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Technological and flavour-generating potential of *Saccharomyces*, *Brettanomyces* and lactic acid bacteria for sour ale production

• Arne Bouchez • Thomas Debing • Frédéric Leroy • Luc De Vuyst

Research Group of Industrial Microbiology and Food Biotechnology, Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium.

frederic.leroy@vub.be

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Abstract

Why was the work done: Sour beers are typically produced using spontaneous fermentation and maturation. This study contributes to the development of a standardised and reproducible approach to sour beer production while retaining the complex and characteristic flavour profile associated with spontaneous fermentation.

How was the work done: Laboratory scale wort fermentations were performed with pure cultures of *Saccharomyces cerevisiae* S2, *Saccharomyces kudriavzevii* S35, *Brettanomyces bruxellensis* B28, and *Brettanomyces anomalus* B43, and the lactic acid bacteria *Pediococcus acidilactici* L13 and *Levilactobacillus brevis* L14. Fermentations were evaluated based on substrate utilisation, microbial growth, production of metabolites and volatile aroma compounds.

What are the main findings: Both *Saccharomyces* yeasts exhibited rapid utilisation of substrates and high ethanol production, together with the formation of ethyl and acetate esters. *Brettanomyces* strains differed markedly, with the formation of volatile phenolic compounds and medium-chain ethyl esters. *B. bruxellensis* B28 produced a broader and more extensive flavour profile than *B. anomalus* B43. The lactic acid bacteria exhibited limited utilisation of saccharides but produced high levels of lactic acid, with minor concentrations of other flavour-active compounds.

Why is the work important: The distinct and complementary fermentation characteristics of the yeasts and lactic acid bacteria provide a basis for their combination in defined mixed starter cultures. Such an approach would allow a more controlled and reproducible fermentation while retaining key characteristics of traditionally produced sour beers.

Keywords

Sour beer; mixed starter culture; flavour; *Saccharomyces*; *Brettanomyces*; *Pediococcus*; *Levilactobacillus*.

Introduction

Sour beers contain high concentrations of organic acids with a low pH and sour taste (Bouchez and De Vuyst 2022). Nonetheless, there is a remarkable variety regarding their production, especially at the level of the microbial fermentation (Snauwaert et al. 2016; Bossaert et al. 2019; Dysvik et al. 2019; Dysvik et al. 2020; Bouchez and De Vuyst 2022). Although there are chemically acidified variants, microbiologically acidified beers are the most widely known and industrially important (Bouchez and De Vuyst 2022). Spontaneously fermented and matured sour beers - Belgian lambic beers, American coolship ales, and their derivatives - are the most well described (Van Oevelen et al 1977; Bokulich et al 2012; Spitaels et al 2014; Tonsmeire 2014; De Roos and De Vuyst 2019; Bongaerts et al. 2021; Bongaerts et al. 2024; Bouchez and De Vuyst 2022; Croonenberghs et al. 2024). The uniqueness of American coolship ales and lambic beers reflects the spontaneous fermentation and maturation performed in wooden barrels (Bokulich et al. 2012; Spitaels et al. 2014; Bossaert et al. 2019; De Roos and De Vuyst 2019; Bongaerts et al. 2021). Typically, the boiled wort is cooled overnight in an open metal vessel (coolship), during which airborne microorganisms 'inoculate' the wort (Spitaels et al. 2014; De Roos et al. 2018 a,b; De Roos and De Vuyst 2019; Bongaerts et al. 2026). The wort is transferred to new or used wooden barrels ('casks' or 'foeders'), with further inoculation of the in-house microbiota (De Roos et al. 2018 b,c; Bongaerts et al. 2021). During the following months, typically up to three years, a multiphasic, heterogenous fermentation and maturation process takes place (Spitaels et al. 2014; De Roos and De Vuyst 2019; Bongaerts et al. 2021; Vermote et al. 2023).

Despite the unique sensory quality and traditional character ('Traditional Specialties Guaranteed' status at the European level, EEG 2301/97), lambic beer brewers face challenges with the long production time and inconsistency of the product (De Roos et al. 2018a; Mulder 2018; Bongaerts et al. 2021, 2024; Bouchez and De Vuyst 2022). To achieve a faster production process and reduced product variability, the addition of starter cultures to the sour beer wort with microorganisms with desired features can be considered (Leroy and De Vuyst 2004). The use of starter cultures is a widespread technology used in the production of spontaneously fermented foods and beverages (Leroy and De Vuyst 2004; Leroy et al. 2006; Marco et al. 2017; Díaz-Muñoz and De Vuyst 2022; Decadt et al. 2023).

In previous work, six microorganisms - *Saccharomyces cerevisiae, Saccharomyces kudriavzevii, Brettanomyces bruxellensis, Brettanomyces anomalus, Pediococcus acidilactici,* and *Levilactobacillus brevis* - isolated from fermenting lambic beer wort (De Roos et al. 2018 a,b; Bongaerts et al. 2026), were screened for their functional properties during fermentation (A. Bouchez, L. De Vuyst, and F. Leroy, unpublished results). The strains used here were selected for their growth dynamics, substrate utilisation, acidification (lactic acid bacteria; LAB), and volatile organic compound production. To provide taxonomic diversity within the yeasts and LAB, the best performing strain was selected, together with the highest performing strain of a different species within the same group, A summary of the screening and the criteria underlying strain selection is provided in Supplementary information Table S1.

Small-scale screening experiments used a simulated media to assess large numbers of strains and prioritise starter culture candidates. Accordingly, further evaluation of performance required more realistic fermentation conditions. Therefore, in this work, the technological and flavour generating potential was determined of six microorganisms derived from lambic - *Saccharomyces* (x 2), *Brettanomyces* (x 2), and LAB (x 2). To this end, fermentations were performed for 14 days in sour beer wort with assessment of the microbiological and physicochemical dynamics, substrate consumption, and metabolite production.

Materials and methods

Sour beer production

Sour beer wort was produced using a 65 L all-in-one brewing system (Brew Monkey, 's-Hertogenbosch, The Netherlands). Two thirds of the grist consisted of malted barley (Bioplanet Pilsmalt, Rhön-Malz, Mellrichstadt, Germany) with one third of unmalted wheat (YP population; Organic Research Centre, Cirencester, Gloucestershire, UK). These grains were milled and ground using a Maltmann 220S malt mill (Sommer, Osnabrück, Germany). Brewing liquor

was used (demineralised water, 54°C) with a grist-to-liquor ratio of 1:4 (w/v). Mashing consisted of four steps: 54°C for 20 min, 62°C for 60 min, 72°C for 30 min, and a 5 min rest at 78°C. Wort filtration was performed gravimetrically, followed by sparging (demineralised water, 90°C) until the initial volume was reached. Overaged hop pellets (1.8 g/L; Hallertau, harvest 2019) were added, and the wort was boiled for 60 min, whirlpooled for 20 min, and cooled to 30°C. Wort (10 L) was transferred to Sartorius Biostat Cplus fermenters (Sartorius, Göttingen, Germany). Antifoam 204 (1 mL/L; Sigma-Aldrich, Saint-Louis, Missouri, USA) was added. The wort was boiled for 60 min to ensure sterility, as the transfer from the brewery to fermenter was not performed aseptically.

The wort produced in this study emulated a traditional lambic beer recipe but without turbid mashing or a shorter boiling step. The former may have resulted in a lower concentration of maltooligosaccharides in the wort. Nevertheless, the overall wort composition was comparable to that of a traditional lambic beer wort (De Roos et al. 2018a; Vermote et al. 2023; Bongaerts et al. 2026).

Inoculation of microorganisms

The fermenters were inoculated with either *S. cerevisiae* S2, *S. kudriavzevii* S35, *B. bruxellensis* B28, *B. anomalus* B43, *P. acidilactici* L13, or *L. brevis* L14. The microorganisms were stored at -80°C using a cryoprotectant (glycerol, 40%, v/v) in either (i) yeast extract-peptone-glucose (YPG) [5 g/L yeast extract (Oxoid, Basingstoke, Hampshire, UK), 10 g/L bacteriological peptone (Oxoid), and 20 g/L glucose (Merck, Darmstadt, Germany)] for yeasts or (ii) for the bacteria, vitamin enriched de Man-Rogosa-Sharpe (MRS) medium [52 g/L of MRS (Oxoid) with 1 mL/L of a vitamin solution containing 200 mg/L of cobalamin, folic acid, nicotinic acid, pantothenic acid, pyridoxal phosphate, and thiamine].

The microorganisms were recovered on agar media (as above with 15 g/L agar, Oxoid). After incubation for 72 h, a single colony was picked using a sterile inoculation loop (Greiner Bio-One, Vilvoorde, Belgium) and transferred to 10 mL of sterile liquid medium (YPG medium for yeasts, vitamin-enriched MRS medium for LAB), and incubated at 30°C for 24 h. Sterile liquid medium (100 mL) was inoculated with 1% (v/v) of the *Saccharomyces* and LAB suspensions, and 5% (v/v) of the *Brettanomyces* yeast strain suspensions, followed by incubation at 30°C for 24 h in a shaking incubator (120 rpm; Certomat® BS-1, B Braun Biotech International, Melsungen, Germany). These cultures were used to inoculate (1%, v/v) the wort in the 10 L fermenters. The temperature (25°C) and agitation (50 rpm) were kept constant during the fermentation.

For each strain, three independent fermentations were performed (biological replicates). Each replicate was performed in wort originating from different brewing sessions, and the experiments were spread over time (with no overlap of biological replicates).

Sampling

Samples (50 mL) were taken after 0, 8, 24, 32, and 48 hours of fermentation, followed by sampling after 5, 7, 9, 12, and 14 days of fermentation. For pH, apparent extract (AE), substrate, and metabolite measurements, the samples were centrifuged at 1,792 x g and 20°C for 10 min.

Analyses

Microbial growth

Growth was assessed by enumerating the counts as colony-forming units (CFU). Samples (in triplicate), diluted in sterile saline (0.85%, w/v, NaCl), were plated on either YPG agar (15 g/L agar) medium (for *Saccharomyces* and *Brettanomyces*) or vitamin-enriched MRS agar medium (for the LAB). The plates were incubated at 30°C for 5 d (*Saccharomyces* and LAB) or 10 d (*Brettanomyces*). Plates with between 30 and 300 colonies were used for the microbial counts.

Physicochemical parameters

pH was measured using an InoLab 7110 pH meter (WTW, Weilheim, Germany). The AE (expressed in °P) was determined using a portable density meter DMA 35 (Anton Paar, Graz, Austria). Physicochemical parameters were determined in triplicate for each biological replicate.

Substrates and metabolites

Quantification of all organic compounds used external calibration curves, constructed by a twofold dilution series and treated identically as the

samples. Every sample of the biological replicates was measured three times (technical replicates).

Saccharides.

The concentration of glucose, fructose, and sucrose were quantified using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (De Roos et al. 2018a; Bongaerts et al. 2024, 2026) using a ICS3000 chromatograph equipped with a CarboPacTM PA20 column (Dionex, Sunnyvale, California, USA) and an ED-40 pulsed amperometric detector (Dionex). Three eluents were used – (i) ultrapure water (MilliQ; EMD Millipore, Billerica, Massachusetts, USA), (ii) sodium hydroxide solution (3.75 g/L; J.T. Baker, Deventer, The Netherlands) in MilliQ, and (iii) NaOH solution (30 g/L) in MilliQ. Rhamnose was used as an internal standard (IS; Fluka Chemika, Buchs, Switzerland). Samples were deproteinated by mixing with an aqueous acetonitrile solution (50/50; Thermo Fisher Scientific), which was vortexed for 3 min, centrifuged (20,817 x g, 15 min), and filtered using Millex LG filters (pore size of 0.2 µm; Merck Millipore, Watford, Ireland). Sample (10 µL) was injected into the column, and a constant flow rate of 0.4 mL/min was applied.

The concentration of maltose and maltooligosaccharides; maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6), maltoheptaose (M7), and maltooctaose (M8)] were quantified similarly, using the same equipment, but with a different column (CarboPacTM PA100, Dionex) and mobile phases, (i) solution A (3.75 g/L of NaOH in MilliQ), (ii) solution B (37.5 g/L of NaOH in MilliQ), and (iii) solution C (3.75 g/L of NaOH and 31.2 g/L of sodium acetate in MilliQ). The injection volume was 10 µL with a flow rate of 0.4 mL/min applied.

Ethanol and organic acids

Ethanol was quantified using gas chromatography coupled to flame ionization detection (GC-FID), with a Trace 1310 gas chromatograph (Thermo Fisher Scientific) equipped with a Stabilwax-DA column (Restek, Bellefonte, Pennsylvania, USA) based on the method of Bongaerts et al (2024; 2026). The samples were deproteinated using acetonitrile [(74.4% (v/v) acetonitrile (Thermo Fisher Scientific) + 24.4% (v/v) MilliQ + 1.2% (v/v) formic acid (Merck)], and 250 µL/L of 1-butanol (Sigma-Aldrich)] in a ratio of 1:10. The mixture was vortexed (3 min), centrifuged (20,817 x g for 10 min) and filtered (0.2 µm Millex LG filters). A volume of 0.8 µL was injected into the column with a split flow of 40 and a helium (Nippon Gasses, Antwerp, Belgium) carrier gas at 1 mL/min.

Lactic and acetic acids were measured using high-performance liquid chromatography with photo diode array detection (HPLC-PDA) and refractive index detection (HPLC-RI) (De Roos et al. 2018a, b). This consisted of a Waters chromatograph with W2996 PDA detector and W410 RI detector (Waters, Milford, Massachusetts, USA) with an ICSep ICE ORH-801 column (Transgenomic North America, Omaha, Nebraska, USA). An isocratic flow rate (0.4 mL/min) was applied, using a mobile phase of 5 mM sulphuric acid (Merck). The samples were deproteinised by mixing them with solutions of Carrez A [36 g/L of potassium ferrocyanide (K4Fe(CN)6.3H2O) in MilliQ] and Carrez B (72 g/L of ZnSO4.7H2O in MilliQ) in a ratio of 2:1:1. The mixture was vortexed for 3 min, centrifuged (20,817 x g, 15 min), and filtered (0.2 µm Millex LG filter units), with 30 µL was injected into the column.

The concentration of citric acid, succinic acid, fumaric acid, malic acid, gluconic acid, p-coumaric acid, and ferulic acid were quantified using ultra-performance liquid chromatography with tandem mass spectrometry detection (UPLC-MS/MS) (Diaz-Munoz et al. 2020). The set-up included an Acquity UPLC system equipped with an HSS T3 column and a triple-quadrupole tandem mass spectrometer (Waters). The samples were deproteinated using 50% (v/v) MilliQ/50% (v/v) methanol (Thermo Fisher Scientific), and 20 mg/L of salicylic acid (Fluka Chemie) in a 1:20 ratio. The mixture was vortexed for 3 min, centrifuged (21,100 × g, 15 min), filtered (0.2 µm Millex LG filter units), with 2 µL was injected into the column.

Two eluents were used, (i) solution A [980 mL of MilliQ, 20 mL of methanol (Thermo Fisher Scientific) and 2 mL/L of formic acid (Merck)] and (ii) solution B [50 mL of MilliQ, 950 mL of methanol (Thermo Fisher Scientific), and 2 mL of formic acid (Merck)], with the following gradients (constant flow rate of 0.23 mL/min): 92/8% of A/B from 0-1.5 min;

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linear decrease to 10.2/89.8% of A/B during 1.5-3 min, 10.2/89.8% of A/B during 3-6 min, linear increase to 92/8% of A/B during 6.-6.5 min, and 92/8% of A/B during 6.5-10 min.

Volatile organic compounds

To quantify VOCs, liquid injection gas chromatography with triple-quadrupole mass spectrometry detection (LI-GC-TQ-MS) was used (Bongaerts et al. 2024) using a Trace 1300 Series gas chromatograph, equipped with a DBwax UI column (Restek, Bellefonte, Pennsylvania, USA), coupled to a TSQ 8000 Evo triple-quadrupole mass spectrometer (Thermo Fisher Scientific). Deproteinisation was performed by mixing undiluted sample with an acetone-based mixture containing three deuterated internal standards - ethyl decanoate-d19, 2,3-butanedione-d6, and 3-methyl-1-butyl-1,1-d2 alcohol (CDN Isotopes, Pointe-Claire, Quebec, Canada). Sample (0.8 µL) was injected into the column with nitrogen used carrier gas, and the oven program with an initial holding phase (40°C for 1.5 min), followed by a heating phase (temperature gradient of 10°C/min, for 20 min).

Statistical analysis

To assess differences over the course of fermentation, linear mixed-effects models were fitted separately for each response variable and microbial group. Strain, time, and their interaction were included as fixed effects, whereas biological fermentation replicate was included as a random effect to account for repeated sampling over time. Time was treated as a categorical variable.

Microbial populations were analysed as log-transformed CFU/mL and treated as continuous response variables. Raw colony counts were not analysed directly. Differences in temporal profiles between microorganisms were evaluated based on the strain × time interaction, the significance of which was assessed using likelihood-ratio tests. Pairwise comparisons between strains at individual sampling points were derived from the fitted models using estimated marginal means (EMMs).

To account for multiple testing, p-values were adjusted using the Benjamini–Hochberg procedure. Adjusted p-values < 0.05 were considered statistically significant. Unless stated otherwise, reported p-values refer to adjusted values. The statistical tests and corresponding adjusted p-values for the comparisons reported in the Results are provided in Supplementary information Table S2. Statistical analyses were performed in Python using the statsmodels, SciPy, and pandas packages.

Results

Microbial growth

Following inoculation, the *Saccharomyces* yeasts grew quickly, reaching maximum population densities of about 8.0 log (CFU/mL) after 32 hours of fermentation (Figure 1). Population dynamics differed significantly over time ($p < 0.001$) with a decrease to 6.0 ± 0.5 log (CFU/mL) for *S. cerevisiae* S2, whereas counts for *S. kudriavzevii* S35 decreased to 7.3 ± 0.8 log (CFU/mL) after two weeks of fermentation. *B. bruxellensis* B28 did not grow during the first two days of fermentation, whereas *B. anomalus* B43 increased from the start, resulting in different population dynamics ($p < 0.001$). Regardless of these differences in initial growth, both *Brettanomyces* strains reached a similar maximum population of about 7.8 log (CFU/mL) after seven days for *B. bruxellensis* B28 and five days for *B. anomalus* B43. The LAB bacteria yielded similar maximal population densities after one day of fermentation, at about 8.8 ± 0.4 log (CFU/mL) for *P. acidilactici* L13 and 9.0 ± 0.2 log (CFU/mL) for *L. brevis* L14. Their population dynamics differed over time ($p = 0.017$), with counts reaching final values of 7.1 ± 0.3 and 7.8 ± 0.1 log (CFU/mL) after two weeks of fermentation.

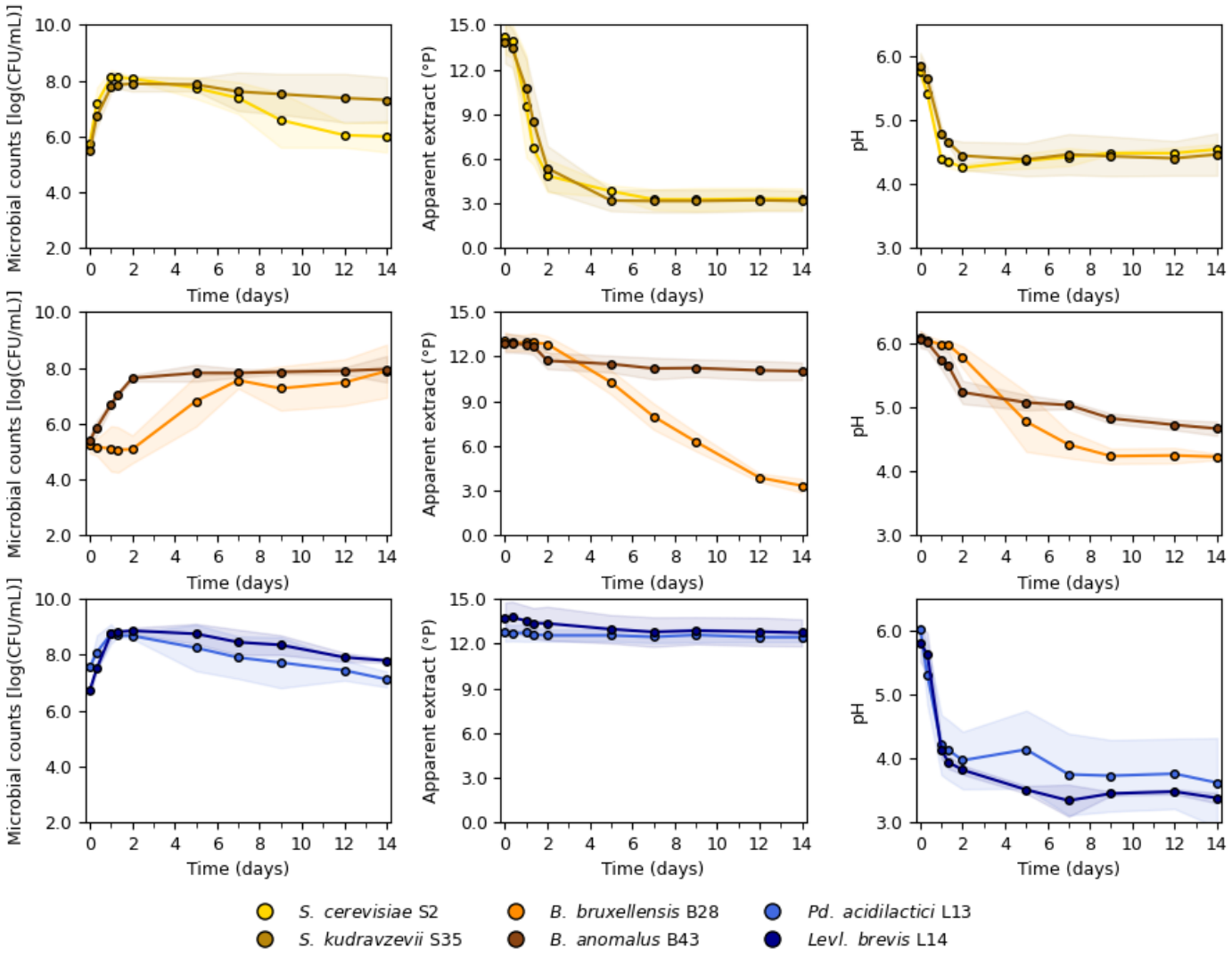
Physicochemical parameters

The AE of the wort was 13.4 ± 0.6°P (Figure 1) and for both *Saccharomyces* strains the AE declined to 3.3 ± 0.1°P after five days of fermentation. The AE value for *B. bruxellensis* B28 did not change during the first two days but then decreased gradually until the end of fermentation to a similar AE value. For *B. anomalus* B43, the AE decreased to 11.7 ± 0.6°P but then did not change during the rest of fermentation. Consequently, the AE profiles of both *Brettanomyces* strains differed significantly over time ($p < 0.001$). Both LAB strains did not lower the AE which remained constant during fermentation.

The initial pH was 5.9 ± 0.1 (Figure 1) and with both the *Saccharomyces* strains, this declined during the

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Figure 1. Microbial counts [log (CFU/mL)], apparent extract (°P), and pH during fermentation over 14 days with six microorganisms from lambic beer - *Saccharomyces cerevisiae* S2 (yellow), *Saccharomyces kudriavzevii* S35 (dark yellow), *Brettanomyces bruxellensis* B28 (orange), *Brettanomyces anomalus* B43 (brown), *Pediococcus acidilactici* L13 (light blue), and *Levilactobacillus brevis* L14 (dark blue). Data shows the average concentrations from three technical replicates of three biological replicates; coloured areas represent the standard deviation.



first two days of fermentation to ca. pH 4.4, and then stabilised until the end of the fermentation. For *B. bruxellensis* B28, the pH decreased slowly reaching 4.2 ± 0.1 after nine days of fermentation. In contrast, with *B. anomalus* B43 the pH lowered to 5.2 ± 0.2 within the first two days of fermentation, followed by a slower further decrease to 4.7 ± 0.1. This was significantly higher ($p < 0.001$) than that with *B. bruxellensis* B28. During the first two days of fermentation, both LAB strains lowered the pH to 4.0 ± 0.5 (*P. acidilactici* L13) and 3.8 ± 0.1 (*L. brevis* L14), which decreased further to 3.6 ± 0.7 (*P. acidilactici* L13) and 3.4 ± 0.1 (*L. brevis* L14).

Saccharide consumption

The wort fermentable sugars used for microbial growth were monosaccharides (glucose and fructose), disaccharides (sucrose and maltose), and maltotriose (Figure 2). None of the strains were able to metabolise longer maltooligosaccharides ('dextrins'), which were present at initially 2.7 ± 0.5 g/L (maltotetraose), 1.2 ± 0.3 g/L (maltoheptaose), 1.1 ± 0.1 g/L (maltohexaose), and 0.6 ± 0.1 g/L (maltopentaose)

Glucose (initial concentration 9.7 ± 1.3 g/L) was utilised by all yeast strains, but not the LAB (Figure 2).

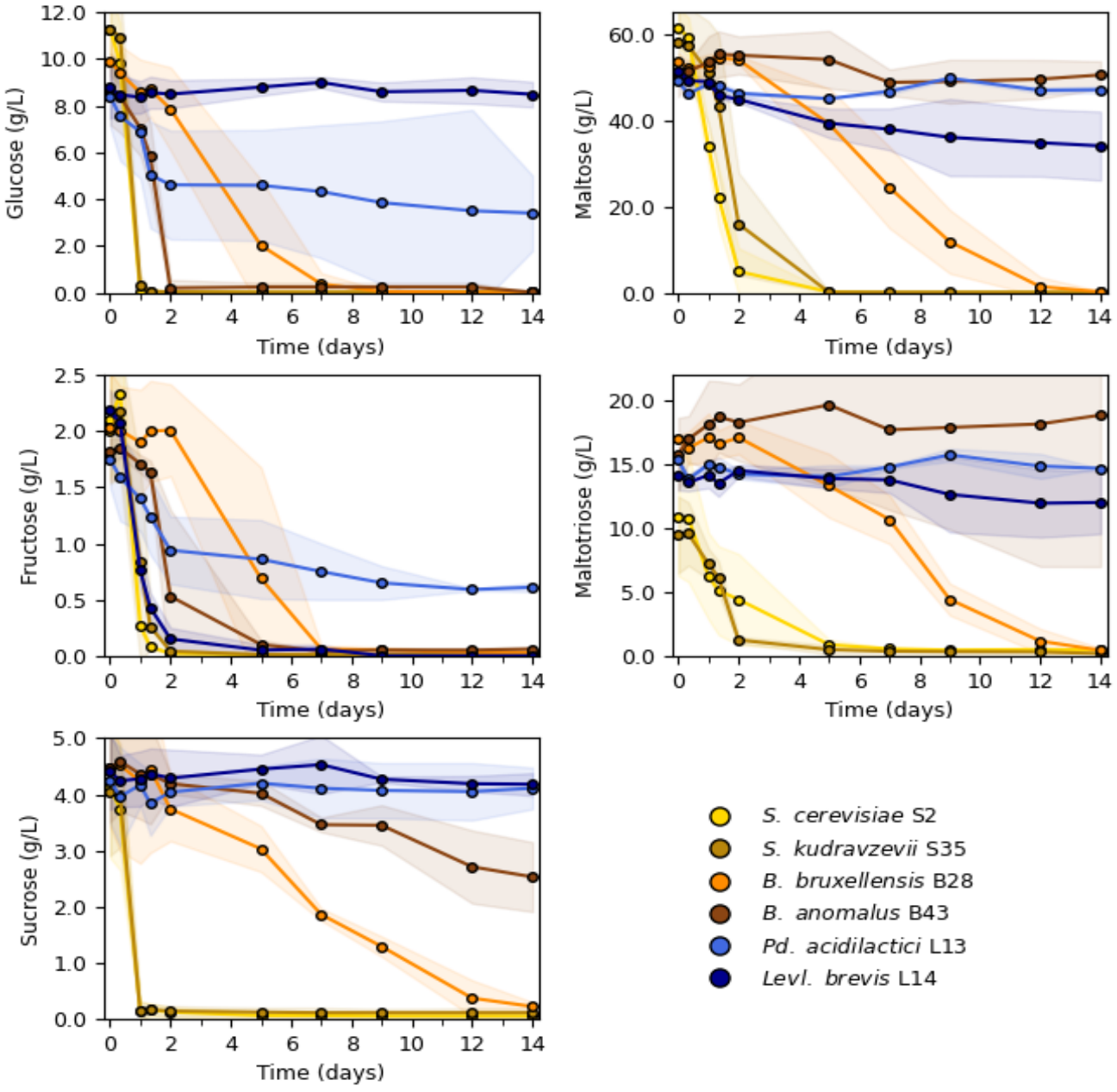
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Both *Saccharomyces* strains utilised the glucose within the first 32 hours of fermentation, whereas *B. anomalus* B43 and *B. bruxellensis* B28 consumed the glucose after 5 and 7 days respectively. After two weeks of fermentation with *P. acidilactici* L13, the glucose concentration remained at 3.4 ± 1.6 g/L, whereas *L. brevis* L14 was unable to utilise glucose.

For fructose (initial concentration of 2.0 ± 0.2 g/L), only *P. acidilactici* L13 of the six microorganisms could not utilise it to completion, with a residual concentration of 0.6 ± 0.0 g/L.

Both *Saccharomyces* strains consumed sucrose (initial concentration 4.3 ± 0.2 g/L) during the first day of fermentation, whereas *B. bruxellensis* B28 achieved this after two weeks. For *B. anomalus* B43, the utilisation of sucrose was significantly different ($p < 0.001$) - slower and incomplete – with both LAB unable to metabolise sucrose. Maltose was utilised

Figure 2. The concentration of glucose, fructose, sucrose, maltose, and maltotriose during fermentations over 14 days with six microorganisms from lambic beer - *Saccharomyces cerevisiae* S2 (light yellow), *Saccharomyces kudriavzevii* S35 (dark yellow), *Brettanomyces bruxellensis* B28 (orange), *Brettanomyces anomalus* B43 (brown), *Pediococcus acidilactici* L13 (light blue) and *Levilactobacillus brevis* L14 (dark blue). Maltooligosaccharide with a DP > 3 did not change over time. Data shows the average concentration from three technical replicates of three biological replicates; coloured areas represent the standard deviation.



by both *Saccharomyces* yeasts within a few days of fermentation, whereas it took up to two weeks for *B. bruxellensis* B28. With *L. brevis* L14, the maltose concentration decreased slowly, whereas no change was observed for *B. anomalus* B43 and *P. acidilactici* L13. Accordingly, maltose utilisation differed significantly between the strains of *Brettanomyces* yeasts and LAB ($p < 0.001$). Only *S. cerevisiae* S2, *S. kudriavzevii* S35, and *B. bruxellensis* B28 could utilise maltotriose (initial concentration 13.8 ± 2.9 g/L) (Figure 2), rapidly in less than a week by the *Saccharomyces* strains, whereas two weeks were required for *B. bruxellensis* B28.

Ethanol and organic acid production

S. cerevisiae S2, *S. kudriavzevii* S35, and *B. bruxellensis* B28 produced ethanol during fermentation (40 - 43 g/L (Figure 3). In contrast, *B. anomalus* B43 produced much less ($p < 0.001$) ethanol (7.6 ± 0.4 g/L). Ethanol formation by the LAB strains was minimal with 0.09 ± 0.15 g/L for *P. acidilactici* L13 and 3.0 ± 1.8 g/L for *L. brevis* L14 (which was significantly higher at $p < 0.001$).

Acetic acid was produced by most of the microbial strains (Figure 3): 0.8 ± 0.1 g/L for *L. brevis* L14, 0.4 ± 0.0 g/L for *B. bruxellensis* B28, 0.3 ± 0.1 g/L for *B. anomalus* B43, 0.3 ± 0.1 g/L for *S. cerevisiae* S2, and 0.1 ± 0.0 g/L for *S. kudriavzevii* S35. No acetic acid was formed by *P. acidilactici* L13. Acetic acid production differed significantly between the *Brettanomyces* strains ($p < 0.001$), whereas for the LAB strains, ethanol production was significantly higher for *L. brevis* L14 compared to *P. acidilactici* L13 at the end of the fermentation ($p < 0.001$). Lactic acid was only detectable with the LAB strains (Figure 3), with *L. brevis* L14 was significantly higher (10.1 ± 0.5 g/L) than that for *P. acidilactici* L13 (6.1 ± 3.6 g/L; $p = 0.002$).

The trends for the organic acids - citric acid, succinic acid, fumaric acid, malic acid, and gluconic acid - differed (Figure 4). Citric acid in the wort (171.1 ± 6.4 mg/L) was utilised by *L. brevis* L14 within two days of fermentation, but not by any of the other strains. Succinic acid in wort (11.1 ± 0.8 mg/L) was impacted by the yeast strains but not by the LAB. The *Saccharomyces* yeasts increased the concentration of succinic acid to 86.6 ± 3.0 mg/L (*S. cerevisiae* S2) and 72.5 ± fermentation, followed by a further increase up to 106.3 ± 1.8 mg/L (*S. cerevisiae* S2) and 85.0 ± 17.8 mg/L (*S. kudriavzevii* S35). Although the *Brettanomyces* strains differed ($p < 0.001$), the increase in succinic acid was slower, with the final concentration similar to the *Saccharomyces* yeasts. The concentration of fumaric acid in wort was 6.9 ± 0.4 mg/L, with *L. brevis* L14 utilising it whereas with *B. bruxellensis* B28 the level increased (45.9 ± 3.0 mg/) initially and then decreased to 34.5 ± 3.4 mg/L

Figure 3. Concentration of ethanol, lactic acid, and acetic acid during fermentation over 14 days with six microorganisms from lambic beer - *Saccharomyces cerevisiae* S2 (light yellow), *Saccharomyces kudriavzevii* S35 (dark yellow), *Brettanomyces bruxellensis* B28 (orange), *Brettanomyces anomalus* B43 (brown), *Pediococcus acidilactici* L13 (light blue), and *Levilactobacillus brevis* L14 (dark blue). Data shows the average concentrations from three technical replicates of three biological replicates; coloured areas represent the standard deviation.

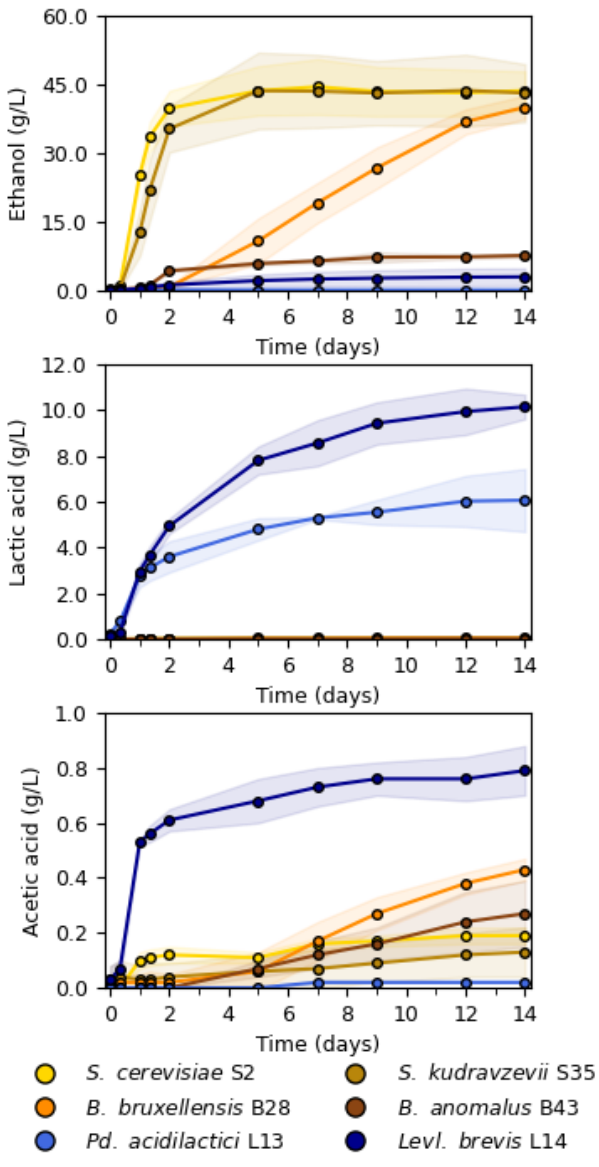
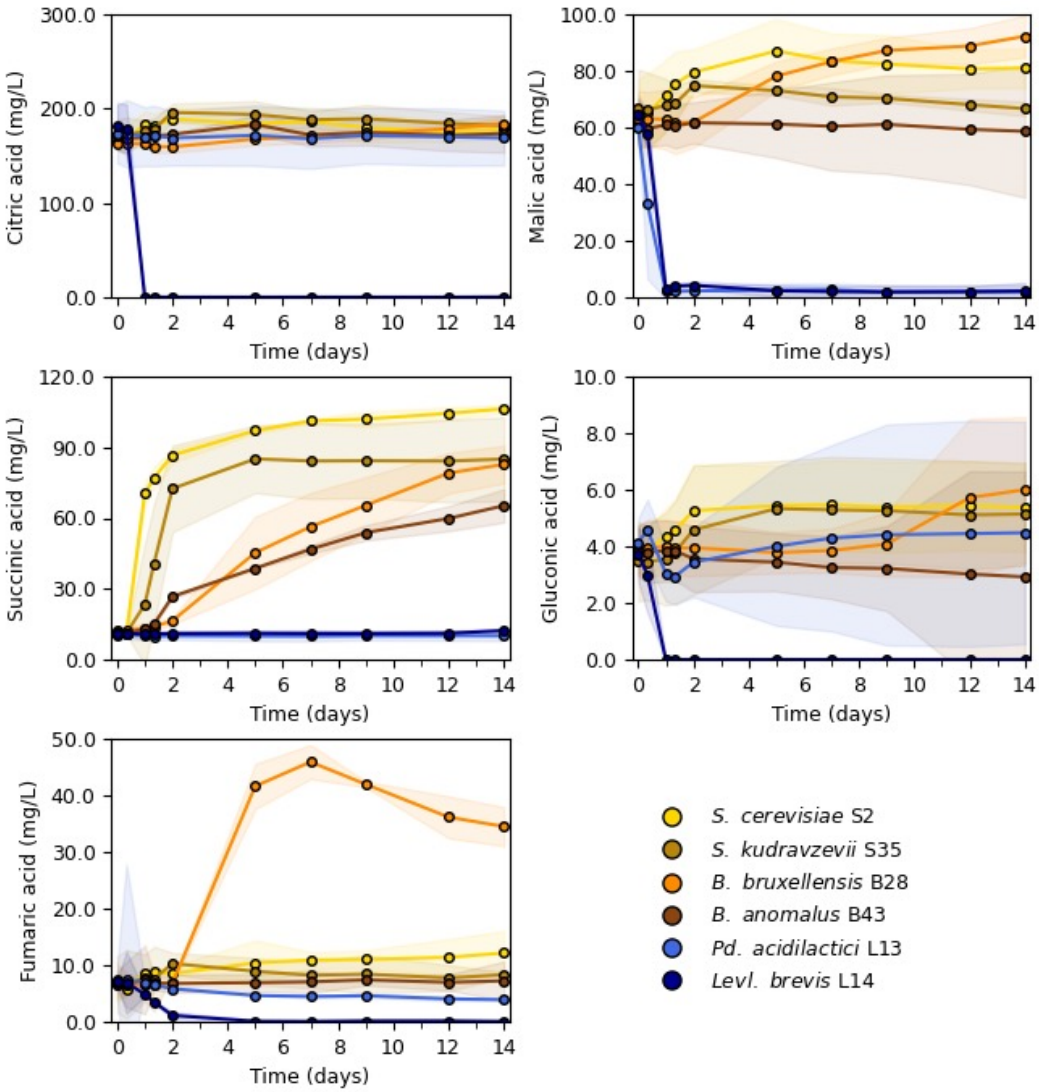


Figure 4. Concentration of citric acid, succinic acid, fumaric acid, malic acid, and gluconic acid during fermentation over 14 days with six microorganisms from lambic beer - *Saccharomyces cerevisiae* S2 (light yellow), *Saccharomyces kudriavzevii* S35 (dark yellow), *Brettanomyces bruxellensis* B28 (orange), *Brettanomyces anomalus* B43 (brown), *Pediococcus acidilactic*i L13 (light blue), and *Levilactobacillus brevis* L14 (dark blue). Data shows the average concentration of three technical duplicates of three biological replicates; coloured areas represent the standard deviation.



18.3 mg/L (*S. kudriavzevii* S35) after two days of at the end of the fermentation. The concentration of fumaric acid was unchanged with the other microorganisms.

Malic acid in the wort (63.5 ± 2.2 mg/L) increased with *S. cerevisiae* S2 (81.0 ± 7.0 mg/L) and *B. bruxellensis* B28 (92.2 ± 7.4 mg/L), but unchanged with *S. kudriavzevii* S35 and *B. anomalus* B43. Both LAB strains utilised malic acid within two days of fermentation. The initial gluconic acid concentration (3.8 ± 0.2 mg/L) was utilised by *L. brevis* L14 within one day of fermentation but remained unchanged with the other microorganism (Figure 4).

Production of volatile organic compounds

Esters and higher alcohols

The yeasts produced ethyl acetate, with the LAB strains making no contribution (Figure 5). With both *Saccharomyces* yeasts, ethyl acetate levels increased during the first two days of fermentation reaching 24.0 ± 6.2 mg/L (*S. cerevisiae* S2) and 18.9 ± 5.6 mg/L (*S. kudriavzevii* S35). The formation of ethyl acetate by *B. bruxellensis* B28 was slow, reaching 15.2 mg/L after two weeks of fermentation, which was higher than that formed by *B. anomalus* B43 (3.8 ± 1.5 mg/L; $p < 0.001$).

Ethyl lactate formation was detectable after two

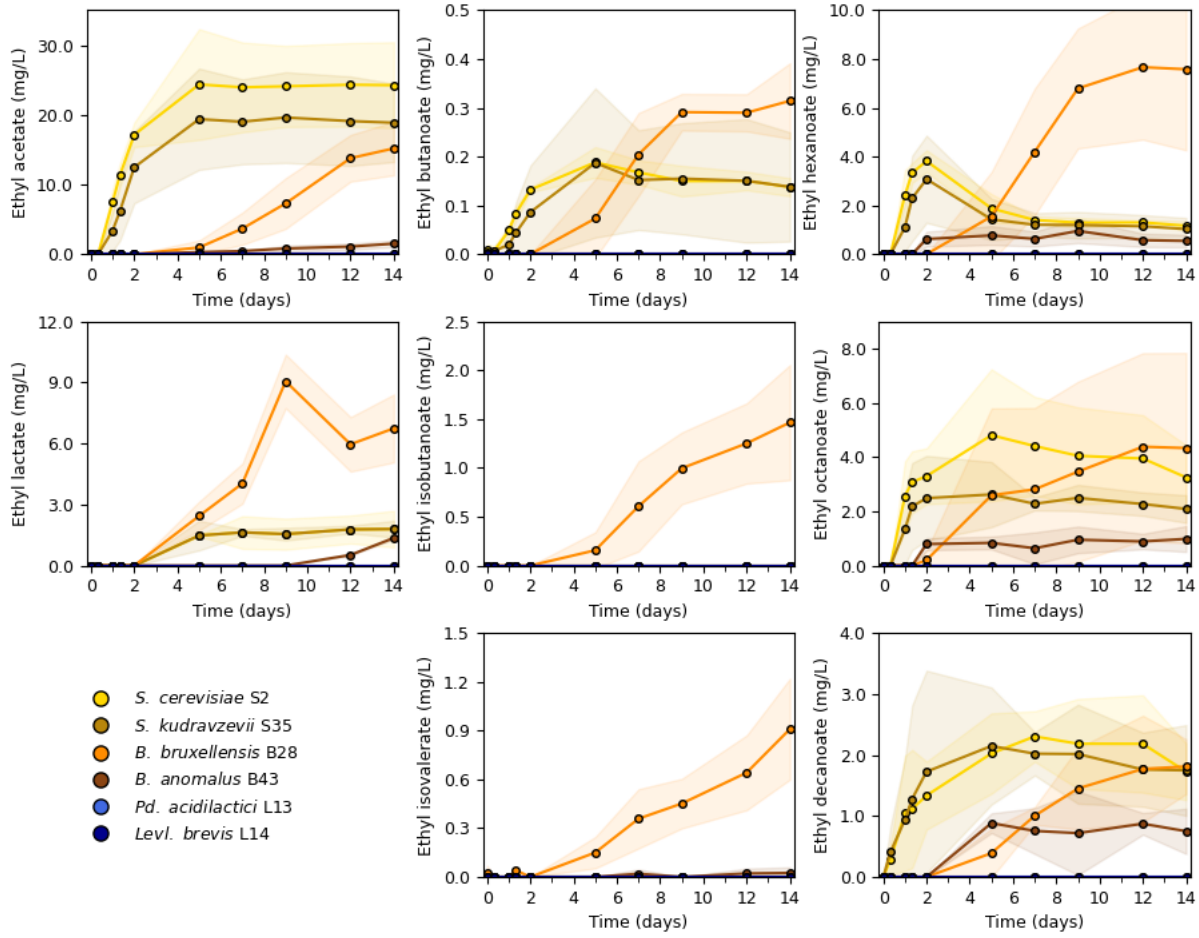
days of fermentation, increasing to 1.8 ± 0.9 mg/L (*S. cerevisiae* S2), 0.8 ± 0.4 mg/L (*S. kudriavzevii* S35), and 1.4 ± 1.4 mg/L (*B. anomalus* B43). *B. bruxellensis* B28 produced ethyl lactate to 6.8 ± 1.7 mg/L, with different production profiles to *B. anomalus* B43 ($p < 0.001$). Ethyl butanoate was formed within the first two days of fermentation by both *Saccharomyces* yeasts (0.1 mg/L) and remained constant thereafter. *B. bruxellensis* B28 formed ethyl butanoate (0.3 ± 0.1 mg/L) more slowly. Of the different microbes, only *B. bruxellensis* B28 formed ethyl isobutanoate (1.5 ± 0.1 mg/L) and ethyl isovalerate (0.9 ± 0.3 mg/L).

Ethyl hexanoate was formed by both *Saccharomyces* yeasts during the first two days of fermentation, yielding 3.8 ± 0.5 mg/L (*S. cerevisiae* S2) and 3.1 ± 2.6 mg/L (*S. kudriavzevii* S35). followed by a slow decline to < 2 mg/L. In contrast, *B. bruxellensis* B28 produced ethyl hexanoate throughout the fermentation process (7.6 ± 1.9 mg/L after 14 days), whereas *B. anomalus* B43 formed 0.6 ± 0.2 mg/L ($p < 0.001$).

Ethyl octanoate was formed by both *Saccharomyces* yeasts - *S. cerevisiae* S2 (3.3 ± 1.1 mg/L) and *S. kudriavzevii* S35 (2.1 ± 0.5 mg/L) - during the first five days of fermentation, and the level stabilised or decreased thereafter. Ethyl octanoate formation by the *Brettanomyces* yeasts was slow achieving 4.3 ± 3.3 mg/L (*B. bruxellensis* B28) and 1.0 ± 0.4 mg/L (*B. anomalus* B43).

Ethyl decanoate was similar to ethyl octanoate with early production by the *Saccharomyces*yeasts and more continuous formation by the *Brettanomyces* strains. The final concentration of ethyl decanoate with *S. cerevisiae* S2 and *S. kudriavzevii* S35 was 1.7 ± 0.7 mg/L and 1.8 ± 0.7 mg/L, with similar concentrations with *B. bruxellensis* B28 (1.8 ± 0.5 mg/L) whereas *B. anomalus* B43 produced less (0.8 ± 0.4 mg/L).

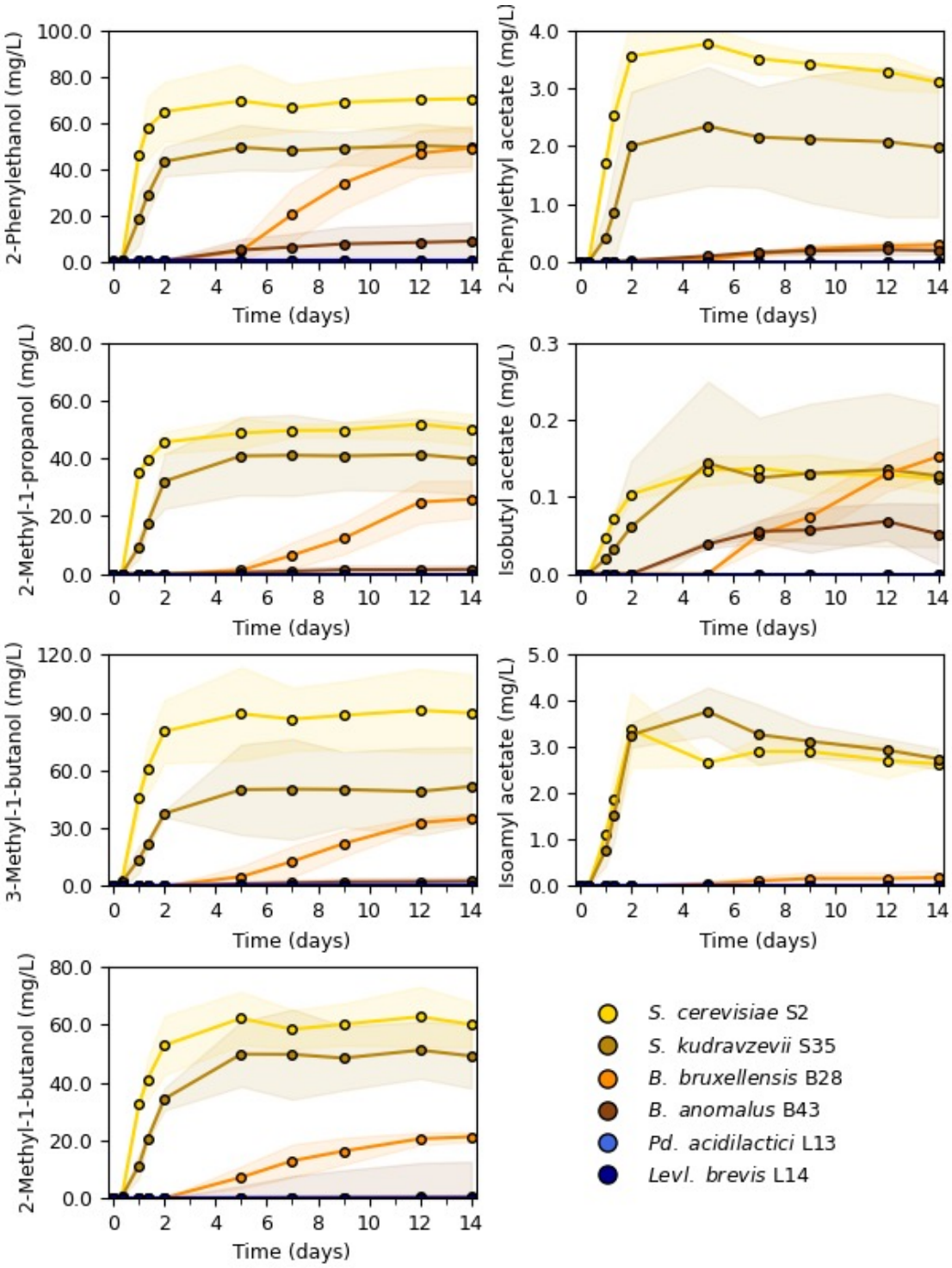
Figure 5. Concentration of ethyl acetate, ethyl lactate, ethyl butanoate, ethyl isobutanoate, ethyl isovalerate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate during fermentation for 14 days with six microorganisms from lambic beer - *Saccharomyces cerevisiae* S2 (light yellow), *Saccharomyces kudriavzevii* S35 (dark yellow), *Brettanomyces bruxellensis* B28 (orange), *Brettanomyces anomalus* B43 (brown), *Pediococcus acidilactici* L13 (light blue), and *Levilactobacillus brevis* L14 (dark blue). Data shows the average concentrations from three technical duplicates of three biological replicates; coloured areas represent the standard deviation.



With both *Saccharomyces* yeasts, the concentration of isobutyl acetate (acetate ester of 2-methyl-1-propanol) increased within the first two days of fermentation, to about 0.1 mg/L. Both *Brettanomyces* strains slowly produced the ester to a comparable final level (0.05-0.15 mg/L).

For isoamyl acetate (acetate ester of 3-methyl-1-butanol) and 2-phenylethyl acetate (acetate ester of 2-phenylethanol), the concentration increased over the first 48 hours of fermentation but decreased thereafter. Both *Saccharomyces* yeasts produced comparable levels of isoamyl acetate (2.7 ± 0.2 mg/L

Figure 6. Concentration of 2-phenylethanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethyl acetate, isobutyl acetate, and isoamyl acetate during fermentation for 14 days with six microorganisms from lambic beer - *Saccharomyces cerevisiae* S2 (light yellow), *Saccharomyces kudriavzevii* S35 (dark yellow), *Brettanomyces bruxellensis* B28 (orange), *Brettanomyces anomalus* B43 (brown), *Pediococcus acidilactici* L13 (light blue), and *Levilactobacillus brevis* L14 (dark blue). Data shows the average concentrations from three technical duplicates of three biological replicates; coloured areas represent the standard deviation.



and 2.8 ± 0.2 mg/L) together with 2-phenylethyl acetate concentrations of 3.1 ± 0.2 mg/L and 2.0 ± 1.1 mg/L (Figure 6). In contrast, the *Brettanomyces* strains produced low concentrations of both acetate esters (< 0.3 mg/L).

The level of 2-methyl-1-propanol increased during the first two days of fermentation to 39.8 ± 12.1 mg/L with *S. kudriavzevii* S35 and 50.0 ± 5.3 mg/L with *S. cerevisiae* S2. With *B. bruxellensis* B28 the concentration was 25.8 ± 6.5 mg/L, whereas *B. anomalus* B43 produced (1.5 ± 0.7 mg/L).

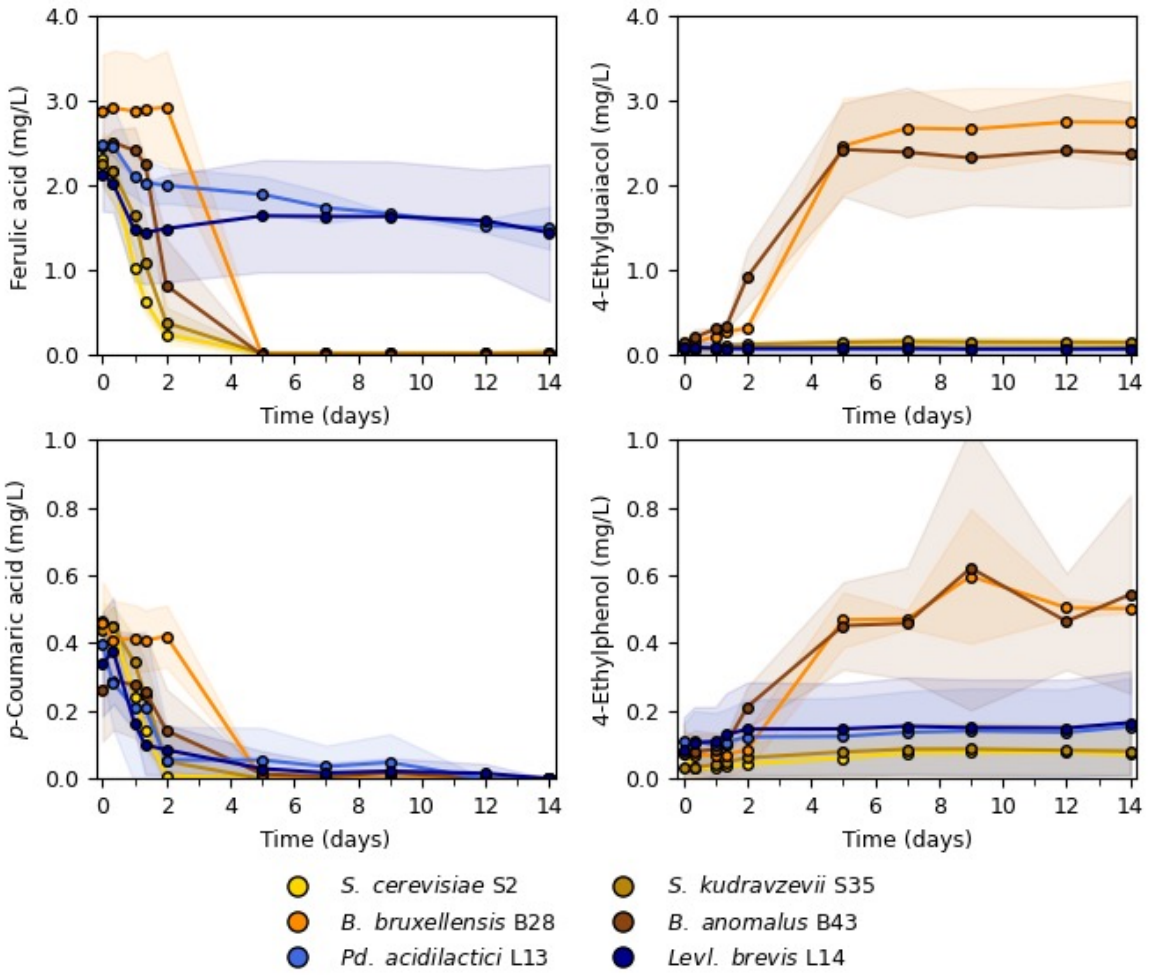
2-Methyl-1-butanol was produced rapidly by the *Saccharomyces* yeasts to levels of 60.8 ± 7.8 mg/L (*S. cerevisiae* S2) and 49.1 ± 11.2 mg/L (*S. kudriavzevii S35*). With *B. bruxellensis* B28, the level of the higher alcohol was 21.2 ± 1.7 mg/L but was not detectable with *B. anomalus* B43.

The concentration of 3-methyl-1-butanol increased during the first two days of fermentation, reaching 51.7 ± 20.3 mg/L (*S. kudriavzevii* S35) and 89.8 ± 20.2 mg/L (*S. cerevisiae* S2). In contrast, *B. bruxellensis* B28 produced 3-methyl-1-butanol more gradually, reaching a final concentration of 35.0 ± 2.4 mg/L. The levels of 2-phenylethanol followed a similar trend of formation by the *Saccharomyces* yeasts - 70.6 ± 13.9 mg/L (*S. cerevisiae* S2), 49.6 ± 8.3 mg/L (*S. kudriavzevii* S35) - and slower accumulation by *B. bruxellensis* B28 (49.2 ± 9.6 mg/L), and *B. anomalus* B43 (8.9 ± 8.1 mg/L) which differed significantly ($p < 0.001$). None of the higher alcohols reported here were produced by *P. acidilactici* L13 or *L. brevis* L14.

Phenolic acids

The initial concentration of (2.4 ± 0.4 mg/L) was unaffected by the LAB strains. However, ferulic acid decreased in the presence of

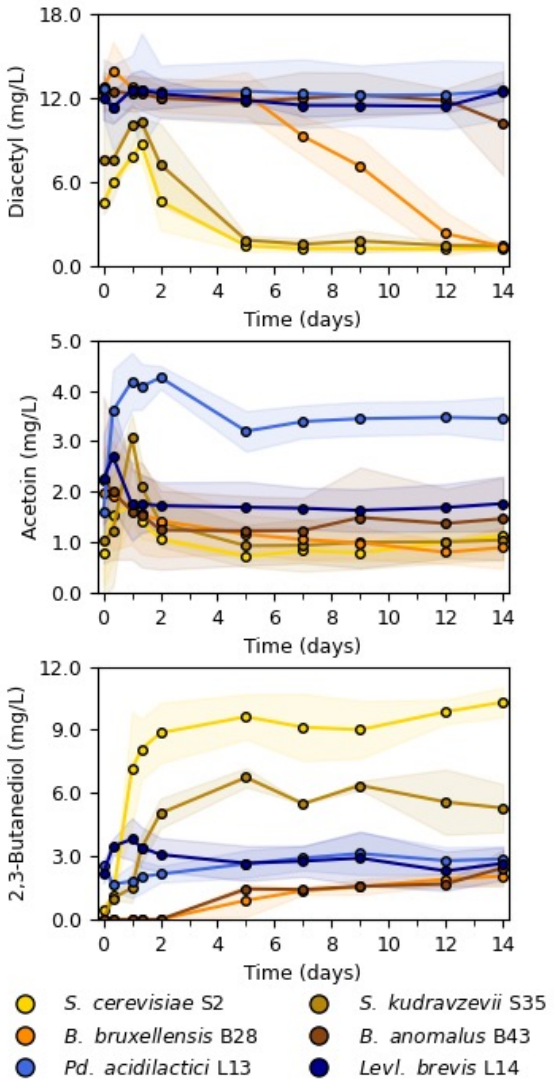
Figure 7. Concentration of ferulic acid, p-coumaric acid, 4-ethylguaiacol, and 4-ethylphenol during fermentation for 14 days with six microorganisms from lambic beer - *Saccharomyces cerevisiae* S2 (light yellow), *Saccharomyces kudriavzevii* S35 (dark yellow), *Brettanomyces bruxellensis* B28 (orange), *Brettanomyces anomalus* B43 (brown), *Pediococcus acidilactici* L13 (light blue), and *Levilactobacillus brevis* L14 (dark blue). Data shows the average concentration from three technical duplicates of three biological replicates; coloured areas represent the standard deviation.



the *Saccharomyces* yeasts, until depletion after five days of fermentation, but more slowly by the *Brettanomyces* yeasts (Figure 7). In contrast, p-coumaric acid (0.4 ± 0.1 mg/L) in wort was utilised within five days by both the LAB and the yeast strains.

Decarboxylation products, 4-ethylguaiacol (from ferulic acid) and 4-ethylphenol (from p-coumaric acid), were produced by the *Brettanomyces* strains, with 2.8 ± 0.5 mg/L (*B. bruxellensis* B28) and 2.4 ± 0.6 mg/L (*B. anomalus* B43) for 4-ethylguaiacol, and 0.5 ± 0.0 (*B. bruxellensis* B28) and 0.5 ± 0.3 mg/L (*B. anomalus* B43) for 4-ethylphenol.

Figure 8. Concentration of diacetyl (2,3-butanedione), acetoin (2-hydroxy-3-butanone), and 2,3-butanediol during fermentation for 14 days fermentation with six microorganisms from lambic beer - *Saccharomyces cerevisiae* S2 (light yellow), *Saccharomyces kudriavzevii* S35 (dark yellow), *Brettanomyces bruxellensis* B28 (orange), *Brettanomyces anomalus* B43 (brown), *Pediococcus acidilactici* L13 (light blue), and *Levilactobacillus brevis* L14 (dark blue). Data shows the average concentration from three technical duplicates of three biological replicates; coloured areas represent the standard deviation.



Diacetyl, acetoin, and 2,3-butanediol

The concentration of diacetyl in the wort was 10.3 ± 3.7 mg/L, which increased with *S. cerevisiae* S2 and *S. kudriavzevii* S35 during first 32 hours of fermentation, and then decreased to 1.3 ± 0.2 mg/L and 1.5 ± 0.3 mg/L. With *B. bruxellensis* B28, the diacetyl concentration did not increase and declined to 1.4 ± 0.1 mg/L. With *B. anomalus* B43 and the LAB, the diacetyl remained constant at 9-12 mg/L.

The concentration of acetoin – the product of diacetyl reduction – in wort (1.8 ± 0.9 mg/L) initially increased and then declined to around 1 mg/L. In contrast, *P. acidilactici* L13 produced acetoin during the first 1-2 days of fermentation, to around 3.5 ± 0.4 mg/L. The concentration of 2,3-butanediol – the product of acetoin reduction - increased with all yeast strains. The level of 2,3-butanediol after fermentation with the *Saccharomyces* strains - 10.3 ± 0.7 mg/L (*S. cerevisiae* S2) and 5.3 ± 1.1 mg/L (*S. kudriavzevii* S35) – were higher than with *B. bruxellensis* B28 and *B. anomalus* B43 (< 3 mg/). *P. acidilactici* L13 and *L. brevis* L14 maintained stable levels (< 4 mg/L) of 2,3-butanediol (Figure 8).

Discussion

Traditional sour beer production involves spontaneous fermentation and maturation in wooden barrels for up to three years (Spitaels et al. 2014; De Roos and De Vuyst 2019; Bouchez and De Vuyst 2022; Bongaerts et al. 2021; 2024; Croonenberghs et al. 2024). Alternatively, sour beers can be produced by using an acidification process (Tonsmeire 2014; Bossaert et al. 2019; Dysvik et al. 2020; Bouchez and De Vuyst 2022; Croonenberghs et al. 2024).

As a first step toward obtaining a more standardised approach to produce sour beer in both a shorter time and without the use of wooden barrels, laboratory-scale wort fermentations were performed with monocultures (isolated from lambic) to evaluate the flavour potential of yeasts (*S. cerevisiae* S2, *S. kudriavzevii* S35, *B. bruxellensis* B28, and *B. anomalus* B43) and lactic acid bacteria (*P. acidilactici* L13 and *L. brevis* L14).

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Both *Saccharomyces* strains exhibited fast fermentation and production of volatile compounds (Briggs et al. 2004; Gallone et al. 2016; De Roos and De Vuyst 2019; Bouchez and De Vuyst 2022). The consumption of sugars reflects efficient HXT, MAL, and AGT1 transport systems and extracellular invertase activity (hydrolysing sucrose to glucose and fructose), coupled with a high glycolytic flux (Özcan and Johnston 1999; Elbing et al. 2004; Marques et al. 2016; Krogerus and Gibson 2020). The *Brettanomyces* strains showed more variability, perhaps due to differences in activity of the sugar permeases together with α-glucosidase (Crauwels et al 2015a,b; Colomer et al. 2020). With *B. bruxellensis* B28 and *B. anomalus* B43, a lack of maltose consumption by the latter strain is a likely due to the absence or low expression of relevant genes, or a preference for alternative substrates, such as sucrose (Gilliland 1961; Smith and Van Grinsven 1984). With the slower fermentation rate and incomplete attenuation, *Brettanomyces* strains are used in combination with *Saccharomyces* yeasts during mixed fermentations for bioflavouring, as is the case for the Belgian Trappist beers (Steensels and Verstrepen 2014; De Roos and De Vuyst 2019).

With the LAB strains, the maximum cell density was reached after one day of fermentation and was accompanied by the pH dropping below 4.0. This rapid acidification is used in kettle-soured beer production, during which LAB strains acidify the wort prior to the yeast fermentation (Bossaert et al. 2019; Dysvik et al. 2019, 2020; Bouchez and De Vuyst 2022). However, whereas *P. acidilactici* L13 utilised only glucose and fructose, *L. brevis* L14 metabolised fructose and, to a limited extent maltose, confirming the narrow range of carbohydrates used by LAB (Holzapfel and Wood 2014; Li et al. 2021). The persistence of glucose during fermentation with *L. brevis* L14 may reflect a balance between consumption and regeneration via maltose hydrolysis.

The most abundant metabolites during sour beer production are ethanol, lactic acid, and acetic acid. Ethanol production, mirroring a decrease in AE was pronounced when using yeasts. Both *Saccharomyces* strains and *B. bruxellensis* B28 achieved ethanol levels of 5.0-5.6% (v/v), consistent with wort with an extract of 13-14°P (Tonsmeire 2014). The lower ethanol production by *B. anomalus* B43 reflected limited sugar utilisation and inability to consume maltose and maltotriose.

In this study, none of the six microorganisms could metabolise maltooligosaccharides with a polymerisation degree greater than three. Although *S. kudriavzevii*, diastatic *S. cerevisiae*, and *Brettanomyces* strains encode extracellular glucoamylases or GH15 α-glucosidases, which break down maltooligosaccharides (Crauwels et al. 2015a; De Roos et al. 2020; Krogerus and Gibson 2020; Vermote et al. 2023; Bongaerts et al. 2026), their activity toward long glucose chains is limited (Kumara et al. 1993). Indeed, the breakdown of maltooligosaccharides leading to over-attenuation (AE < 1.5 °P), requires extended maturation, as in the production of traditional lambic beer (De Roos et al. 2018a; Vermote et al. 2023; Bongaerts et al. 2026).

Lactic acid and acetic acid are key to the production of sour beers, with levels of lactic acid between 1.5 and 10 g/L (Bongaerts et al. 2021), and acetic acid typically below 1.5 g/L (Vermote et al. 2023; Bongaerts et al. 2026), although 5 g/L has been reported (De Roos et al. 2018a). Whereas *P. acidilactici* performs homolactic fermentation, *L. brevis* metabolises saccharides through the phosphoketolase pathway, yielding equimolar amounts of lactic acid, ethanol or acetic acid, carbon dioxide, and ATP (Holzapfel and Wood 2014; Gänzle 2015). The higher lactic acid concentration obtained with *L. brevis* L14 may reflect increased overall carbohydrate conversion and redox balancing, supported by efficient reoxidation of NADH. This may be facilitated by using fructose as an alternative external electron acceptor, which is then reduced to mannitol to maintain redox homeostasis during maltose metabolism (Wisselink et al. 2002; Axelsson 2004; Martínez-Miranda et al 2022).

This mechanism has been documented in obligate heterofermentative lactobacilli, including *L. brevis*, for whom maltose serves as the primary energy source, while fructose acts as a hydrogen acceptor (Kandler and Weiss 1986). This dual-substrate strategy explains the simultaneous consumption of fructose and maltose by *L. brevis* L14, contrasting with the more restricted substrate utilisation of *P.*

acidilactici L13. This metabolic flexibility not only enhances acidification by *L. brevis* but stabilises redox metabolism during prolonged fermentation, a property that underlies spontaneous and mixed beer fermentation (Vaughan et al. 2005; De Roos and De Vuyst 2019). Acetic acid accumulated during fermentation with yeasts and *L. brevis* L14, the latter leading to the highest yield, followed by *B. bruxellensis* B28. The level with *L. brevis* L14 (around 200 mg/L), were above the reported flavour threshold in beer (Zhang et al. 2013), suggesting a vinegar-like acidity. The *Saccharomyces* strains produced acetic acid primarily by oxidising acetaldehyde through acetaldehyde dehydrogenase activity (Remize et al. 2000).

The metabolism of other organic acids - citric acid, succinic acid, malic acid, and gluconic acid - was explored. Citric acid was utilised by *L. brevis* L14, perhaps as alternative energy and redox substrate in glucose-limited conditions through citrate lyase and oxaloacetate decarboxylase, resulting in the flavour active carbonyl compounds diacetyl and acetoin (García-Quintáns et al. 2008). In contrast, the yeasts showed little utilisation of citric acid, consistent with the limited anaplerosis in the tricarboxylic acid (TCA) cycle during anaerobic fermentation (Li and Liu 2015). Succinic acid was not affected by the LAB strains, due the absence of a complete TCA cycle, but accumulated with the *Saccharomyces* and *Brettanomyces* yeasts owing to active TCA cycling and a reductive branch flux under microaerobic conditions (Gibson et al. 2007; Vilela et al. 2013). In *Saccharomyces*, succinic acid is primarily formed from α-ketoglutarate via the oxidative branch and from fumaric acid via the reductive branch of the TCA cycle, contributing to buffering capacity. Although succinic acid has been linked with sour, salty, bitter, and umami-related flavour, the levels were below the threshold in beer (200 mg/L; Engan, 1973), making a sensory contribution less likely (Saerens et al. 2010). During the *Brettanomyces* fermentation, succinic acid accumulation reflecting the activity of the reductive branch of the TCA cycle, in which fumarate is converted into succinate under microaerobic conditions since succinic acid assimilation requires FADH2, which requires the presence of oxygen to link with the electron transport chain (Rosenfelt and Beauvoit 2003). Malic acid was completely utilised by both LAB strains, consistent with malolactic fermentation of malate to lactate and carbon dioxide. This decarboxylation reaction contributes to deacidification of the cytoplasm and generates a proton motive force (Lonvaud-Funel 1999). The mechanism is well known in winemaking, during which LAB species - *Oenococcus oeni*, *L. plantarum*, and *L. brevis* - perform malolactic fermentation as a secondary process, improving the microbial stability of wine and softening of its acidity (Lonvaud-Funel 1999; Bartowsky and Henschke 2004). Malolactic fermentation has been reported during the spontaneous production of cider and lambic (Zhang and Lovitt 2006; De Roos et al. 2018a, 2020; Bongaerts et al. 2021; Vermote et al. 2023). Malic acid was produced by *S. cerevisiae* S2 and *B. bruxellensis* B28, likely linked due to anaplerotic reactions involving decarboxylation of pyruvate to oxaloacetate, followed by oxaloacetate reduction to malate (Zelle et al. 2008). Finally, gluconic acid concentrations only declined during fermentation with LAB, acting as a co-substrate and entering the phosphoketolase pathway via gluconate kinase and 6-phosphogluconate dehydrogenase. Such metabolism generates reducing power, supporting anabolic reactions and protecting the cells against oxidative stress during the early exponential growth (Axelsson 2004; Gänzle et al. 2007; Li et al. 2021a, b). In contrast, yeasts show no or a very limited capacity to assimilate gluconic acid, which is strain or condition-dependent (Boulton and Quain 2001; Colomer et al. 2020; Ogawa et al. 2021).

The link between the different starter cultures and the production of volatile organic compounds (VOC) was explored. The first major VOC class investigated consisted of esters which potentially confer fruity notes to the sour beer. Ethyl acetate was produced rapidly by *Saccharomyces* strains and, more gradually, by the *Brettanomyces* strains. With beer maturation, this gradual accumulation by *Brettanomyces* continued over time (Verachtert and Derdelinckx 2005; De Roos et al. 2018a; Colomer et al. 2020; Vermote et al. 2023; Bongaerts et al. 2026). Ethyl acetate production depends both on enzymatic potential and the availability of precursors (ethanol and acetic acid). Although *Brettanomyces* is associated with acetic acid formation, under microaerobic conditions, *S. cerevisiae* S2 and *S. kudriavzevii* S35 produced similar levels of acetic acid. Higher ethyl acetate formation can be

attributed to the expression of alcohol acetyltransferases (Verstrepen et al. 2003b; Saerens et al. 2008; Colomer et al. 2020). Ethyl lactate formation was minor with the *Saccharomyces* strains but notable for *B. bruxellensis* B28. This is a known feature of *Brettanomyces*-led spontaneous sour beer fermentation and maturation processes (De Roos et al.2018a 2020; Vermote et al. 2023; Bongaerts et al. 2026). The LAB strains did not produce esters, as they have limited esterase activity and cannot obtain precursors from metabolism unlike yeasts (Pradal et al. 2025). Nonetheless, ethyl acetate and ethyl lactate formation by heterofermentative LAB - *L. brevis* and *Limosilactobacillus fermentum* - has been reported to a limited extent during sourdough production (Kaseleht et al. 2011; Di Renzo et al. 2018; Pradal et al. 2025).

Ethyl butanoate, ethyl hexanoate, and ethyl octanoate accumulated primarily during fermentation with *Saccharomyces* yeasts. These esters originate from the medium chain fatty acid (MCFA) metabolism, during which MCFAs (intermediates of the fatty acid biosynthesis pathway) are esterified with ethanol by alcohol acetyltransferases, mainly ATF1p and EEB1p (Lilly et al. 2006; Saerens et al. 2008). The biosynthesis of these ethyl esters is closely linked to the *de novo* fatty acid biosynthesis and the intracellular acyl-CoA pool, which in turn depends on oxygen availability, lipid biosynthesis, and redox state (Mason and Dufour 2000; Dufour et al. 2003). In contrast, the *Brettanomyces* strains produced these esters more gradually but steadily until the later stages of fermentation, consistent with their capacity to oxidise and reutilise MCFAs via β-oxidation and subsequent re-esterification of regenerated acyl-CoA intermediates (Crauwels et al. 2015a; Colomer et al. 2020). Esterification is a notable activity of *Brettanomyces* during extended lambic maturation, when oxidative metabolism and acyl-CoA recycling remain active under low nutrient conditions. Consequently, *Brettanomyces* contributes to the fruity and complex aroma profile of mature sour beers through the production of ethyl esters. Ethyl hexanoate exceeded its flavour threshold in beer (ca. 0.2 mg/L; Engan 1972), and ethyl octanoate reached concentrations above the threshold (ca. 1.0 mg/L; Engan 1972). Ethyl acetate, ethyl butanoate, and ethyl decanoate reached concentrations near their respective reported thresholds, while ethyl lactate was below its threshold. Such levels infer the sensory relevance of ethyl esters, particularly ethyl hexanoate and ethyl octanoate. However, their contribution was not assessed, requiring the use of a sensory panel (Meilgaard 1975; Bongaerts et al. 2024, 2026).

Higher alcohols, including 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol, originate from amino acid catabolism via the Ehrlich pathway (Hazelwood et al. 2008; Holt et al. 2019). The formation of acetate esters, mainly isoamyl acetate and 2-phenylethyl acetate, results from alcohol acetyltransferase activity, which esterifies higher alcohols with acetyl-CoA (Saerens et al. 2008). The *Brettanomyces* strains produced lower levels which may relate to slower amino acid assimilation and a preference for oxidative metabolism (Crauwels et al. 2015a; Steensels et al. 2015). However, 2-phenylethanol, 3-methyl-1-butanol, and 2-methyl-1-butanol reached concentrations at or above their reported thresholds, with 2-phenylethyl acetate at its threshold. The ester, isoamyl acetate exceeded its reported beer flavour threshold (1.2-2.0 mg/L) (Meilgaard 1975; Verstrepen et al. 2003a). These compounds contribute floral, fruity, banana, or alcoholic notes to the aroma profile. However, excessive accumulation is undesirable in sour beers, as the European regulation for Oude Gueuze (European Commission 2016) limits isoamyl acetate to 0.5 mg/L after six months of bottle maturation, which is below the threshold of 1.2-2.0 mg/L in 'conventional' beers (Meilgaard 1975; Verstrepen et al. 2003a). In a balanced mixed fermentation, moderate levels of higher alcohols and esters enhance the aromatic complexity, whereas an excess can mask the desired acidic and phenolic character of *Brettanomyces* driven fermentation and maturation. However, high concentrations of acetate esters during fermentation need not be problematic, as *Brettanomyces* can hydrolyse isoamyl acetate and other esters during maturation and bottle refermentation (Spaepen and Verachtert 1982; Steensels et al. 2015; Bongaerts et al. 2024).

Volatile phenolic compounds are associated with spicy/smoky (4-ethylphenol) and barnyard/leathery (4-ethylguaiacol) aromas. In this study, 4-ethylguaiacol reached concentrations above

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its flavour threshold in beer (ca. 0.1 mg/L; Thompson-Witrick et al. 2017), whereas the highest 4-ethylphenol concentration approached the threshold value (ca. 0.4 mg/L; Thompson-Witrick et al. 2017). Both compounds were found when using the *Brettanomyces* yeasts, reflecting their phenolic off-flavour (POF^+) phenotype, and the presence of phenolic acid decarboxylase and vinyl phenol reductase activities (Crauwels et al. 2015a; Steensels et al. 2015; Colomer et al. 2020). *Saccharomyces* yeasts and some LAB species are known to possess the phenolic acid decarboxylase (*PAD*) and ferulic acid decarboxylase (*FDC1*) genes, which catalyse the decarboxylation of ferulic acid and coumaric acid to 4-vinyl guaiacol and 4-vinyl phenol (Couto et al. 2006; Mukai et al. 2010; Richard et al. 2015). However, the second step - the reduction of the vinyl compounds into the ethyl derivatives by vinyl phenol reductase activity - is typical of *Brettanomyces* and not other yeasts or lactic acid bacteria (Crauwels et al. 2015a). However, phenolic acid decarboxylase activity and vinyl phenol reductase activity has been reported in LAB as a rare, strain-dependent trait (in *L. plantarum*), and is insufficient to generate ethyl phenol compounds in detectable concentrations (Cavin et al. 1997; Rodriguez et al. 2009).

Finally, the formation of diacetyl and other vicinal diketones provided insight into flavour differentiation and redox balancing. With the *Saccharomyces* strains there was an early increase in diacetyl concentration, followed by its reduction to acetoin and then 2,3-butanediol. This reflects high α-acetolactate synthase activity during valine biosynthesis and the subsequent reductive conversion under fermentative redox conditions (Dufour et al. 2003). With both *Brettanomyces* strains, the reduction of diacetyl to 2,3-butanediol was less, indicating a slow or incomplete reductive capacity. Reduction to acetoin without further conversion is consistent with low butanediol dehydrogenase expression, constrained NADH availability, and a more oxidative metabolism (Crauwels et al. 2015a; Colomer et al. 2020). Residual diacetyl concentrations remained above the reported flavour threshold in beer (0.014-0.15 mg/L; Saison et al. 2009), suggesting that diacetyl would have contributed a buttery aroma. In contrast, acetoin reached concentrations around its sensory threshold, whereas 2,3-butanediol remained several orders of magnitude below its threshold. Reduction of diacetyl not only reflects redox balancing but also converts a highly flavour active compound into products with reduced sensory impact. The LAB strains maintained almost a constant diacetyl and acetoin concentration, the net result from accumulation via the citrate metabolism and partial reduction to acetoin (García-Quintáns et al. 2008; Gänzle 2015).

Overall, the microorganisms used in this work displayed distinct functional profiles that extended beyond their capacity for attenuation, acidification, or aroma compound production. *S. cerevisiae* S2 combined rapid consumption of fermentable sugars with ethanol production, reduction of diacetyl, and early formation of higher alcohols and esters, supporting its role as a primary fermentative strain. In contrast, *B. bruxellensis* B28 exhibited slower more prolonged metabolic activity, combining attenuation with gradual ester formation, the production of volatile phenolic compounds, and a complementary role during later fermentation. *B. anomalus* B43 had a more restricted substrate range and lower metabolite production, which may limit its contribution to mixed-culture conditions. The LAB strains contributed through acidification and organic acid formation, with *L. brevis* L14 having a broader metabolic profile than *P. acidilactici* L13 with strong lactic acid production and the conversion of organic acids.

Taken together, the complementary profiles of *S. cerevisiae* S2, *B. bruxellensis* B28, and *L. brevis* L14 may be relevant during mixed-strain fermentation. Although their combined performance cannot be confirmed from single culture fermentations as microbial interactions may alter growth, substrate consumption, and metabolite formation (Peyer et al. 2017; Ciosek et al. 2019; Dysvik et al. 2019, 2020). Further, substrate competition as well as inhibitory and/or stimulatory effects produced by other microorganisms (e.g., ethanol towards LAB) may modify their behaviour. In addition, the sequence of inoculation can alter microbial succession and the final balance between attenuation, acidification, and flavour formation (Dysvik et al. 2019, 2020).

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Conclusions

The functional and potential flavour generation for sour beer by yeast and bacterial strains differed during the fermentation of wort in monocultures. Both *Saccharomyces* strains showed rapid attenuation and ester formation, with *S. cerevisiae* S2 displaying a favourable flavour generation and fermentation efficiency. Of the *Brettanomyces* strains, *B. bruxellensis* B28 combined attenuation with the production of esters and volatile phenolic compounds, whereas *B. anomalus* B43 was more limited in flavour generation and was unable to consume maltose and maltotriose. The lactic acid bacteria contributed through acidification and organic acid conversion rather than attenuation or aroma formation, with *L. brevis* L14 showing broader metabolic activity than *P. acidilactici* L13. These strain specific differences in monoculture highlight complementary functional traits that may be useful in the design of defined mixed strain starter cultures. Taken together, *S. cerevisiae* S2, *B. bruxellensis* B28, and *L. brevis* L14 are promising candidates for evaluation in mixed-strain fermentation. However, further analysis and validation through co-culture experiments will be required for standardised sour ale production, while identifying which monoculture properties can (or cannot) be extrapolated to more complex microbial groupings.

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Conflict of interest

The authors declare no conflict of interest.

CRediT author contributions

Arne Bouchez: conceptualisation, methodology, investigation, data curation, writing (original draft), writing (review and editing), and visualisation.
Thomas Debing: investigation, data curation, and visualisation.
Frederic Leroy: writing (review and editing), and supervision.
Luc De Vuyst: conceptualisation, methodology, writing (review and editing), supervision, project administration, and funding acquisition.

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