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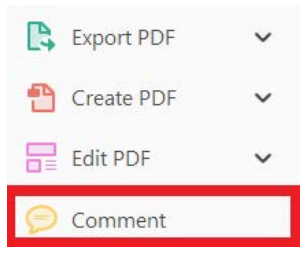

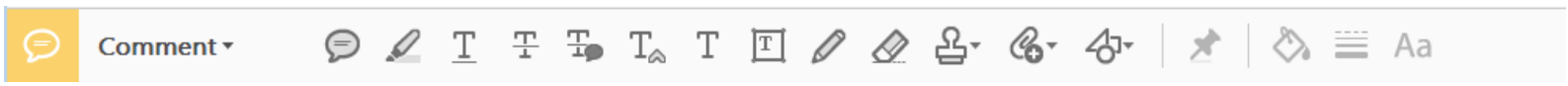

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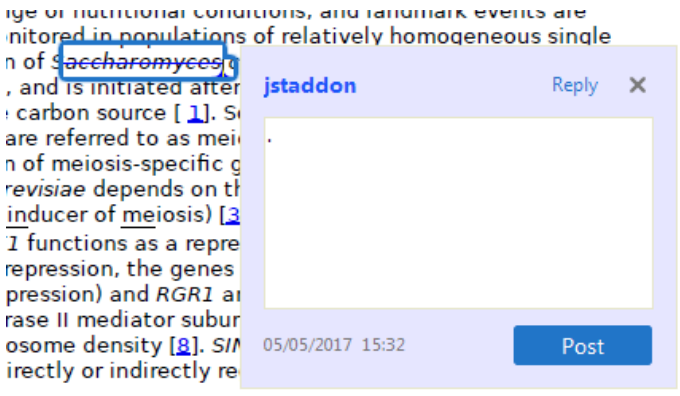

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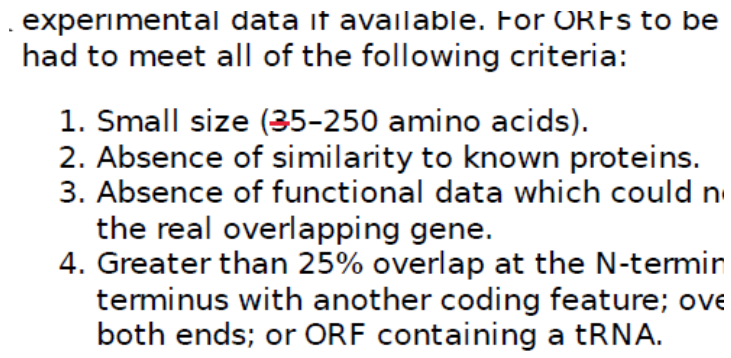
experimental data if available. For ORFs to be had to meet all of the following criteria:

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3. Absence of functional data which could n the real overlapping gene.
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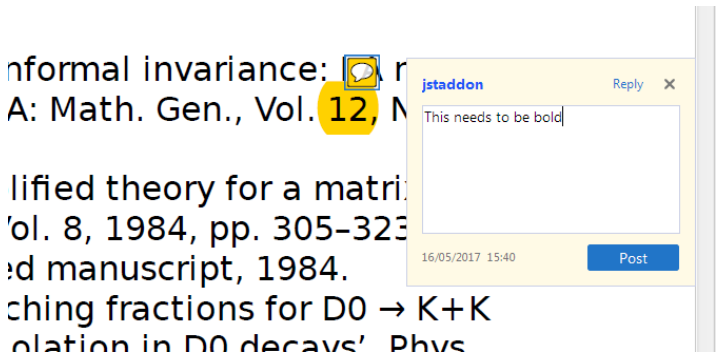

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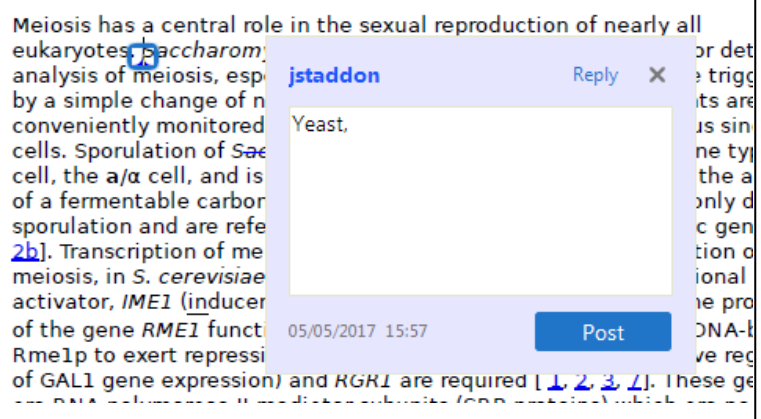

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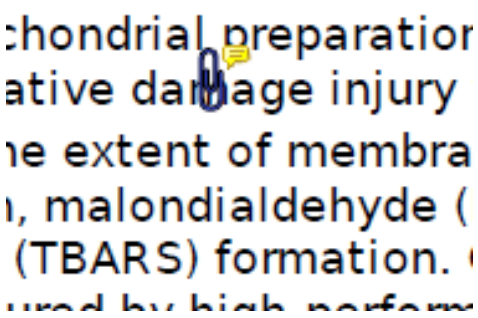

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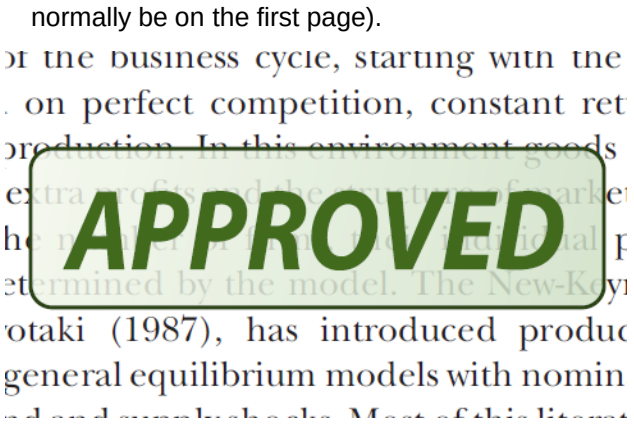

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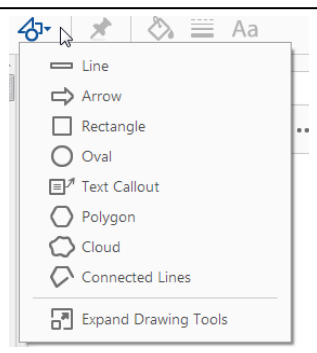

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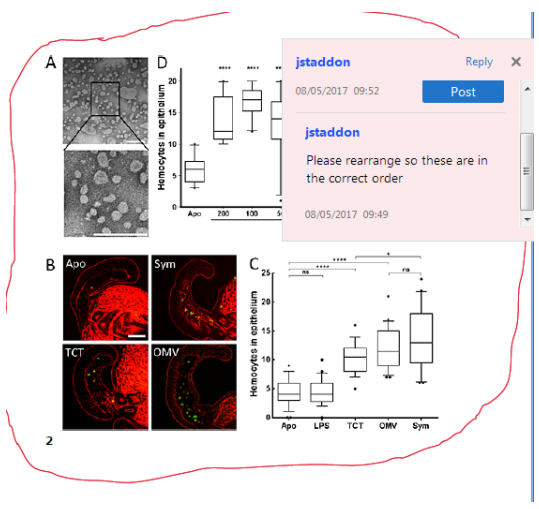

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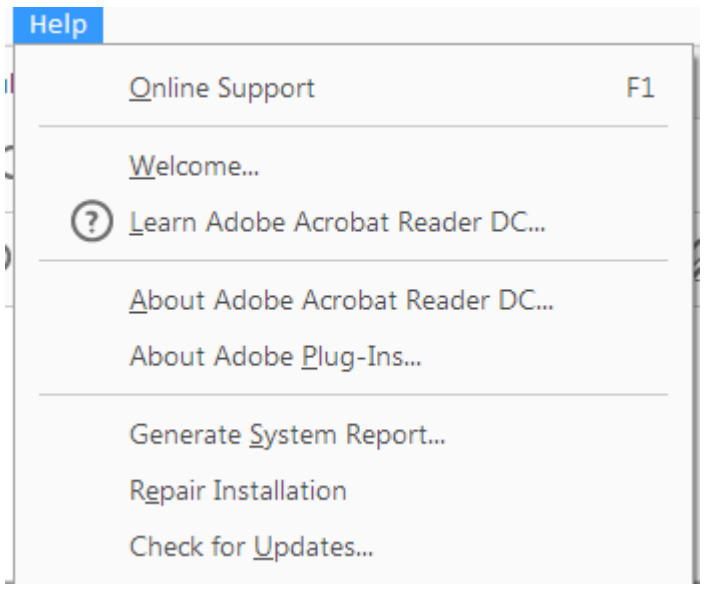

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Pre-fermentation with lactic acid bacteria in sour beer production

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Sour beer is beer with an intentionally sour taste. In traditionally produced sour beer, the acidic character results from spontaneous, mixed fermentation where different bacteria and yeast species participate. These complex fermentations take years to complete and can be difficult to control. Owing to increasing interest in sour beer in recent years and challenges related to commercial sour beer brewing, alternative production methods are being explored. In the current paper, pre-fermentation with lactic acid bacteria (LAB) is investigated as a timesaving and highly controllable strategy for the production of sour beer. Four beers were produced with either biological or chemical acidification of wort, and a reference beer was produced with no acidification. Volatile compounds and organic acids were analysed by headspace gas chromatography and high-performance liquid chromatography to explore any contribution from LAB to the composition of beer. Finally, descriptive sensory analysis was carried out to evaluate the sensory contribution from LAB. A significant effect was observed from LAB pre-fermentation, with respect to both volatile compounds (e.g. restraining effect on production of 2-methyl-1-butanol) and organic acids (e.g. production of acetic acid). Biological acidification by LAB pre-fermentation had a significant impact on the sensory character of beer. This sensory impact did, however, not surpass that obtained by chemical acidification, as few significant differences were found between biologically and chemically acidified beers. © 2019 The Institute of Brewing & Distilling

Keywords: sour beer; mixed fermentation; *Lactobacillus*; lactic acid bacteria; sequential fermentation

Introduction

During beer fermentation, yeast metabolise sugars to ethanol, carbon dioxide and other flavour-active metabolites *(1)*. A selection of metabolites important to beer flavour is given in T1 Table 1. *Saccharomyces pasteurianus* and *Saccharomyces cerevisiae*, both referred to as brewers' yeast, are used for the production of lager and ale, respectively. Sour beer is beer with an intentionally sour taste, where the sour character results from mixed fermentation *(12)*. Brewers' yeast usually plays an important part in such fermentations, but accompanied by non-*Saccharomyces* yeast strains as well as various bacterial species *(13–15)*. Belgian brewing culture is famous for its long traditions for sour beer production. Most of the well-known classic styles within the genera originate from Belgium, such as lambic, gueuze and kriek *(16)*. No active addition of microbes is carried out in traditional sour beer production; rather the wort is exposed to an environment by which it is spontaneously inoculated. Boiled wort is cooled down in open vessels where microbes from the air come into contact with the wort, before it is transferred to wooden barrels used in previous fermentations. Remaining microbes living on the surface of the porous wood *(17)* then further inoculate the brew. These two steps result in a multitude of microorganisms being introduced in the wort. A vast consortium of different microbes metabolise simultaneously and successively as fermentation progresses. The involvement of more than 2000 different yeast and bacterial strains in a lambic fermentation has been documented *(18)*. Species considered important for the final beer character include strains of *Saccharomyces*, *Brettanomyces* and lactic acid bacteria (LAB) such as *Lactobacillus* and *Pediococcus*. Other microbes, e.g Enterobacteriaceae, can also be involved, and multiple strains of each species can take part *(16,18)*.

The path from unfermented wort to stable, mature beer is far more time-consuming and complex for sour beer compared with cleanly fermented ale or lager. A commercially produced ale or lager can be ready for consumption in a few weeks, while the fermentation and maturation of sour beer can take many years *(19)*. The lack of active microbial pitching restrains fermentation speed, as does the progressively inhospitable growth environment. Ethanol, pH, carbon dioxide, substrate deprivation and inter-microbial competition for nutrients all contribute to decelerated microbial growth and metabolism *(20)*. Commercial sour beer production can be quite challenging owing to the long time demand, as long-term storage of barrels takes up space and reduces beer output per time unit. The high number of microbial strains involved in mixed fermentations can also represent a challenge, as it is complicated to control the collective metabolism of a vast microbial consortium. This makes it difficult to obtain a consistent product.

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Table 1. Flavour-active metabolites in beer, with reported taste thresholds and flavour characteristics

Compound	Sensory threshold in beer (mg/L)	Flavour characteristic
1-Propanol	600[1]	Alcohol, sweet,[1] fruity[2]
2-Methyl 1-butanol	65[2]	Alcoholic, winey,[2] Malty[10]
2-Methyl 1-propanol	200[2]	Fruity,[3] whiskey, winey[2]
Phenylethyl alcohol	125[2]/40 [1] (lager beer)	Rosey,[2] honey[3]
Ethyl heptanoate	0.17[4]/0.4[5]	Berries, melon, peach, pineapple, plum[3]
Ethyl octanoate	0.9–1.0[1]	Apricot, banana, flowery, pear, winey, pinapple[3] Apple, aniseed,[1] Sweet, fruity[2]
Lactic acid	400[6]	Acrid[7]
Acetic acid	200[6]	Tart, sour[6]
Pyruvic acid	250[8]	Sour[8]
Formic acid	83[9]	Sour[9]
Citric acid	60[6]	Sour, lemon juice[6]

References:
[1] *(2)*,
[2] *(3)*,
[3] *(4)*,
[4] *(5)*,
[5] *(6)*,
[6] *(7)*,
[7] *(8)*,
[8] *(9)*,
[9] *(10)*,
[10] *(11)*

A substantial increase in interest for sour beer has emerged during recent decades, as has an enhanced understanding of the contributions from the various microorganisms typically involved *(16)*. This has led to the development of new techniques for sour beer production, with improved process control and shortened production time *(21,22)*. Pre-fermentation with LAB is an example. Pre-fermentation can be carried out at different stages prior to yeast addition. In 'sour mashing' it occurs in the mashing kettle, in 'kettle souring' in the brewing kettle and in the 'sour wort' method the pre-fermentation occurs after the wort has been transferred from the brew kettle. LAB, e.g. a *Lactobacillus* strain, is pitched in unhopped wort, and when the desired level of acidity is reached, the LAB can be stopped by boiling the wort before brewers' yeast is added and ethanol fermentation can transpire *(22)*. *Lactobacilli* are useful in pre-fermentations owing to their rapid lactic acid production and low yield of flavour-potent metabolites associated with unwanted sensory properties. Most strains are sensitive towards ethanol and antimicrobial hop components present in beer *(23)*. By having LAB fermentation before yeast, and by adding hops during the boil between LAB and yeast fermentations, the ethanol and hop hurdles are circumvented, and sour beer can be produced in a highly controlled and swift manner. A drawback to this method is the lack of flavour complexity typically found in beer resulting from mixed fermentations. The contribution by *Lactobacillus* beyond acid production is assumed to be limited *(21)*.

LAB has the ability to produce organoleptically active metabolites besides lactic acid in barley malt-based beverages. These metabolites include other organic acids such as acetic acid *(24)* and formic acid *(25)*, esters such as ethyl acetate *(26)* and a wide range of higher alcohols, aldehydes, ketones, phenolic and heterocyclic compounds. This aspect has been extensively reviewed with respect to fermented malt-based beverages *(27)*. However, most of the research is focused on non-alcoholic, probiotic drinks, and not on the contribution of lactic acid bacteria in sour beer. Even though pre-fermentation with LAB is a commercially utilised technique for sour beer production *(22)*, little research on this method can be found within the scientific literature. The sensory contribution from *Lactobacillus* in beer produced through this two-step fermentation process seems to be a partly unresolved issue. Here we present the contribution from *L. buchneri* CD034 to the organoleptic character of sour beer produced through a two-step fermentation where the LAB precedes yeast. Furthermore, we identify the metabolic compounds constituting this contribution and assess how these affect the sensory properties of the beer.

Materials and methods

Wort preparation

Wort was produced using a 60 L PRO pilot-scale brewery with separate brew kettle and lauter tun delivered by CoEnCo (Oostkamp, Belgium, 2014). Pilsner malt (66.6%, BestMalz, Germany) and wheat malt (33.3%, Weyermann, Germany) were crushed, mixed with water at a rate of 1:4 (w/v) and mashed according to the following scheme: step 1, 45 min at 65°C; step 2, 15 min at 72°C; step 3, 2 min at 78°C. The mash was transferred to the lauter tun where liquid was recirculated for 10 min, before the wort was separated from the spent grain. The spent grain was sparged with water (76°C) until a specific gravity of 1.032 was obtained in the wort. A short boiling step (15 min) yielded unhopped wort serving as a base in the brewing experiment, for propagation and preparation

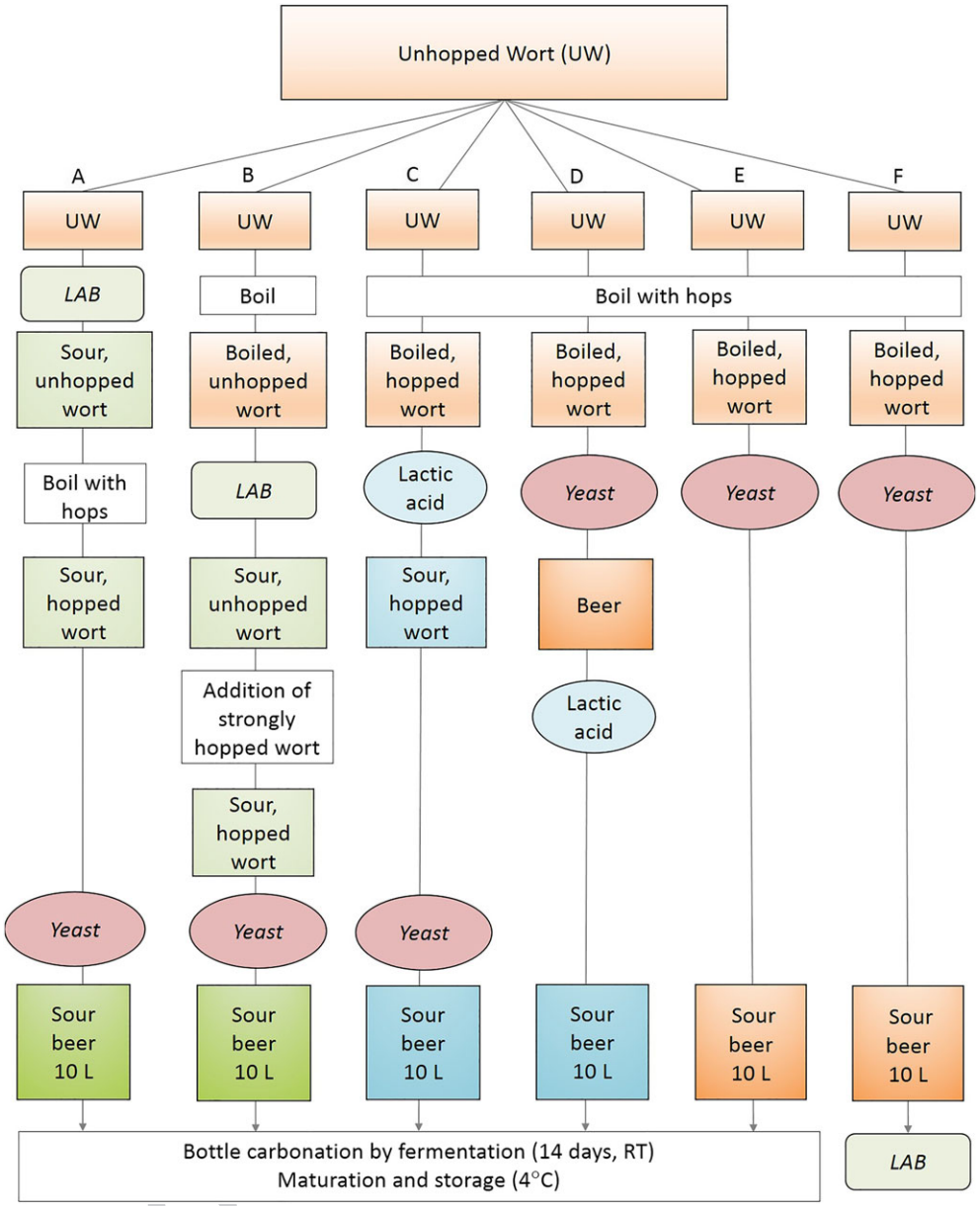

Figure 1. Experimental setup for production of beers A–F. UW, Unhopped wort; LAB, lactic acid bacteria; and RT, room temperature. [Colour figure can be viewed at wileyonlinelibrary.com]

of the LAB starter culture and in the microbial growth experiment. Hopped wort was prepared by boiling the unhopped wort with 1 g/L hop pellets (Fuggles, 4% α acids) for 45 min, and highly hopped wort was prepared in the same manner but at 10 times the hopping rate (10 g/L). A specific gravity of 1.036 (9°P) was obtained in the unhopped, hopped and highly hopped worts by adjustments with water.

Preparation of starter cultures

The LAB strain, *L. buchneri* CD034 used in this experiment was originally isolated from silage grass *(28)*. The culture was kindly donated by the Department of Biotechnology at the University of Natural Resources and Life Sciences, Vienna, Austria. The bacteria were propagated in unhopped wort at 30°C in glass bottles (1 L) for 24 h, before the cells were harvested by centrifugation (9000***g***, 10 min). The cell pellet was resuspended in unhopped wort supplemented with glycerol (15%) to yield 10% of the volume of the original culture. The LAB starter culture was stored at −80°C and thawed at 4°C prior to use. Viability was checked after freezing and thawing by Lactobacillus Selection Agar (also known as Rogosa Agar, Becton, Dickinson and Company, Sparks, USA) plate counts. The yeast strain was a commercial strain of *S. cerevisiae*, Safale US-05 purchased from Fermentis (Gabriel Perl, France). Yeast starter culture was prepared by suspending dry yeast 1:10 (w/v) in sterile water, allowing rehydration at room temperature (RT) for 30 min before inoculation.

Beer production

Six portions of wort of approximately 10 L each, A and B unhopped and C–F hopped, were subjected to different downstream

processing steps as illustrated in Fig. 1. Each step is described in the following section.

Portions A and B – acidification by pre-fermentation with LAB. Portion A was inoculated with LAB directly while portion B was inoculated after a second boiling step (45 min). Both portions were inoculated with 10^6 cells/mL. The fermentation was allowed to proceed for 24 h at 18°C. At this point a pH-reduction from about 5.7 to 4.1 had been obtained. When the desired pH was reached, portion A was boiled (45 min) and hopped at a rate of 1 g/L. Portion B was subjected to the addition of highly hopped wort. Boiling of A and addition of highly hopped wort to B were carried out to halt lactic acid fermentation when the desired level of acidity was reached.

Portions C and D – acidification by lactic acid addition. Lactic acid (80% Vinoferm, Beverlo, Belgium) was added to hopped wort portion C to obtain a pH drop corresponding to that resulting from the LAB fermentation in A and B (pH 4.1). Then yeast was added. A corresponding amount of lactic acid (1 mL/L) was added to portion D, but after yeast fermentation.

Portion E – reference. Yeast was added to hopped wort portion E, without acidification.

Portion F – acidification by secondary fermentation with LAB. Corresponding process steps to portion E were carried out for portion F, up until the end of yeast fermentation. After yeast fermentation, beer F was inoculated with LAB (10^6 cells/mL) in an attempt to carry out secondary lactic acid fermentation. The LAB strain was not able to survive in the beer, and as no further development occurred owing to LAB, beer F was taken out of the study.

Beers A–E. The wort portions were inoculated with yeast (3×10^6 cells/mL) and fermentation proceeded for 21 days (18°C) before the beers were bottled with sucrose (5 g/L) and left at room temperature (14 days) to ensure bottle carbonation by fermentation. After bottle carbonation, all beers were kept at 4°C for maturation and storage. The pH in all beers were monitored through the process using a PHM92 lab pH meter (Radiometer, Copenhagen, Denmark). The beer production was done in triplicate. Samples (50 mL) were drawn from all beers throughout the production process and stored at −20°C until metabolic compound analysis. Samples were drawn at the following times: T0, the common wort; T1, at yeast addition; T2, after yeast fermentation; and T3, matured beer (after 3 weeks storage at 4°C). Samples were kept frozen and thawed overnight at 4°C before analysis.

Analysis of metabolites

Headspace gas chromatography–volatile compounds. Volatile compounds in the samples were analysed by headspace gas chromatography (HSGC) according to the method described by Grønnevik *et al. (29)*. The samples were filtrated using grade 602h½ folding filters (pore size <2 μm, Schleicher & Schuell, Dassel, Germany) at 4°C to remove CO_2. The filtrate was then centrifuged (1960***g***, 20 min, 4°C) using a Kubota 2010 centrifuge (Kubota Corporation, Tokyo, Japan) to remove yeast cells before 10.00***g*** of the supernatant was transferred to headspace vials (Machery Nagel, Dueren, Germany). The vials were sealed with Teflon-coated septa with aluminium rings (PFTA/Si septa, Agilent Technologies, Wilmington, DE, USA) and placed in a 7679A automatic headspace sampler connected to a 6890 GC system with flame ionisation detector (Agilent Technologies). The system was operated through Open LAB EZChrom software (version A.04.05, Agilent Technologies). Helium 6.0 (Aga, Norway) was used as the carrier gas at a flow rate of 5.0 mL/min. A headspace bath temperature of 50°C and manifold temperature of 60°C were applied. Samples were mixed (70 shakes/min) during equilibration (45 min) before the application of pressure (10 psi) and injection (0.5 min injection time). Components were separated based on volatility and affinity of the column stationary phase, this on a

Table 2. Attributes (odour, O, texture, taste, T, and flavour, F) and descriptions used in descriptive sensory analysis of the beers

Attribute	Description	Attribute	Description
Odour		*Taste and flavour*	
Total intensity-O	The strength of all odours in the sample	Total intensity-F	The strength of all flavours in the sample
Sour-O	Related to a fresh, balanced odour owing to the presence of organic acids	Sour-F	Related to a fresh, balanced flavour owing to the presence of organic acids
Hoppy-O	Odour of hops	Sweet-T	Related to the basic taste sweet (sucrose)
Malty-O	Odour of malt	Acidic-T	Related to the basic taste acidic (citric acid)
Fruity-O	Odour of fruits (citrus, pineapple, pears, apple and rhubarb)	Bitter-T	Related to the basic taste bitter (caffeine)
Perfume-O	Odour of flowers and perfume	Hoppy-F	Flavour of hops
Yeasty-O	Odour of yeast	Malty-F	Flavour of malt
		Fruity-F	Flavour of fruits (citrus, pineapple, pears, apple and rhubarb)
Texture		Perfume-F	Flavour of flowers and perfume
Fullness	Mechanical textural attribute relating to resistance to flow	Yeast-F	Flavour of yeast
Foaminess	Mechanical textural attribute related to a foaming, sparkling sensation in the mouth	Alcohol-F	Flavour of alcohol, spirits (ethanol)
Astringency	Organoleptic attribute of pure substances or mixtures which produces the astringent sensation	After-F	Flavour which occurs 30 s after elimination of the product

CP-SIL 5CB GC column (Varian, Middelburg, Netherlands) of 25 m × 0.53 m i.d. with film thickness 5 μm. The following GC temperature programme was applied: 35°C for 5 min; increase of 10°C/min until 40°C for 2 min; increase of 30°C/min until 130°C for 4 min; increase of 30°C/min until 160°C for 4 min; increase of 10°C/min until 180°C for 2 min; and increase of 10°C/min until 200°C for 2 min. The volatile components were identified and quantified based on calibration with standard solutions with known concentrations.

High-performance liquid chromatography – organic acids. Organic acids in the samples were analysed by high-performance liquid chromatography (HPLC) according to the method described by Grønnevik *et al. (29)*. A 2.5 mL aliquot of MilliQ water, 200 μL H_2SO_4 (0.5 M) and 8 mL acetonitrile were added to a 1.00 g sample. The blend was mixed (30 rpm, 30 min) in a MultiRS-60 BIOSAN turner (Montebello Diagnostics A/S, Oslo, Norway) followed by centrifugation (15 min, 1470***g***) using a Kubota 2010 centrifuge (Kubota Corporation, Tokyo, Japan). The supernatant was filtered using a 0.2 μm PTFE membrane (Acrodise CR 13 mm Syringe Filter, PALL, UK) into a HPLC vial (VWR, USA). Samples were analysed using an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA), held at 30°C. The column was connected to a 1260 Infinity HPLC instrument (Agilent Technologies, Singapore) with pump, autosampler, column oven, DAD-UV detector and RI-detector. Q5 The system was operated through Openlab CDS software (Agilent Technologies). H_2SO_4 (5 mM, Merck, USA) was used as mobile phase at a flow rate of 0.4 mL/min. The organic acids were identified and quantified based on calibration with standard solutions. The RI-detector was used for detection of acetic acid, and the DAD-UV detector was used for detection of the remaining organic acids.

Beer analysis

Standard beer analysis was carried out using a DMA 4500M density meter, connected to a PBA sampling unit, an Alcolyzer Beer ME module and a CarboQC ME module. The equipment was all operated through Generation M instrument software version V2.42 (all delivered by Anton Paar, Graz, Austria, 2014). Matured beer was sampled directly from bottles. Extract, ethanol, carbon

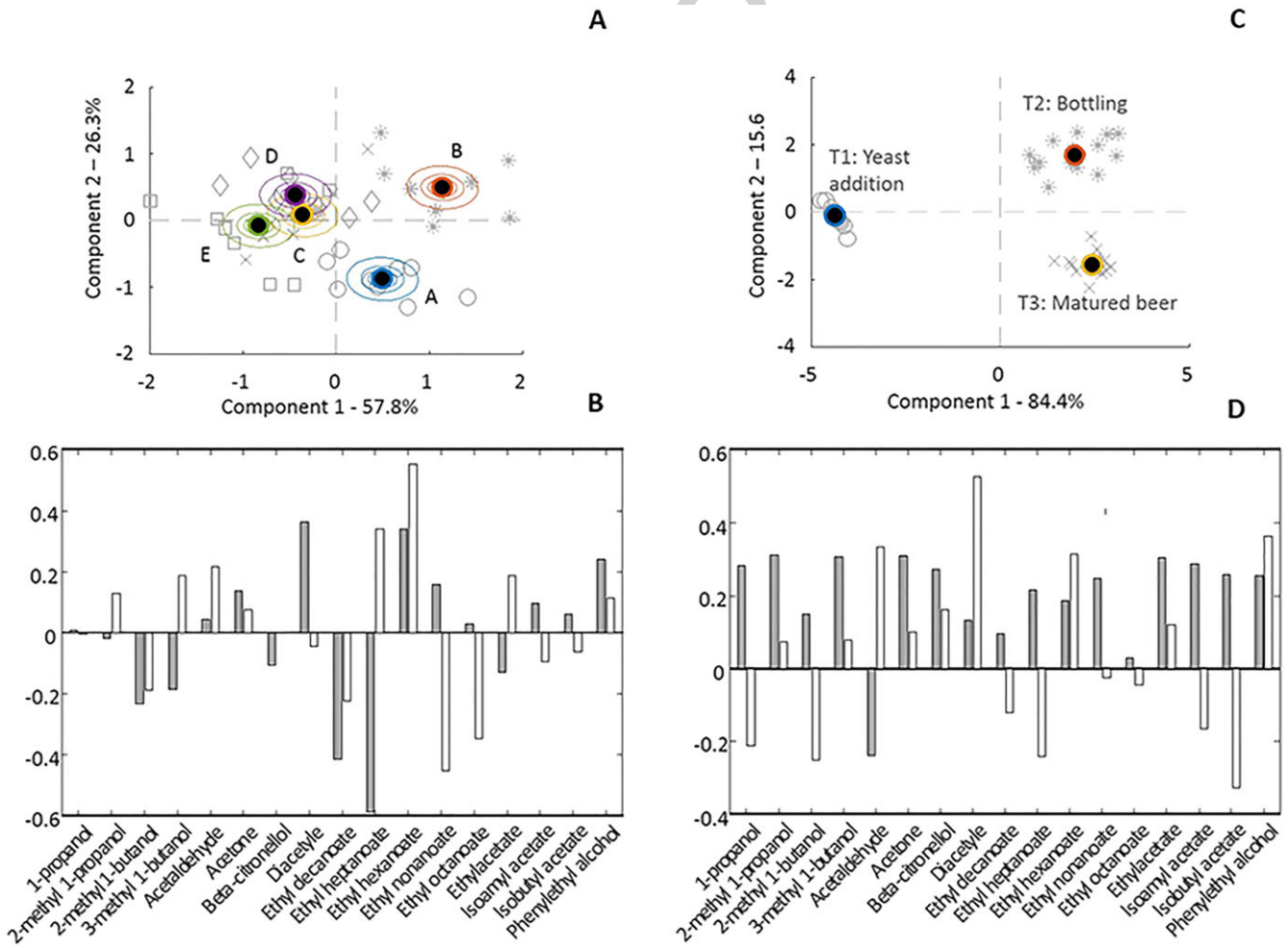

Figure 2. Variation in volatile compounds related to brewing method and time. (a) Variation in samples owing to brewing method (between the five beers A–E) and replicate variation, described by analysis of variance simultaneous component analysis (ASCA) scores. Brewing method explains 5.3% of the variation in volatile compounds. (b) Loading weights for ASCA model in (a). Grey bars show loadings for component 1 (57.8%). White bars show loadings for component 2 (26.3%). (c) Variation in samples owing to time (for sampling points T1, T2 and T3 in the process) and replicate variation, described by ASCA scores. Time explains 68.5% of the variation in volatile compounds. (d) Loadings for ASCA model in (c). Grey bars show loadings for component 1 (84.6%). White bars show loadings for component 2 (15.6%). [Colour figure can be viewed at wileyonlinelibrary.com]

Colour online, B&W in print

dioxide, colour and turbidity were determined. Two bottles of each beer were analysed.

Microbial growth

The entire experimental setup from the beer production (except beer F) was repeated on a small scale to monitor the microbial growth from production to beer bottling. Beers A–E were produced at 400 mL scale. Samples were drawn from beers A and B at LAB addition and after 24 h, that is before the boiling step for beer A, and before the addition of strongly hopped wort to beer B. Further samples were drawn from all beers at yeast addition, after 24 h and 5 7, 14 and 21 days. Plate counts on Man, Rogosa and Sharpe broth (MRS, Merck, Darmstads, Germany) supplemented with 15% agar (VWR Chemicals, Leuven, Belgium) and 25 mg/L cycloheximide (Sigma-Aldrich, St. Louis, USA) were used to monitor LAB growth in beers A and B. The same method was used to verify the absence of LAB in beers C and D/E throughout the fermentation. MRS plates were incubated at 30°C for 3 days. Plate counts on Rose-Bengal Chloramphenicol agar (RBC, Oxoid, Basingstroke, UK) were used to monitor the yeast growth in all beers throughout the fermentation. RBC agar plates were incubated at 30°C for 5 days. The microbial growth experiment was done in triplicate.

Sensory evaluation by trained panel

The professional sensory panel consisted of eight trained assessors at Nofima AS, Norway. The panel was screened for sensory ability (basic tastes, colour vision, odour detection, tactile sensibility) as well as ability to communicate sensory descriptions of products recommended in ISO 8586:2012 in a sensory laboratory designed in accordance with ISO8589 (ISO, 2007). Each assessor evaluated all samples using EyeQuestion for direct recording of data (v4.10.4, Logic8, Holland). A list of attributes was developed based on previous experiments with beer and in a separate brain storming session where the assessors generated relevant words for the selected beer products. Before profiling, one session was used to train the assessors in the definition of 21 selected odour, flavour, taste and texture attributes (Table 2), and agree on the consensus list for the profiling and on the definition of each attribute. T2

Descriptive analysis (DA), as recommended in ISO 13299:2016, was used to evaluate five beers in duplicate. The evaluation of 10 samples in total was conducted in three sessions with a warm-up sample served at the beginning of the first serving. All beers were from two-different batches for the respective beer types. Two bottles of beer (from the same batch) for each replicate were poured into a beaker, avoiding the sediment in the bottles. A 30 cL sample of beer was served in clear plastic glasses, tagged with three-digit random codes and monadically evaluated at individual speed and registered continuously. All samples in a session were placed in the sensory evaluation booths at the same time. Serving temperature was 15 ± 2°C. Each assessor evaluated samples within each session in individual randomised order. The assessors were instructed to take a sip of the beer and rate the intensity of all attributes on a non-structured continuous scale. The endpoints of the scale corresponded to 1 (lowest intensity) and 9 (highest intensity) and the scores were converted to a number between 1 and 9 by the Eye Question software. All samples were expectorated

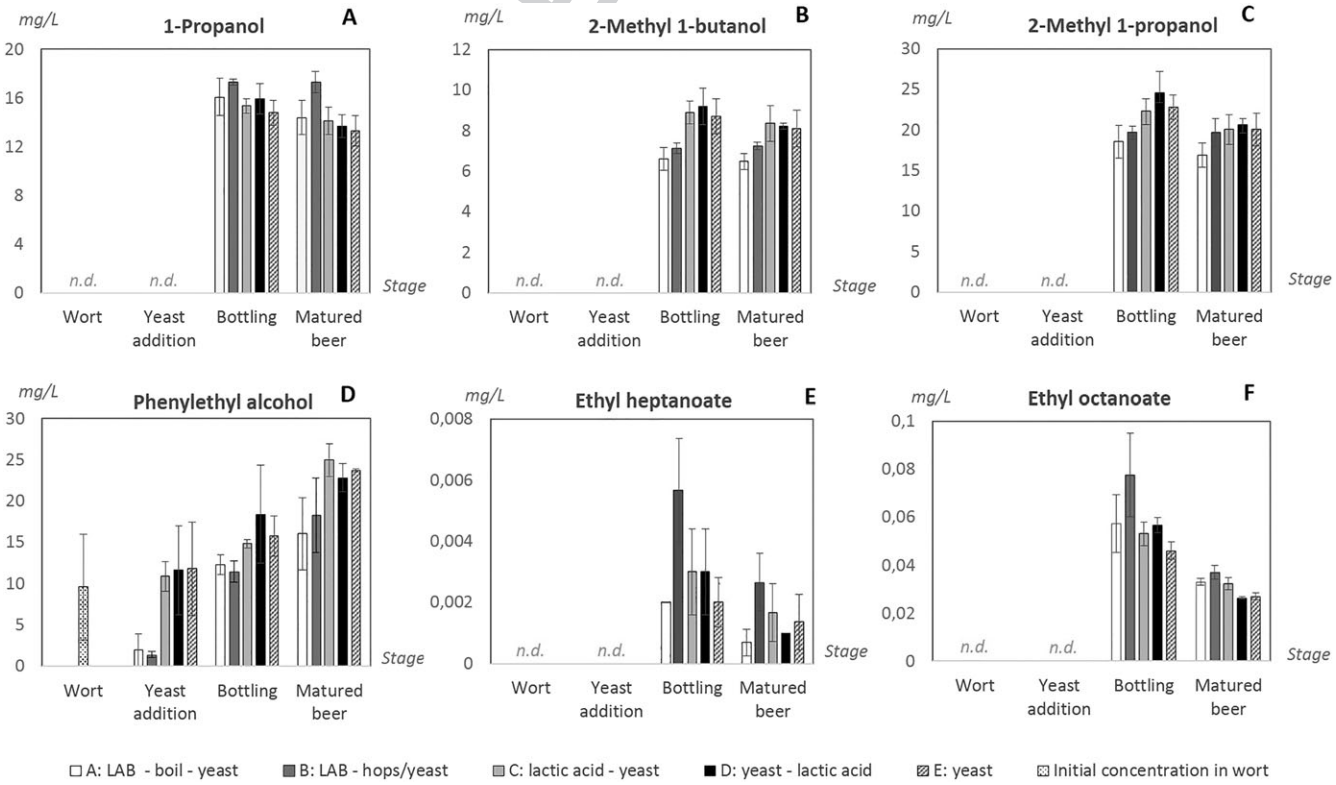

Figure 3. Volatile compounds with different development ($p < 0.01$) related to brewing method. The concentration at the sampling times (T0, wort; T1, yeast addition; T2, bottling; T3, matured beer) for each beer (A–E) is given with standard deviation as error bars. Non-detected values are indicated with 'n.d.' Note that the range in mg/L is different for each volatile compound. (a) 1-Propanol; (b) 2-methyl-1 butanol; (c) 2-methyl 1-propanol; (d) phenylethyl alcohol: (e) ethyl heptanoate; and (f) ethyl octanoate.

and unsalted crackers and warm and cold water for rinsing were available. Table 2 shows the list of sensory attributes. EyeOpenR (v4.10.4, Logic8, Holland) was used to analyse the data in an ANOVA combined with Tukey's test for pairwise differences. Significantly different attributes ($p < 0.05$) were selected based on the ANOVA with Tukey's test and analysed further by principal component analysis (PCA) using PanelCheck V1.4.2 (Norway).

Statistical analysis

Analysis of variance simultaneous component analysis (ASCA) *(30)* was used to examine differences in the volatile compounds and organic acids related to 'brewing method' and 'time'. Separate ASCA models were created for volatile compounds and organic acids using MATLAB (2017, The Mathworks, Natick, MA). ASCA is a multivariate ANOVA combined with compression. This means that variation owing to the design variables is first summarised across all measured properties, and the associated explained variances are calculated. Then each block associated with a design variable is analysed using PCA and visualised as two-dimensional score plots and loading plots (bar plots). The former shows how the samples are grouped according to the design, while the latter shows how the beer properties are affected by the design. In the ASCA score plots one can display the uncertainty of the effect level means, similar to Tukey's test in ANOVA, using confidence ellipsoids *(31)*. In the current experiment, the design variables were 'brewing method' with five levels (brewing methods A–E) and 'time' with four levels (T0, wort; T1, yeast addition; T2, bottling; and T3, matured beer). All five levels of the 'brewing method' design variable were incorporated into the ASCA models. As the primary focus of the current experiments was differences related to microbial metabolism, level 'T0, wort' of the time variable was used as baseline and subtracted from all other time points. ANOVA was

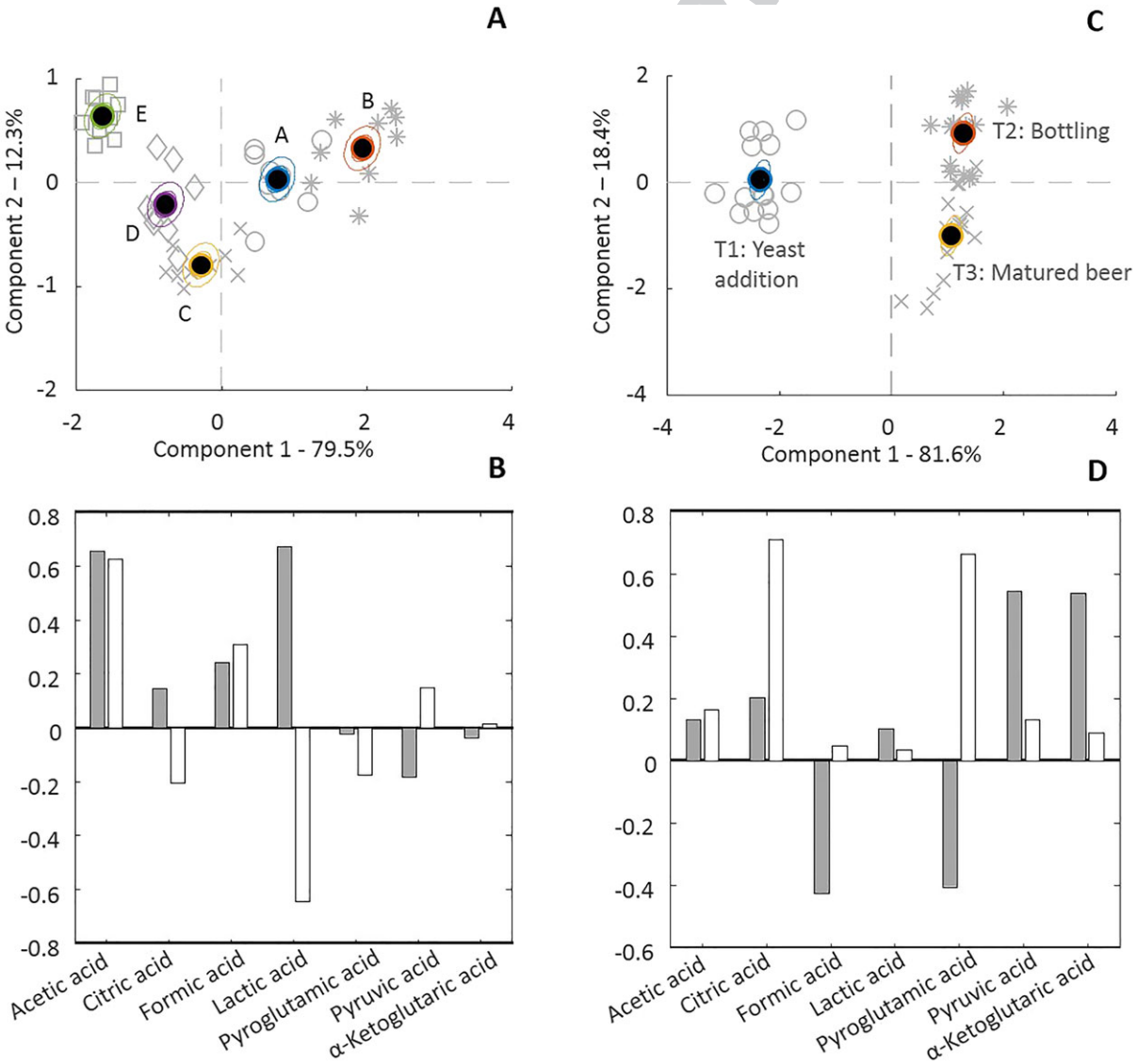

Figure 4. Variation in organic acids related to brewing method and time. (a) Variation in samples owing to brewing method (between the five beers A–E) and replicate variation, described by ASCA scores. Brewing method explains 28.4% of the variation in organic acids. (b) Loadings for ASCA model in (a). Grey bars show loadings for component 1 (79.5%) and white bars show loadings for component 2 (12.3%). (c) Variation in samples owing to time (for sampling points T1, T2 and T3 in the process) and replicate variation, described by ASCA scores. Time explains 48.3% of the variation in organic acids. (d) Loadings for ASCA model in (c). Grey bars show loadings for component 1 (81.6%) and white bars show loadings for component 2 (18.4%) [Colour figure can be viewed at wileyonlinelibrary.com]

used to estimate to what degree the variation in each volatile compound or organic acid was associated with the design variables 'brewing method' or 'time'. The ANOVA was combined with Tukey's test for honestly significant differences. This was done to obtain groups of effect levels, e.g. groups of beers, which are not significantly different with respect to a chosen measured property. The ANOVA with Tukey's test was carried out using R 3.5.0 (R Core Team 2018, Austria, Vienna), and the statistical significance level was set at $p < 0.01$.

Results and discussion

Metabolic compounds

F2 The ASCA score plot in Fig. 2a displays variation in volatile compounds that can be attributed to brewing method. The brewing method factor explain 5.3% of the variation in the volatile compounds in beers A–E. The five beers are grouped in three significantly different groups in the ASCA model: beer A, beer B and beers C–E. Beers C–E are not significantly different from each other based on volatiles, suggesting no substantial influence from the presence of lactic acid alone during yeast fermentation. The C–E group is separated from beers A and B in component 1 in the model, where beer B is furthest away from the C–E group. Component 1 explains 57.8% of the brewing method-related variation in volatiles. As can be seen from the loadings plot in Fig. 2b, the most important drivers of this component are diacetyl, ethyl hexanoate, ethyl decanoate and ethyl heptanoate. The separation of beers A and B from C–E in the ASCA model in Fig. 2a indicates that pre-fermentation with LAB affects the volatile composition of beer. Beers A and B are separated from each other along component 2, explaining 26.3% of the brewing method related variation. The most important compounds driving this component are ethyl heptanoate, ethyl hexanoate, ethyl nonanoate and ethyl octanoate. The ASCA score plot in Fig. 2c displays variation attributed to the time factor. The time factor, which encompass variation in the samples at the different process steps, explains 68.5% of the variation in volatiles. A clear separation of the sampling points can be observed, where each sampling forms its own significantly different group. The yeast addition group is separated from the bottling group and the mature beer group in component 1, explaining 84.4% of time related variation. The bottling group and the mature beer group are separated along component 2, explaining 15.6% of the time-related variation. A far greater portion of the variation in volatiles in the sample set is explained by the time factor than the brewing method factor. The changes occurring in the beers during fermentation are greater than the differences attributed to the various brewing methods in this experimental setup. Brewing method is, however, also significant.

Six of 17 measured volatile compounds developed differently at significance level $p < 0.01$ for the different brewing methods, according to the ANOVA with Tukey's test. Four of these were higher alcohols: 1-propanol (Fig. 3a), 2-methyl 1-propanol (Fig. 3b), 2- F3 methyl 1-butanol (Fig. 3c) and phenyl ethyl alcohol (Fig. 3d). Two were esters: ethyl heptanoate (Fig. 3e) and ethyl octanoate (Fig. 3-f). The effect from LAB pre-fermentation was most pronounced on

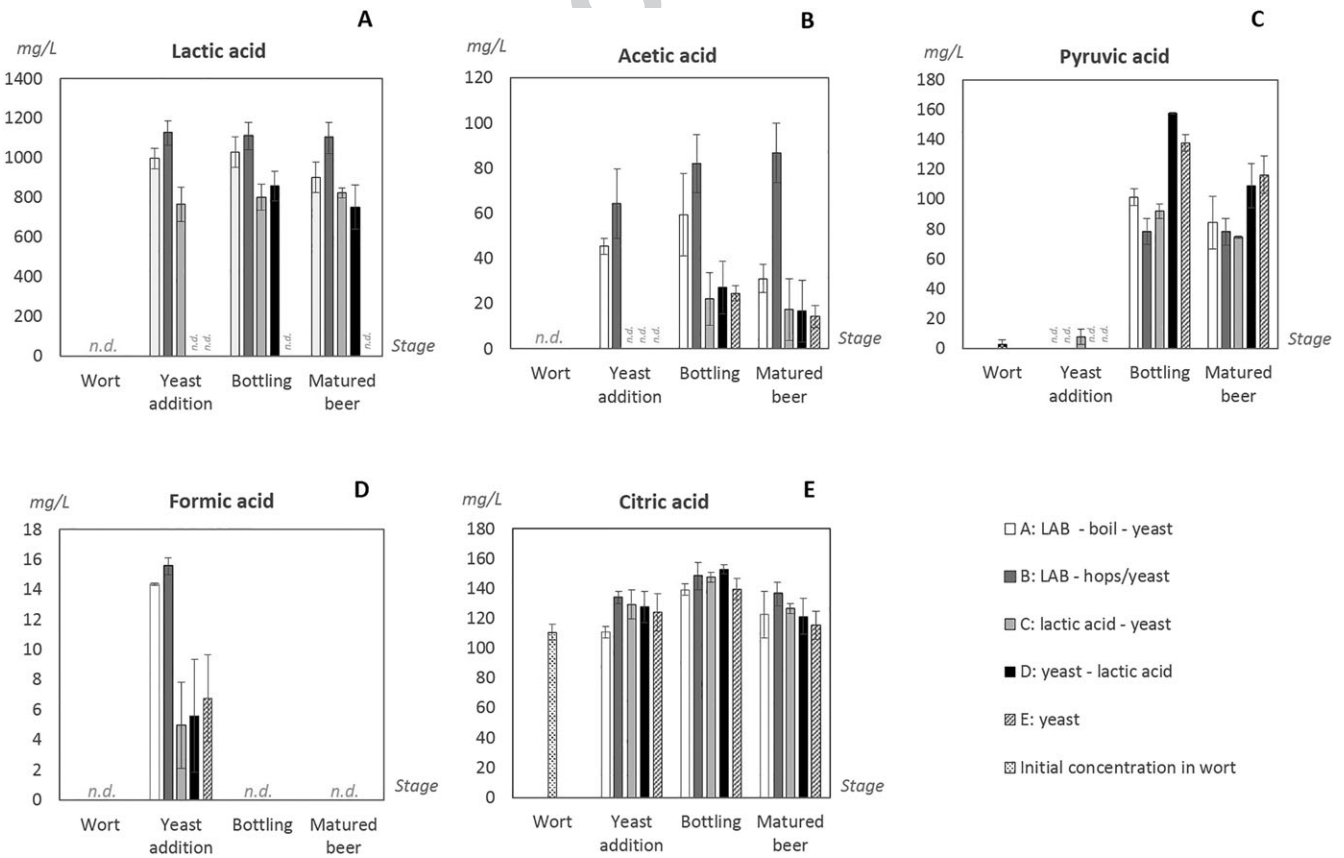

Figure 5. Organic acids with different development ($p < 0.01$) related to brewing method. The concentration at the sampling times (T0, wort; T1, yeast addition; T2, bottling; T3, matured beer) for each beer (A–E) is given with standard deviation as error bars. Non-detected values are indicated with 'n.d.' Note that the range in mg/L is different for each organic acid. (a) Lactic acid; (b) acetic acid; (c) pyruvic acid; (d) formic acid; and (e) citric acid.

2-methyl-1-butanol, 2-methyl-1-propanol and phenylethyl alcohol, all lower in beers A and B, compared with beers C–E. The concentrations of ethyl octanoate, associated with apricot and other fruity notes, were slightly higher in beers A–C compared with beers D and E. The higher concentrations in beers A–C point to a stimulating effect from lactic acid on the production of ethyl octanoate during yeast fermentation. The levels of the volatiles were all below reported taste thresholds in beer (Table 1), making it difficult to pinpoint a direct sensory impact. Their presence could, however, influence overall sensory perception, as multiple compounds below threshold can influence flavour through synergistic effects *(32)*. 2-Methyl-1-butanol can contribute alcoholic and malty notes, 2-methyl-1-propanol is associated with fruity and winey notes and phenylethyl alcohol is linked to rosy and honey flavours.

F4 The ASCA score plot in Fig. 4a displays the variation in organic acids attributed to brewing method. The brewing method factor explains 28.4% of the variation in organic acids in beers A–E. Each of the five beers forms its own group, significantly different from all other beers. Component 1 in the model explains 79.5% of the brewing method-related variation in organic acids. Beers C–E are located on the same side in component 1, opposite to beers A and B, suggesting an effect from pre-fermentation with LAB on the organic acid composition of beer. Beers E and B are furthest apart along component 1, primarily driven by acetic and lactic acid (Fig. 4B). Component 2 explains 12.3% of brewing method-related variation. Beers E and C are most different in component 2, for which acetic and lactic acid are also the most important drivers (Fig. 4b). The ASCA score plot in Fig. 4c displays the variation in organic acids attributed to the time factor. Time explains 49.3% of the variation in organic acids, and clear separation of the sampling points is apparent. The yeast addition group is separated from the bottling group and the mature beer group in component 1, explaining 81.6% of the variation. The bottling group and the mature beer group are separated along component 2, explaining 18.4% of the time-related variation in the model.

Five of seven measured organic acids developed differently owing to the brewing method, according to the ANOVA with Tukey's test at significance level $p > 0.01$. These were lactic acid (Fig. 5a), F5 acetic acid (Fig. 5b), pyruvic acid (Fig. 5c), formic acid (Fig. 5d) and citric acid (Fig. 5e). Lactic acid (about 1000 mg/L), acetic acid (about 50 mg/L) and formic acid (about 15 mg/L) were produced during LAB fermentation in beers A and B, and the effect of LAB pre-fermentation was most pronounced on these acids. Beers A and B contained higher levels of lactic acid and acetic acid at the yeast addition, bottling and matured beer stages compared with beers C–E. Lactic acid was well above the reported taste threshold, while acetic acid was below in both A and B. Both of these acids are associated with acidic taste. The presence of lactic acid seemed to inhibit production of pyruvic acid during yeast fermentation, as beers A–C were lower in pyruvic acid compared with beers D and

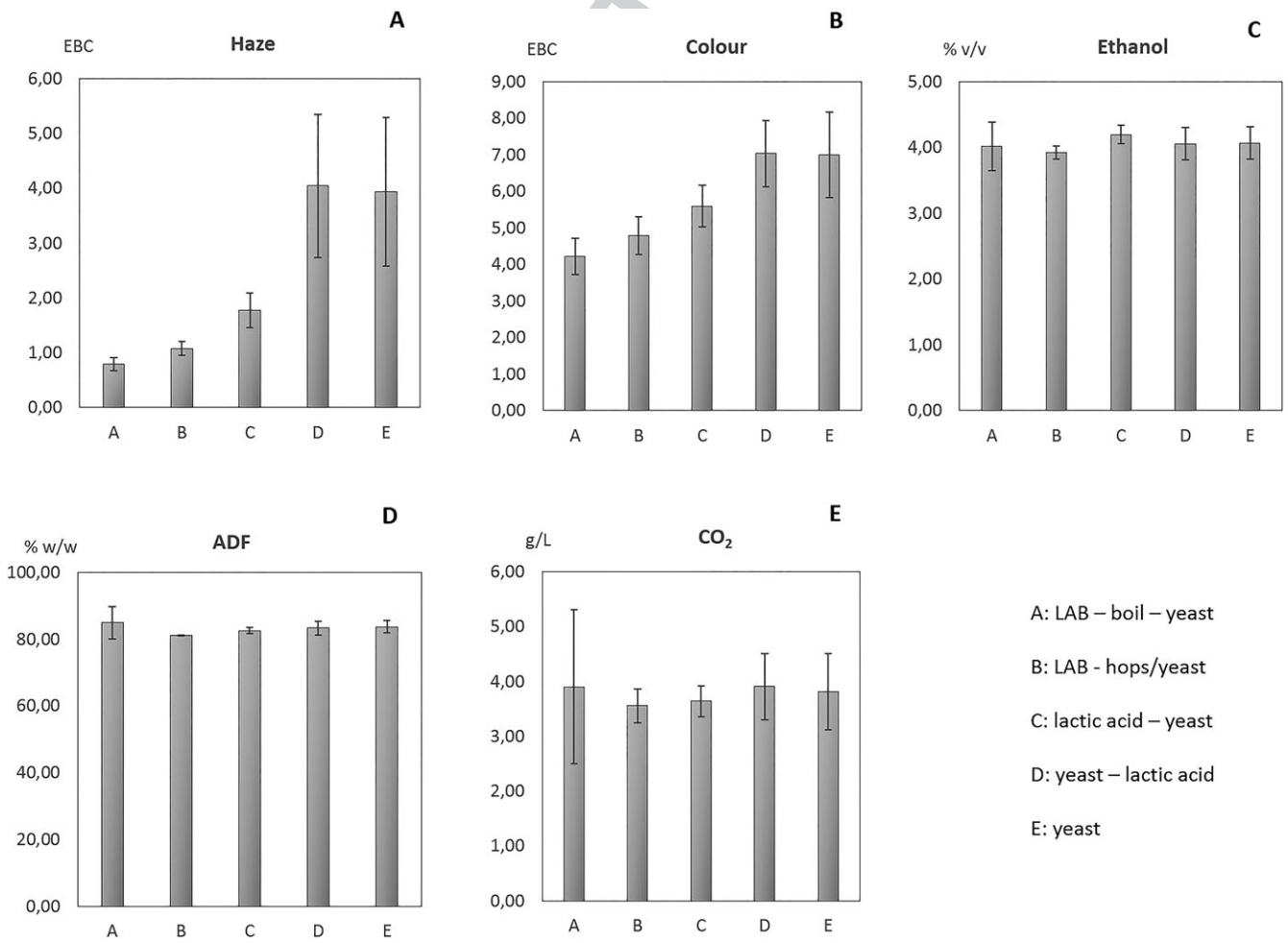

Figure 6. Properties for beers A–E. (a) Haze; (b) colour; (c) ethanol; (d) apparent degree of fermentation (ADF); amd (e) carbon dioxide (CO_2).

Table 3. pH[a] at the various process stages for beers A–E

Beer A		Beer B		Beer C		Beer D		Beer E	
Stage	pH	Stage	pH	Stage	pH	Stage	pH	Stage	pH
LAB addition	5.81 ± 0.02	LAB addition	5.81 ± 0.2	Lactic acid addition	5.80 ± 0.02	Yeast addition	5.80 ± 0.02	Yeast addition	5.82 ± 0.01
Yeast addition/24 h after LAB addition	4.11 ± 0.01	Yeast addition/24 h after LAB addition	4.04 ± 0.02	Yeast addition	4.10 ± 0.02	Lactic acid addition/3 weeks after yeast addition	3.99 ± 0.04	Bottling/3 weeks after yeast addition	4.02 ± 0.03
Bottling/3 weeks after yeast addition	3.68 ± 0.02	Bottling/3 weeks after yeast addition	3.64 ± 0.01	Bottling/3 weeks after yeast addition	3.67 ± 0.01	Bottling/after lactic acid addition	3.51 ± 0.01		
Matured beer	3.66 ± 0.01	Matured beer	3.63 ± 0.01	Matured beer	3.69 ± 0.01	Matured beer	3.52 ± 0.03	Matured beer	4.03 ± 0.02

[a]Average pH values with standard deviations.

E. Pyruvic acid contributes to sour and tart flavours. The level is below the reported taste threshold in all beers, but an impact on the overall flavour cannot be excluded, owing to potential synergistic interactions between multiple subthreshold constituents. Beers D and E were the same for all volatiles and organic acids except lactic acid, suggesting no influence from lactic acid during re-fermentation on the production of metabolic compounds.

Beer B was highest in all organic acids at the matured beer stage, except for pyruvic acid. A pronounced difference was observed for acetic acid, as a 3- to 4-fold higher concentration was measured in beer B compared with the others. This beer also contained the highest concentrations of the volatile esters ethyl heptanoate and ethyl octanoate, both associated with fruity notes. The largest contribution from LAB was conveyed when applying method B, where LAB is present longer. LAB is known to be important for the flavour properties in other fermented alcoholic beverages, such as wine *(33)* and whiskey *(34)*. LAB is also known to be crucial for flavour formation when involved in mixed LAB and yeast fermentations of sour dough. Sensory quality was compromised in bread baked from chemically acidified dough *(35)*, despite the majority of the flavour precursors being present in the flour and the majority of the flavour formation occurring during the baking step *(36)*.

Beer properties

Large differences were observed for the different beers in haze (Fig. 6a). Beers A and B had significantly less haze compared with beer C, and all of these had significantly less haze compared with beers D and E. This points to an impact both from the pre-fermentation with LAB and from the presence of lactic acid during yeast fermentation. The same pattern was observed for colour value (Fig. 6B), with lower levels for beers A–C

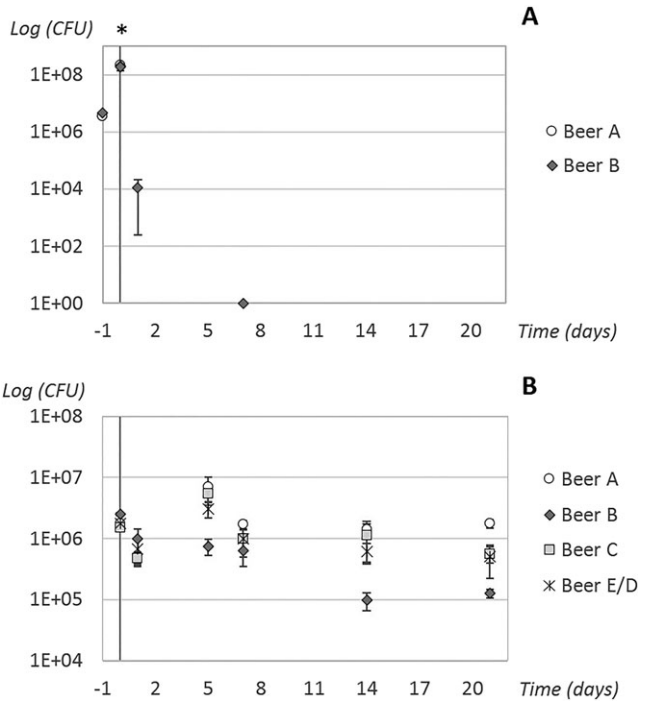

Figure 7. Microbial growth during 3 weeks fermentation of beers A–C and D/E. (a) LAB growth in beers A and B. (b) Yeast growth in beers A–C and D/E. Note that the range of the *y*-axis is 1–108 in (a) and 104–108 in (b). * Beer A, boiling with hops and yeast addition; beer B, addition of strongly hopped wort and yeast.

compared with beers D and E. The major causes of haze in beer are proteins *(37)* and yeast cells *(38)*. Potential explanations for the lower haze values in beers A–C could be more efficient yeast flocculation at lower pH, or fewer protein–polyphenol complexes in solution at lower pH. Proper yeast flocculation is important to obtain clear beer *(39)* and pH affects the flocculation behaviour in yeast *(40)*. Lower pH was associated with increasing flocculation tendencies in a study by Rogers *et al. (41)*. Proteins contribute to haze in beer by forming light-scattering complexes with polyphenols. This complex formation is influenced by pH, and less haze formation is associated with lower pH *(42)*. The lower haze value observed for beers A and B compared with beer C also suggests that LAB fermentation affects the haze in some manner additional to the pH effect alone. None of these aspects has been pursued in the current study, but are subjects for future research.

No significant differences were detected between the beers in ethanol, apparent degree of fermentation (ADF) or CO_2 concentration. The obtained ethanol concentration was ~4% (Fig. 6C), the ADF ~80% (Fig. 6D) and the CO_2 concentration ~4 g/L (Fig. 6E) for all beers. The similar values for ADF in beers A, B and E suggests that LAB does not contribute to higher ADF when used in pre-fermentation. As the levels of CO_2 and ethanol in beers A–C corresponded to that of the reference beer E, the presence of lactic acid and a reduced pH does not seem to influence the yeasts production of these during primary fermentation. The levels in beer D also correspond to reference beer E, suggesting that reduced pH during re-fermentation in bottles does not affect the yeast's ability to produce CO_2. The conditions in this study did not result in 'terminal acidic shock' to the yeast CO_2 production, as reported by Rogers *et al. (41)*. This is probably due to more moderate stressor conditions (ethanol 4% and pH 3.6, as opposed to ethanol 8.4% and pH 3.17 in Rogers *et al. (41)*).

The pH development in beers A–E is given in Table 3. During 24 h of LAB fermentation, the pH of both beers A and B was reduced from 5.8 to about 4. A corresponding pH reduction was obtained for beer C by lactic acid addition. For beers A–C the pH was about 3.7 at both bottling and matured beer stages. The initial wort pH for beers D and E was 5.8. After 3 weeks of yeast fermentation, a pH of about 4.0 was obtained in both. At this point, lactic acid was added to beer D. The final pH at the matured beer stage was 3.5 for beer D and 4.0 for beer E.

Microbial growth

The microbial growth from the small-scale fermentation is displayed in Fig. 7. There was a 10^2-fold increase in colony forming units per volume (CFU/mL) during the 24 h fermentation by LAB (Fig. 7a). LAB were not detected in beer A after boiling and yeast addition. In beer B, the level of LAB was reduced from 10^8 to 10^4 CFU/mL 24 h after the addition of highly hopped wort and yeast, and LAB viability was below 1 CFU/mL at all later sampling times. LAB fermentation was efficiently stopped by boiling in beer A, and a pronounced reduction in the LAB was observed in beer B, 24 h after the addition of highly hopped wort and yeast. The loss of LAB viability in wort was less efficient in method B, but the acid production from the bacteria was very low after addition of highly hopped wort and yeast. In a study by Carvalho *et al. (43)*, where the interactions

Table 4. The average scores for the sensory attributes assessed in the descriptive analysis

Sensory attributes[a]	Beer A	Beer B	Beer C	Beer D	Beer E	*p*-Value
Total odour intensity	5.2 A	5.4 A	5.4 A	5.2 A	5.3 A	0.929
Sour odour	4.0 A	3.5 A	4.0 A	4.1 A	4.4 A	0.284
Hoppy odour	4.1 A	3.8 A	4.2 A	4.2 A	4.2 A	0.737
Malty odour	2.7 A	2.8 A	2.8 A	3.2 A	2.7 A	0.810
Fruity odour	3.5 A	2.6 A	3.0 A	2.6 A	3.6 A	0.054
Perfume odour	2.1 A	1.7 A	1.8 A	1.9 A	2.0 A	0.968
Yeasty odour	2.9 A	2.6 A	2.6 A	2.9 A	2.2 A	0.230
Total flavour intensity	5.5 A	5.7 A	5.5 A	5.5 A	5.5 A	0.866
Sour flavour	3.5 AB	*3.3 B*	3.9 AB	3.8 AB	**4.4 A**	0.042
Sweet taste	3.2 AB	*2.8 B*	*3.0 B*	*3.2 B*	**3.8 A**	0.003
Acidic taste	**4.4 A**	**4.4 A**	**4.4 A**	**4.3 A**	*3.0 B*	0.01
Bitter taste	4.3 A	4.8 A	4.8 A	4.5 A	5.1 A	0.073
Hoppy flavour	4.7 A	4.4 A	4.8 A	4.3 A	5.0 A	0.136
Malty flavour	*2.4 B*	3.0 AB	2.9 AB	**3.2 A**	**3.2 A**	0.006
Fruity flavour	3.6 A	2.8 A	3.4 A	3.1 A	3.7 A	0.217
Perfumed flavour	1.8 A	1.5 A	1.5 A	2.2 A	1.7 A	0.237
Yeasty flavour	2.6 A	2.9 A	2.6 A	2.8 A	2.6 A	0.860
Alcoholic flavour	3.2 A	3.3 A	3.7 A	3.0 A	3.8 A	0.043
Foaminess	*3.7 B*	*4.5 B*	*4.4 B*	*4.4 B*	**5.7 A**	<0.001
Astringency	4.5 A	4.1 A	4.7 A	4.5 A	4.4 A	0.204
Aftertaste	5.3 A	5.8 A	5.6 A	5.6 A	6.0 A	0.242

[a]The intensity of the attributes were scored on a non-structured continuous scale where the endpoints corresponded to 1 (lowest intensity) and 9 (highest intensity). Significantly different beers according to ANOVA ($p < 0.05$) with Tukey's test are assigned different letters and different groups are highlighted with bold (higher value) and italic (lower value).

between *S. cerevisiae* and *Lactococcus lactic* during fermentation of sugar cane were studied, reduction in LAB growth was observed as a response to co-fermentation with yeast. Competition for nutrients was proposed as an explanation, along with inhibitory effects from produced ethanol. In a study by Dongmo *et al. (44)* on the growth of six LAB strains in barley malt wort, they observed a rapid decrease in CFU/mL after a 24 h exponential growth phase for some strains. Dongmo *et al. (44)* concluded that depletion of key amino acids coupled with low buffering capacity was the limiting factor for LAB growth in wort. It is unknown whether the loss of LAB viability in beer B in the current study was caused by depletion of key amino acids, competition for nutrients, dropping pH, increasing ethanol or the introduction of hops. In all likelihood, all of these factors influenced the LAB growth. The failure of beer F in the experimental brewing setup indicates that the LAB strain used (*L. buchneri* CD034) was vulnerable to beer stressors and unsuited for secondary fermentation of beer. A controlled secondary fermentation with LAB diverges from the traditional spontaneous fermentation by which sour lambics are usually produced, but the fermentation conditions for LAB would be closer to those in a spontaneous process. Further research with other LAB strains more robust to harsh beer conditions is necessary to explore this subject.

The yeast growth patterns (Fig. 7b) in the experiment were similar for all beers except for beer B. A slight decrease in CFU/mL was observed for all beers 24 h after yeast addition. An ~10-fold increase in CFU/mL was observed 4 days later for beers A, C and D/E. One week after yeast addition, the CFU/mL was back to the original pitching level and the number of yeast cells was stable after this for beers A, C and D/E. Reduced pH and the presence of lactic acid in beers A and C did not seem to affect the growth kinetics of the yeast. Rogers *et al. (41)* found that the effect of pH and the presence of lactic acid on yeast growth was highly dependent on yeast strain. The yeast strain used in the current study (Safale US-05) is seemingly robust towards reduced pH and/or increased lactic acid concentrations. The growth pattern for beer B diverged from the others, as no real increase in CFU/mL was observed, and about a 10-fold lower CFU/mL compared with beers A, C and D/E was observed 2 and 3 weeks into the fermentation. This contradicts the findings of Carvalho *et al. (43)*, where yeast growth was seemingly unaffected by LAB presence. In the study by Carvalho *et al. (43)*, the growth was however monitored

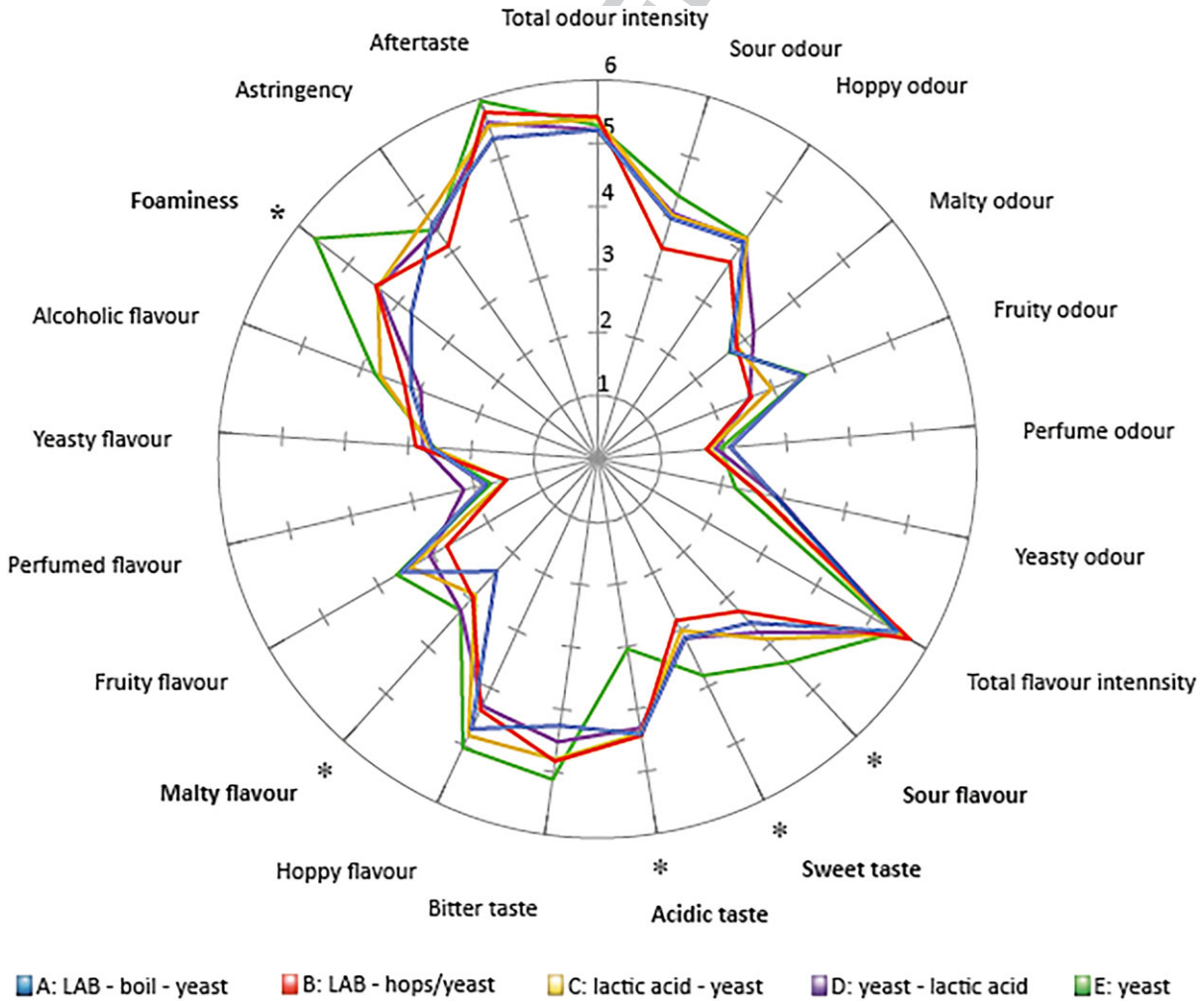

Figure 8. Average scores for sensory attributes in the descriptive analysis. Attributes scored significantly differently ($p > 0.05$) for two or more beers are marked by * and indicated in bold. The intensity of the attributes was scored on a non-structured continuous scale where the endpoints corresponded to 1 (lowest intensity) and 9 (highest intensity) [Colour figure can be viewed at wileyonlinelibrary.com]

during a shorter period. The diverging yeast growth pattern observed for beer B suggests that the presence of viable LAB at yeast addition does influence the yeast growth pattern despite the rapid decline in LAB viability after yeast addition.

Descriptive sensory analysis

Sensory averages obtained in the descriptive sensory analysis are T4 given in Table 4, and spider plot profiles based on these are F8 displayed in Fig. 8. Five of 21 assessed attributes scored significantly differently for two or more beers. Four of these were related to the reference beer E compared with other beers. Beer E scored highest in sweet taste, significantly higher than beers B–D. The concentration of residual sugars in the beers was not measured in the current experiment, but the ADF was similar for all beers, suggesting that the same amount of extract had been utilised. Beer E was higher in pH compared with all other beers, and lower in most organic acids. This is not necessarily tantamount to higher sweetness, but it could contribute to amplification of the sensory impact from the sugars present. Beer E was significantly lower in acidic taste compared with all other beers, in accordance with expectations, as beer E was the only beer without LAB pre-fermentation or acid addition. Beer E scored highest in 'sour flavour', significantly higher than beer B. 'Sour flavour' is often associated with organic acids, but should not be confused with acidic taste. 'Sour flavour' is a highly complex sensory property related to both freshness and sour–sweet balance. The different composition of organic acids in E could explain the difference in perceived level of 'sour flavour'. If higher 'sour flavour' is desired, modifications to the currently explored brewing methods could be necessary. A more optimal composition of organic acids could potentially be achieved by using a different LAB strain *(45)*, changing the fermentation conditions *(46)*, expanding the LAB fermentation period, using multiple LAB strains for the pre-fermentation or using a different LAB and yeast combination for the production *(47,48)*.

Beer E scored significantly higher than all other beers in sensory foaminess. Investigations of the effect of LAB or lactic acid on the foaming properties of beer were not part of the objective of the current study, but the higher sensory foaminess for beer E is noteworthy. The presence of CO_2 is important for the foaminess of beer, but no differences were observed for the beers with respect to CO_2 levels. Protein content is also important for foaming properties. The effect of pH on the foaming properties of beer is highly complex and dependent on the nature of the polypeptides present *(49)*. We speculate that the lower scores in sensory foaminess were due to some foam-stabilising proteins present behaving differently as a response to lowered pH in beers A–D. This is a subject for further research. Beer A received the lowest score in 'malty flavour', significantly lower compared with beers D and E. 2-Methyl 1-propanol is associated with malty flavour, and the significantly lower concentration in beer A compared with beer E and D corresponds well with the sensory difference between the beers. 'Malty flavour' was the only attribute for which a biologically acidified (beer A) and a chemically acidified (beer D) beer received significantly different scores in this study.

A PCA bi-plot based on the sensory attributes scored significantly different for two or more beers is displayed in Fig. 9, with F9 sensory attributes as scores and beers as loadings. Beer E is separated from beers A–D along PC1, explaining 86.3% of the variation. Beer E is positively correlated with foaminess, sweet taste and sour flavour, and negatively correlated with acidic taste in this component. Beers A–D are negatively correlated with foaminess, sweet taste and sour flavour and positively correlated with acidic taste.

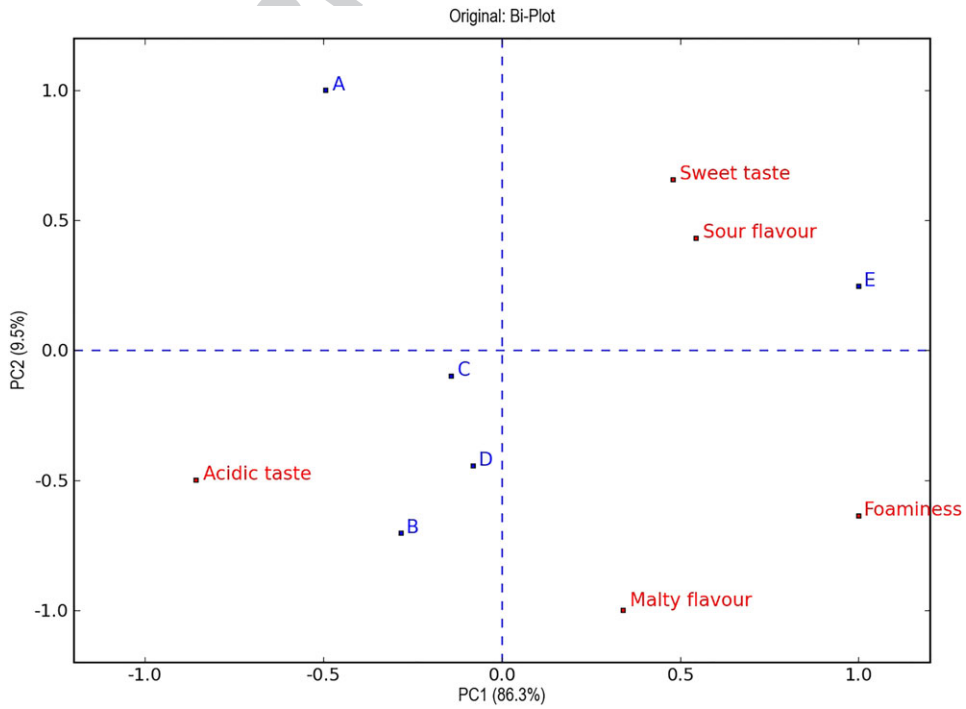

Figure 9. PCA bi-plot based on attributes with significantly different levels ($p < 0.05$) with beers as loadings and attributes as scores. PC1 explains 86.3% and PC2 explains 9.5% of the variation in the sample set [Colour figure can be viewed at wileyonlinelibrary.com]

Colour online, B&W in print

Beers A and B are located on opposite sides along PC2, explaining 9.5%. Beer A is negatively correlated with malty flavour. As can be seen from the sensory PCA plot (Fig. 9), the majority of the variation in the sensory data is related to the reference beer E being different from the soured beers. The model points to a sensory impact from LAB pre-fermentation (beers A and B), however not surpassing that obtained by chemical acidification (beers C and D). Despite the contribution from LAB going beyond lactic acid production, looking at metabolic compounds, the sensory impact from this seems very slight. A potential drawback to the current study is the final compound analysis being carried out 3 months before the descriptive sensory analyses. However, we consider this to have only marginal effects on stable flavour properties as the beer was kept cold (4°C), dark and still.

Conclusion

Few studies have been conducted on sour beer production in general or on alternative, non-spontaneous production techniques. Osburn *et al. (50)* recently published a paper on an alternative method for sour beer production named 'Primary souring' where alternative yeast strains with high production of organic acids produced acidity during primary fermentation. Besides the work by Osburn *et al. (50)*, there is to the best of our knowledge, no published research looking at alternative approaches to sour beer production. Extensive work has been done on the ability of LAB to ferment wort and produce flavour-active compounds *(26,27,51,52)*. Some research can be found on LAB in beer, but then most frequently with LAB as an unwanted beer spoiler *(53–56)*. The importance of microbial symbiotic coexistence between LAB and yeast for flavour formation in traditional fermented foods has been reviewed *(57)*, and emphasis is placed on the importance of interplay between LAB and yeast for the desired flavour formation. Pre-fermentation with LAB in sour beer production is referred to within the scientific literature *(50)*, but to date no papers have been published where the actual contribution from LAB in this method to the composition and sensory properties of beer is explored. In the current study, sour beer was successfully produced through a two-step fermentation where LAB fermentation preceded yeast. The results suggest that LAB makes a significant contribution beyond the lactic acid production, to the composition of beer. This is both with respect to volatile compounds and organic acids. Biological acidification by LAB pre-fermentation has a significant impact on the sensory properties of beer. This contribution does, however, not seem to exceed the sensory effect obtained by chemical acidification.

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