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Exploring the diversity of native Lachancea thermotolerans strains isolated by sugary extracts from manna ash to modulate the flavour of sour beers

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Abstract:	The craft beer industry is becoming increasingly interested in the production of innovative beers. A novel approach, designated as "primary souring," employs diverse yeast species, including Lachancea thermotolerans, to produce sour beers. Furthermore, there is a growing interest in utilising unconventional yeasts to produce beers with distinctive flavours. For the first time, yeast strains of L. thermotolerans, isolated from sugar extracts of manna ash, were evaluated for their ability to produce and improve the sensory properties of sour beers. In particular, five strains exhibited notable resistance to ethanol, sugar and hops, as well as comparable lactic acid production (ranging from 0.33 to 0.45 g/L). Experimental beers produced using MNF105 (T1) were perceived as the most “fruity". This is the first study to examine the impact of this novel indigenous strain, derived from unconventional matrixes such as manna, on the organoleptic quality of craft sour beers. Consequently, elevated levels of ethyl decanoate (165.30 mg/L), ethyl hexanoate (29.4 mg/l), ethyl octanoate (29.7 mg/l) and ethyl acetate (36.1 mg/l) were found in T1 beer, exceeding the perception threshold. The ability of this strain to perform light bio-acidification is a valuable feature for the development of new brewing techniques, particularly for the creation of sour beers with balanced acidity and innovative flavours. The yeast L. thermotolerans

	MNF105, which is related to manna, has excellent technological properties and is a promising starter for beer production with the ability to light bio-acidify and modulate flavour.

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SAAF
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Palermo, 19/06/2024

Dear Editor,

I am pleased to submit our paper titled: "*Exploring the diversity of native Lachancea thermotolerans strains isolated by sugary extracts from manna ash to modulate the flavour of sour beers*" to your attention.

The present work was carried out to investigate fifteen strains of *L. thermotolerans*, isolated by sugary extracts from manna ash, for their brewing capacity.

Very limited information has been published to date on yeast biodiversity characterizing the mentioned sugary extracts from *Fraxinus* spp. tree and no data have been reported on *L. thermotolerans* as starter to produce sour beers.

The craft beer industry is increasingly interested in sour beers produced without inoculum of lactic acid bacteria, thus the sour flavour is gained by single yeast starter fermentation. In the present research, a technological screening of the 15 strains has been applied to evaluate their behaviour (hydrogen sulphide production, sugar and ethanol tolerance assays, glucose, fructose and maltose assimilation test, cross resistance to hop and ethanol, flocculation assay and microfermentation). Subsequently, the strain that showed the best fermentation capacity was applied under real wort conditions. The microbiological, physico-chemical and sensory parameters as well as the composition of volatile organic compounds of the beers were carried out. The *L. thermotolerans* MNF105 strain, isolated from manna sugary extracts, showed better results in several aspects than the fermentation conducted by the commercial controls. This strain has been shown to be capable of producing sour beer with innovative flavour with large amount of ethyl hexanoate, ethyl octanoate and ethyl acetate correlated to an improved fruit aroma than control trial. The yeast *L. thermotolerans* MNF105, which is related to manna, has excellent technological properties and is a promising starter for beer production with the ability to lightly bio-acidify and modulate flavour. To our knowledge, no scientific researches have been carried out on these topics based on modulation of light acidity and improvement of volatile and sensory composition of craft beers.

From recent issues, we believe that our research article may be of interest for Food Research International readers.

The manuscript has been prepared following FRI authors' guidelines.

I hope the paper could be revised by FRI reviewers.

With my best personal regards,
Nicola Francesca

- *L. thermotolerans* yeast strains from manna present optimal brewing characteristics
- MNF105 produced moderate amount of lactic acid (0.47 g/L)
- MNF105 showed improvement of aromatic perception
- The overall organoleptic investigation showed a preference for MNF105
- MNF105 is able to produce sour beer with innovative flavours

Exploring the diversity of native *Lachancea thermotolerans* strains isolated by sugary extracts from manna ash to modulate the flavour of sour beers

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ABSTRACT

The craft beer industry is becoming increasingly interested in the production of innovative beers. A novel approach, designated as "primary souring," employs diverse yeast species, including *Lachancea thermotolerans*, to produce sour beers. Furthermore, there is a growing interest in utilising unconventional yeasts to produce beers with distinctive flavours. For the first time, yeast strains of *L. thermotolerans*, isolated from sugar extracts of manna ash, were evaluated for their ability to produce and improve the sensory properties of sour beers. In particular, five strains exhibited notable resistance to ethanol, sugar and hops, as well as comparable lactic acid production (ranging from 0.33 to 0.45 g/L). Experimental beers produced using MNF105 (T1) were perceived as the most “fruity". This is the first study to examine the impact of this novel indigenous strain, derived from unconventional matrixes such as manna, on the organoleptic quality of craft sour beers. Consequently, elevated levels of ethyl decanoate (165.30 mg/L), ethyl hexanoate (29.4 mg/L), ethyl octanoate (29.7 mg/L) and ethyl acetate (36.1 mg/L) were found in T1 beer, exceeding the perception threshold. The ability of this strain to perform light bio-acidification is a valuable feature for the development of new brewing techniques, particularly for the creation of sour beers with balanced acidity and innovative flavours. The yeast *L. thermotolerans* MNF105, which is related to manna, has excellent technological properties and is a promising starter for beer production with the ability to light bio-acidify and modulate flavour.

Keywords: Alcoholic fermentation; Beer aroma; *Lachancea thermotolerans*; Sour beer; Innovative beer; Volatile organic compounds.

1. Introduction

In recent years, the brewing industry has seen an increase in production, not only in terms of volume, but also in terms of new beer styles and products (Aquilani, Laureti, Poponi, & Secondi, 2015). Drinkers around the world are increasingly seeking innovative, tasty, and complex craft beers (Betancur, Motoki, Spence, & Velasco, 2020). To achieve new beer styles, numerous studies have been conducted on the main materials of beer, such as malt (Gugino et al., 2023; Zdaniewicz, Pater, Hrabia, Duliński, & Cioch-Skoneczny, 2020a), hops (Paguet et al., 2024), and other ingredients such as fruit (Pirrone et al., 2022). Another alternative for creating new beers with distinctive flavours is the use of non-conventional yeasts. In fact, many researches are focused on selecting, characterizing, and applying non-conventional yeasts (Burini, Eizaguirre, Loviso, & Libkind, 2022; Larroque et al., 2021; Sampaolesi et al., 2023; Simões et al., 2023).

Moreover, sour beer is a very diverse genre of beer that defies any specific definition based on production process, raw material or geographical origin. A common denominator of sour beers is a higher concentration of organic acids, resulting in a lower pH (pH 3.0 to 3.9) compared to 'normal' beers. Sour beers are intentionally designed to be acid and can also be fermented with wild microorganisms or fruit, barrel-aged, or blended with younger beers (Bossaert, Crauwels, De Rouck, & Lievens, 2019; Tonsmeire, 2014). There are different microbiological approaches for brewing sour beers, through spontaneous fermentation or through inoculum of acidifying bacteria, such as acetic acid or lactic acid bacteria or yeasts of the genus *Brettanomyces* or *Lachanchea* (Dysvik et al., 2020). Osburn et al. (2018) introduced the concept of 'primary souring', which focuses on using different yeast species, including strains of *Lachancea thermotolerans*, to brew sour beer without bacteria, resulting in a significant reduction in the duration and variability of the transformation process. This species is capable of heterolactic fermentation of sugar into lactic acid, ethanol, CO_2, and at the same time, producing pleasant aromatic and flavour compounds (Domizio et al., 2016; Osburn et al., 2018). Using lactic acid bacteria in breweries is widely acknowledged to result in significant contamination risks, along with escalating sanitisation expenses (Maifreni et al., 2015).

Recently, researches have conducted studies on *L. thermotolerans* for brewing application. Postigo, Esteban, & Arroyo, (2023) determined the fermentation capacity of 10 yeast strains of *L. thermotolerans* and showed that beer fermented with strain CLI 1232 had a balanced acidity with a fruity flavour profile and honey notes, whereas strain 1-8B had a balanced acidity but less fruity and citrus flavour than strain CLI 1232. Canonico et al. (2019) confirmed that *L. thermotolerans* strains can lower the pH of the inoculation medium due to the formation of significant amounts of lactic acid, as well as they consistently produce ethyl butyrate and ethyl acetate. However, Zdaniewicz, Satora, Pater, & Bogacz (2020b) demonstrated that certain strains of *L. thermotolerans* have poor lactic acid production and have a slight effect on pH lowering, although they generated higher amounts of ethyl lactate than *S. cerevisiae*.

Some authors (Guarcello et al., 2019; Matraxia et al., 2021; Sinacori et al., 2014) have recently conducted research on the selection of yeasts from high-sugar matrices such as honey, honey by-products and manna. The findings indicate that the strains isolated from these matrices have the potential to enhance the organoleptic quality of fermented beverages, with particular relevance to beer. Manna is a sugary product obtained from the solidification of the processed sap that emerges from incisions made in the stem and main branches of certain species of the genus *Fraxinus* sp. during the summer season (Schicchi, Camarda, Di Stefano, Spadaro, & Pitonzo, 2007; Yücedag and Sen, 2008). Manna hosts microorganisms that are osmophilic, meaning they can survive in a viable form under intense stress conditions caused by osmotic pressure due to the high sugar content. The most abundant yeast species found in manna is *L. thermotolerans* (Guarcello et al., 2019). Yeast strains isolated from this matrix, both *Saccharomyces* and non-*Saccharomyces*, have great technological properties and can improve the flavour profile of beers produced (Francesca et al., 2023; Pirrone et al., 2022). This is the first study to investigate the impact of these indigenous *L. thermotolerans* from an unconventional matrix, as manna, on the organoleptic quality of craft sour beers.

The research aimed to achieve the following objectives: (i) characterizing for the first-time indigenous *L. thermotolerans* strains isolated from manna for their brewing properties; (ii) selecting the yeast strain that has demonstrated the best effective fermentation performance in the production of innovative craft beer; and (iii) investigate the impact of this novel strain on the organoleptic quality of craft sour beers.

2. Materials and methods

2.1. Yeast strains and media

The yeasts used in this study, were previously isolated from Manna and identified in a precedent work by Guarcello et al. (2019). These strains were stored in glycerol stocks at -80 °C at the microbial collection of Department of Agricultural, Food and Forest Sciences (SAAF; University of Palermo, Italy). The strains were recovered and grown on YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose] at 30 °C for 2 days. All media components were purchased from Thermo Fischer (Milan, Italy). The investigation focuses on fifteen different strains (MN28, MN136, MN93, MN400, MNF104, MNF105, YS186, YS1, YS42, YS45, YS55, XV11, XV22, XV34, and XV47) belonging to the *L. thermotolerans* species. Commercial yeast strains *L. thermotolerans* Philly Sour and *S. cerevisiae* US-05 (both sourced from Lallemand Inc., Montreal, Canada) were utilized as controls in this study.

2.2. Technological screening of Lachancea thermotolerans strains

2.2.1. Hydrogen sulphide production

The production of hydrogen sulphide (H_2S) was evaluated by culturing the strains onto bismuth sulphite agar (Biggy Agar), Wilson-Blair medium (Merck, Darmstadt, Germany; Jiranek, Langridge, & Henschke, 1995). The results were evaluated by measuring the degree of colony blackening after 3 days of incubation at 28 °C, using a five-level scale: 0 = white, 1 = beige, 2 = light brown, 3 = brown, 4 = dark brown, 5 = black (Matraxia et al., 2021). The positive control was represented by *S. cerevisiae* GR1 (SAAF Department collection), which exhibited a brown colouration (3 = brown).

2.2.2. Sugar and ethanol tolerance assays

To evaluate ethanol tolerance, dilutions of pure exponential cultures were transferred onto tubes containing liquid YPD media supplemented with 6, 8 and 10% (v/v) ethanol. All samples were incubated at 28 °C for 3 days. Sugar stress tolerance assays were obtained using the same procedure as described by Binati et al. (2019). Stress conditions were tested in YPD medium containing different doses of stress agent. YPD medium was added (1%) to a previously grown culture and incubated overnight at 27 °C with agitation to reach the initial stationary phase. Accordingly, the following glucose concentrations were used: 220, 270 and 320 g/L. YPD medium without stress agent was used as a negative control. A commercial *S. cerevisiae* US-05 was used as a positive control strain. All analyses were carried out in triplicate.

2.2.3. Glucose, fructose, and maltose assimilation test

The ability of the strains to grow in the presence of different sugars was assessed using the procedure illustrated by Kurtzman, Fell, Boekhout, & Robert (2011) with the following modifications: tests were conducted in rimless tubes (16 × 180 mm), each one containing 10 mL of Yeast extract – Malt extract medium (YM composition: yeast extract, 3 g/L, triptone, 5 g/L; glucose, fructose or maltose, 200 g/L) and inoculated with pure strain cultures as reported by Hall et al. (2014). Growth was assessed by visual inspection (Kurtzman et al., 2011). The growth of the pure strain cultures in wort was further investigated by optical density (OD) measurement at 600 nm wavelength into a 96-well microtitre plate (Michel et al., 2016). The measurement was performed at 24 h intervals for 4 d using the ScanReady Microplate photometer P-800 (Life Real Biotechnology Co., Ltd, Hangzhou, China). Incubation temperature was set at 25 °C. Blank measurement was subtracted from each OD reading. The variables describing the growth curves were represented by the sum of the values of the subtended areas of the curves measured daily until the end of the experiment. A commercial *L. thermotolerans* (Philly sour) was used as a control strain, and a same medium without inoculum was used as a negative control. All analyses were performed in triplicates in two independent experiments.

2.2.4. Cross resistance to hop and ethanol

The five strains of *L. thermotolerans* that showed the best technological performances, i.e. low H_2S production, ethanol resistance, sugar stress tolerance, and excellent sugar assimilation ability, were evaluated for their ability to hop resistance, flocculate, and ferment beer wort. The tolerance of *L. thermotolerans* strains to hop was evaluated applying the procedure illustrated by Matraxia et al. (2021). Growth was assessed by visual inspection (Kurtzman et al., 2011; Michel et al., 2016). A commercial *S. cerevisiae* (US-05) was used as a control strain, and the same medium without inoculum was used as a negative control. All analyses were performed in triplicates.

2.2.5. Flocculation assay

The flocculation assay was carried out as previously described by Tofalo et al. (2014). Flocculation was also evaluated using the Helm's assay. In this assay, flocculation type and sedimentation volume were measured and evaluated in a calcium sulphate solution buffered at pH 4.5 according to Casey et al. (1994). A commercial strain of *S. cerevisiae* (US-05) and *L. thermotolerans* (Philly sour) were used as control strains, and the same medium without inoculum was used as a negative control. All analyses were executed in triplicates.

2.2.6. Microfermentation: monitoring of weight loss, strain concentration and physicochemical analysis

To ensure standardized conditions for all trials, the wort fermentation medium was prepared as described by Matraxia et al. (2021). Aliquots of 200 mL of wort were placed in 300 mL flasks, sealed with a Müller valve to allow CO_2 produced during fermentation to leave the system and autoclaved at 110 °C for 15 min. After sterilization, the extracted wort was allowed to cool to 18 °C and then inoculated with each yeast strain. Fermentation was performed at 18 °C under static conditions and was monitored daily by measuring weight loss until day 12. To facilitate CO_2 removal, the flasks were occluded with a Müller valve (Ciani and Rosini, 1987) and the weight loss was monitored until a daily decrease below 0.01 g (end of fermentation process). According to Ciani and Maccarelli (1998), the fermentation rate was determined as the daily production of CO_2 after 3 days and at the end of alcoholic fermentation, and fermentation vigour as the maximum amount of ethanol produced (as % v/v).

A commercial yeast of *L. thermotolerans* (Philly sour) was used as a positive control, and a wort without inoculum was used as a negative control. The cell density of *L. thermotolerans* strains was monitored at different stages of the fermentation process: at the first inoculum (D_0), at the 3rd day (D_3), at the 6th day (D_6), at the 9th day (D_9), at the 12th day (D_{12}), and at the end of alcoholic fermentation (D_{15}). The samples were serially diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy) and spread-plated (0.1 mL) into Wallerstein Laboratory (WL) nutrient agar to evaluate *Saccharomyces* populations and Lysine Agar medium (LA) for non-*Saccharomyces* (Di Maio et al., 2011; Iris, Antonio, Antonia, & Antonio, 2020). Lysine Agar is a medium that does not allow the growth of *S. cerevisiae* (Lin, 1975), and was used for the evaluation of theses with *L. thermotolerans* strains. All different colonies were identified as putative *Saccharomyces* and *Lachancea* after microscopic examination (Cavazza, Grando, & Zini, 1992). Total yeast counts were determined by incubating the samples at 28 °C for 48–72 h. At the end of fermentation, the beers were analysed for pH, residual sugar, lactic acid, acetic acid, and glycerol content using standard methods. The pH of the several samples collected was conducted using a pH meter Mod.70 XS/50010162 (Cheimika, Pellezzano, Italy). Brix degrees (°Bx) of the wort were determined with a refractometer DBR Salt (Zetalab srl, Padova, Italy). The determination of lactic acid, acetic acid, and glycerol was performed through the enzymatic analyser iCubio iMagic M9 (Shenzhen iCubio Biomedical Technology Co. Ltd. Shenzhen, China) following the procedure reported by Matraxia et al. (2021). All reagents and standards were purchased from R-Biopharm AG (Darmstadt, Germany). All analyses were performed in triplicates.

2.3. Experimental design and sample collection for beer production

The role of the inoculum during fermentation was followed on experimental top-fermented beers produced at a medium-scale level (5 L batch) using an all-in-one microbrewing plant Klarstein mod.

10031629 (Chal-Tec GmbH Berlin, Germany). For beer production, 4 kg wheat malt and 4 kg pilsen malt were used (BestMalz, Heidelberg, Germany). The malts were ground through a roller mill placed at 1.20 mm. The malts were then added to 30 L of water with 8 g of calcium sulfate ($CaSO_4$) and 8 g of calcium chloride ($CaCl_2$) for pH correction (Marconi, Rossi, Galgano, Sileoni, & Perretti, 2016). The mash was carried out according to the procedure of Francesca et al. (2023). Subsequently, the grains were rinsed with 16 L of water previously heated to 78 °C during the lautering phase, yielding in a total wort volume of 35 L. After filtration, the liquid portion of the wort was boiled and hops (Mandarina Bavaria - pellets, 40 g, 9.7 % w/w α-acids) were added at the beginning of this stage. At the end of this process, the total volume was 33 L. The wort was then clarified with a whirlpool involving a 10-min recirculation and a 10-minute rest. Finally, the wort was cooled to the yeast inoculation temperature of 21 °C using a stainless-steel wort cooling coil. Three experimental trials were inoculated as follows: T1 with *L. thermotolerans* MNF105, T2 with *L. thermotolerans* Philly sour and T3 with *S. cerevisiae* US05.

Therefore, to better evaluate the behaviour of the native strain, it was compared with a commercial strain of *L. thermotolerans* and one of *S. cerevisiae*.

Yeast strain was inoculated at a cell density of approximately 2.0×10^6 cells/mL (Holt, Mukherjee, Lievens, Verstrepen, & Thevelein, 2018). Fermentation was conducted at 20 °C in glass fermenters with hermetic closure and compensation valve. Samples were collected at different stages of beer production, including uninoculated must, after the inoculum of yeast strains, at 3, 6, 9 and 12 d. At the end of the fermentation process, the beer was transferred into 0.33 L bottles. At the end of the fermentation process, the beer was transferred into 0.33 L bottles by adding 6 g/L of dextrose. The bottles were conditioned at 20 °C for 25 days (Francesca et al., 2023). At the end of this process, the beers were subjected to volatile organic compounds and sensory analysis. All fermentation experiments were carried out in triplicate.

2.4. Microbiological counts and dominance of the inoculated strains and determination of physicochemical parameters

Microbiological counts and determination of physicochemical parameters were performed as described at paragraph 2.2.6. For each treatment and sampling point, isolates were obtained from the highest dilutions of the respective culture medium (Lysine Agar for non-*Saccharomyces* and WL agar for presumptive *Saccharomyces*) in order to verify the dominance of *L. thermotolerans* MNF105, Philly Sour and *S. cerevisiae* US-05. Five yeast colonies with the same macroscopic morphology were purified to obtain axenic colonies. These colonies were then observed under a light microscope to determine their cell morphology. Yeasts with a cell morphology similar to that of the genus *Lachancea* (Lachance and Kurtzman, 2011) and *Saccharomyces* (Vopálenská et al., 2005) were subjected to DNA extraction. The identification of the species *L. thermotholerans* and *S. cerevisiae* was confirmed through Restriction Fragment Length Polymorphism (RFLP) analysis, in accordance with the methodology described by Esteve-Zarzoso et al. (1999), utilising the restriction enzymes C*fo*I, H*in*f, and H*ae*III. The strain typing of *L. thermotholerans* strains was carried out by DNA (RAPD)-PCR analysis using the primer M13 (Binati et al., 2019), while for *S. cerevisiae*, interdelta analysis (Legras and Karst, 2003) was used. Amplification, visualisation of bands and result analysis were conducted using Gelcompar II software, version 6.5 (Applied-Maths, Sint-Martens-Latem, Belgium), in accordance with the methodology described by Alfonzo et al. (2021). The comparison of the polymorphic and interdelta profiles of the isolates from the different trials and the inoculated strains (*L. thermotholerans* MNF105, Philly Sour and *S. cerevisiae* US-05) enabled the percentage of dominance to be determined.

BeerFoss™ FT Go (FOSS Italia srl, Padova, Italy) was used to measure alcohol (% vol), density (FG), real extract (°P), energy (kcal/100g), apparent extract (°P), original extract (°P), specific gravity (°P) and real attenuation (%) of the final beers (Gugino et al., 2024; Francesca et al., 2023).

2.5. Analysis of Volatile organic compounds (VOCs) of beer samples

2.5.1 *Standard solutions*

Limonene (Fisher Scientific S.L.C, 28108 – Alcobendas-Madrid) was used as standard for calibration line. Standard solutions were prepared at five different concentrations (31.2 mg/L, 62.5 mg/L, 125 mg/L, 169 mg/L and 250 mg/L)

2.5.2. SPME analysis

Beer samples (10 mL) were placed in a 20 mL SPME glass vial (Gerstel, 75.5 × 22.5 mm) together with 1 g of sodium chloride. The column temperature was initially kept constant at 40 °C for 2 min (during splitless injection), subsequently, increasing the temperature by 4 °C/min was set to 60 °C, at which it was kept constant for 2 min. Increasing the temperature by 2 °C/min., it was raised to 90 °C, from 190 °C to 230 °C, increasing by 5 °C/min and finally left at 230 °C for 15 min. The analyses in the fiber were automatically injected at 250 °C with the splitless mode. The mass spectrometer was set in MS mode to acquire all mass-to-charge ratios from 35 to 450 amu (0.1 amu). Identification of compounds was carried out using Adams, NIST 11, Wiley 9 and FFNSC 2 mass spectral database. These identifications were also confirmed by other published mass spectra. Quantification was carried out using limonene calibration line.

2.5.3. Identification and quantification of VOCs by GC-MS

Gas chromatographic analyses were performed using an Agilent 7000C GC system, fitted with a fused silica Agilent DB-5MS capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness), coupled to an Agilent triple quadrupole Mass Selective Detector MSD 5973; ionization voltage 70 eV; electron multiplier energy 2000 V; transfer line temperature, 295 °C. Solvent Delay: 3.5 min. Helium was the carrier gas (1 mL/min). The odour activity value (OAV) was calculated for each VOC detected, following the approach proposed by Butkhup et al. (2011), to determine the VOCs that contributed significantly to the odour series characterising each beer.

2.6. *Sensory analysis*

Fifteen judges (10 men and 5 women) aged between 26 and 46 were selected from the University of Palermo to evaluate the beer produced. All panelists had experience in brewing and participated in previous studies as sensory judges. The sensory analysis in this study adhered to ethical standards for sensory research. All participants provided voluntary consent and were informed about the study. Participant information and privacy were protected through anonymization and appropriate measures. Prior to publication of experimental data, informed consent was obtained from all participants. Moreover, no ethical permission is required from the institution and/or country for this study. The beer evaluation procedure was carried out as described by Francesca et al. (2023) with some modifications to the descriptors as follows: odour (intensity, complexity, fruity, floral, hoppy, wheat/cereal, honey/caramel, acetic, oxidized/aged, sulphury, alcohol and DMS) and taste (intensity, complexity, sweet, bitter, acid, astringent, fruity, spicy, hoppy, sapidity, wheat/cereal, burnt/cooked, alcohol, body, DMS and oxidized/aged) and overall acceptance. The results were presented in an unstructured scale of 9 cm in length, with the endpoints demarcated by the descriptors "none/weak" and "strong", respectively (Barry, Metz, Hughey, Quirk, Bochman et al., 2018; Jackson, 2016). The average of the three assessments was used to obtain the final scores.

2.7. Statistical analysis

The results of physicochemical parameters of micro-fermentation and fermentation were statistically processed by analysis of variance (ANOVA), and homogeneous groups were identified by Tukey’s test (statistical significance: $P < 0.05$). Sensory product characterization was performed to differentiate between the different treatments based on data collected during sensory analysis, conducted following the methodology outlined by Alfonzo et al. (2023). The objective of this study is to evaluate the correlation between the aromas identified in the VOCs with an odour activity value exceeding 1 and the sensory analysis (only aroma), a principal component analysis (PCA) was performed using the XLstat software version 2019.2.2 (Addinsoft, New York, NY, USA) for Excel.

3. Results and Discussion

3.1 Technological characteristics of Lachancea thermotolerans yeast strains for beer production

3.1.1 H_2S production, alcohol resistance and sugar stress tolerance

The results of technological screening are reported in Table 1. All strains were found to be devoid of H_2S production, confirming the findings of Porter, Divol, & Setati (2019a). The evaluation of H_2S production revealed no strain variability. However, these results are in contrast with those of Comitini et al. (2011), who demonstrated that some strains belonging to the genus *Lachancea* can high amounts of this compound (3 to 5 on a scale of 0 to 5). It is important to note that this trait is not dependent on the genus or species, but on the strain. All strains of *L. thermotolerans* showed growth on lysine agar (25 °C). Additionally, the strains exhibited tolerance to ethanol. In our study, all strains of *L. thermotolerans* showed the ability to resist alcohol at 6 % and 8 % (v/v), and five strains showed high resistance at 10% (v/v). Moreover, *L. thermotolerans* spp. is known to be more resistant to ethanol than other non-*Saccharomyces* species. However, this characteristic depends on the strain, with some strains showing greater resistance than others. For example, some strains of *L. thermotolerans* have been shown to be more resistant than other species of this genus, including *Lachanchea lanzarotensis* and *Lachanchea fermentati* (Porter et al., 2019a; Porter, Divol, Setati, 2019b). Similarly, it has been shown to be more resistant than other species such as *H. uvarum*, *Metschnikowia pulcherrima* or *Starmerella Bacillaris* (Aponte & Blaiotta, 2016). The *L. thermotolerans* strains chosen for this investigation may be used as starters to produce beer with up to 10% (v/v) ethanol. Regarding sugar stress tolerance, all isolates were able to grow at the three concentrations tested and showed no difference in growth at the increased osmotic pressure caused by high glucose concentrations. This contrasts with Binati et al. (2019), who showed a decrease in yeast culture growth with increasing sugar content. This may be related to strain and sampling matrix, as yeast strains isolated from manna, a high sugar matrix, may be more resistant to osmotic pressure (Guarcello et al., 2019).

3.1.2 Glucose, fructose and maltose assimilation test

The strains were tested for their ability to assimilate fructose, glucose, and maltose during the fermentation of the main sugar in the wort (Fig. 1a, 1b, 1c). The results showed that all 15 isolates grew in the presence of single sugar as the sole carbon source, but some of them showed more growth than others. Strain XV47 showed the highest total growth during fermentation, in terms of fructose consumption (Fig. 1a), followed by strain XV22. However, in terms of glucose consumption (Fig. 1b), the highest total growth was achieved by strain MN400, while the second-best strain was MN28. As demonstrated, this species is characterized by the ability to ferment sugars such as glucose and fructose, confirming numerous studies (Comitini et al., 2011; Toh, Chua, Lu, & Liu, 2020; Zdaniewicz et al., 2020b). After fructose and glucose uptake, yeast assimilates maltose, the most abundant fermentable sugar in brewing wort (Boulton & Quain, 2006). *L. thermotolerans* is known to be able to consume maltose, but not all strains are able to do so (Domizio, House, Joseph, Bisson, & Bamforth, et al., 2016; Toh et al., 2020; Postigo et al., 2022). In this case, all strains were able to consume maltose, with the highest total growth regarding maltose consumption found for strain MNF105 (Fig. 1c), followed by strain MN400. These results suggest that the strains selected for this research can be used as starter strains for brewing applications, with some of these strains being particularly suited to beer production.

3.1.3. Cross resistance to hop and ethanol

The study evaluated the growth of yeasts in the presence of iso-α-acid and in the cross presence of iso-α-acid and ethanol to select the most resistant strains (Table 2). All strains were able to grow in liquid medium containing 0, 25, 50, and 90 IBU. In terms of cross-resistance to ethanol and hops, all strains showed vigorous growth in the presence of all the limiting conditions present (Table 2). Accordingly, to Domizio et al. (2017) demonstrated that this species is not affected by IBU levels, at least up to 60 IBU.

3.1.4. Flocculation assay

The mean sedimentation volumes ranged from 0.45 to 2.70 mL as measured by the Helm’s assay. All yeasts, with the exception of PC2, exhibited type II flocculation with an ascending interface near the bottom of the tubes, a phenomenon that is characteristic of non-flocculating yeasts, as described by Casey et al. (1994). Furthermore, PC2 demonstrated type I flocculation, and the suspension rapidly separated into two distinct layers near the top of the liquid, which is a characteristic of flocculent yeasts. The flocculation capacity of brewer's yeast is directly related to the yield, clarity and filtration performance of green beer (Govender, Kroppenstedt and Bauer, 2011; Varela et al., 2020). Furthermore, low flocculation can result in higher attenuation (Panteloglou, Smart and Cook, 2012). Consequently, yeasts with low flocculation tend to remain in suspension, resulting in a cloudy appearance of the finished beer. Nevertheless, beer turbidity may also be a desired quality in unfiltered or naturally turbid beers, such as wheat beer (Kahle, Zarnkow and Jacob, 2021).

3.1.5. Microfermentation

The strains were evaluated for their ability to grow under technological conditions simulating beer production. The results of the fermentation kinetics of wort weight loss as a function of CO_2 production (Fig. 2) showed that the MNF105 strain lost slightly more weight than the control strain Philly Sour. After 3 days of alcoholic fermentation, strain MN28 showed the greatest weight loss (2.80 g). From day 3 to day 15, the greatest weight loss was measured for strain MN93 (10.39 g). At the end of the alcoholic fermentation process, the greatest weight losses, ranging from 10.80 g to 11.15 g, were observed for strains MN28 and MNF105, respectively. Yeast strain MN28 showed a higher value for fermentation rate (2.80 v/v), while MNF105 showed a higher value for fermentation vigour (4.61 g of CO_2/day). In general, both strains exhibited lower values than the control strain. These results contrast with those obtained by Zdaniewicz et al. (2020b), who reported a high fermentation intensity in the first days of the process.

The growth kinetics of the different inoculated strains are shown in Fig. 3. The microbiological counts of the wort without inoculation were below the detection limit. After inoculation, yeast cell densities varied between 6.4 and 7.0 Log CFU/mL, which increased just after 1 d for all trials. Starter yeasts increased by about 1 Log cycles after 3 days for all trials. At the next sampling point (day 6 of alcoholic fermentation), trials MN93, MN400, and XV47 started to decrease the presumed populations of *Lachancea* spp., while the other trials continued to increase. Instead, on day 9, all trials started to decrease except trial MN28, which started from the next sampling point. The presumptive *L. thermotolerans* strains in the different trials showed similar dynamics, with an increase in the first few days with a peak between 7.7 and 7.2 (Log CFU/mL), followed by a subsequent decrease. The trial inoculated with the MNF105 strain showed higher values than the control strain. At the end of the alcoholic fermentation, the highest cell counts were registered for the MNF105 trial (7.7 Log CFU/mL).

The yeast growth dynamics during the alcoholic fermentation of beer were like to those described by (Domizio et al., 2016). The physicochemical parameters of the final beers are presented in Table 3. Yeast strain MNF105 proved to be more capable to produce lactic acid than the other strains. In terms of final density, MNF105 was also the strain with the closest values to the control strain and these results are particularly encouraging. The analysis of the physicochemical parameters and the evolution of the glycerol, lactic acid, and acetic acid concentrations showed several differences between the strains (Table 3). The strain XV47 produced the highest amount of acetic acid (0.32 g/L). In contrast, MNF105 exhibited the highest glycerol production (2.17 g/L), exceeding the control (2.10 g/L). This strain demonstrated a higher lactic acid production (0.45 g/L) than the other strains, yet remained below the control (2.55 g/L).

3.2. Beer production

3.2.1. Yeast growth during fermentation

The evolution of the concentrations of yeasts during the alcoholic fermentation of beer wort fermentation is shown in Fig. 4. On both WL and LA medium, the uninoculated wort's microorganism concentrations were below the detection limit. The inoculation rate showed a slight difference between trials, 6.2 Log CFU/mL for T1 and 6.1 and 6.5 Log CFU/mL for T2 and T3, respectively. These yeast strains showed a similar fermentation trend, which followed general fermentation dynamics of beer wort (Toh et al., 2020; Francesca et al., 2023). For both tests, the microbial load values increased until day 6 and then started to decrease. This phenomenon could be imputable to several factors, including the lack of appropriate nutrients (Domizio et al., 2016; Michel et al., 2016). All strains remained viable until the end of fermentation with high values. In fact, on the last day of fermentation (day 12), the microbial load values of the trials were 6.1 Log CFU/mL (trial T1) and 5.9 Log CFU/mL (control trial T3). The trial with the highest score was T3 on day 6 with a score of 7.2 Log CFU/mL, which was higher than the other trials. Thus, the new strain MNF105 in the T1 trial demonstrated a fermentation growth curve comparable to that of both the T2 and T3 control strains. A total of 225 yeast isolates were collected. Specifically, 75 yeast isolates were obtained from each trial (T1, T2 and T3). DNA analysis (RAPD-PCR) demonstrated that all *L. thermotolerans* strains exhibited an identical RAPD profile to the inoculated strains (*L. thermotolerans* MNF105 and Philly Sour) in the respective trials (T1 and T2). In the T3 treatment, the interdelta profiles of the *S. cerevisiae* were comparable to those of the inoculated *S. cerevisiae* strain US-05.

3.2.2 Physicochemical analysis obtained during fermentation

The wort had a pH of 5.70 and 12.20 °Bx. The sugar composition was: 4.60 g/L of fructose, 7.50 g/L of glucose, 37.50 g/L of maltose, and 15.95 g/L of sucrose. The data recorded by the FOSS analysis is reported in Table 4. The *L. thermotolerans* strain MNF105 was able to reduce the pH from 5.70 to 3.87. Furthermore, the beers produced with this strain showed a slightly higher pH than that showed by the commercial control *L. thermotolerans* (T2) but lower than that determined by the *S. cerevisiae* strain used (T3), which reached a final value of 3.56 and 4.12, respectively. Our results are consistent with those of Domizio et al. (2016); indeed, *L. thermotolerans* 101 was able to produce beers with a final pH of 3.77. In contrast, Zdaniewicz et al. (2020b) showed that the strain MN477031 only marginally influences the drop of pH compared to *S. cerevisiae*. In terms of sugar consumption, the results of the new strain were comparable to those of the commercial control *L. thermotolerans* (T2), with a final density of 1.016 compared to 1.014. However, none of them equalled the outcomes of the *S. cerevisiae* strain in the T3 trial. There is a correlation between ethanol production, residual sugar amount, and attenuation, with consistent differences between the trials inoculated with *L. thermotolerans* (T1 and T2) and those inoculated with *S. cerevisiae* (T3). However, the results among the strains for the main sugar, acids and glycerol strains were encouraging (Table 5). Consequently, trial T1 (6.00 g/L) demonstrated a higher final sugar content than trials T2 and T3, with values of 5.51 g/L and 0.98 g/L, respectively.

In terms of sugar consumption kinetics, the control strain *S. cerevisiae* US-05 (T3) demonstrated superior results. The MNF105 strain demonstrated comparable behaviour to the Philly Sour strain in the T2 trial, both of which exhibited a slower rate of sugar consumption than *S. cerevisiae*. Finally, MNF105 exhibited a lower rate of sugar consumption than the other trials. After 3 days of fermentation, the strains have almost consumed all the sugars except maltose. Subsequently, maltose was the only sugar remaining even after the completion of alcoholic fermentation. In contrast, Toh et al. (2020) showed that yeast strain of *L. thermotolerans* Concerto consumed all sugars present in the wort, including maltose.

Our results show that *L. thermotolerans* could assimilate maltose, but this capacity is strain-dependent and overall lower than that of *S. cerevisiae* (Callejo et al., 2019). The two strains of *L. thermotolerans* produced higher levels of lactic acid than the *S. cerevisiae* strain, confirming the acidifying capacity of this species. However, differences were found between the two strains, with the novel strain producing 0.47 g/L at the end of alcoholic fermentation compared to 1.62 g/L for the control strain. These results are significantly higher than those demonstrated by Domizio et al. (2016) and Zdaniewicz et al. (2020b) and particularly encouraging as the low lactic acid content makes this strain interesting to produce low-sour beers, which, unlike sour beers, appeal to a wider range of consumers. The results of the trials indicated that the values for acetic acid, malic acid and tartaric acid were notably low. Regarding glycerol, the concentrations registered in our study (3.16 – 3.47 g/L) are higher than the range of values reported by Domizio et al. (2016) and Viana et al. (2021), but lower to those reported by Zdaniewicz et al. (2020b).

3.2.3 Determination of volatile organic compounds

The experimental beers contained a diverse range of aromatic organic compounds, as shown in Table 6. The composition of VOCs in the final beer showed great complexity, characterised by five classes: alcohols, esters, carboxylic acids, terpenes and others. The use of the novel MNF105 (T1) strain for beer fermentation resulted in the highest concentration of VOCs, with a higher amount of esters compared to the other control trials.

The ester class was the most abundant, with a particularly high concentration in T1 (324.5 mg/L) followed by T3 inoculated with *S. cerevisiae* US-05 (259.7 mg/L). The ester class was characterised by twenty-one different compounds. Of these, T1 trial yielded particularly interesting results, demonstrating higher values for eleven compounds. The presence of esters is responsible for the fruity flavour of fermented beverages, and their concentration depends on the enzyme activity of the yeast strains (Pires, Teixeira, Bràanyik, & Vicente, 2014). The main ester compound identified in this study was ethyl decanoate, which provided rich fruity and floral properties (Liu et al., 2022). The T1 trial yielded highly encouraging results, with a value of 165.3 mg/L compared to 12.1 mg/L and 93.0 mg/L for the control strains T2 and T3, respectively. This compound has been detected in stout, lager and wheat beers (De Flaviis, Santarelli, Mutarutwa, Giuliani, & Sacchetti, 2022). The other most abundant esters were ethyl hexanoate (29.4 mg/L in T1 followed by 18.0 in T2), ethyl acetate (36.1 mg/L in T1 followed by 29.1 mg/L in T3), and ethyl octanoate (29.7 in T1 followed by 16.0 mg/L in T2). These compounds are secondary metabolites of yeasts and contribute to the aroma of beer. It should be noted that the main active esters in beer are phenylethyl acetate (flowers, rose, honey), isoamyl acetate (fruit, banana), ethyl acetate (fruit, sweet), and ethyl hexanoate and ethyl octanoate (sour apple) (Verstrepen et al., 2003). These compounds were also found in other studies where they brewed beers with certain strains of *L. thermotolerans* (Gobbi et al., 2013; Postigo et al., 2023). Ethyl acetate was also noted in barley beers brewed with a yeast strain belonging to the *Lachancea* genus (Galaz & Franco, 2023), and in sorghum beers fermented with *S. cerevisiae* (Tokpohozin, Fischer, & Becker, 2019). However, it has also been detected in fruit lambic beers, which are spontaneously fermented and usually associated with *Brettanomyces* activity (Bongaerts, De Roos, & De Vuyst, 2021). Regarding alcohols, the results of the two trials inoculated with *L. thermotolerans* are comparable, with the T2 control trial (127.0 mg/L) showing a higher value than T1 trial (100.0 mg/L). The trial T3 inoculated with *S. cerevisiae* exhibited greater amounts of alcohols (216.9 mg/L) in contrast to the other two trials. The class of alcohols is characterised by alcoholic, floral or solvent aromas (Eβlinger, 2009).

Compounds belonging to the terpenoid class, such as limonene and β-myrcene, have been found in beers and can be attributed to hops. Limonene is the second most abundant terpenoid in nature and is an important aroma compound associated with hops that gives beer a citrus or pine flavour (Ramírez and Viveros, 2021; Jiang et al., 2023). This compound is mainly found in lemons and other citrus fruits. However, it is generally found in more than 300 plants (Jongedijk et al., 2016). β-myrcene is also a compound derived from hops (Brendel, Hofmann, & Granvogl, 2020). Brendel et al. (2020) detected its presence in beer made with different variety of hops. Among the thirty-one VOCs, seventeen compounds showed an odour activity value greater than one, specifically one alcohol (1-Pentanol), fifteen esters (ethyl acetate, isobutyl acetate, isoamyl acetate, isoamyl isobutyrate, ethyl hexanoate, ethyl heptanoate, ethyl octanoate, isoamyl caproate, phenylethyl acetate, ethyl nonanoate, ethyl decanoate, isoamyl caprylate, ethyl laurate, and ethyl palmitate) and two terpenes (β-myrcene and limonene). Esters are the principal and significant group of compounds generated by yeasts metabolism during alcoholic fermentation. These compounds exert a considerable influence on the beer's ultimate flavour profile (Holt et al., 2019).

3.2.4 Sensory analysis

The resulting beers were evaluated by sensory analysis to determine the sensory suitability of the strain tested. Fig. 5 shows histograms of the sensory analysis results for aroma and taste. The differences between the experimental beers were significant, and the novel yeast had a different effect on the sensory characteristics of the final products. No off-flavours or off-odours were reported by the panellists. Consequently, these descriptors have not been included in Fig. 5. These results agree with those obtained by Francesca et al. (2023), who tested the strain MNF105 during fruit beer production and was shown to enhance the aromatic characteristics. The sensory characterisation of the beers defined coefficients for 9 aroma descriptors and 15 flavour descriptors. These coefficients were quantified by how much the calculated value was significantly above or below the overall average. The three trials were characterized by different descriptors above the mean values. According to many studies, the use of *L. thermotolerans* increases the perceived acidity of beers due to its ability to produce lactic acid determining a drop of pH (Osburn et al., 2018; Peces-Pérez, Vaquero, Callejo, & Morata, 2022; Postigo et al., 2023; Romero-Rodríguez et al., 2023).

The overall organoleptic study showed a preference for T1 beer inoculated with strain MNF105, which presented fruity notes and a balanced acidity. Instead, it showed different descriptors above the mean values, five for aromas (intensity, complexity, fruity, honey/caramel and overall acceptance) and eleven for flavours (intensity, complexity, sweet, bitter, fruity, spicy, hoppy, sapidity, burnt/cooked, body and overall acceptance). Beers brewed with commercial *L. thermotolerans* (T2) were too acidic and therefore unbalanced, while those brewed with *S. cerevisiae* (T3) were drier and with a higher alcohol content. These results confirm the research of Postigo et al. (2023), who showed that the yeast strain *L. thermotolerans* CLI 1232 has a balanced acidity with a fruity flavour profile. Furthermore, these analyses showed that the new strain isolated from manna can produce beers with a low-acidity level, which are more balanced and have flavours that could be appreciated by a larger number of consumers.

3.3. Correlation between volatile organic compounds and sensory profiles

A PCA was conducted to evaluate the relationship between VOCs and sensory profile (aroma attributes). The results (Fig. 6) showed that the F1 factor contributed 65.67% of the total variance, whereas the F2 factor explained 34.33% of the total variance. The biplot graph indicated that each beer was distinct from the others. The T1 beer was associated with ethyl acetate, ethyl decanoate, ethyl heptanoate, ethyl octanoate, and ethyl hexanoate, which produced fruity and sweet aromas

(Verstrepen et al., 2003). These esters exceeded the odour threshold and were also identified in the sensory analysis, with higher fruity and honey/caramel scores than in the other trials. The hop aroma, higher in the T2 beer, is associated with limonene and β-myrcene, which is attributable to hops (Jiang et al., 2023). Instead, the T3 beer was associated with four odour descriptors (wheat/cereals, alcohol and floreal). Phenylethyl acetate is responsible for the floral in T3 beer, as it can produce floral aromas (Verstrepen et al., 2003).

Conclusion

In conclusion, for the first time this study examines indigenous *L. thermotolerans* strains that originate from high-sugar matrices, specifically manna ash, for their potential brewing applications. The research enriches our limited scientific knowledge of sugar extracts from manna ash on a microbial level and sheds new light on the potential role of indigenous *Lachancea* yeasts as starters for sour beer production. Five strains demonstrated significant resistance to 10 % ethanol and 320 g/L of sugar and interesting weak lactic acid production (between 0.33 g/L and 0.45 g/L). The strain MNF105 has demonstrated excellent fermentation performances and has been chosen for use in brewing conditions. During sensory analysis, beers produced using MNF105 (T1) were the most preferred and perceived as the most “fruity” with an acid taste lower than control. As a results, elevated levels of ethyl decanoate (165.30 mg/L), ethyl hexanoate (29.4 mg/L), ethyl octanoate (29.7 mg/L) and ethyl acetate (36.1 mg/L) were found in T1 beer, exceeding the perception threshold. The use of this strain in the beer production process resulted in improvements in physicochemical parameters, VOCs, and sensory characteristics of beer produced. This evidence indicates that the isolated strain from an unconventional matrix, as manna, has the potential to serve as a brewing starter for a diverse range of beer production styles. The species *L. thermotolerans* is typically employed in the acidification of wort. However, in this instance, in addition to acidification, this strain has demonstrated the ability to aromatically enhance beverages. Moreover, the capacity of this strain to generate limited quantities of lactic acid could be a valuable trait for the development of novel beer production techniques, particularly in the creation of sour beers with a balanced sour taste. This would result in beer with a well-balanced acid or sour taste and innovative flavours. Manna sugary exudate remains an innovative source of potential starter yeasts for fermented alcoholic beverage. Further research is required to assess the impact of these strains on wort fermentation in industrial settings and under varying wort compositions.

Declarations of Competing Interest

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

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Table 1. Technological characteristics of *Lachancea thermotolerans* strains for beer production

	Production of H_2S[a]	**Growth on LA[b]**	**Ethanol resistance**			**Sugar test tolerance**		
			6%	**8%**	**10%**	**220 g/L**	**270 g/L**	**320 g/L**
MN28	-	+	+	+	+	+	+	+
MN136	-	+	+	+	-	+	+	+
MN93	-	+	+	+	+	+	+	+
MN400	-	+	+	+	+	+	+	+
MNF104	-	+	+	+	-	+	+	+
MNF105	-	+	+	+	+	+	+	+
YS186	-	+	+	+	-	+	+	+
YS1	-	+	+	+	-	+	+	+
YS42	-	+	+	+	-	+	+	+
YS45	-	+	+	+	-	+	+	+
YS55	-	+	+	+	-	+	+	+
XV11	-	+	+	+	-	+	+	+
XV22	-	+	+	+	-	+	+	+
XV34	-	+	+	+	-	+	+	+
XV47	-	+	+	+	+	+	+	+
PC[c]	+/-	-	+	+	+	+	+	+
NC[d]	-	-	-	-	-	-	-	-

Symbols: +, positive growth; –, no growth; +/- , weak growth.

Abbreviations: [a] H_2S, Hydrogen sulphide; [b] LA, lysine agar, [c] CP, positive control, [d] NC, negative control.

Table 2. Cross resistance to hop and ethanol of the different yeast strains

	Resistance to hop				Cross resistance				**Flocculation assay**[a]	**Sedimentation volume**[b]
Strain code	0 IBU	25 IBU	50 IBU	90 IBU	0 IBU/5% ethanol	25 IBU/5% ethanol	50 IBU/5% ethanol	90 IBU/5% ethanol		
MN28	+	+	+	+	+	+	+	+	0	0.75
MN93	+	+	+	+	+	+	+	+	0	0.65
MN400	+	+	+	+	+	+	+	+	0	0.55
MNF105	+	+	+	+	+	+	+	+	0	0.45
XV47	+	+	+	+	+	+	+	+	0	0.60
PC1	+	+	+	+	+	+	+	+	0	0.75
PC2	-	-	-	-	-	-	-	-	1	2.10
NC	-	-	-	-	-	-	-	-	0	0

Symbols: +, positive growth; −, no growth; +/- , weak growth.

Abbreviations: IBU, International Bitterness Unit; PC, positive control; NC, negative control;

[a] Flocculation degree after 22 days of incubation;

[b] Mean sedimentation volume (mL) expressed according to Helm's Assay.

Table 3. Physicochemical analysis the different trials of microfermentation samples and main fermentation properties.

	MN28	**MN93**	**MN400**	**MNF105**	**XV47**	**TC**	**S.S.**
Fermentation vigour as ethanol % (v/v)	2.80 ± 0.10^{ab}	2.48 ± 0.17^{b}	2.54 ± 0.13^{ab}	2.55 ± 0.14^{ab}	2.50 ± 0.07^{b}	2.85 ± 0.11^{a}	**
Fermentation rate[a] (g of CO_2/day)	4.52 ± 0.14^{ab}	4.34 ± 0.12^{b}	4.53 ± 0.11a^{b}	4.61 ± 0.08^{ab}	4.40 ± 0.13^{ab}	4.76 ± 0.20^{a}	*
pH	3.85 ± 0.10^{a}	3.89 ± 0.11^{a}	3.79 ± 0.08^{a}	3.75 ± 0.11^{a}	3.78 ± 0.15^{a}	3.60 ± 0.05^{a}	N.S.
Density (FG)	1.017 ± 0.007^{a}	1.019 ± 0.008^{a}	1.017 ± 0.003^{a}	1.016 ± 0.009^{a}	1.018 ± 0.007^{a}	1.015 ± 0.006^{a}	N.S.
Acetic acid (g/L)	0.11 ± 0.01^{bc}	0.07 ± 0.00^{c}	0.12 ± 0.00^{bc}	0.18 ± 0.01^{b}	0.32 ± 0.05^{a}	0.17 ± 0.01^{b}	***
Lactic acid (g/L)	0.33 ± 0.03^{b}	0.34 ± 0.03^{b}	0.39 ± 0.04^{b}	0.45 ± 0.07^{b}	0.41 ± 0.05^{b}	2.55 ± 0.12^{a}	***
Glycerol (g/L)	1.85 ± 0.12^{ab}	1.80 ± 0.13^{b}	2.04 ± 0.08^{ab}	2.17 ± 0.07^{a}	1.97 ± 0.07^{ab}	2.10 ± 0.12^{a}	**

[a] Time period of 3 days.

Values are expressed as average of three measurements ± standard deviation.

Abbreviations: N.S., not significant.

Data in the same line followed by the same letter are not significantly different according to Tukey’s test. Symbols: ***, $P < 0.001$; **, $P < 0.01$; * $P < 0.05$.

Table 4. Physicochemical parameters identified in the beers by FOSS.

	T1	T2	T3	S.S.
Alcohol % (v/v)	4.07 ± 0.11^c	4.33 ± 0.09^b	4.86 ± 0.08^a	***
Density (FG)	1016 ± 1.20^a	1014 ± 1.00^a	1010 ± 1.15^a	**
Real extract (°P)	5.45 ± 0.12^a	5.04 ± 0.09^b	4.65 ± 0.10^c	***
Energy (kcal/100g)	50.34 ± 0.15^a	45.04 ± 0.13^b	44 ± 0.11^c	**
Apparent extract (°P)	4.08 ± 0.11^a	3.57 ± 0.12^b	2.91 ± 0.09^c	**
Original extract (°P)	11.67 ± 0.09^b	11.70 ± 0.12^b	12.20 ± 0.10^a	***
Real attenuation (%)	52.9 ± 0.24^c	56.4 ± 0.36^b	62.80 ± 0.42^a	***
pH	3.87 ± 0.12^b	3.56 ± 0.10^c	4.12 ± 0.17^a	***

Values are expressed as average of three measurements ± standard deviation
Abbreviations: S.S., statistical significance; N.S., not significant.
Beer fermented by: *L. thermotolerans* MNF105 (T1); *L. thermotolerans* Philly Sour (T2), *S. cerevisiae* US-05 (T3). Data in the same line followed by the same letter are not significantly different according to Tukey's test.
Symbols: ***, $P < 0.001$; **, $P < 0.01$; * $P < 0.05$.

Table 5. Monitoring of the chemical composition of samples during the alcoholic fermentation of beer.

	T1	T2	T3	S.S.
D-fructose (g/L)				
3d	0.22 ± 0.01^{a}	0.15 ± 0.02^{b}	0.12 ± 0.02^{b}	***
6d	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.02 ± 0.00^{a}	N.S.
9d	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.02 ± 0.00^{a}	N.S.
End AF	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	N.S.
D-glucose (g/L)				
3d	0.58 ± 0.04^{a}	0.47 ± 0.05^{b}	0.15 ± 0.01^{c}	***
6d	0.48 ± 0.06^{a}	0.09 ± 0.01^{b}	0.04 ± 0.00^{b}	***
9d	0.04 ± 0.01^{a}	0.03 ± 0.00^{a}	0.02 ± 0.01^{a}	N.S.
End AF	0.00 ± 0.00^{a}	0.01 ± 0.00^{a}	0.00 ± 0.00^{a}	N.S.
Maltose (g/L)				
3d	25.85 ± 0.36^{a}	23.58 ± 0.28^{b}	7.74 ± 0.15^{c}	***
6d	16.88 ± 0.48^{a}	15.58 ± 0.26^{b}	3.38 ± 0.05^{c}	***
9d	7.89 ± 0.16^{a}	6.58 ± 0.18^{b}	1.45 ± 0.07^{c}	***
End AF	6.47 ± 0.18^{a}	5.49 ± 0.09^{b}	0.95 ± 0.15^{c}	***
D-sucrose (g/L)				
3d	3.24 ± 0.09^{a}	2.04 ± 0.11^{b}	0.35 ± 0.03^{c}	***
6d	0.15 ± 0.01^{a}	0.14 ± 0.02^{a}	0.18 ± 0.02^{a}	N.S.
9d	0.09 ± 0.01^{b}	0.11 ± 0.01^{b}	0.17 ± 0.02^{a}	***
End AF	0.03 ± 0.00^{a}	0.01 ± 0.00^{a}	0.02 ± 0.00^{a}	N.S.
Lactic acid (g/L)				
3d	0.07 ± 0.01^{a}	0.07 ± 0.00^{a}	0.05 ± 0.01^{a}	*
6d	0.41 ± 0.06^{b}	1.49 ± 0.12^{a}	0.09 ± 0.00^{c}	***
9d	0.45 ± 0.06^{b}	1.56 ± 0.09^{a}	0.12 ± 0.01^{c}	***
End AF	0.47 ± 0.07^{b}	1.62 ± 0.09^{a}	0.08 ± 0.00^{c}	***
Acetic acid (g/L)				
3d	0.03 ± 0.00^{b}	0.14 ± 0.01^{a}	0.01 ± 0.00^{c}	***
6d	0.06 ± 0.01^{b}	0.15 ± 0.02^{a}	0.02 ± 0.00^{c}	***
9d	0.15 ± 0.02^{a}	0.17 ± 0.02^{a}	0.04 ±0.01^{b}	***
End AF	0.10 ± 0.01^{b}	0.13 ± 0.01^{a}	0.04 ± 0.01^{c}	***
L-Malic Acid (g/L)				
3d	0.13 ± 0.01^{ab}	0.1 ± 0.01^{b}	0.16 ± 0.02^{a}	**
6d	0.15 ± 0.03^{ab}	0.11 ± 0.01^{b}	0.18 ± 0.01^{a}	*
9d	0.17 ± 0.01^{b}	0.14 ± 0.01^{b}	0.22 ± 0.02^{b}	***
End AF	0.18 ± 0.02^{ab}	0.15 ± 0.03^{b}	0.22 ± 0.03^{a}	N.S.
Glycerol (g/L)				
3d	2.57 ± 0.10^{b}	3.11 ± 0.08^{a}	2.96 ± 0.05^{a}	***
6d	3.14 ± 0.17^{a}	3.16 ± 0.09^{a}	3.04 ± 0.15^{a}	N.S.
9d	3.20 ± 0.22^{a}	3.28 ± 0.08^{a}	3.08 ± 0.09^{a}	N.S.
End AF	3.47 ± 0.09^{a}	3.32 ± 0.11^{ab}	3.16 ± 0.04^{b}	*
Tartaric acid (g/L)				
3d	0.15 ± 0.07^{a}	0.16 ± 0.03^{a}	0.18 ± 0.05^{a}	N.S.
6d	0.16 ± 0.01^{b}	0.17 ± 0.01^{b}	0.22 ± 0.03^{a}	*
9d	0.15 ± 0.03^{a}	0.17 ± 0.04^{a}	0.21 ± 0.02^{a}	N.S.
End AF	0.18 ± 0.01^{b}	0.20 ± 0.01^{b}	0.24 ± 0.02^{a}	**

Values are expressed as average of three measurements ± standard deviations.

Abbreviations: S.S., statistical significance; AF, alcoholic fermentation; N.S., not significant.

Beer fermented by: *L. thermotolerans* MNF105 (T1); *L. thermotolerans* Philly Sour (T2), *S. cerevisiae* US-05 (T3). Data in the same line followed by the same letter are not significantly different according to Tukey's test. Symbols: ***, $P < 0.001$; **, $P < 0.01$; * $P < 0.05$.

Table 6. Volatile organic compound detected in beer samples. Compounds detected by SPME (all values in mg/L).

RT	Compounds	Aroma Description	Odour Threshold	T1 (OAV)	T2 (OAV)	T3 (OAV)	S.S.
	∑Alcohols			100.0 ± 3.2^{c}	127.0 ± 4.1^{b}	216.9 ± 8.5^{a}	***
5.748	2-Methyl-1-propanol	Alcoholic	100[1]	0.0 ± 0.0^{b} (<1)	0.0 ± 0.0^{b} (<1)	32.4 ± 1.6^{a} (<1)	***
8.402	1-Pentanol	Alcoholic, iodoform-like	80[1]	45.1 ± 1.6^{c} (<1)	66.3 ± 2.3^{b} (<1)	122.5 ± 4.6^{a} (1.53)	***
8.502	2-Methyl-1-butanol	Alcoholic, banana, vinous	65[1]	19.8 ± 0.7^{a} (<1)	19.3 ± 0.8^{a} (<1)	0.0 ± 0.0^{b} (<1)	***
19.549	Phenethyl alcohol	Rose, perfumy	125[1]	35.1 ± 1.0^{c} (<1)	41.4 ± 1.1^{b} (<1)	62.0 ± 2.4^{a} (<1)	***
	∑Esters			324.5 ± 11.0^{a}	110.1 ± 5.0^{c}	259.7 ± 9.3^{b}	***
5.403	Ethyl acetate	Fruity, sweet	30[1]	36.1 ± 0.8^{a} (1.20)	20.6 ± 0.7^{c} (<1)	29.1 ± 0.8^{b} (<1)	***
9.048	Pentyl lactate	Acetic acid, vinegar, milky	100[2]	0.0 ± 0.0^{b} (<1)	0.0 ± 0.0^{b} (<1)	3.6 ± 0.1^{a} (<1)	***
9.497	Isobutyl acetate	Banana, sweet, fruity	1.6[2]	0.0 ± 0.0^{b} (<1)	0.0 ± 0.0^{b} (<1)	3.7 ± 0.1^{a} (2.31)	***
10.347	Ethyl butanoate	Papaya, apple, perfumed	0.40[1]	0.0 ± 0.0^{b} (<1)	0.0 ± 0.0^{b} (<1)	3.6 ± 0.2^{a} (9.00)	***
12.701	Isoamyl acetate	Fruity, banana, pear	0.50[2]	4.3 ± 0.3^{a} (8.60)	4.9 ± 0.2^{a} (9.80)	28.0 ± 0.8^{a} (56.00)	***
13.800	Isoamyl isobutyrate	Green, rummy, cocoa	0.03[2]	2.0 ± 0.1^{a} (66.66)	1.7 ± 0.1^{b} (56.66)	0.0 ± 0.0^{c} (<1)	***
16.200	Ethyl hexanoate	Apple, fruity, aniseed	0.17[1]	29.4 ± 1.8^{a} (172.94)	18.0 ± 1.2^{b} (105.88)	7.5 ± 0.4^{c} (44.11)	***
16.700	Isoamyl butanoate	Fruity, melon, berry	unknown	6.3 ± 0.2^{b} (n.d.)	7.0 ± 0.2^{a} (n.d.)	0.0 ± 0.0^{c} (n.d.)	***
17.050	Methyl-3-ethyl-2-pentanoate	unknown	unknown	2.2 ± 0.1^{a} (n.d.)	2.1 ± 0.1^{a} (n.d.)	0.0 ± 0.0^{b} (n.d.)	***
18.799	Ethyl heptanoate	Fruity, perfumed, fatty	0.40[2]	7.8 ± 0.2^{a} (19.50)	5.8 ± 0.2^{b} (14.50)	5.8 ± 0.3^{b} (14.50)	***
18.949	Linalyl butanoate	Citrus, bergamot, berry	unknown	10.2 ± 0.3^{a} (n.d.)	9.2 ± 0.3^{b} (n.d.)	5.3 ± 0.2^{c} (n.d.)	***
21.198	Ethyl octanoate	Apple, sweet, fruity	0.37[1]	29.7 ± 1.3^{a} (99.00)	16.0 ± 0.8^{b} (43.24)	9.52 ± 0.7^{c} (25.72)	***
22.394	Isoamyl caproate	Perfumed, tropical fruits	0.90[2]	0.0 ± 0.0^{b} (<1)	0.0 ± 0.0^{b} (<1)	5.1 ± 0.4^{a} (5.66)	***
22.644	Phenylethyl acetate	Roses, honey, apple, flowery	3.80[2]	0.0 ± 0.0^{b} (<1)	0.0 ± 0.0^{b} (<1)	24.0 ± 1.2^{a} (6.31)	***
23.498	Ethyl nonanoate	Fruity, fatty acids, sweet	1.20[2]	9.9 ± 0.2^{b} (8.25)	7.0 ± 0.3^{c} (5.83)	9.5 ± 0.2^{a} (7.91)	***
24.148	Methyl geraniate	Floral, rose-fatty	unknown	3.5 ± 0.1^{a} (n.d.)	2.6 ± 0.2^{b} (n.d.)	3.2 ± 0.1^{a} (n.d.)	**
25.547	Ethyl-trans-4-decenoate	Green, pineapple, pear	unknown	13.0 ± 0.6^{a} (n.d.)	1.2 ± 0.1^{b} (n.d.)	0.0 ± 0.0^{c} (n.d.)	***
25.647	Ethyl decanoate	Caprylic, fruity, apple	0.57[2]	165.3 ± 4.8^{a} (290.00)	12.1 ± 0.7^{c} (21.22)	93.0 ± 2.4^{b} (163.15)	***
26.593	Isoamyl caprylate	Fruity, orange, pear, melon	2.00[2]	0.0 ± 0.0^{b} (<1)	0.0 ± 0.0^{b} (<1)	8.9 ± 0.8^{a} (4.45)	***
29.596	Ethyl laurate	Caprylic, estery	2.00[2]	4.8 ± 0.2^{b} (2.40)	1.9 ± 0.1^{c} (<1)	14.2 ± 0.5^{a} (7.10)	***
36.290	Ethyl palmitate	Fatty acids, fruity, sweet	1.50[2]	0.0 ± 0.0^{b} (<1)	0.0 ± 0.0^{b} (<1)	2.8 ± 0.1^{b} (1.86)	***
	∑Carboxylic acids			0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	13.2 ± 0.6^{a}	***
20.944	Octanoic acid	Caprylic	15.00[1]	0.0 ± 0.0^{b} (<1)	0.0 ± 0.0^{b} (<1)	13.2 ± 0.6^{a} (<1)	***
	∑Terpenes			4.80 ± 0.4^{b}	7.00 ± 0.5^{a}	0.0 ± 0.0^{c}	***
16.000	β-Myrcene	herbs, resinous, spicy	0.20[2]	1.8 ± 0.2^{b} (9.00)	4.5 ± 0.4^{a} (22.50)	0.0 ± 0.0^{c} (<1)	***
17.149	Limonene	Citrus, fruity	0.1[1]	3.0 ± 0.2^{a} (30.00)	2.5 ± 0.1^{b} (25.00)	0.0 ± 0.0^{c} (<1)	***

	∑Other			16.20 ± 0.8^{a}	14.10 ± 0.5^{b}	6.0 ± 0.3^{c}	***
5.703	3-Methyl butanolide	unknown	unknown	10.8 ± 0.5^{a} (n.d.)	9.5 ± 0.2^{b} (n.d.)	0.0 ± 0.0^{c} (n.d.)	***
8.102	Glycerol formal	viscosity	10000[2]	1.2 ± 0.1^{a} (<1)	0.7 ± 0.1^{b} (<1)	0.0 ± 0.0^{c} (<1)	***
13.201	Styrene	balsamic	20[2]	4.2 ± 0.2^{b} (<1)	3.9 ± 0.2^{b} (<1)	6.0 ± 0.3^{a} (<1)	***

Values are expressed as average of three measurements ± standard deviation.

Concentrations are calculated using limonene as the standard for the calibration line.

Compounds in each class are ordered according to their retention time.

Odour threshold as reported in literature.

Abbreviations: S.S., statistical significance; RT, retention time using a non-polar DB-5MS column; OAV, odour activity value; n.d., not determinable; N.S., not significant.

Beer fermented by: *L. thermotolerans* MNF105 (T1); *L. thermotolerans* Philly Sour (T2), *S. cerevisiae* US-05 (T3). Data in the same line followed by the same letter are not significantly different according to Tukey's test. Symbols: ***, $P < 0.001$; **, $P < 0.01$; * $P < 0.05$.

[1] Maarse, H. (2017). Volatile compounds in foods and beverages. Routledge.

[2] Zunkel, M., Gastl, M., Schoenberger, C., Sedin, D., Becker, T. (2011). Beer flavor database. In ASBC 74th Annual Meeting. Ft. Myers.

Figure 1. Fructose, glucose and maltose (a, b and c) consumption monitored during the alcoholic fermentation of the wort, inoculated with the different yeast strains, over 4 days of sperimentation (Values are expressed as average of three measurements ± standard deviation).

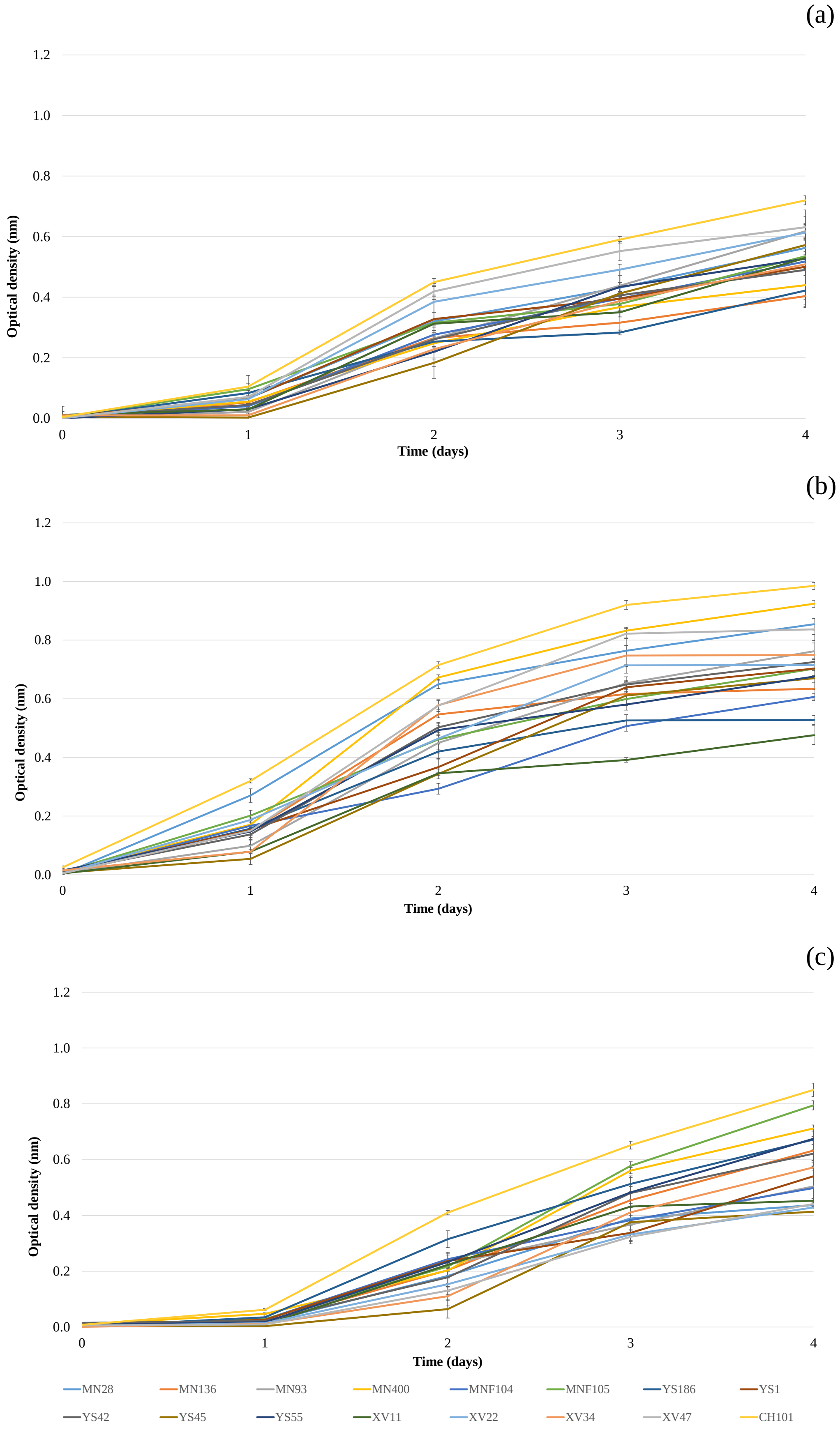


Figure 2. Fermentation curves measured as CO_2 emission of different samples inoculated with the five best strains. Synthetic beer wort fermented by: MN28, MN93, MN400, MNF105, XV47 and *L. thermotolerans* Philly Sour (TC) (Values are expressed as average of three measurements ± standard deviation).

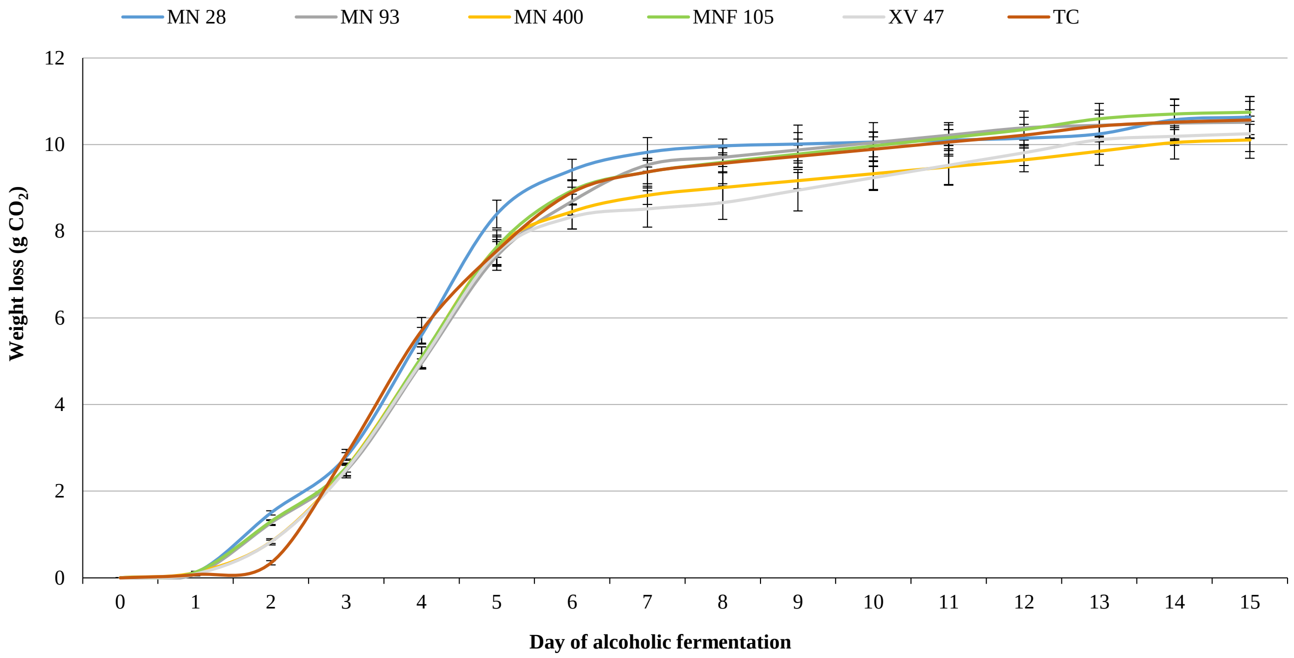


Figure 3. Evolution of *L. thermotolerans* concentrations during alcoholic fermentation. Synthetic beer wort fermented by: MN28, MN93, MN400, MNF105, XV47 and *L. thermotolerans* Philly Sour (TC) (Values are expressed as average of three measurements ± standard deviation).

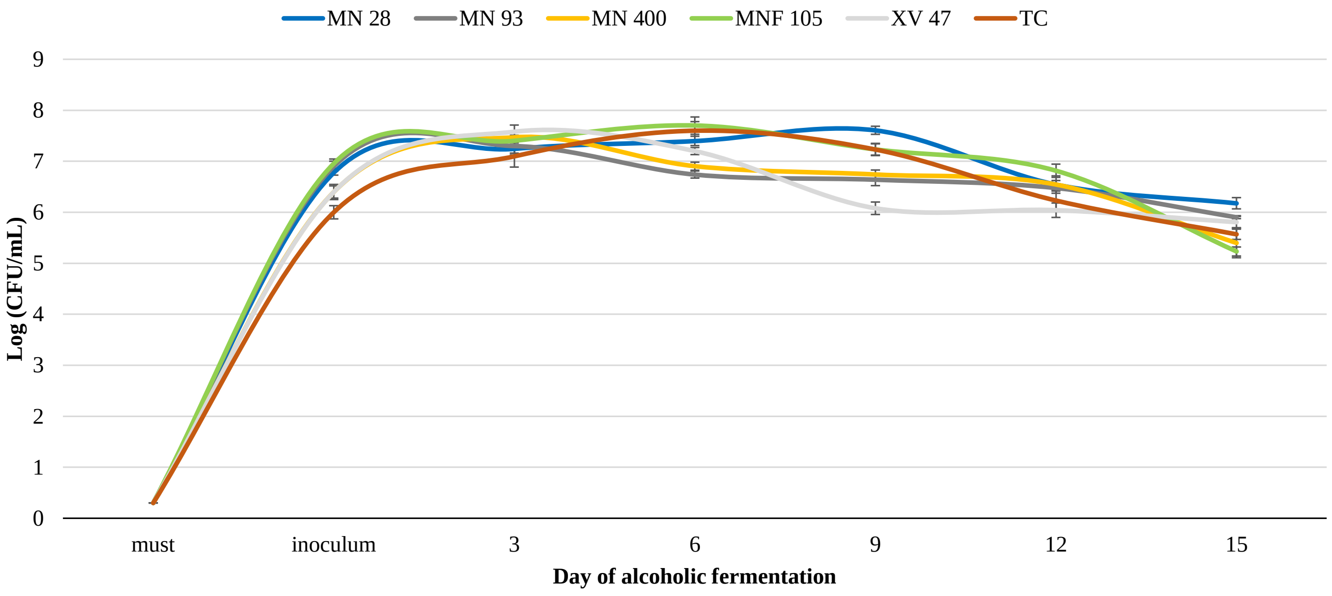


Figure 4. Evolution of yeasts concentrations during wort fermentation under real conditions. Beer fermented by: *L. thermotolerans* MNF105 (T1); *L. thermotolerans* Philly Sour (T2), *S. cerevisiae* US-05 (T3).

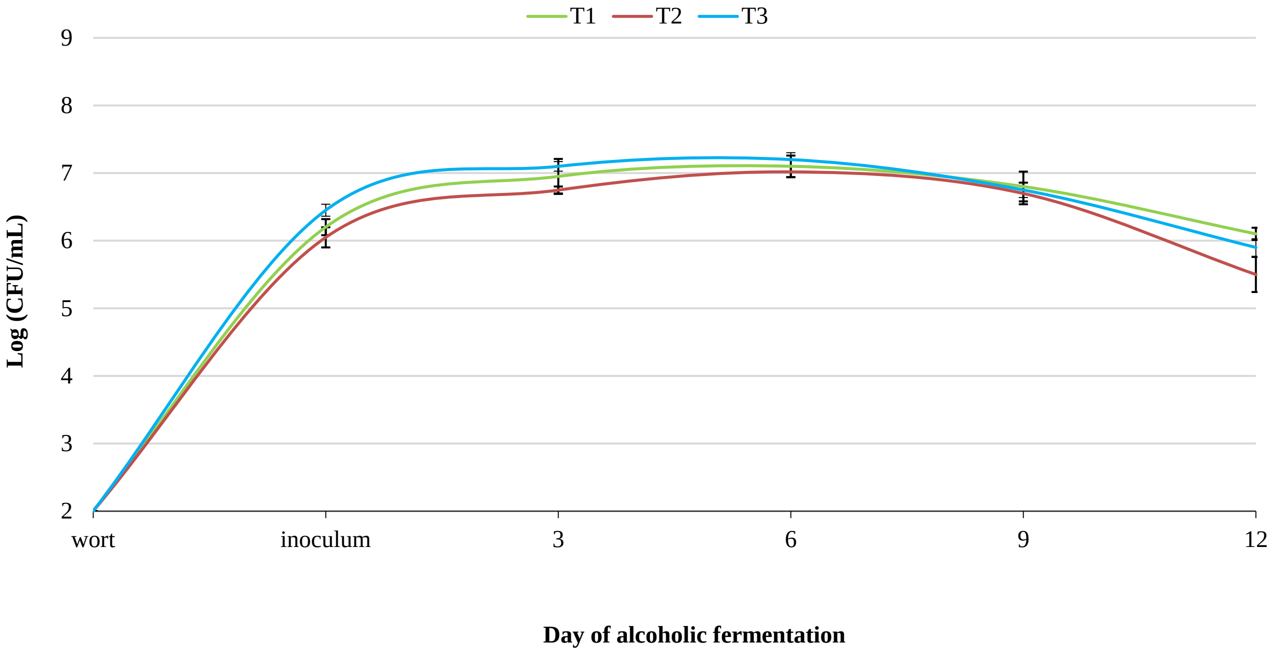


Figure 5. Sensory profiles of experimental beers: (a) *L. thermotolerans* MNF105 (T1) (aroma); (b) *L. thermotolerans* Philly Sour (T2) (aroma); (c) *S. cerevisiae* US-05 (T3) (aroma); (d) *L. thermotolerans* MNF105 (T1) (taste); (e) *L. thermotolerans* Philly Sour (T3) (taste); (f) *S. cerevisiae* US-05 (T3) (taste). Blue-colored histograms are associated with coefficients that have a significantly positive value, while red-colored histograms are associated with coefficients that have a significantly negative value.

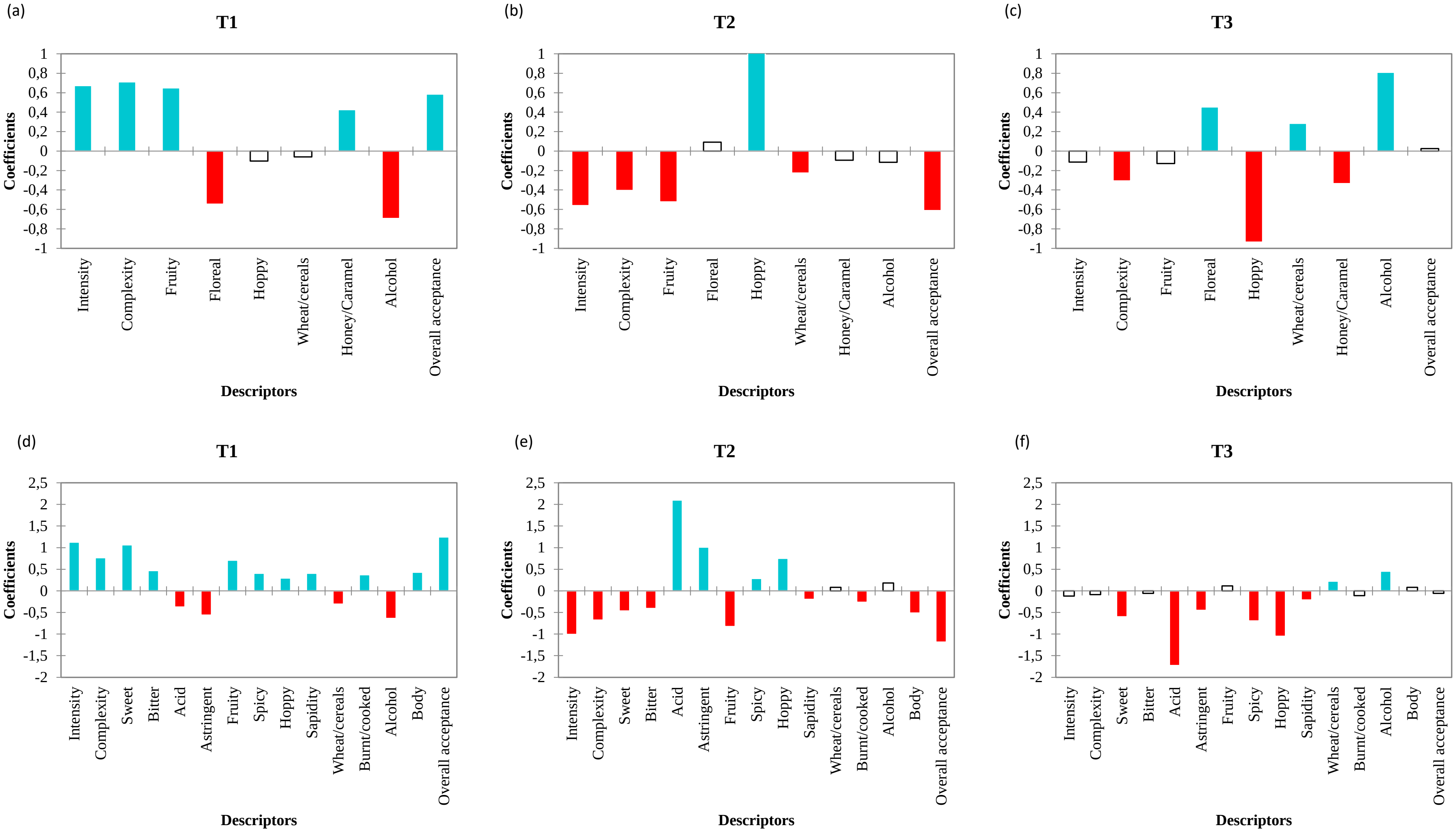
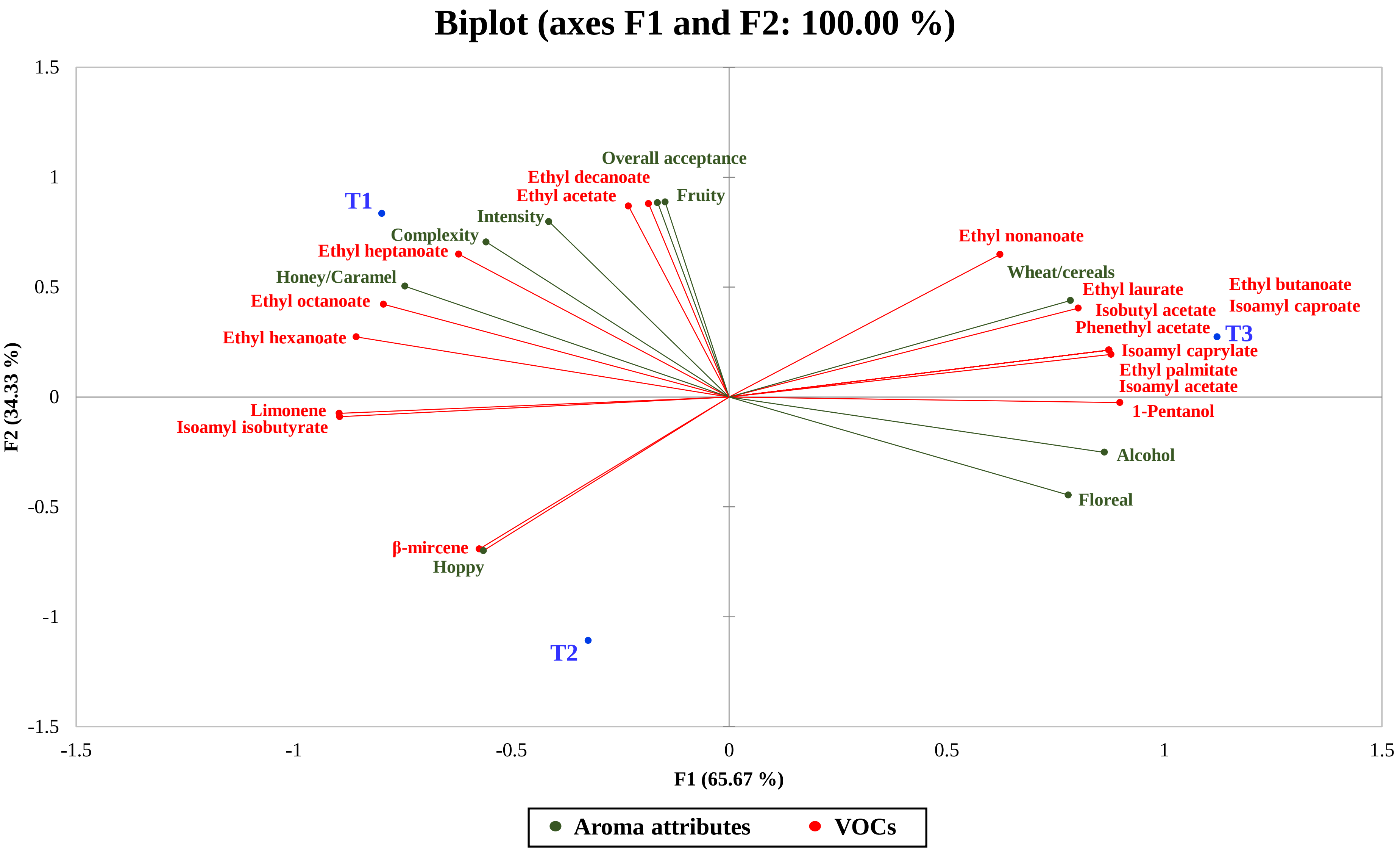


Figure 6. Principal component analysis (PCA) biplot for VOCs and aroma attributes. Abbreviations: *L. thermotolerans* MNF105 (T1); *L. thermotolerans* Philly Sour (T2); *S. cerevisiae* US-05 (T3).



Graphical Abstract

Use of yeasts isolated from manna

Technological screening

Micro-fermentation

Production of experimental top-fermented beers

Fermentation

Beer analysis

- Physico-chemical analysis
- Analysis of volatile organic compounds
- Sensory evaluation

Declaration of Interest Statement

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest for this research.