins, both acylated and non-acylated; pyranoanthocyanins, including vitisins and vinylphenolic anthocyanins; and polymeric pigments. Two solvents were used: solvent

A (water/formic acid 95:5 v/v) and solvent B (methanol/formic acid 95:5 v/v) with a gradient of 0–2 min, 85% A (working flow 0.8 mL/min); 2– 10 min, 85–50% A linear; 10–12 min, 50% A; 12–13 min, 50–85% A linear; and 13–15 min until steady-state or re-equilibration. Malvidin-3-O-glucoside (Merck Life, Madrid, Spain) has been used as an external standard for the quantification of all pigments at a wavelength of 525 nm. The wavelength of maximum absorbance was used to identify the anthocyanins (He, Liang, Mu, Pan, Wang, Reeves, & Duan, 2012; He, Liang, Mu, Pan, Wang, Reeves, Duan, et al., 2012). The anthocyanins identified with this technique are: Delphinidin-3-O-glucoside (D3G), Cyanidin-3-O-glucoside (C3G), Petunidin-3-O-glucoside (Pn3G), Peonidin-3-O-glucoside (Pt3G), Malvidin-3-O-glucoside (M3G) grouped under the label non-acylated anthocyanins; Malvidin-3-O-glucoside-acetaldehyde adduct (vitisin B, Vit B), Malvidin-3-O-glucoside-acetic acid adduct (vitisin A, Vit A), Malvidin-3-O-glucoside-4-vinylphenol (M3GVPh) and Malvidin-3-O-glucoside-4-vinylguaiacol (M3GVg) grouped under the label pyranothanocyanins; Delphinidin-3-O-(6”-acetylglucoside) (D3GAc), Cyanidin-3-O-(6”-acetylglucoside) (C3GAc), Petunidin-3-O-(6”-acetylglucoside) (Pt3GAc), Malvidin-3-O-(6”-acetylglucoside) (M3GAc) and Malvidin-3-O-(6”-p-coumaroylglucoside) (M3GCm) grouped under the label acylated anthocyanins. The detection limit has been set to 0.1mg/L (Morata, Loira, Heras, et al., 2016).

2.7 Colour assessment

The colour of the wines was determined with a UV-visible spectrophotometer 8453 from Agilent Technologies™ (Palo Alto, CA, USA) and a DNA Phone Smart Analysis (Biosystems, Barcelona, Spain) for wine. The spectrophotometer has a photodiode array detector and uses a 1 mm path length quartz cuvette for the determination. A discrete wavelength mode was selected to acquire the absorbance at 420, 520, and 620 nm. The colour intensity is expressed as the sum of the absorbance of all three wavelengths multiplied by a dilution factor of 10, and the hue is expressed as the quotient from dividing the absorbance at 420 nm by the absorbance at 520 nm (Ribéreau-Gayon, 1974). The colour representation obtained with the DNA Phone is given in CIELab coordinates, which assess the luminosity or lightness (L), the green-red (a) and the blue-yellow (b) components, and CIELCh$_{uv}$ cylindrical coordinates that compare chroma (C)

and hue (h). The samples were placed in a 1cm path length polymethyl methacrylate (PMMA) cuvette. No sample preparation was needed to perform these two analyses.

2.8 Sensory evaluation

The analysis done for the sensory evaluation of the different wines obtained in this experiment was assessed with a panel of ten wine-tasting experts. The sensory evaluation was performed at the Chemistry and Food Technology Department of the School of Agricultural, Food and Biosystems Engineering (ETSIAAB) at Universidad Politécnica de Madrid (Spain). The panel was composed of five females and five males whose ages ranged from 25 to 55. The panel evaluated 15 basic wine descriptive attributes agreed upon by consensus earlier, two of which assessed the quality of the wines. The parameters were rated on a five-point scale from low perception (1) to high perception (5). The hue was rated on a separate scale from red (1) to orange (5). The descriptive attributes shown in the sensory evaluation form were evaluated in three distinctive sections: appearance (the intensity of the colour, the hue, and the transparency); aroma (intensity of the aroma, quality of the aroma, flowers, herbs, fruitiness, as well as reduction and oxidation notes); mouth (general acidity, astringency, body, bitterness); and a final general overall note. The pour size used for this tasting was 60mL (approximately 2 ounces). The results were treated with statistical analysis, and the average values were plotted in a radar chart.

2.9 Statistical analysis

Multivariate analysis was used to describe the differences observed in the wines produced, although the data is not extensive. All samples were compared to assess the production of lactic acid by the different fermentative media, and also to elucidate the profile of the wines produced by each fermentative condition. The means and the standard deviations were calculated, and the differences were examined using one-way ANOVA and the least significant difference (LSD) test. A principal component analysis (PCA) was obtained with the average concentration of oenological parameters, aromas, and pigments to illustrate the composition of wines produced in each fermentative

condition. The calculations previously mentioned were made using PC Statgraphics v.XI software (Graphics Software Systems, Rockville, MD, USA). The significance has been set at $p < 0.05$.

3. Results and Discussion

3.1 Microbial populations

The use of PL as a treatment for the reduction of native microbiota on grapes for winemaking is uncommon. The effect of the PL treatments on the reduction of initial populations found on the pruina of the grapes in this experiment was not powerful enough to eliminate the yeast strains in comparison to other non-thermal technologies such as ultra-high pressure homogenization (UHPH) (Loira et al., 2018). As a matter of fact, the reduction observed on the sprayed yeast after the treatment was 1.2 $\log_{10}$ CFU/mL. Nonetheless, this result is comparable to what has been achieved by authors on other food matrices, such as spinach leaves and fresh-cut lettuce (Agüero et al., 2016; Tao et al., 2019), but the reduction was lower than what was observed on tomato fruit (Aguiló-Aguayo et al., 2013). The PL treatments have greater results on the reduction of bacteria on foodstuff than reducing moulds and yeasts, as observed in avocado, watermelon and mushrooms (Ramos-Villarroel et al., 2015). One reason why this treatment is not as powerful may be that UV photons used in PL are less energetic than other photons (Martín-Belloso et al., 2014) and they may also reduce their energy when the must is released from the grapes under treatment (Bhavya & Umesh Hebbar, 2017).

Regarding the energy dose used in this experiment, the density produced by the pulses was 0.8 J/cm^2, similar to the total fluence used in spinach leaves to reduce populations of *Listeria innocua* and *Escherichia coli* (Agüero et al., 2016), but considerably lower than total fluences of 2.2 J/cm^2 and 4 J/cm^2 used for the reduction of *Saccharomyces cerevisiae* and moulds/yeasts in tomato and fresh-cut tomatoes, respectively (Aguiló-Aguayo et al., 2013; Valdivia-Nájar et al., 2018). Besides the fluence produced in the PL chamber, the effectiveness of the pulses might also be limited by the amount of must released from the grapes during the treatment, as the grapes were destemmed, and shadows were created by the geometry of the berries, as geometry is important for the efficiency of PL treatments (Heinrich et al., 2016).

In terms of the effectiveness of the treatment on the fermentation of the Tempranillo must, there are no significant differences in the populations observed between treated and untreated grapes for the span of the experiment, except for the total counts found at day 0. At day 0, the populations were larger in all untreated musts due to the reduction of counts produced in the treated grapes. From this time on, all musts behaved similarly. In fermentative scenarios F1 and F2, the bacterial counts remained constant at ~7 $\log_{10}$ CFU/ml since day 0 in the first case, and from day 8 in the second. In these two scenarios, the population of *S. cerevisiae* decreased to ca. 4.4 $\log_{10}$ CFU/ml after the fourth day. Fermentative scenario F3 did not show any significant difference in the population of *L. thermotolerans* at any time of the fermentation. All these results suggest that the microbial populations had similar development over the different stages, and the behaviour was similar for treated and untreated grapes for each fermentative scenario.

3.2 Wine composition

As shown in Table 2, the composition of the finished wines has no significant statistical differences in terms of volatile acidity, and although all wines are dry and have less than 2.1 g/L total sugars, the wines produced in scenario F3 had consumed the largest proportion of the sugars available in the must. The volume of ethanol reached (% v/v) is between 13% and 14.4%. This parameter is significantly higher in the fermentations with treated grapes, except for scenario F3, which obtained similar values to those with untreated grapes. This last result is in line with the fact during the early stages of fermentation, part of the sugars is consumed by non-*Saccharomyces* yeasts with lower fermentative power, whose populations are higher at the start of fermentation in untreated grapes. This slightly reduces the content of ethanol in finished wines (Contreras et al., 2015). Lastly, the main differences among fermentative treatments are visible in the pH values, the lactic acid produced, and the total acidity expressed as tartaric acid (g/L). As expected, the fermentations where strain 3.1 of the species *Lachancea thermotolerans* was used produced the highest concentration of lactic acid, up to 6 g/L, the lowest pH values (3.4), and the highest concentration of total acids with more than 8 g/L. The differences are significant between fermentative scenarios, but

also between PL treatments. In this last matter, the samples that had PL treatment prior to the inoculation of Lt strain 3.1 stood out from those untreated trials. Fermentative wise, it has been observed that the strain L3.1 reduces the production of lactic acid when in the presence of other non-*Saccharomyces* yeast strains, especially with the apiculate genus *Hanseniaspora* spp. This phenomenon is stronger in the species *H. vineae* (Vaquero et al., 2021). Therefore, the reduction of microbial counts with the PL treatment may lead to a less competitive fermentative environment for the *L. thermotolerans* to predominate, avoid inhibition, and increase the production of lactic acid.

Table 2. Chemical composition of finished wines from treated and untreated grapes. Average and standard deviation; n = 3. Different letters indicate statistical differences ($p < 0.05$) between treatments of each fermentative scenario.

ID.	Ethanol (% v/v)	Glucose/Fructose (mg/L)	pH[1]	Total Acidity (mg/L)	Malic acid[2] (mg/L)	Lactic acid[2] (mg/L)	Volatile acidity[3] (mg/L)
F1-C	13.7 ± 0.1*b*	1.9 ± 0.3*a*	4.1 ± 0.0*a*	4.2 ± 0.3*c*	0.1 ± 0.1*b*	1.7 ± 0.2*bc*	0.2 ± 0.0*a*
F2-C	13.7 ± 0.1*b*	1.7 ± 0.2*ab*	4.1 ± 0.0*a*	4.1 ± 0.3*c*	0.1 ± 0.1*b*	1.4 ± 0.2*c*	0.2 ± 0.0*a*
F3-C	13.2 ± 0.3*c*	1.4 ± 0.3*c*	3.6 ± 0.1*b*	6.1 ± 0.6*b*	1.3 ± 0.4*a*	2.6 ± 1.3*b*	0.3 ± 0.0*a*
F1-PL	14.4 ± 0.1*a*	2.0 ± 0.1*a*	4.1 ± 0.0*a*	4.3 ± 0.1*c*	0.2 ± 0.1*b*	1.6 ± 0.0*bc*	0.2 ± 0.0*a*
F2-PL	14.2 ± 0.2*a*	1.9 ± 0.1*a*	4.2 ± 0.0*a*	4.1 ± 0.2*c*	0.1 ± 0.1*b*	1.3 ± 0.0*c*	0.2 ± 0.0*a*
F3-PL	13.7 ± 0.3*b*	1.3 ± 0.2*bc*	3.5 ± 0.1*b*	7.4 ± 0.8*a*	1.4 ± 0.1*a*	4.2 ± 0.3*a*	0.2 ± 0.1*a*

[1]pH electrode; [2]enzymatic analyser; [3]expressed as acetic acid.

3.3 Fermentative volatile compounds

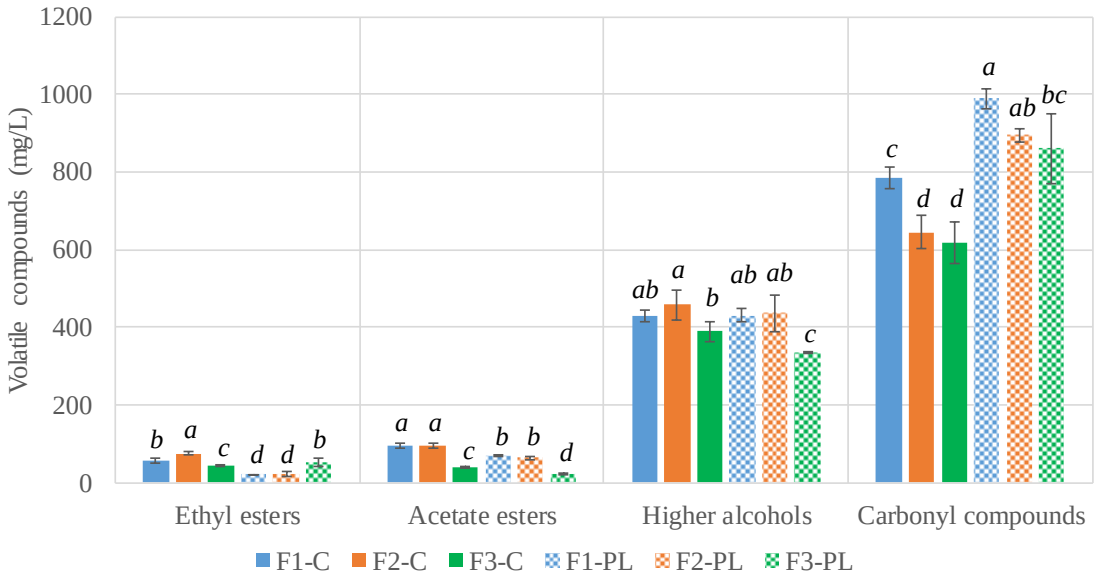
Besides the differences observed in the oenological parameters, the wines produced also showed variations in the fermentative volatile compounds in accordance with the microbial populations thriving in the different fermentative scenarios. In this way, the untreated grapes had produced larger amounts of ethyl esters and acetate esters than their counterpart scenarios with treated grapes (see Figure 1). This observation has been reported in either mixed or sequential inoculations of *H. vineae* and *S. cerevisiae* for the production of icewine with more floral and fruity aromas (Zhang et al., 2018). A larger initial population of apiculate *H. opuntiae* in untreated grapes may be responsible for

this accumulation of aromatic metabolites. This metabolic characteristic is present in all species of the genus *Hanseniaspora* spp., and it is related to a high enzymatic activity that leads not only to the production of aroma compounds but also to a reduction of the concentration of higher alcohols (Martin et al., 2018). This last feature was not noticeable in this experiment, since the concentration of higher alcohols is similar for the counterpart scenarios with treated and untreated grapes. Despite having a reduced amount of esters in the scenarios with treated grapes, the concentration achieved in these wines is above the threshold; therefore, it is expected for these wines to contribute to the aromatic complexity as well. For instance, the lowest concentration of ethyl acetate yielded in fermentative scenario F3-PL (12.1 mg/L – data not shown) can provide pear-like or banana-like aromas (Fan et al., 2019). This ester, which odour threshold is 7.5 mg/L (Petronilho et al., 2020), is more abundant in wines that underwent MLF (scenarios F1 and F2) since *S. cerevisiae* strains have been shown to have alcohol acetyl transferase activity encoded by the *ATF1* gene (Rojas et al., 2001). Lastly, the concentration of carbonyl compounds, comprising acetaldehyde, acetoin, diacetyl and mainly 2,3-butanediol, is more frequently produced in the wines with treated grapes than their fermentative counterparts. The main contribution to this group of compounds is given by the 2,3-butanediol, which is produced in large quantity by *S. cerevisiae* from the reduction of acetoin by yeast metabolism (Romano et al., 1998). The concentration of 2,3-butanediol yielded in the wines from treated grapes ranged from 785 mg/L to 954 mg/L. Apiculate yeasts, mainly present in early fermentative yeasts and more abundant in untreated grapes, yield concentrations of 2,3-butanediol between 54 and 221 mg/L in pure fermentations (Romano et al., 1998). In this experimental set up, the difference in the concentration of this volatile molecule found in wines with untreated grapes goes from -20% to -25% with the respective treated counterpart. Due to the contribution of this last volatile compound, whose wine tasting descriptor can be perceived as creamy or oily on the palate, the concentration of total volatile compounds is also higher in the wines with treated grapes.

3.4 Anthocyanins characterisation

The PL may induce damage to the cellular structures located at the surface of the grape's skin. This damage is due to the photophysical effects, which produce the disruption of the vacuoles, among other structures, with the possible freeing of anthocyanins (Unni & Chauhan, 2019). This phenomenon has not been reported as significant, although some minor changes in colour intensity have been described in previous experiments (Escott et al., 2017). The reason why this technology does not increase pigment extraction considerably may be that PL has a low penetration depth in comparison to other technologies (Fava et al., 2011). This characteristic can be reduced even further when liquid must is released from the grapes, since the propagation of the photons is less effective in liquid media (Oms-Oliu et al., 2010). The structural damage is then expected to be limited. Nonetheless, and despite this evidence, there is a tendency to have more pigments in wines from treated grapes when comparing treatments from the same fermentative scenarios (see Figure 2). The effect has no statistical significance, and thus, it cannot be considered decisive.

Figure 2. The volatile fraction of major fermentative metabolites by GC-FID. Different letters indicate a significant difference between means ($p < 0.05$).



Having said that, if the fermentative scenarios are considered, it is evident that scenario F3 has retained the least concentration of anthocyanins after fermentation, and the

difference is significant when compared to scenarios F1 and F2. It is important to note that scenario F3 carried out a co-inoculation of *L. thermotolerans* with *S. cerevisiae* and did not undergo MLF. Different yeast strains behave differently towards anthocyanin reduction due to the interactions between the microbial wall and the molecules of anthocyanins (Morata, Loira, & Suárez Lepe, 2016). In this way, the populations developed in fermentative scenario F3 interacted more with the anthocyanins in solution during fermentation, and thus, the concentration of pigments was reduced considerably. The effect was more intense in the wines produced with untreated grapes, where the initial inoculated populations increased the viable cells and the interactions between cell walls and anthocyanins. The reduction of anthocyanins in scenario F3-C reaches 18%, while the reduction is around 9% for scenario F3-PL. This interaction seemed less intense in scenario F1, followed by scenario F2, where LAB were used in co-inoculation and sequential fermentation, respectively. The use of PL reduces the chances of losing colouring matter as the initial yeast population decreases.

3.5 Colour assessment

The effect of the PL treatments on grapes is also evident in the colour intensity and the total polyphenol index values. Both parameters have consistently higher values on the wines elaborated with treated grapes (see Table 3) than their untreated counterparts. Analysing in more detail, it may be inferred that there was a higher polyphenol extraction with the PL. The pigment concentration was higher in wines where *L. thermotolerans* was not used and slightly higher, with no significant difference, in the three fermentative scenarios using treated grapes. The extraction is probably due to modifications caused on cellular structures, such as the vacuoles containing anthocyanins, as photophysical changes occur as a consequence of the irradiation with PL (Nowacka et al., 2021). This increment corresponds to the increase got in the absorbance measured at 520 nm, which indicates the presence of anthocyanins that absorb in the red range of the visible spectrum. The increment in CI also has an impact on the TPI, which is, as expected, also higher in the wines produced with treated grapes. Finally, the tonality expressed as the ratio between the absorbance of yellow (⍰$_{420}$) divided by the absorbance of red (⍰$_{520}$) has no statistical differences in the fermentative

scenarios F1 and F2, where *O. oeni* was inoculated regardless of the use of treated or untreated grapes. Significant differences appeared when *L. thermotolerans* was used in fermentative scenario F3. Both assays, with treated and untreated grapes, had a lower hue; the wine with treated grapes (F3-LP) had the lowest value of all. This can be explained by the lactic acid accumulated in these wines decreasing the pH value to a higher extent, which has increased the quantity of anthocyanins in the ionised conformation or flavylium ions (Porter et al., 2019). The higher the quantity of ionised anthocyanins, the more reddish a hue the wines would have. As per the CIEL*ab* and CIECh values, the vinifications produced with untreated grapes had higher values of chroma, *a* and *b* which relates to the colour and the saturation. On the other hand, treated grapes had lower luminosity values (L) which correlates with higher colour intensity (CI).

Table 3. Total polyphenol index (TPI), colour intensity (CI), tonality, CIE*Ch* and CIE*Lab* coordinates of all finished wines. Average and standard deviation; n = 3. Different letters indicate statistical differences ($p < 0.05$) between treatments of each fermentative scenario.

ID.	TPI	CI	Tonality	Chroma	Hue (°)	L	a	b
F1-C	34.8 ± 0.5*d*	7.1 ± 0.2*c*	0.9 ± 0.0*a*	63.0 ± 0.8*ab*	32.3 ± 0.7*b*	27.0 ± 1.2*a*	53.1 ± 0.3*ab*	33.8 ± 1.0*ab*
F2-C	35.1 ± 0.4*cd*	7.2 ± 0.4*c*	0.9 ± 0.0*a*	61.6 ± 1.4*b*	32.9 ± 0.3*ab*	25.8 ± 0.2*a*	51.9 ± 0.9*b*	33.3 ± 1.1*bc*
F3-C	32.5 ± 0.8*e*	7.6 ± 0.2*c*	0.8 ± 0.0*b*	65.0 ± 2.2*a*	33.6 ± 0.9*a*	26.0 ± 0.5*a*	54.1 ± 1.3*a*	36.1 ± 2.0*a*
F1-PL	37.8 ± 0.6*b*	8.2 ± 0.3*b*	0.9 ± 0.0*a*	58.7 ± 1.5*c*	33.4 ± 0.3*a*	21.1 ± 0.5*b*	49.1 ± 1.5*c*	32.2 ± 0.6*bc*
F2-PL	39.3 ± 1.2*a*	8.7 ± 0.1*b*	0.9 ± 0.0*a*	54.9 ± 1.4*d*	32.1 ± 0.6*b*	18.6 ± 1.0*c*	46.5 ± 0.9*d*	29.1 ± 1.2*d*
F3-PL	36.2 ± 0.4*c*	9.5 ± 0.2*a*	0.7 ± 0.0*c*	57.9 ± 1.6*cd*	32.1 ± 0.8*b*	18.9 ± 1.0*c*	49.1 ± 1.1*c*	30.7 ± 1.3*cd*

CI = $\lambda_{420} + \lambda_{520} + \lambda_{620}$; Tonality = $\lambda_{420}/\lambda_{520}$

3.6 Wine sensory evaluation

To determine whether the differences observed through the analytical analyses can be perceived by consumers, the wines were submitted to a sensory evaluation. Figure 3 summarises the results obtained from the winetasting. Attributes such as aromatic intensity, herbaceous and floral notes, reduction, oxidation, and sweetness did not have any significant statistical differences. On the other hand, the parameters aromatic quality, fruity, bitterness, acidity, colour intensity, hue, and global perception yielded statistical differences. The wines produced in scenario F3 with *L. thermotolerans* were

identified as wines with fruity aroma and better aroma quality, higher acidity, higher colour intensity, and better global perception. In particular, as previously seen in the colour assessment, wines produced in scenario F3 with treated grapes were perceived with a lower hue (reddish appearance), the highest acidity, and the best aroma quality, with fruiter scents. These two wines were perceived as fresher, as the attributes of acidity and fruitiness contribute to enhancing this characteristic, which is usually appreciated in white wines (Morata et al., 2020). Freshness related to acidity is less common in red wines as it is in white wines, and it is difficult to achieve through MLF. At the same time, the wines elaborated with scenarios F1 and F2 produced wines with a flatter aroma, with more bitterness, and with a more yellowish tone as well. Nonetheless, and despite these results, the market's acceptance of this type of red wines, fresher than current commercially available wines, is yet to be assessed (Figure 4).

Figure 3. Comparison of pigment content determined with HPLC-DAD and grouped by anthocyanin families. Different letters indicate a significant difference between means ($p < 0.05$).

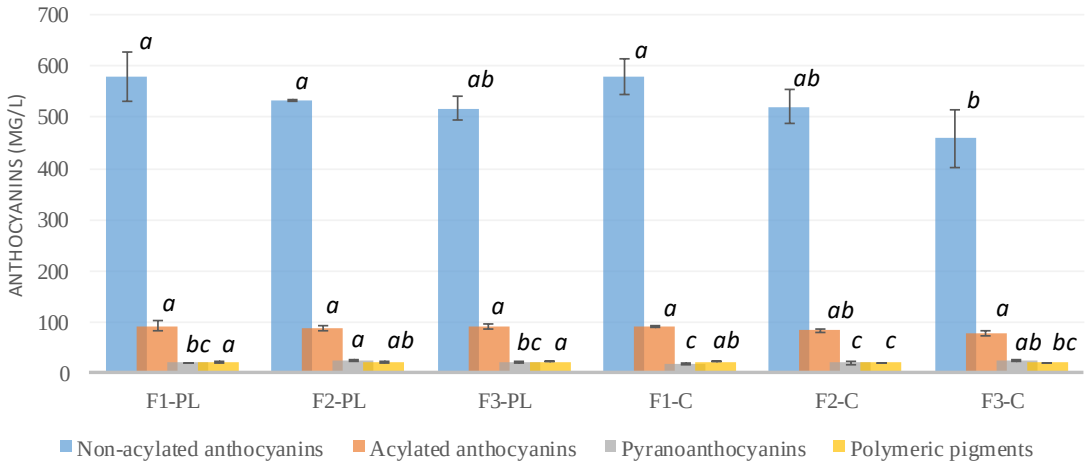
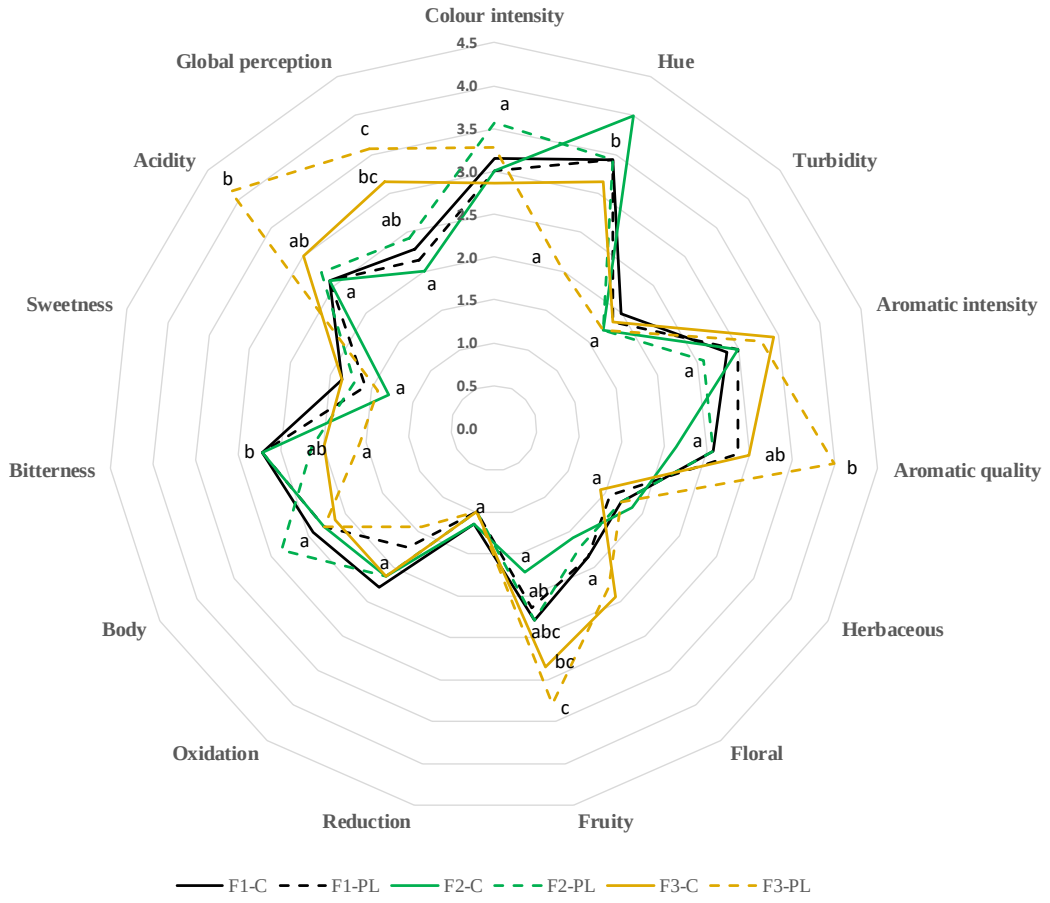


Figure 4. Two-dimensional star plot of winetasting descriptors of a 60mL pour size. Different letters indicate a significant difference between means ($p < 0.05$).



4. Conclusion

The effect of PL on the reduction of native microbial populations found in grapes is not as noticeable as with the use of other non-thermal technologies, such as UHPH or PEF. Nonetheless, the pretreatment of musts with PL, even at low energy doses, can reduce the yeast populations enough to allow inoculated strains to thrive in such a way as to positively contribute to the overall perception of wines. The co-inoculation of *Lachancea thermotolerans* and *Saccharomyces cerevisiae* stands out from the rest of the fermentative biotechnologies in this matter. The reduction of native yeast populations allowed the production of higher concentrations of lactic acid. The concentration of lactic acid increased the freshness of wines which also increased the perception of floral and fruity aroma, although the concentration of volatile compounds responsible for floral and fruity aromas is slightly lower than the untreated counterpart wines. The

fermentation of treated grapes also increased the amount of 2,3-butanediol, which enhanced the palate and balanced the lactic acid produced largely by *L. thermotolerans*. Lastly, biological acidification also improved the colour intensity in fermentations where the quantity of anthocyanins is reduced after their interaction with the lees cell walls. It is important to guarantee this effect in treatments with moving grapes in selection tables, as this may be the ideal time to treat the grapes during winemaking before the grapes are crushed and the tanks filled.

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