Norwegian University of Life Sciences
Faculty of Chemistry Biotechnology and Food Science

Philosophiae Doctor (PhD)
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Modern sour beer production: Mixed fermentations with yeast and lactic acid bacteria

Moderne produksjon av surøl: Blanda fermenteringar med gjær og mjølkesyrebakteriar

Anna Dysvik

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Abstract

Traditional sour beers are produced by spontaneous, mixed fermentations where numerous yeast and bacterial species are involved. One of the traits that separate sour beers from other beers is the high concentration of organic acids (e.g. lactic acid) which results in reduced pH and increased acidic taste. Traditional production of sour beer is associated with several issues, including poor process control, lack of consistency in product quality and lengthy fermentation time-frames (1-3 years). The current PhD work is based on the notion that application of pure cultures of key microorganisms, responsible for important features resulting from spontaneous fermentations, can be used to produce sour beer in a rapid and highly controlled manner. The three papers/manuscripts presented in the current thesis explore different approaches for pure culture, mixed fermentations with *Lactobacillus* strains and *Saccharomyces cerevisiae*.

In paper I, pre-fermentation with *L. buchneri* prior to yeast fermentation was tested for production of sour beer. Sour beers (pH 3.5-3.7) with high lactic acid concentrations (~1000 mg/L) were produced, within 3 weeks of fermentation. Although *L. buchneri* made a significant contribution to the metabolite composition of beer, the sensory influence of this did not surpass the influence obtained with chemical acidification. Resistance of three different Lactobacilli (*L. brevis*, *L. plantarum* and *L. buchneri*) to beer-related stress factors were explored in paper II, where co-fermentations with *S. cerevisiae* was evaluated for sour beer production. Sour beers (pH 3.6-3.8) with high lactic acid concentrations (~1800-2600 mg/L) were successfully produced within 3 weeks of fermentation. *L. plantarum* contributed to the sensory properties of beer by causing increased intensity in fruity odour and dried fruit odour, while the *L. brevis* fermented beer was assessed as sensory similar to a commercial sour beer in acidic taste and astringency. The *Lactobacillus* strain displaying the highest robustness towards beer-related stress in paper II (*L. brevis*) was used in paper III to explore secondary LAB fermentation in sour beer production. A specific substrate consisting of xylooligosaccharides (XOS) derived from birch wood was introduced in beer to expedite LAB fermentation. Sour beer was produced (pH 3.3-3.6) within 2-4 weeks containing lactic acid concentration of 1750-3900 mg/L. XOS induced secondary fermentation shifted multiple sensory properties significantly, and the produced XOS sour beer was assessed as sensory similar to a commercial sour beer in dried fruit odour, total flavour intensity, astringency and acidic taste.

Based on the experiments presented in the current thesis, pre-fermentation, co-fermentation and secondary fermentation with LAB and yeast, all seem viable options for rapid production of sour beer with a high level of process control.

Samandrag

Tradisjonelle surøl vert produsert gjennom spontane, blanda fermenteringar der talrike artar av gjær og bakteriar deltek. Høgt innhald av organiske syrer (til dømes mjølkesyre) skil surøl frå andre ølslag, og fører til låg pH og høg sursmak. Fleire problem er knytt til tradisjonell produksjon av surøl, blant anna dårleg prosesskontroll, ujamn produktkvalitet og svært langvarige fermenteringar (1-3 år). Arbeidet bak denne doktorgradsavhandlinga, spring ut frå ein tanke om at det kan vere mogleg å produsere surøl raskt og med høg prosesskontroll. Dette ved å nytte reinkulturar av nøkkelmikroorganismar som er ansvarlege for viktige eigenskapar ved tradisjonelt surøl. Dei tre inkluderte artiklane/manuskripta, utforskar ulike tilnærmingar for blanda fermenteringar med reinkulturar der ulike *Lactobacillus* stammar er nytta saman med *Saccharomyces cerevisiae*.

I artikkel I, vert pre-fermentering med *L. buchneri* før gjæring, nytta for produksjon av surøl. Surøl (pH 3.5-3.7) med høgt mjølkesyreinnhald (~1000 mg/L) vart laga. Fermenteringa tok 3 veker. *L. buchneri* bidrog signifikant til samansetjinga av metabolske produkt og smakseigenskapar, men tilsats av mjølkesyre hadde liknande smakseffektar som fermentering med *L. buchneri*. I artikkel II, vart motstandsevna til tre Lactobacillar (*L. brevis*, *L. plantarum* og *L. buchneri*) testa. Mjølkesyrebakteriane vart utsette for ulike stressfaktorar knytt til øl, og co-fermentering med *S. cerevisiae* vart nytta for produksjon av surøl. Surøl (pH 3.6-3.8) med høgt innhald av mjølkesyre (~1800-2600 mg/L) vart produsert etter 3 veker med fermentering. *L. plantarum* bidrog til ølsmaken ved å auke fruktlukt og lukt av tørka frukt. Ølet produsert ved co-fermentering med *L. brevis* vart vurdert som liknande eit kommersielt surøl i sursmak og astringens. I artikkel III, vart den mest robuste mjølkesyrebakterien (*L. brevis*) nytta for å undersøkje sekundærfermentering med mjølkesyrebakteriar i produksjon av surøl. Xylooligosakkarider (XOS) frå bjørk vart blanda inn i øl for å fremja mjølkesyrefermentering. Etter 2-4 veker var surøl (pH 3.3-3.6) med mjølkesyreinnhald mellom 1750 og 3900 mg/L produsert. Sekundærfermenteringa med XOS førte til endra intensitet i fleire sensoriske eigenskapar, og det produserte XOS surølet vart vurdert som liknande eit kommersielt surøl i lukt av tørka frukt, total smaksintensitet, astringens og sursmak.

Basert på forsøka som er lagt fram i denne avhandlinga, er det mogleg å lage surøl ved å nytte pre-fermentering, co-fermentering og sekundærfermentering med gjær og mjølkesyrebakteriar.

Abbreviations

AAB	Acetic acid bacteria
ACA	American coolship ale
ADF	Apparent degree of fermentation
ADI	Arginine deaminase pathway
BSG	Brewers' spent grain
CFU	Colony forming units
DEFT	Direct epifluorescence technique
DGGE	Denaturing Gradient Gel Electrophoresis
EPS	Exopolysaccharide
GABA	γ-Aminobutyrate
GAD	Glutamate decarboxylase
LAB	Lactic acid bacteria
MALDI-ToF MS	Matrix-assisted lased desorption/ionization time-of-flight spectrometry
OTU	Operational taxonomic unit
Spp.	Species
VNBC	Viable but not culturable
XOS	Xylooligosaccharides
XP1	XOS preparation 1
XP2	XOS preparation 2

List of papers

Paper I. Pre-fermentation with lactic acid bacteria in sour beer production

Dysvik, A., Liland, K. H., Myhrer, K. S., Westereng, B., Elling-Olav Rukke, E. O., De Rouck, G. and Wicklund, T. (2019).. *Journal of the Institute of Brewing, 125*(3), 342-353. https://doi.org/10.1002/jib.569

Paper II. Co-fermentation with *Saccharomyces cerevisiae* with *Lactobacillus* strains tolerant to brewing-related stress factors for controlled and rapid production of sour beer. Manuscript in preparation

Dysvik, A., La Rosa, S. L., Liland, K. H., Myhrer, K. S., Østlie, H. M., De Rouck, G., Rukke, E. O., Westereng, B., and Wicklund, T. 2019. *Manuscript in preparation.*

Paper III. Secondary lactic acid bacteria fermentation with wood-derived xylooligosaccharides as a tool to expedite sour beer production

Dysvik, A., La Rosa, S. L., Buffetto, F., Liland, K. H., Myhrer, K. S., Rukke, E. O., Wicklund, T. and Westereng, B. 2019. *Submitted to the Journal of Agricultural and Food Chemistry. In review.*

Introduction

1. Beer

1.1 Introduction

Beer is a malt-based, alcoholic beverage consumed worldwide. The earliest written records of beer-consumption are dated to 2800 BC, but historians believe beer or beer-like beverages were consumed much earlier. Billions of litres are consumed each year, making beer among the most popularly consumed beverages today. According to the German Beer Purity Law (1516), beer should only contain water, malt, hops and yeast. The purity law (with some modifications) is still applied in some countries (e.g. Germany), but the use of non-malt carbohydrate sources in beer production is applied extensively in others (Pires and Brányik, 2015).

In beer production, malt (usually wheat or barley) is milled and mixed with hot water in a mashing step. During the mashing, enzymes (α-amylase and β-amylase are the most important) degrade starch to fermentable sugars. After the mashing the insoluble fraction, referred to as Brewer's spent grain (BSG) is separated from the resulting sugar-rich liquid, referred to as wort, in a process called lautering. The wort is then boiled with hops, before it is cooled down and inoculated with yeast. A schematic illustration of the brewing process is displayed in figure 1. The most commonly used yeast species for beer fermentation is *Saccharomyces pastorianus*, used for the fermentation of lager beer, followed by *S. cerevisiae*, used in ale fermentation. *S. pastorianus* and *S. cerevisiae* are both referred to as brewer's yeasts, and single-strain cultures are commonly used in beer fermentations. During fermentation, yeast utilizes available sugars, amino acids, and other nutrients in wort, and generates ethanol, carbon dioxide, higher alcohols, esters and other metabolites in the resulting beer (Pires and Brányik, 2015).

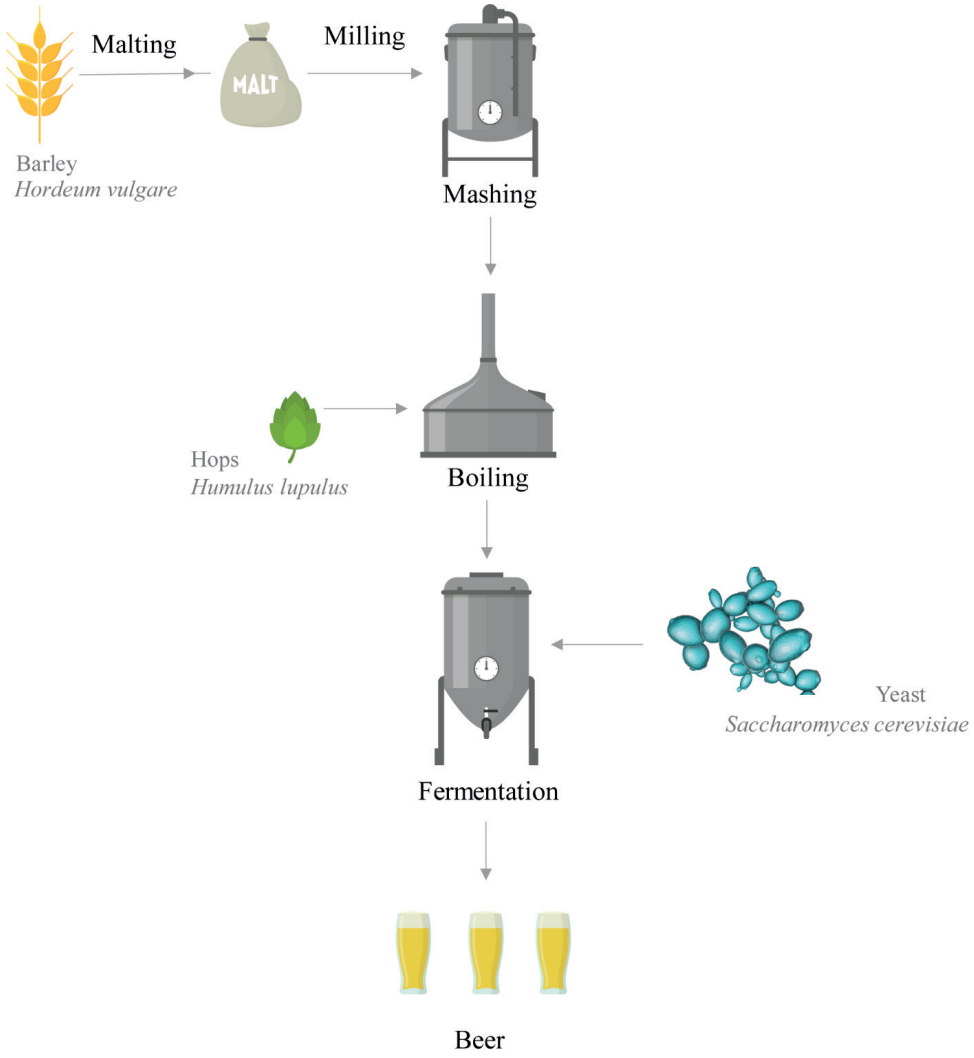

Figure 1. Schematic illustration of the beer production process. Grain is malted, milled and mashed, before wort is separated from Brewer's spent grain and boiled with hops. Yeast is added to chilled wort and ferments the sugary wort to ethanol containing beer.

1.2 Microbiological stability of beer

Different processing steps in beer production reduce the beer's proneness to unwanted microbial growth. Examples of such processing steps include malt acidification, application of high temperatures during mashing, boiling and pasteurisation, in addition to filtrations and application of low temperatures during storage (Vriesekoop et al., 2012). Furthermore, hops containing antimicrobial iso-α acids (typically 17-55 mg/L in beer) also act preserving. By going through a fermentation process, beer typically obtains a number of properties that also protect against microbial spoilage. These include ethanol, typically in the range of 3.5-5 % (can be higher), acidic pH in the range 3.9-4.4, low oxygen content, high carbon dioxide content and low quantities of available nutrients. Collectively, all these factors make beer an inhospitable environment, and reduce the probability of microbial infections (Vriesekoop et al., 2012, Menz et al., 2009). This type of food preservation exemplifies hurdle technology, where multiple factors with relatively mild preserving effects, together produce substantial conservation.

Ethanol in beer provides an antimicrobial hurdle in beer. In 1935, Shimwell (Shimwell, 1935) showed that beers with higher ethanol content were more resistant towards growth of *Lactobacillus brevis,* which was referred to as *Saccharomacillus pastorianus* at that time. Decreasing microbial survival rate with increasing ethanol concentration has been shown for a number food-borne pathogens (Menz et al., 2010). The antimicrobial mode of action of ethanol is through inhibition of cell membrane function (Casey and Ingledew, 1986) and through induction of cell membrane leakage (Eaton et al., 1982). Ethanol induced increase in membrane permeability causes increased influx of protons to the cell cytoplasm, which makes it difficult for bacterial cells to maintain pH homeostasis (Barker and Park, 2001). This is especially important in low pH environments, such as beer. Cell morphology and a variety of cellular functions can be affected by ethanol (Fried and Novick, 1973, Kalathenos and Russell, 2003).

Low **pH** represents an additional hurdle that microorganisms need to circumvent to grow in beer. Beer pH generally ranges between 3.4 and 4.7 depending on beer style, but most beers have a pH ranging from 4.0 and 4.5 (van Leeuwen et al., 2006). Acidic pH causes increased influx of organic acid and acidification of the cytoplasm. This can destroy various enzyme systems and disturb uptake of nutrients and thereby interrupt cellular metabolism in general (Neal et al., 1965). If microorganisms subjected to acidic environments are unable to maintain steady intracellular pH, cell death will follow (Booth and Stratford, 2003). In addition to the direct effect of low pH, acidic environment affects microbial cells synergistically with hop compounds (Simpson and Hammond, 1991, Suzuki, 2011).

When **hops** are added to beer, they introduce various antimicrobial compounds (α-acids, iso-α acids and β-acids). **Iso-α acids** are perhaps the most important, and they exert their antimicrobial effects in a number of different ways (Schurr et al., 2015), and an important mode of action is through their activity as

ionophores (Simpson, 1993). Being week acids, undissociated iso-α acids can cross cell membranes and dissociate intracellularly where the pH is higher (Simpson and Smith, 1992). The release of protons causes the intracellular pH to decrease, which disrupts the transmembrane proton gradient and demolishes the proton motive force. This can inhibit uptake of nutrients (Ye et al., 1994) and disturb cellular metabolism in general by, causing decreased enzyme activity (Simpson and Smith, 1992). Other antimicrobial actions inherent to iso-α acids include induction of membrane leakage (Teuber and Schmalreck, 1973) as well as infliction of oxidative stress in the presence of manganese at low pH (Behr and Vogel, 2009, Behr and Vogel, 2010).

Carbon dioxide is formed during yeast fermentation of beer, and the presence of CO_2 contributes to making it microbiologically stable. The presence of CO_2 lowers pH, which has an antimicrobial effect. Further, the presence of CO_2 can create an anaerobic environment which inhibits the growth of aerobic bacteria that need **oxygen** for their metabolism (Vriesekoop et al., 2012). The conserving effect of CO_2 has been known for long and is utilised in the preservation of a multitude of different food products (Dixon and Kell, 1989, Daniels et al., 1985). CO_2 acts preserving through pH reduction and oxygen displacement, but also due to an inherent antimicrobial effect not yet fully elucidated (King Jr and Nagel, 1967). An inhibitory effect of CO_2 on a number of different metabolic enzymes has been suggested as an important mode of action (King Jr and Nagel, 1975, Damon Swanson and Ogg, 1969), as has disturbance of cell membrane function (Sears and Eisenberg, 1961). Regardless of the mechanism, CO_2 exposure inhibits growth in both gram-positive and gram-negative bacteria (Martin et al., 2003), and higher levels of CO_2 in beer has been associated with reduced growth of beer spoilers (Hammond et al., 1999). During fermentation, yeast will consume the majority of **nutrients**, and the available quantities of carbohydrates and amino acids in most beers are low (Sakamoto and Konings, 2003). Increased nutrient content has been correlated with increased susceptibility to bacterial growth (Fernandez and Simpson, 1995).

Due to the collective effect exerted by the hurdles described above, beer can be considered as relatively stable with respect to microbial growth. There are, however, microorganisms capable of contaminating beer, and causing deterioration in product quality. The presence of microorganisms with beer spoilage potential can cause loss of colloidal stability, ropiness, aroma and taste defects among others (Esmaeili et al., 2015). Lactic acid bacteria (LAB) (Garofalo et al., 2015)(24), acetic acid bacteria (AAB)(VAN VUUREN et al., 1979), *Enterobacteriaceae* (Vuuren et al., 1980), *Zymomonas*, *Pectinatus* spp (Lee et al., 1980), *Megasphaera* spp. (Satokari et al., 1998) are all bacteria associated with beer spoilage. Some yeasts also have beer spoilage potential, these include B*rettanomyces*, *Candida*, *Hanseinaspora*, *Torulaspora*, *Pichia* and *Saccharomyces* (Jespersen and Jakobsen, 1996). It has been a common belief that beer is resistant towards food borne pathogens, and this has been supported by research. Some studies have,

however, suggested that some food borne pathogens (strains of *Eschericia coli* and *Bacillus cereus*) are able to survive in beer (Kim et al., 2014), and that attention should be paid to this. In the context of sour beers, microbes with "beer spoilage potential" can be viewed in a different light, as the involvement of microbes beyond conventional brewer's yeast is essential in sour beer production.

2. Sour beer

2.1 Introduction

Sour beer is a highly diverse genre of beer, not restricted to one specific definition based on production process, raw material or geographic origin. A common denominator for sour beer is higher concentrations of organic acid and reduced pH (pH 3.0-3.9) compared to "regular beers", and elevated intensity of corresponding sensory attributes such as acidic taste (Tonsmeire, 2014). Generally, the elevated levels of organic acids in sour beer originate from the involvement of acid producing bacteria in the fermentation process. While the fermentation of regular beer is generally limited to single strain yeast fermentations, sour beer is typically a product of mixed fermentation by both yeasts and bacteria (Van Oevelen et al., 1977). Belgian brewing culture is famous for its sour beer traditions, and classic sour beer styles of Belgian origin include Lambic, Geuze, Kriek, Flanders Red Ale and Old Brown. Berliner Weisse and Gose are examples of sour beer styles of German origin (Bossaert et al., 2019). American Coolship Ale is a product from the American craft beer culture, but with a production process heavily inspired by the Belgian classic styles (Bokulich et al., 2012).

2.2 Traditional sour beer products

Lambic beers are produced through spontaneous mixed fermentations where no active inoculation of microbial starter cultures is carried out. After the wort is produced and boiled, it is transferred hot to open vessels (coolship) and left to cool down, completely open to the air, typically overnight (Van Oevelen et al., 1977). This exposure is assumed to facilitate inoculation by environmental microorganisms present in air in the brewhouse (Martens et al., 1991, Verachtert et al., 1995, De Roos et al., 2018). In order to ensure that the cooldown occurs within a reasonable amount of time, and as a means for some level of control of the involved microbes, the traditional Lambic brewing is only carried out during winter months (Van Oevelen et al., 1977, Verachtert and Iserentant, 1995). After the wort has reached the suitable temperature of approximately 20°C, the wort is transferred to wooden barrels for fermentation and maturation (Van Oevelen et al., 1977). Some microbial inoculation may as well occur from the barrels, which potentially host a large number of microbes that are in a dormant stage in microcavities in the wood surface (De Roos et al., 2019, Malfeito-Ferreira, 2018). After transfer to wooden barrels, a microbial succession takes place consisting of four microbial phases. The first phase is the enterobacteria phase, where enterobacteria dominate. Enterobacterial domination can prevail for a week (De Roos et al., 2018) or up to a month (Van Oevelen et al., 1977, Spitaels et al., 2014). The following phase is the main fermentation phase, where *Saccharomyces* spp. dominate for 3-4 months, followed by an acidification phase dominated by LAB and AAB. The final phase is the maturation phase, where *Brettanomyces* yeast and LAB dominate, usually from ~8 months and onward (Van Oevelen et al., 1977). Lambic is the base beer for a variety of different beer styles. **Geuze** (also referred to as Gueuze) is a highly carbonated beer that is made by mixing young (1

year) and old (2 years or more) Lambic and allowing refermentation in bottles. **Faro** is made by mixing old Lambic with ale and sugar (Verachtert and Derdelinckx, 2014). **Kriek** is a fruit Lambic, made by mixing sour cherries with a young Lambic, and allowing a second round of fermentation on the fruit sugars (De Keersmaecker, 1996). Raspberries can also be used in the same way in Lambic beer referred to as **Framboise** (Verachtert and Derdelinckx, 2014). A schematic illustration of the Lambic production process is displayed in figure 2.

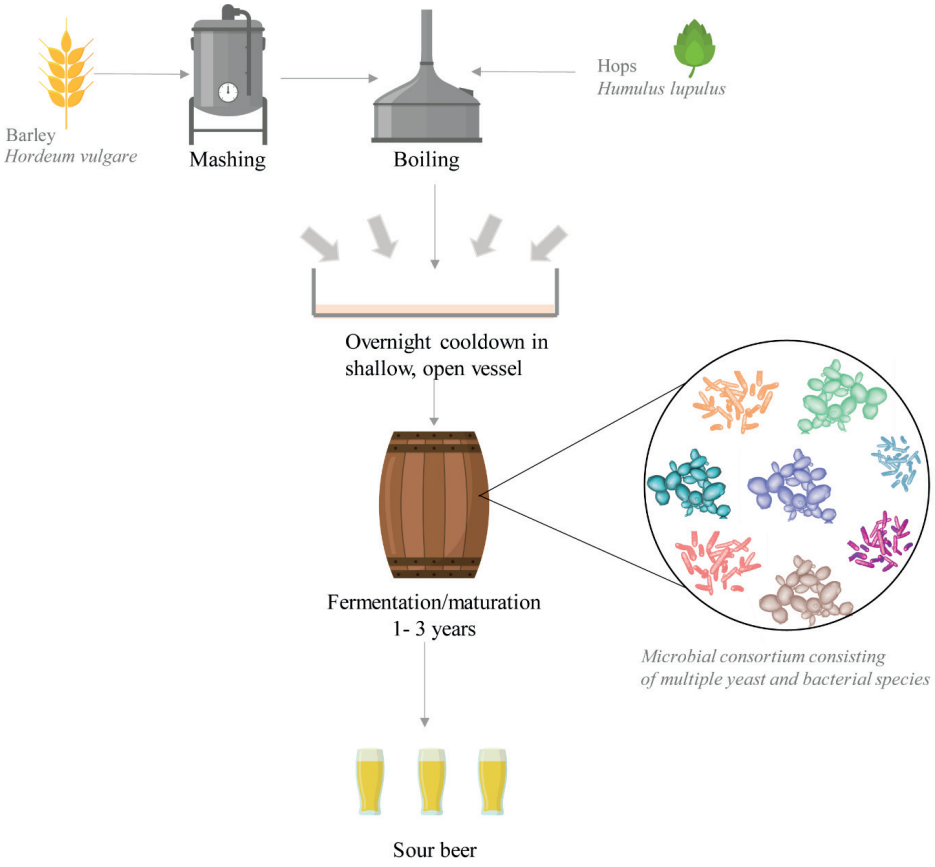

Figure 2. Schematic illustration of the Lambic beer production process. Active inoculation of wort is not carried out. The boiled wort is cooled down in a shallow, open vessel (coolship), where it is spontaneously inoculated by being exposed to the environment. The wort is transferred to wooden casks where mixed fermentation by a variety of yeasts and bacteria can transpire.

American coolship ales (ACA) is a product of the American craft brewing culture, heavily inspired by the traditional Belgian production method for Lambic beers. In ACA production, wort is cooled down in open, shallow cooling vessels to favour spontaneous inoculation by the environment before transfer to wooden barrels. The microbial succession is similar to that of Belgian Lambic, although some differences can be found (Bokulich et al., 2012).

Flanders red ale and **Old Brown** are also products of mixed fermentation, and year-long maturations. These beers have historically also been produced through spontaneous processes, and some still are. Modern production is, however, also carried out as controlled mixed fermentations in which inoculated yeast and bacteria ferment the wort, before young beer is matured (Alworth, 2015). Flanders red ale originates from West Flanders, is red coloured and is said to be "wine-like". Flanders red ale is matured for up to two years in barrels of oak. The maturation in oak separates Flanders red ale from the Old Brown ales indigenous to Eastern Flanders. The Old Browns can be described as more malt-driven and less acidic, but they represent sour beers and emerge from mixed fermentation (Preedy, 2009).

Gose and **Berliner Weisse** are examples of German sour beer styles in which wheat malt makes up a substantial fraction of the malt bill, and lactobacilli can play important roles in the fermentation. Both these beer styles, originating from Goslar and Berlin, respectively, and they both represent traditional products that now can be produced both through traditional, and also with more modern methods. An important difference between Berliner Weisse and Gose, is the spiciness in the latter, imposed by addition of salt and coriander (Bossaert et al., 2019).

2.3 History of sour beer research

In 1976 and 1977 two papers of great importance to the scientific literature on sour beer were published by a research group at the University of Leuven in Belgium. One focused on the microbiological aspects of spontaneous Lambic and Geuze fermentations (Van Oevelen et al., 1977), the other focused on the formation of aroma compounds during the same fermentation (Van Oevelen et al., 1976). The authors gathered Lambic samples originating from different casks (200-650 L) and different beer productions from a Lambic brewery. These samples were used to establish a pattern of aroma compound development and microbial succession during spontaneous fermentation spanning over 24 months. In the 1976 paper, the authors stated that studies had been carried out previously looking at the microbiology of spontaneous fermentations, but that insight with respect to the fermentation progression over time was missing. Van Oevelen et al. reported on a study published in 1900 (Van Laer, 1900), describing rope-forming bacteria in Lambic fermentation. Further, they stated that super-attenuative yeasts (*Brettanomyces bruxellensis* and *B. lambicus*) producing smells resembling aged Lambic had been described in 1921 (Kufferath and Van Laer, 1921), and that a symbiotic fermentation including LAB, AAB and yeast, had been suggested for Lambic fermentations in 1935 (Kufferath, 1935) and 1936 (Kufferath, 1936). Van Oevelen et al. also mentioned that in 1953 Steenberghen and Simonart (1953) found that coli-aerogenes bacteria emerged during the initial Lambic fermentation stage and disappeared as alcohol evolved in 1953. Finally, Van Oevelen et al. mentioned a study by De Keersmaeker (1974), where high ethyl acetate and low iso-amyl acetate in Geuze in which attributed to *Brettanomyces*, and acetic acid production and ropiness were attributed to acetic acid bacteria.

In the landmark studies of Van Oevelen et al., (1977) four fermentation phases were established during 24 months of fermentation, each characterised by microorganisms and metabolites. They also pointed at lactic acid, acetic acid, ethyl lactate and ethyl acetate as the most characteristic aroma compounds. Another study (Spaepen et al., 1978), from the same research group looked at fatty acids and esters in the same samples, and production of free fatty acids from pure cultures isolated from the above mentioned samples. Their results supported their previously reported microbial succession pattern in Lambic, as fatty acid synthesis from isolated pure cultures, corresponded to production in the Lambic fermentation, at the point where the isolated microbes were active. The authors also reported Lambic and Geuze beers as especially rich in caprylic and capric acids, which were attributed to *Brettanomyces* and *Saccharomyces*, and suggested ethyl caprate as a typical aroma component of these beers (Spaepen et al., 1978). Other studies on microflora (Verachtert et al., 1995), compound formation (Spaepen et al., 1979) and important microbes (Spaepen and Verachtert, 1982) were published by the same laboratory in the following years. About 15 years later, researchers working in the same laboratory (Laboratory of Microbiology and Biochemistry at the University of Leuven, Belgium) published more extensive characterisations of the *Enterobacteriaceae* phase, with

respect to microbial populations (Martens et al., 1991), and formation of aroma compounds (Martens, 1992). The maturation phase was also more thoroughly studied (Kumara and Verachtert, 1991). In 1997, the same research group published a study on the microbial development in industrial fermentation of Flanders acidic ales, which had not been studied extensively previously, according to the authors (Martens et al., 1997). A three-stage microbial succession was described, where *Saccharomyces* dominated the first stage and lactobacilli the second. The final stage, a two-year fermentation in wooden casks, where *Brettanomyces* as well as *Lactobacillus*, *Pediococcus* and acetic acid bacteria were all present. The resemblance to the microbial succession in Lambic/Geuze fermentation was highlighted (Martens et al., 1997).

In the above-mentioned research, microbial analysis was done using classical culture-dependent methods. Currently, culturing methods are not considered the best option for obtaining information about microbial communities, both due to enrichment media bias and the possibility of microbes being present in a viable but not culturable (VBNC) state. A study by Gorski (2012) showed how different microbial patterns emerged from culturing the same mixed strain culture in different culture media, demonstrating how culture bias can emerge with culturing methods. The study (Gorski, 2012) was looking at *Salmonella* strains, and the authors emphasized that multiple methods and enrichment media should be used to favour identification of strains of different culture-fitness characteristics in tested samples. Attention should, however, be paid to this when working with any mixed microbial community. Culturing methods can give false negative results with respect to presence of live microorganisms, if microorganisms are present as VBNC. When microorganisms enter the VBNC state, i.e. in response to environmental stress, they are unable to produce colonies on culture media but capable of metabolic activity (Rahman et al., 1994), respiration (Oliver et al., 1995), gene transcription (del Mar Lleò et al., 2000) and protein synthesis (Rahman et al., 1994).

The viable but non-culturable state for *Escherichia coli* and *Vibrio cholerae* was first described in 1982 by Xu et al. (1982). The condition has since been described for a wide range of bacteria, and the VBNC research on bacteria has been reviewed extensively (Pinto et al., 2015). Furthermore, VBNC has also been proven in yeasts (Divol and Lonvaud-Funel, 2005), including *Brettanomyces bruxellensis* (Serpaggi et al., 2012) and *Saccharomyces cerevisiae* (Salma et al., 2013). An example of how the VBNC state can introduce error was demonstrated by Millet and Lonvaud-Funel (2000) using SO_2. Treatment with SO_2 is an acknowledged method for killing bacteria, which is used in the food industry for antimicrobial purposes, both in wine and beer production. In their study, Millet and Lonvaud-Funel (2000) demonstrated how SO_2 treatment efficiently "killed" LAB in wine, by reducing the CFU/mL obtained by culturing methods from 1.2×10^6 CFU/mL to below 1 CFU/mL. Using the direct epifluorescence technique (DEFT) method, in which cell counting is based on capability for metabolic activity, the same samples showed that the LAB

population had merely been reduced from 3×10^6 to $4.4\pm0.5\times10^5$ CFU/mL. These results clearly suggested that LAB had entered the VBNC state in response to the SO_2 treatments, not been killed. In the same study, Millet and Lonvaud-Funel (2000) showed how the VBNC state was induced in acetic acid bacteria by oxygen deprivation (>1 CFU/mL by culturing, 3 $\log_{10}$ CFU/mL by DEFT), and reversed by reintroduction of oxygen (corresponding CFU/mL in both methods). Multiple other studies have also demonstrated how stress-removal can cause microbial cells to regain their ability to multiply, after the VBNC state has been induced by stress in both bacteria (Roth et al., 1988, Oliver et al., 1995, Maalej et al., 2004, Lleo et al., 2001, Su et al., 2015) and yeast (Serpaggi et al., 2012, Salma et al., 2013). It is worth acknowledging that VBNC has been observed for *Enterobacter* (Pedersen and Jacobsen, 1993), AAB (Millet and Lonvaud-Funel, 2000), LAB (Liu et al., 2017), *Saccharomyces* (Salma et al., 2013) and *Brettanomyces* (Serpaggi et al., 2012), which are all important in the natural microbial succession occurring in Lambic fermentations.

More recent studies on the microbial biodiversity have been carried out using culturing methods in conjunction with culture-independent techniques in order to obtain higher quality information. The study by Spitaels et al. (2014) is an example of this, where they acquired samples from two batches from a Lambic brewery throughout the fermentation process. Denaturing Gradient Gel Electrophoresis (DGGE) of amplified DNA fragments were used for microbial community fingerprinting. Traditional culturing methods were also used to isolate involved microbial strains which were subsequently identified by matrix-assisted lased desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) as well as sequencing of different molecular markers. The authors also obtained samples from the brewery environment (coolship, roof above coolship, cellar walls and ceiling), and used enrichment cultures to obtain isolates that were identified by MALDI-ToF-MS and sequencing methods. The authors found a similar microbial succession to that reported by Van Oevelen et al. (1977), with an initial *Enterobacteriaceae* phase the first month, followed by dominance by *Saccharomyces* spp. and *Pediococcus damnosus,* until *Dekkera bruxellensis* dominated after 6 months. This study, however, suggested that acidification and main fermentation occurred simultaneously, rather than as an extended acidification phase as described previously (Verachtert et al., 1995, Van Oevelen et al., 1977). These results corresponded well with those of Bokulich et al. (2012), where samples representing a three-year fermentation period of spontaneously fermented American Coolship ale were analysed using classical culturing methods together with modern culture-independent methods. Microbiology of Belgian red-brown acidic ales has also been studied recently, by 454 pyrosequencing of 16S rRNA (bacteria) and the internal transcribed spacer region (yeast) in beers at the end of the maturation phase (Snauwaert et al., 2016). In their study, Snauwaert et al., (2016) used sequencing in conjunction with classical culture-dependent methods to obtain overview of metabolic diversity, as well as identifying dominant community members. Comparison of the operational

taxonomic units (OTUs) revealed that *Pediococcus*, *Acetobacteraceae*, *Lactobacillus*, *Dekkera* and *Pichia* were present in all three beers. Targeted isolation using classical culturing methods and subsequent sequencing of these, revealed *Pediococcus damnosus*, *Dekkera bruxellensis* and *Acetobacter pasteurianus* as the most important species. The same study pointed at lactic acid and ethanol as main metabolites, and ethyl acetate, isoamyl acetate, ethyl hexanoate and ethyl octanoate as the main aroma compounds (Snauwaert et al., 2016).

In Geuze beers, where young (1 year) and aged (>2 years) Lambic are mixed and bottled, the young Lambic provides residual carbohydrates (dextrin), while the aged beer is rich in yeast and bacteria able to ferment the residual carbohydrates and produce CO_2. In this manner, Geuze beers are bottle carbonated without addition of external substrate (Verachtert and Iserentant, 1995). After this, the Geuze bottles can be aged for more than ten years by traditional breweries, and in 2015 Spitaels et al. (2015b) published a study assessing the impact of aging (a few months to 17 years) on metabolites and microbiota in Geuze beers. The authors suggested ethyl lactate as a positive Geuze biomarker for aging, as concentrations increased with time. Ethyl decanoate decreased with aging, and its disappearance was suggested as an additional identifier of aging. Further, the authors Spitaels et al. (2015b) stated that Geuze beers should be aged for less than 10 years and that substantial metabolite variation was observed between bottles, preventing generalization of aging effects.

Some industrial breweries produce Lambic beers at a larger scale in a process that diverges somewhat from the traditional one. These breweries usually use modern processing such as pasteurisation, filtration and forced carbonation also for their Lambic products (Spitaels et al., 2015c). By using modern equipment to chill wort, these breweries can carry out production the entire year, not depending on low winter temperatures for overnight cooldown in shallow vessels (even though they still use cooling tuns for wort exposure). Industrial Lambic breweries also use wooden casks, but generally these are custom-made and far greater in size (170-200 hL) compared to the retired wine or cognac casks used in traditional Lambic breweries (Spitaels et al., 2015c). Spitaels et al. (2015c) characterised the microbial succession during a one-year fermentation in an industrial Lambic brewery, and compared it to their findings from 2014 looking at a traditional production (Spitaels et al., 2014). The authors concluded that a core microbiota, common for both traditional and industrial Lambic production was observable, where *S. cerevisiae*, S. *pastorianus*, *D. bruxellensis* and *P. damnosus* where the main performers in the main fermentation and maturation. Revealed differences between the traditional and industrial fermentations included absence of the *Enterobacteriaceae* phase (explained by reduced initial pH due to lactic acid addition) and larger variety of AAB in the industrial production.

Two recent studies have looked at Lambic beer fermentation and maturation. One looked at consumption of wort substrate and production of metabolites, and attempted to explain the successive microbial growth through this (De Roos et al., 2018). The authors attempted to dissect microbial identities, roles, evolution and metabolite formation systematically, through a two-year fermentation process using culture-dependent, culture-independent, as well as targeted metabolite analyses. The results highlighted the importance of the application of the coolship, as high CFU/mL were obtained from sampling after overnight cool-down, prior to transfer to the wooden casks. The initial microbiota present in the coolship included both LAB (10^3 CFU/mL) and yeasts (10^4 CFU/mL). Furthermore, they describe a distinct four-phase microbial succession with an enterobacteria phase (first week), a main fermentation (24 h - 7 weeks), acidification (week 7 – 9 months) and maturation (6 months and onward) (De Roos et al., 2018). The authors obtained more than 2000 microbial isolates throughout the two-year fermentation, about 400 bacterial strains and more than 1700 yeast strains. The occurrence of an enterobacterial phase was contradictory to the observations by Spitaels et al. (2015c), where lactic acid addition prevented the growth of enterobacteria. Wort acidification by lactic acid addition was also carried out in the study by De Roos et al. (2018), resulting in a shortening of the enterobacterial phase to a week. For comparison, the enterobacterial phase lasted for a month in traditional Lambic production without wort acidification (Spitaels et al., 2014).

In the second study they, looked closer into Lambic barrels using both culture-dependent and culture-independent techniques (De Roos et al., 2019). This study clearly demonstrated that the microbiota present at the inner surface of wooden casks used in a traditional Lambic brewery varied with both cleaning procedure of the barrels, as well as the general condition of the casks with respect to age, wood thickness and wood porosity. Based on 16s rRNA sequencing, De Roos et al. (2019) identified a wide variety of bacteria, including important species in Lambic fermentations such as *Pediococcus*, *Lactobacillus* and *Acetobacter*. Further, sequencing of the internal transcribed spacer region (ribosomal DNA), a number of different yeasts were identified, including *Saccharomyces*, *Dekkera* and *Pichia*. The authors conclude that microbes present in the barrels (also non-detected microbes, perhaps present in a VBNC state) could act as a source for microbial inoculation, in addition to that of the brewery air and brewing equipment. A complete review of all existing sour beer literature is beyond the scope of the current PhD thesis. Additional studies do exist, and these can be read about in the recent reviews by Bossaert et al. (2019) and De Roos and De Vuyst (2019).

2.4 Challenges with traditional sour beer production

The production of sour beer through spontaneous fermentation is associated with several challenges. These include inconsistency in product quality, wastage due to failed fermentations and time consumption. The study by Spitaels et al. (2015a) looking at microbiota and metabolites of aged Geuze clearly demonstrated inconsistency in production, as the bottle-to-bottle metabolite variation made it impossible to generalize age effects on Geuze. As the authors state, this "*illustrated the unique character of Lambic beers*". The product variations that arise through the traditional process can be seen as a positive attribute, and are greatly appreciated by some consumers, as they represent a mark of authenticity and natural production. The inconsistency in production can also be considered negative, as it imposes the need to discard substantial quantities of beer due to failed organoleptic characteristics. The issue of inconsistency is also clearly demonstrated in the study by Spitaels et al. (2015c), on industrially produced Lambics, where there were substantial differences between the fermentation progression in the two batches that were monitored. The time-demand is an additional challenge with traditional sour beer production methods, especially if product has to be discarded after multiple years of fermentation/maturation due to quality defects.

The idea of using pure cultures in controlled, mixed fermentations is an appealing idea. Not only because it could offer improved process control, production consistency and potentially reduced production time for sour beers, but also because controlled mixed fermentations offer a tremendous potential for generation of novel products. The application of mixed cultures and non-conventional microbial strains in beer fermentation represents vast possibilities for flavour generation. In addition, the capacity of non-conventional brewing microbes for diverse carbohydrate utilization, offers the inclusion of non-conventional raw materials in beer production. This could be used as a tool to improve process control, but also represents a method for conversion of non-food carbohydrate sources to food products directly through fermentation. Lactobacilli are highly interesting in this regard, and the role of lactobacilli in beer, their adaption to beer-related stress, their potential for flavour generation and carbohydrate degradation are described in the following section.

3. *Lactobacillus*

3.1 Introduction

Lactobacillus are rod-shaped bacteria belonging to the lactic acid bacteria (LAB). LAB have lactic acid as the main metabolic product of carbohydrate metabolism as a common denominator (Wright and Axelsson, 2019). Lactobacilli are gram-positive and can be anaerobic or aerotolerant. Their metabolism is generally classified either as obligately homofermentative, meaning that they convert hexose sugars to lactic acid almost exclusively, or as obligately/facultatively heterofermentative, meaning that they convert hexose sugars to lactic acid as well as CO_2 and ethanol or acetic acid. Lactobacilli have a great record for safety and are used as health-promoting probiotics as well as starter cultures for fermentation of a vast variety of food products. They are associated with fermented dairy products such as yogurts (McFarland, 2015) and cheeses (Carafa et al., 2015), fermented vegetables (Petrović et al., 2012) and fermented meat products (Cocolin et al., 2009, Fontana et al., 2016). Lactobacilli are also vital contributors in production of a number of food products through mixed fermentations where both bacteria and yeast participate, including kefir (Guzel-Seydim et al., 2011, Vardjan et al., 2013), water kefir (Gulitz et al., 2011), sour dough bread (Minervini et al., 2014) and alcoholic beverages such as wine (Mtshali et al., 2012), sake (Tsuji et al., 2018) and beer (Vriesekoop et al., 2012).

In beer, lactobacilli can be terrible spoilers or vital fermentation contributors, depending on the beer style and the strain properties. In ales and lagers, the ideal fermentation usually transpires without the involvement of anything but *S. cerevisiae* or *S. pastorianus*, respectively. Lactobacilli are considered spoilers in this context, associated with unwanted haze and sedimentation, off-flavours and acid formation as well as ropiness (Suzuki, 2011). Ropiness occurs when exopolysaccharide (EPS) producing bacteria cause increased viscosity and “sliminess”. EPS are high molecular weight homopolysaccharides, composed of one repeating monosaccharide unit, or heteropolysaccharides composed of repeating units of different monosaccharides (Fraunhofer et al., 2017). This is an unwanted phenomenon that can occur in wine (Llauberes et al., 1990, Lonvaud-Funel et al., 1993), cider (DueÑAs et al., 1995) and beer (Shimwell, 1947), due to the presence of different bacteria (Sutherland, 1972, Sutherland, 1985, Cerning, 1990), also including lactobacilli (Fraunhofer et al., 2017). In sour beer, where production of acid is welcomed, lactobacilli can represent appreciated contributors, vital for the development of the wanted organoleptic characteristics through fermentation. Regardless of their presence as spoilers or as needed fermenters in beer, lactobacilli need to overcome the comprehensive sum of hurdles constituted by beer in order be involved. A wide set of systems for detection and adaption to stress are involved in this (van de Guchte et al., 2002, Sakamoto and Konings, 2003, Geissler et al., 2016).

3.2 Adaption to stress

The antimicrobial effect of hops in beer can largely be attributed to the iso-α acids. Lactobacilli are generally inhibited from growing in beer by the presence of **iso-α acids**, however, some strains are resistant to the antimicrobial actions by hops and are thus able to survive in beer (Vriesekoop et al., 2012, Suzuki, 2011). Genes associated with hop resistance in LAB include *horA*, *horC* and *hitA* (Bergsveinson et al., 2015). The *horA* gene encodes an ABC transporter capable of expelling hop bitter acids from cells (Sakamoto et al., 2001, Sami et al., 1997). The *horC* gene presumably encodes a PMF-dependent multidrug effluence pump (Iijima et al., 2009, Iijima et al., 2006, Suzuki et al., 2005). Products from *horA* and *horC* contribute to hop resistance by lowering the net influx of hop bitter acids into cell cytoplasm and thereby restrict their actions as antibacterial protonophores. The *hitA* gene is assumed to encode a divalent cation transporter that aids hop-sensitive bacteria by transporting divalent cations, e.g. Mn^{2+}, into cells where the proton gradient has been dissipated (Hayashi et al., 2001). Other cellular adaptions are also involved in hop resistance in LAB, including modifications of the cell wall (Behr et al., 2006) and cell morphology (Asano et al., 2007). This has been reviewed extensively by Suzuki (2011). The presence of *horA* and/or *horC* is, however excellent genetic marker for ability to survive in beer, as 94% of 51 tested beer spoilers had *horA*, 96 % had *horC* and 100% had at least one of them in a study by Suzuki et al. (2005).

Lactobacilli are generally resistant to **ethanol**, which gives them competitive advantages in fermentation environments (G-Alegría et al., 2004, Gold et al., 1992, Ingram and Dombek, 1987). They do, however, display huge variation in their resistance, as some (e.g. strains of *L. plantaurm*) stop growing at 5-6% ethanol, while others can sustain environments of much higher concentrations (Wibowo et al., 1985, Suzuki et al., 2008). While most LAB are inhibited above 13 % ethanol (Nojiro, 1984), reports exist of sake spoilers able to grow at 20 % ethanol (Momose, 1989). Kleynmans et al. (1989) reported of lactobacilli able to resist 16 % ethanol, even at pH as low as 3.3. Even though lactobacilli are generally able to sustain the ethanol levels in many beers, this does not mean that they are able to grow in beer, and the role of ethanol tolerance on beer spoilage potential is not well characterised (Pittet et al., 2011). In a study by Pittet et al. (2011) no correlation was found between ability to grow in beer and ethanol tolerance,

The end product of carbohydrate metabolism by lactic acid bacteria is organic acids. Metabolism by lactobacilli causes accumulation of organic acids and reduction in **pH** in the environment in which they reside, making in inhospitable for many potential microbial competitors. Extracellular, undissociated acids can pass cell membranes, where they dissociate in response to the higher intracellular pH. Decrease in the intracellular pH due to increase in protons can dissipate the proton motive force and thereby inhibit transport of nutrients across it, as well as affecting enzyme activity and damaging DNA (Guchte et al., 2002). Lactobacilli are not immune to acidic environments even though they inflict it upon themselves, and several

different strategies are involved in their response towards acidic stress. The glutamate decarboxylase (GAD) system is one of these. In the GAD system, an extracellular amino acid (glutamate) is internalised, decarboxylated (to γ-aminobutyrate/GABA) in a reaction where a proton is consumed before the decarboxylated product is transported to the extracellular environment again. The consumption of an intracellular proton contributes to increased intracellular pH. In addition, the decarboxylation can be coupled to an electrogenic transporter, which allows ATP generation through the proton motive force (Cotter and Hill, 2003, van de Guchte et al., 2002, Higuchi et al., 1997). The arginine deaminase pathway (ADI)(Cunin et al., 1986) is another system for maintaining pH homeostasis in lactobacilli (Champomier Verges et al., 1999) and other LAB (Arena et al., 1999). In the ADI pathway, arginine is converted to ornithine, ammonia (NH_3) and carbon dioxide (CO_2), and ATP is generated. NH_3 is generated in the conversion and reacts with intracellular protons and thus contributes to alkalize the cytoplasm. F_0F_1-ATPase is an ubiquitous enzyme among bacteria, which can facilitate the production of ATP in a reaction sustained by the transmembrane proton motive force, or expel protons from cells in an energy consuming process sustained by ATP consumption (Boyer, 1997, Stock et al., 1999). Active proton expulsion increases in acidic environments, and is vital for maintaining pH homeostasis in lactobacilli (Corcoran et al., 2005) and other LABs (Futai et al., 1989). Several other systems are known to be involved in the acid stress response of LABs. A complete review of these are beyond the scope of this thesis, and further details are comprehensively covered in the review by van de Guchte et al. (2002).

Lactic acid bacteria are known as robust towards **CO_2** compared to many other bacteria (Borch et al., 1996). In a study by Devlieghere et al. (1998), increased CO_2 levels were associated with decreased growth rate for *Lactobacillus sake*. The effect was, however, minor, and the presence of CO_2 is likely not the major hurdle for lactobacilli in beer. Neither are low levels of **oxygen**, as lactobacilli are anaerobic or aerotolerant (Wright and Axelsson, 2019).

Regarding **carbohydrate depletion,** conventional brewer's yeast will utilize sucrose, fructose, glucose, maltose and maltotriose in their fermentation of wort. Maltotriose, which is assimilated after maltose is depleted, is the largest sugar degraded by conventional brewer's yeast. Poly-/oligosaccharides are also present in wort, often referred to as dextrins (Boulton and Quain, 2001). Dextrins can contribute to the sensory perception, e.g. fullness, in ale or lager beer, but in mixed fermentations, these higher Poly-/oligosaccharides can serve as substrate for microorganisms with carbohydrate degrading capabilities exceeding those of conventional brewer's yeast. In traditional Lambic production, higher content of such polysaccharides is promoted by the inclusion of unmalted wheat in the grain bill (≤30%), and the application of turbid mashing. Both these contribute to reduce the enzymatic carbohydrate degradation mashing and promote a higher content of dextrins in wort, which is assumed important for sustaining the prolonged

fermentation phases occurring after the main fermentation in Lambic production (De Roos and De Vuyst, 2019). Many lactobacilli have enzymes facilitating utilization of residual carbohydrates in wort, not degradable by conventional brewer's yeast. Maltotetraose (Møller et al., 2017), maltopentaose and more complex maltodextrins can sustain growth of *Lactobacillus* (Spear et al., 2014) and genes encoding enzymes necessary for cellular import of maltodextrin, as well as degradation, have been identified (Nakai et al., 2009). Amylolytic lactobacilli can also degrade starch (Mukisa et al., 2012, Reddy et al., 2008), and some lactobacilli can degrade cellobiose (Gänzle and Follador, 2012). *Lactobacillus* involvement in super-attenuation of Lambic beer has been implicated, where a larger carbohydrate fraction has been fermented, than the one that is degradable by brewer's yeast (Andrews and Gilliland, 1952, De Cort et al., 1994).

As previously stated, lactobacilli must overcome the sum of hurdles in beer, posed by ethanol, low pH, presence of iso-α acids (and other hop compounds) and nutrient depletion (Fig. 3), in order to carry out metabolism in beer. If a *Lactobacillus* "infection" is wanted, e.g. in sour beer production, this can perhaps be promoted by removing or reducing the level of one of the hurdles discussed above, e.g. nutrient depletion. A specific substrate, known to promote metabolism of a limited number of microbes, could for instance be added to beer, to promote a rapid acidification phase in mixed or sequential fermentations. An example of such a substrate could for instance be lactose, not promoting growth of *S. cerevisiae* (Domingues et al., 2010) but supporting *Lactobacillus* metabolism.

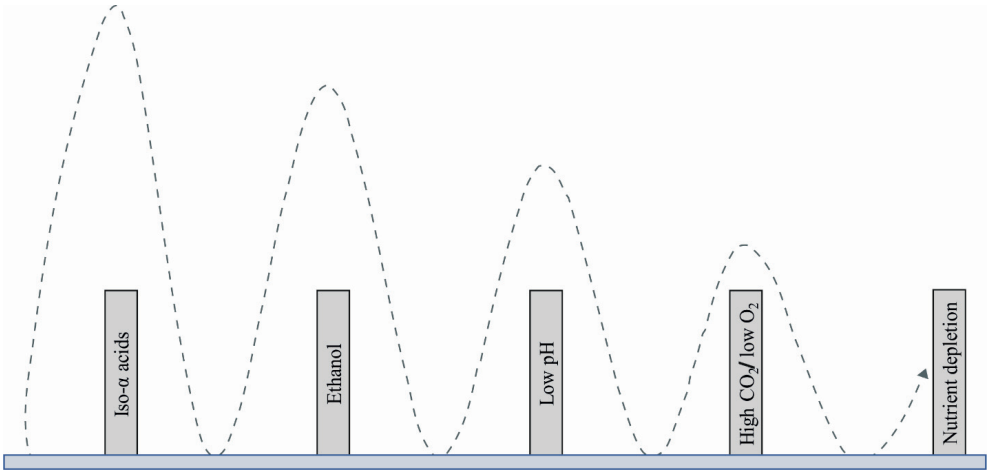

Figure 3. Illustration of the hurdle effect in beer, where relatively low intensity of hurdles such as iso-α acids, ethanol, low pH, high CO_2, low O_2 in sum pose a substantial antimicrobial effect.

4. Modern methods of sour beer production

4.1 Introduction

Producing sour beers in controlled fermentations with pure cultures is by no means a new idea. In late seventies, Van Oevelen et al. (1977) concluded in their paper on microbiology of spontaneous wort fermentation by suggesting a number of questions for further research, one of which was "*Can Lambic be made with pure cultures*?". Even though this question was raised in 1977, there is little evidence of it being pursued within the scientific literature until quite recently. As is evident from the "history of sour beer research" section above, the majority of scientific literature is focused on characterizing the microbiology and metabolite formation in spontaneous fermentation, rather than investigating alternative production methods offering improved process control and/or reduces fermentation times. An exception is the study by Kumara and Verachtert (1991), which is discussed more thoroughly below.

Experimentation with and development of alternative production methods have emerged in industry, and different modes of spontaneous, semi-spontaneous and pure-culture fermentations are carried out for commercial production. An example of this is the "sour worting" method where *Lactobacillus* fermentation for acid production is carried out prior to yeast fermentation (either by *Saccharomyces*, *Brettanomyces* or both) in oak barrels (Tonsmeire, 2014). Descriptions of other strategies developed and employed by different craft beer breweries can be reviewed in the book by Tonsmeire (2014) "American Sour Beers: Innovative Techniques for Mixed Fermentations" which is based on interviews with different commercial breweries. The spiked interest in sour beers, both in traditional production and alternative production methods is evident from the publication of books such as the above mentioned Tonsmeire (2014), "Wild Brews: Beer Beyond the Influence of Brewer`s Yeast" (Sparrow, 2005) and "Gose: Brewing a Classic German Beer for the Modern Era" (Allen, 2018). It is evident from the publications discussed in the following section, that the question stated by Van Oevelen et al. in 1977 about the possibility of producing Lambic using pure cultures, remains highly relevant.

4.2 Research on modern methods for sour beer production

A strategy for simplifying and shortening the production process, as well as achieving improved process control was tested by Kumara and Verachtert (1991) in 1991. They fermented wort from a Lambic brewery for a short period (≤48h) at high temperature (28°C) with *S. cerevisiae* in order to obtain a wort without *S. cerevisiae* fermentable sugars. The yeast cells were then removed, and the pre-fermented wort was pasteurised before it was inoculated with a mixed population from spontaneously fermenting, 1-year old Lambic. In the same manner, a Lambic at an earlier fermentation stage (higher carbohydrate content) was pasteurised and reinoculated with the same mixed population from the further progressed Lambic fermentation. The fermentations were incubated at 28°C. The authors observed that over-attenuation

occurred in 30 days, resulting in beers with more than 4000 mg/L lactic and 800 mg/L acetic acid in both fermentations. Sensory testing is not described in the publication, and the authors conclude by stating that their approach with inoculating clarified and pasteurized 1-year old Lambic and applying 28°C as fermentation temperature "*might be a first step in rendering the fermentation a little more economic without affecting the notion of it being a natural fermentation*".

Single-strain fermentations with non-conventional, acid-producing yeast is one suggested strategy. Domizio et al. (2016) tested three different strains of *Lanchancea thermotolerans* in three week-long fermentations of wort at 14°C, where they compared the *L. thermotolerans* performance to that of a conventional *S. cerevisiae* brewing strain. All the non-conventional strains were able to degrade maltose but not maltotriose. They were also able to produce comparable quantities of ethanol (approximately 5 % v/v) and higher quantities of lactic acid when compared to the *S. cerevisiae*. A substantial increase in acidity was obtained with one of the tested strains (final pH 3.77 compared to 4.24 for the *S. cerevisiae*). Even though the lactic acid content was higher for all *L. thermotolerans* compared to the *S. cerevisiae* fermentation, it only ranged between approximately 100 and 300 mg/L, which is substantially lower compared to most sour beers. Osburn et al. (2018) also tested 284 (54 species, 26 genera) yeasts isolated in small scale beer fermentations for their fermentation performance. Through sensory testing of the resulting beers, the authors observed that many of the strains generated beers that were described as tart or sour. They identified multiple yeast strains capable of producing lactic acid and used four of these (strains of *Hanseniaspora vineae*, *Lachancea fermentati*, *Schizosaccharomyces japonicus* and *Wickerhamomyces anomalus*) in brewing experiments where ~ 1×10^{11} cells were inoculated in 19L wort (corresponding to ~5×10^{6} cells/mL) and incubated at 21.7°C for 1 month. A reference fermentation with *S. cerevisiae* was included in the trial. The beers generated in the trial with lactic acid producing yeasts, had final pH values ranging from 3.20-3.74, substantially lower compared to pH 4.35 in the *S. cerevisiae* beer. Quantification of the lactic acid in the beers ranged from 10 and 50 mM (corresponding to ~900-4500 mg/L) and examples of sensory descriptors used for the *W. anomalus* fermented beer are "Very sour, pear, apple and apricot". Osburn et al. (2018) propose their method, named "primary souring", as an alternative production route for sour beer, solely relying on fermentation with yeast that produce lactic acid as well as ethanol and CO_2.

The application of an initial biological acidification step of wort, is another alternative production method for sour beer that has been explored both in industry (Tonsmeire, 2014) and in research (Peyer et al., 2017). The biological acidification can be carried out in the mashing tun (sour mash), in the brewing kettle (kettle sour) or after the wort has been transferred to the fermentation vessel (sour wort). The concept is to utilize the ability of LAB to produce high quantities of lactic acid within a short time frame (typically 24-48h) in wort (typically unhopped), boil the wort to stop bacterial fermentation (typically with addition of hops) and

then carry out a single strain fermentation with conventional brewer's yeast. In a study by Peyer et al. (2017), *Lactobacillus amylovorus* was used for biological acidification at different stages of the production in order to obtain a better understanding of biological wort acidification as a production method for sour beer. The authors inoculated *L. amylovorus* in mash (post-mashing, pre-lautering), pre-boil wort and post-boil wort. The mash, pre-boil and post-boil worts were incubated for 18 hours at 40°C, before subsequent processing steps were carried out and acidified worts were inoculated with yeast (*S. cerevisiae,* Safale US-05) and incubated for 14 days at 20°C. The produced beers were matured for 14 days at 0°C before analysis with respect to compound composition and sensory properties. The authors showed how biological acidification at different time points in the pre-yeast fermentation process led to differences in the obtained beer product. The produced beers had final pH values in the range of 3.44-3.75, substantially lower than the yeast control beer (pH 4.12). They concluded that reduced pH during yeast fermentation delayed yeast metabolism, but ultimately did not affect the attenuation. The authors further stated that acidification of pre-boil wort emerged as the superior method to ensure "*high acidity and minimal organoleptic failure*".

Another approach has been investigated, where co-fermentation with a *Lactobacillus* and yeast is used in beer production (Alcine Chan et al., 2019). *L. paracasei* L26 was inoculated (~1%) together with *S. cerevisiae* US-05 (~0.5%) in unhopped wort, and fermentation progressed at 30°C for 48 hours before the temperature was adjusted to 20°C for the next 8 days (10 days fermentation period in total). Pre-isomerised hop extract was added after fermentation to obtain an iso-α acid content of 27 mg/L before the beers were stored at 5°C or 25°C to assess the temperature effect on lactobacilli survival. The colder temperature was proven as a better option for preserving the highest possible CFU during storage. The main objective of Alcine Chan et al. (2019) was to develop a novel sour beer beverage with sufficiently high lactobacilli count to represent a legitimate delivery vehicle for probiotics. The authors succeeded in this, producing a sour beer (pH 3.62) containing 10^9 CFU probiotic lactobacilli per serving (100 mL) and containing more than 5000 mg/L lactic acid.

Outline and aim of the thesis

Interest towards sour beer has increased substantially in recent decades. Sour beer is traditionally produced through spontaneous fermentations where complex microbial consortiums are involved (Fig. 4). These can include different yeast (*Saccharomyces* spp. and *Brettanomyces* spp.) and bacterial species (*Lactobacillus* spp, *Pediococcus* spp, *Acetobacter* spp). A diverse range of metabolites are formed through the successive microbial progression of such fermentations, resulting in highly complex products, with respect to sensory properties. High quantities of organic acids, such as lactic and acetic acids, results in low pH and high intensity in sourness and acidic taste compared to ales and lagers fermented by pure, single cultures of *Saccharomyces cerevisiae* and *S. pastorianus*, respectively. Several issues are related to production of sour beer through traditional methods. These include poor process control, lack of consistency in product quality and lengthy time-frames for fermentations. Selected pure-cultures of microorganisms with ideal properties, in conjunction with careful application of processing steps, could facilitate production of sour beer with a higher level of process control and rapid fermentation compared to traditional methods. The collective metabolism of the microorganisms involved in traditional spontaneous fermentations of sour beer, represents fermentation properties exceeding those of single-strain brewer's yeast. This applies both with respect to formation of flavour-active metabolites and to potential for substrate utilization. Some microorganisms accounts for key fractions of this collective potential. Application of these key strains could perhaps facilitate production of sour beer in a controlled and rapid manner without losing the unique flavour-complexity of traditional sour beer products. Utilization of such strains could also open possibilities for using non-conventional sources of carbohydrates in food production through fermentation.

The majority of sour beer research has been focused on understanding the complex spontaneous fermentation process, originating from traditional Belgian brewing culture. This research is presented in the introduction of the current PhD thesis. Only a few publications on alternative, pure-culture fermentations for sour beer production have been published in recent years. The overall scientific literature on the subject is, however, quite narrow. The three papers/manuscripts included in this thesis attempt to expand on this knowledge. The main objective of the current PhD project was to explore different strategies for sour beer production and investigate whether pure-culture fermentations with strains of *Lactobacillus* and *S. cerevisiae* could generate sour beer products through rapid, highly controlled fermentations by utilizing novel substrates, preferably generating beer with desired flavour characteristics.

In **paper I**, the explored strategy was pre-fermentation with lactic acid bacteria (LAB) (Fig. 4). *Lactobacillus buchneri* CD034 was used in a kettle sour approach, where biological acidification of unhopped wort was carried out prior to yeast fermentation. The objective was to evaluate the contribution by LAB to the metabolite composition and sensory properties of beer. This was done by comparing beers produced through biological acidification to beers produced through chemical acidification. Mixed fermentations with pure cultures were further investigated in **paper II** (Fig. 5). Three different strains of *Lactobacillus* (*L. buchneri* CD034, *L. plantarum* WildBrew™ Sour Pitch, *L. brevis* BSO464) were subjected to various beer-related stress factors (ethanol, low pH, iso-α acids etc.), to investigate the influence of these on lactobacilli metabolism. The three strains were also used in separate co-fermentations with yeast. The aim of this, was to evaluate co-fermentation with lactobacilli and *S. cerevisiae* as a time-saving strategy for sour beer production. The *Lactobacillus* strain displaying the highest robustness towards beer-related stress in paper II (*L. brevis* BSO 464), was used for further experiments in **paper III** (Fig. 5). In paper III, secondary LAB fermentation of beer was carried out. A wood-derived specific substrate was added to beer after yeast fermentation, to expedite acidification through LAB fermentation. The objective of paper III was to demonstrate how hemicellulosic biomass can be used in food production directly through fermentation, and to evaluate the potential for using selective substrates for promoting secondary fermentations in rapid sour beer production.

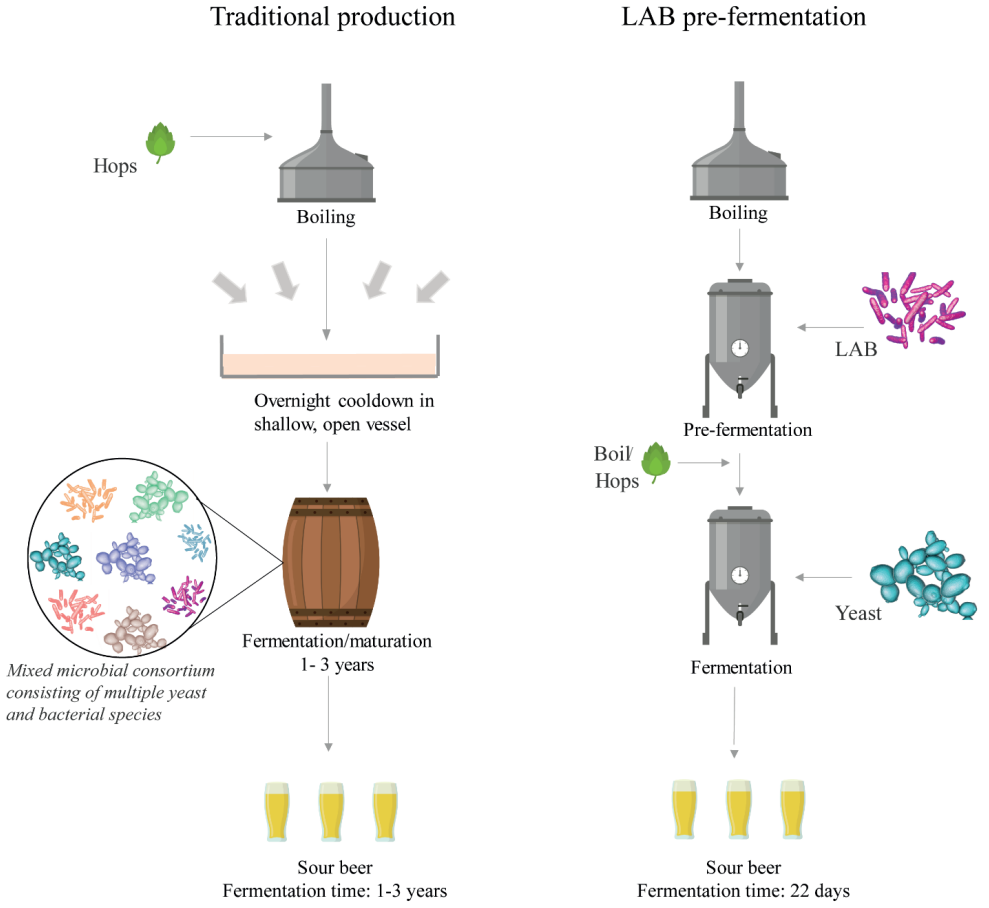

Figure 4: Approaches for sour beer production. Traditional production process with spontaneous fermentation (left). Pre-fermentation with LAB followed by yeast fermentation, as described in paper I (right).

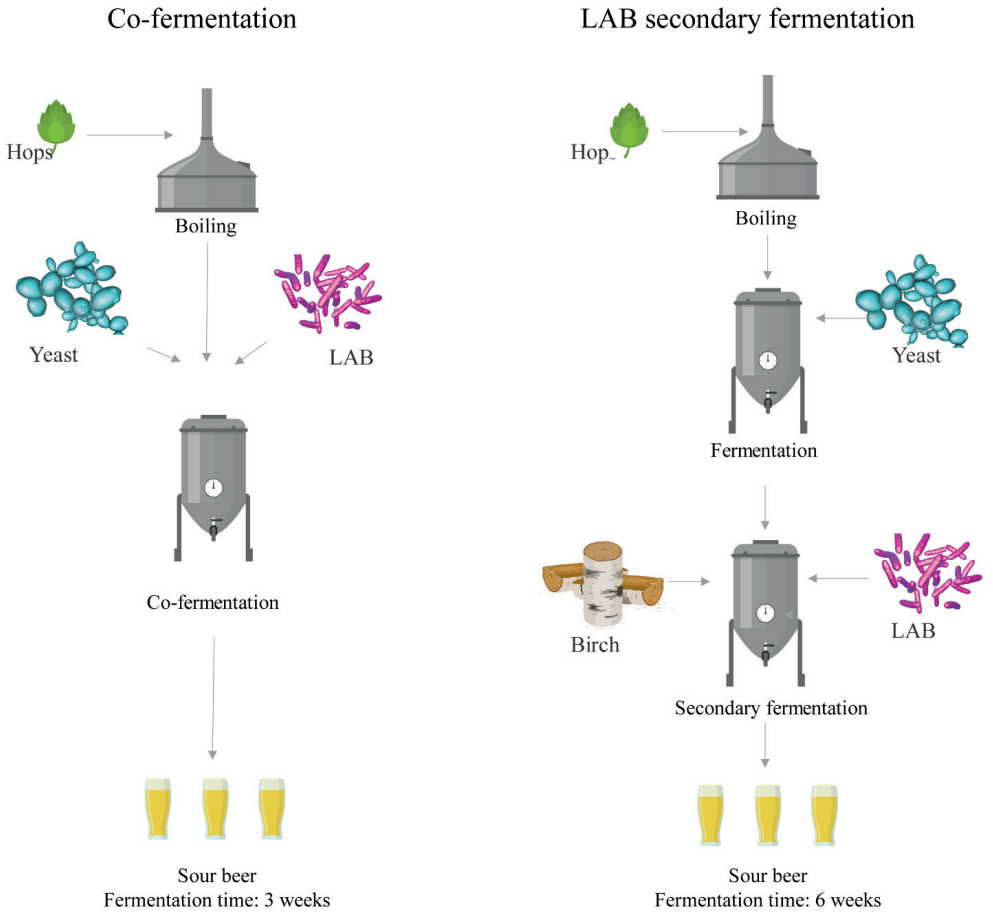

Figure 5: Approaches for sour beer production. Co-fermentation with yeast and LAB as described in paper II (left). Secondary fermentation with LAB, expedited by wood-derived carbohydrates, as described in paper III (right).

Main results and discussion

The rationale behind the current PhD work was that application of pure cultures of key microorganisms, responsible for important features resulting from spontaneous fermentations, can be used to produce sour beer in a rapid and highly controlled manner. Traditional sour beer products are lower in pH and higher in lactic acid content compared to "regular" ales and lager beers. In a study on commercial Lambic beers for instance, a pH ranging from 3.2-3.6, and lactic acid concentrations ranging from ~3700 mg/L to ~17 500 were found (Thompson Witrick et al., 2017). For comparison, non-sour beer typically is in the range of pH 3.9-4.4 (Vriesekoop et al., 2012). Lactic acid bacteria (LAB), such as *Lactobacillus* spp. and *Pediococcus* spp. are among the microbes associated with spontaneous, sour beer fermentations (Van Oevelen et al., 1977, Bokulich et al., 2012, Spitaels et al., 2014, Spitaels et al., 2015c, De Roos et al., 2018, De Roos et al., 2019). These LABs have lactic acid as their main product from carbohydrate metabolism and contribute to the high lactic acid quantities found in Lambic beers. Due to the more frequent associations between *Pediococcus* and formation of ropiness/slime (Van Oevelen and Verachtert, 1979, Fernandez et al., 1996) compared to *Lactobacillus* (DueÑAs et al., 1995), the latter was chosen for this current PhD study.

Paper I – Pre-fermentation with lactic acid bacteria in sour beer production

Pre-fermentation with lactic acid bacteria (LAB) is a method used for production of sour beer in the brewing industry (Tonsmeire, 2014). By carrying out LAB fermentation in unhopped wort, prior to yeast fermentation, the hurdle effect imposed by yeast fermentation (ethanol, nutrient depletion, low pH etc.) and iso-α acids on LAB metabolism can be circumvented, and the ability of LAB to rapidly produce high quantities of lactic acid is exploited. By boiling the wort when the desired level of lactic acid has been obtained, and thus stopping LAB metabolism, a high level of control is achieved. In this process, volatiles produced by the LAB can be stripped off. This is fortunate if they confer unappealing sensory properties, but unfortunate if they contribute positively. An alternative to the inter-fermentational boiling step is addition of highly hopped wort at yeast addition, to introduce antimicrobial iso-α acids after the wanted bacterial activity has transpired. The contribution by LAB, when applied in this type of method, is a partly unresolved issue, and the experiments described in **paper I** attempted to expand on this knowledge. In this study, we used a *Lactobacillus* strain (*L. buchneri* CD034, hereafter referred to as *L. buchneri*) in pre-fermentation, either separated from yeast fermentation by a boiling step in beer A (with hop addition), or by addition of strongly hopped wort in beer B. The produced beers (~10 L) were compared to beers produced by chemical acidification of wort (pre yeast addition) in beer C or beer (post yeast addition) in beer D, as well as to a reference produced without biological or chemical acidification. This was done to control for the contribution of pure lactic acid, both with respect to influence on yeast performance, and with respect to final sensory properties. A schematic illustration of the experimental setup can be reviewed in figure 1 (paper I). The produced beers were analysed with respect to composition of flavour active organic acids and volatiles and sensory properties. Furthermore, a small-scale (400 mL) version of the experiment was carried out to monitor microbial growth.

Metabolic activity by *L. buchneri* was efficiently stopped by boiling, as no CFU/mL was detected after the boiling (Fig. 7A, paper 1). A rapid decline in LAB viability was also observed in response to addition of yeast and strongly hopped wort in beer B. 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. Reduced viability of LAB in response to co-fermentation with yeast has been found previously, and has been explained by nutrient depletion and ethanol production (Carvalho et al., 2015). This was likely also contributing factors in the loss of viability in beer B in this study, along with the introduction of antimicrobial hop compounds. The failure of *L. buchneri* fermentation in beer F in the experimental brewing setup indicated that this strain was unable to sustain the harsh beer environment and was unsuited for secondary beer fermentation. Even though controlled secondary LAB fermentation would diverge from spontaneous fermentation of sour Lambics, 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 was deemed necessary to explore the secondary LAB fermentation approach.

Based on the volatile compounds, the five produced beers were separated in three distinct groups; beer A, beer B and beers C/D/E (Fig. A and B, paper I). No effect was found by lactic acid alone, on the production of volatiles by yeast, as beers C, D and E were in the same group. The separation of beers A and B from the C/D/E group, points to an effect from pre-fermentation with *L. buchneri* on the volatile composition of beer. The separation of beers A and B from each other, also suggests that the choice of a post-acidification boiling step versus addition of strongly hopped wort, is influential with respect to composition of volatiles. However, only 5.3 % of the variation in volatiles were explained by the brewing method, and time/fermentation progression accounted for a much higher fraction of the variation (68.5%) in the metabolites (Fig. 2C and D, paper I). The brewing method was more important with respect to organic acid content, accounting for 28.4 % of the variation (Fig. 4A and B, paper I). All five brewing methods generated significantly different beers with respect to organic acid content. Lactic acid (Fig. 5A, paper I) was present in all beers except the reference (beer E), with final quantities slightly higher in the biologically acidified beers (~1000 mg/L in beers A and B) compared to the chemically acidified beers (~800 mg/L in beers C and D). Acetic acid (Fig 5B, paper I) was present in all matured beers, but the obtained levels were higher in the biologically acidified beers. The final concentration was higher in beer B compared to beer A at yeast addition. It should be noted that beer B contained the highest concentration of all organic acids at the matured beer stage (except pyruvic acid). This beer also contained highest concentrations of the volatile esters ethyl heptanoate (Fig. 3E, paper I) and ethyl octanoate (Fig. 3F, paper 1), both associated with fruity notes (Library, 2019b, Library, 2019a). The largest contribution from LAB was conveyed when applying method B, where LAB is present longer.

In the descriptive sensory analysis, five of 21 assessed attributes were scored significantly differently between two or more beers (Table 4, paper I). Acidic taste was one of the most pronounced sensory differences, where Beer E was scored significantly lower compared to all other beers. The lower score in acidic taste in beer E, corresponds well with the metabolic data, as beers A-D contained lactic acid in the range ~800-1000 mg/L (Fig. 5A, paper I) while lactic acid was not detected in beer E. Lactic acid is associated with sourness and acidic taste (Van Oevelen et al., 1976, Da Conceicao Neta et al., 2007). With reported sensory threshold in beer of 400 mg/L (Engan, 1974) beers A-D were all well above the sensory threshold. The results from the sensory analysis suggest that although biological acidification with *L. buchneri* had a significant contribution (beyond lactic acid production) to metabolite composition of beer, the contribution did not exceed that of chemical acidification, with respect to sensory properties.

Paper II – Co-fermentation of *Saccharomyces cerevisiae* with *Lactobacillus* strains tolerant to brewing-related stress factors for controlled and rapid production of sour beer

Due to a failed LAB secondary fermentation (beer F, Fig. 1, paper I) and rapid decline in LAB viability observed after introduction of yeast and iso-α acids in beer B in paper I (Fig. 7A, paper I), **paper II** was initiated to evaluate lactobacilli resistance towards beer-related stress factors. Three strains of Lactobacillus (*L. buchneri*, *L. plantarum* WildBrew™ Sour Pitch, hereafter referred to as *L. plantarum* and *L. brevis* BSO464, hereafter referred to as *L. brevis*) were exposed to different stress factors. This was done both to evaluate their general resistance towards the harsh beer environment, but also to investigate if and how production of flavour active metabolites was affected. *L. brevis* was the most robust strain, displaying the same growth pattern and obtaining the same final pH (3.7-3.8) in wort, regardless of stress factors (Fig. 1A, paper II). *L. plantarum* generated the lowest final pH at the reference trial (pH 3.2), but growth and ability to reduce pH in response to multiple stressors (especially iso-α acids) suggested high vulnerability to stress (Fig. 1B, paper II).

The results suggested iso-α acids as the most influential stress factor. Lactobacilli are sensitive to iso-α acids acting as ionophores, dissipating the transmembrane proton gradient and impairing the cell metabolism (Simpson, 1993, Ye et al., 1994, Simpson and Smith, 1992). A multi-stress trial was carried out where the lactobacilli fermented wort in presence of 5 mg/L iso-α acids, 5 % ethanol and low initial pH 4. Both *L. plantarum* and *L. buchneri* were severely inhibited in the multi-stress trial. This clearly demonstrated how the hurdle effect, where multiple low-level hurdles, contribute to a substantial inhibition of bacterial metabolism (Leistner, 2000). Iso-α acids affect microbial cells synergistically with low pH (Simpson and Hammond, 1991, Suzuki, 2011), and increased inhibition was therefore expected. The greater robustness observed for *L. brevis* was also expected, as this strain has a number of genes associated with resistance towards iso-α acids and is known to be able to grow in beer (Bergsveinson et al., 2015, Bergsveinson et al., 2016). Strain, environmental stress, and interactions between strain and environmental stress were all found important with respect to metabolite formation (Fig. 2, paper II).

L. brevis and *L. plantarum* protruded as the most and least stress tolerant strains, respectively, and were selected for upscaled beer production (22°C, 21 days). *L. brevis* generated the largest pH-drop, with a final pH of 3.6, compared to 3.8 for *L. plantarum* and 4.1 in the reference beer (Figure 3B, paper II). Ethanol was produced all throughout the different fermentations (Fig. 4D, paper II), reaching final concentrations of 4.2% (v/v) in the reference, 4.1% in the *L. plantarum* beer and 3.8 % for *L. brevis* beer. It could be argued that the minor reduction in ethanol production by yeast in the *L. brevis* beer was merely a result of reduced fermentation pH, as the final pH was lower in the *L. brevis* co-fermentation. However, in a study by Alcine Chan et al. (2019) looking at co-fermentation with *L. paracasei* L26 and *S. cerevisiae* S-04, no significant

effect from bacterial co-fermentation on ethanol production was found, even though the pH in that study was correspondingly low (final pH in co-fermentation 3.62). Resistance towards acidic pH is highly strain-dependent in yeasts (Rogers et al., 2016), and there could be differences in this regard between *S. cerevisiae* S-04 and S-05. However, no pH-effect by lactic acid alone was found on production of ethanol in paper I on *S. cerevisiae* S-05 (beer C, figure 6C, paper I). This points to an interfering effect on yeast fermentation performance, specific to the used *L. brevis* strain. The results in the current study also illustrate how important choice of strain can be, in order to obtain wanted properties in the final product. Although, *L. brevis* generated higher quantities of organic acids (Table S3, paper I), and represented the better choice if low pH/high organic acid content in beer was wanted, this strain also impaired the yeast metabolism in some way, resulting in a beer where fermentable sugars in the wort was exploited to a lower extent (more residual maltose and maltotriose compared to the reference and *L. plantarum* beers, Table 1, paper II).

The metabolite composition of the final beers was analysed, and the beers were evaluated sensorially. *L brevis* was more influential with respect to metabolites compared to *L. plantarum*. This was evident from variation in organic acids and volatile compounds in the finished beers (Fig. 5A and B, paper II), where the *L. brevis* beer was separated from the reference beer in both components 1 and 2, while the *L. plantarum* beer only in component 2. The highest concentration of organic acids was obtained in the *L. brevis* beer (2598±56 mg/L lactic, 942±11 mg/L acetic acid and 196±14 mg/L succinic acid) (Table S3, paper II). Substantial quantities of organic acid were also obtained in the *L. plantarum* beer as well (1791±94 mg/L of lactic and 89±26 mg/L acetic acid), while only a low amount of acetic acid (31±4 mg/L acetic acid) was generated in the reference beer. Lactic acid is associated with acidity and sourness (Van Oevelen et al., 1976) and has a reported taste threshold 400 mg/L (Engan, 1974). Acetic acid is associated with acidity, sour (Engan, 1974) and vinegary flavours (Van Oevelen et al., 1976) and has a reported sensory threshold of 200 mg/L (Engan, 1974). Both lactic and acetic acids were well above reported sensory thresholds in the *L. brevis* beer, which corresponds well with this beer being perceived as significantly higher than the *L. plantarum* and the reference beers in acidic taste in the sensory analysis (Fig. 5C, Table S4, paper II). The *L. brevis* beer was also scored as significantly higher in astringency compared to the reference and *L. plantarum* beers, corresponding well with the higher organic acid concentrations, as astringency is partly related to organic acid content (Da Conceicao Neta et al., 2007), and higher perception of astringency is correlated with decreasing pH (Lawless et al., 1996). When compared to a commercial sour beer product (Geuze, Mariage Parfait, 2015, Boon Brewery, Belgium), the *L. brevis* beer received similar scores to this product in both acidic taste and astringency (Table S5, paper II)

Even though the effect by *L. plantarum* while co-fermenting with *S. cerevisiae* was less pronounced with respect to metabolite composition, the *L. plantarum* presence had a substantial effect to the sensory

properties. Examples of this include a significant increase in intensity of fruity odour and dried fruit odour (Fig. 5C, Table S4, paper II). This corresponded with the *L. plantarum* beer being higher in the fruity esters ethyl hexanoate and ethyl octanoate (Table S3). Ethyl hexanoate is associated with fruit, fennel and solvent flavours (Xu et al., 2017) and has a sensory threshold in beer of 0.3 mg/L (Harrison, 1970). Ethyl octanoate is associated with sweet and fruity flavours (Yonezawa and Fushiki, 2002), and a sensory threshold of 0.9-1.0 mg/L in beer (Pires and Bránayik, 2015). At 0.11±0.01 mg/L ethyl hexanoate and 0.03 mg/ ethyl octanoate in the *L. plantarum* beer (Table S3), both esters were below the sensory threshold. Their presence could, however, be influential to the sensory properties through synergistic, sub-threshold effects (Dalton et al., 2000). The sensory influence by the different lactobacilli was different, as the *L. brevis* and *L. plantarum* beers were perceived as significantly different from each other in multiple sensory attributes (sour odour, fruity odour, perfumed odour, yeasty odour, sweet taste, acidic taste, astringency) (Fig. 5C, Table S4).

Paper III - Secondary lactic acid bacteria fermentation with wood-derived xylooligosaccharides as a tool to expedite sour beer production

Due to the superior tolerance towards beer-related stress factors displayed by *L. brevis* BSO 464, both previously (Bergsveinson et al., 2016) and in paper II, this strain represented a promising candidate for a new attempt at the failed secondary fermentation approach (Fig. 1, Beer F, paper I) from paper I. In order to promote rapid secondary fermentation in beer, which is low in readily available carbon substrate, a secondary substrate was added to beer. To ensure specific promotion of the *L. brevis* fermentation, the substrate had to be non-degradable by yeast. Wood-derived xylooligosaccharides (XOS) were chosen for this purpose, both to promote *L. brevis* fermentation, and as an attempt at demonstrating how hemicellulosic carbohydrates can be utilized directly in food products through fermentation.

In **paper III**, XOS were extracted from birch (*Betula pubescens*) and added to beer, which was inoculated with *Lactobacillus brevis* BSO 464. Growth, pH, XOS degradation and metabolic products were monitored throughout fermentations (2-4 weeks), and the final beer was evaluated sensorially. Two XOS preparations were tested, XP1 and 2 (both described in Fig. 1). XP1 was prepared through enzymatic digestion followed by alkaline deacetylation, while XP2 was prepared through alkaline deacetylation followed by enzymatic digestion. XP2 proved the better choice for promoting LAB secondary fermentation, as it contained larger quantities of short oligosaccharides (Fig. 1, paper III), and generated lower final pH (Fig. 2B compared to figure 3B, paper III) in fermentation experiments. XP2 promoted *L. brevis* growth in beer (Fig. 3A, paper III), pH reduction from above pH 4 to final pH 3.3-3.4 depending on the XP2 dosage (Fig. 3B, paper III). Simultaneously to this, xylobiose provided in XP2 was depleted (Fig. 3C), and lactic and acetic acids were produced (Fig. 3D, paper III) along with other metabolites (Fig. 3E and F, paper III). *L. brevis* generated lactic and acetic acids in the negative control (beer without additional substrate) as well (560 mg/L and 360 mg/L, respectively), but the quantities after 14 days of secondary fermentation at 25°C were substantially higher with 0.5% XP2 (2280 mg/L and 1740 mg/L, respectively) and even higher with 2% XP2 (3940 mg/L and 2930 mg/L, respectively).

XP2 also supported *L. brevis* fermentation in larger scale (5L) secondary fermentations in beer brewed with higher hop dosage (estimated iso-α content of 10 mg/L, compared to 5 mg/L in the experiment described above). During 28 days of incubation at 25°C, a pH reduction from 4.1 to 3.6 (Fig. 4B, paper III) occurred concurrently with depletion of xylobiose (Fig. 4C, paper III) and production of lactic and acetic acids (Fig. 4D, paper III). The final composition of metabolites in the produced XOS sour beer, the non-sour base beer and a commercial sour beer reference (Geuze, Mariage Parfait, 2015, Boon Brewery, Belgium) were analysed, and the three beers were subjected to sensory analysis. Comparing the metabolites in the XOS sour beer and the base beer, XOS promoted *L. brevis* fermentation influenced the composition significantly

(Fig. 5A, paper III). Substantial differences were observable in metabolites such as lactic and acetic and acid (Fig. 5C, paper III). At 1750 mg/L and 1100 mg/L, both lactic and acetic acids, respectively, were well above their respective sensory detection thresholds (Table S1, paper III) and likely influential to sensory properties. This complies with this beer being perceived as higher in sour odour, sour flavour and acidic taste.

In the sensory analysis, 15 out of 22 sensory attributes were scored significantly different between the XOS sour beer and the base beer (Fig. 6A, paper III), proving a substantial sensory impact from XOS promoted *L. brevis* secondary fermentation. The beer increases in complexity by going through the secondary process as most of the attributes, including total intensity odour and flavour, and perfumed odour and flavour, increase in intensity. Some of the changes in these sensory properties might be brought about by the addition of the secondary substrate itself. It is however clear, looking at the metabolic data, that the XOS-induced secondary fermentation by *L. brevis* causes multiple significant shifts in the concentration of metabolites known to have sensory relevance (Fig 5C-F, paper III).

The commercial sour beer reference (Boon Geuze) was different from both the XOS sour beer and the base beer, with respect to metabolite composition (Fig. 5, paper III), properties such as alcohol, apparent degree of fermentation (ADF), colour (Table 2, paper III) and sensory properties (Fig. 6B, Table S3, paper III). The commercial sour beer had a different organic acid composition to that of the XOS sour beer, containing more than 5000 mg/L lactic acid and 700 mg/L acetic acid. Despite the vastly higher content of lactic acid in the commercial sour beer, the XOS sour beer and the commercial sour beer were perceived with similar intensity in acidic taste. Lactic, acetic and succinic acids are all associated with acidic taste. The higher concentrations of succinic and acetic acids (Fig. 5C, paper III) in the XOS sour beer might compensate for the lower lactic acid, with respect to the acidic taste. Differences in characteristics and sensory attributes were expected between the commercial sour beer and the XOS sour beer, as the commercial sour beer is produced through a completely different process and based on a different recipe. While the XOS sour beer resulted from secondary fermentation by a single *L. brevis* strain, the commercial sour beer reference was produced through a traditional Lambic/Geuze fermentation by a complex microbial consortium. The active fermentation time in the XOS sour beer production was less than 2 months, compared to multiple years for the commercial sour beer reference. However, the XOS induced secondary *L. brevis* fermentation generated a sour beer assessed as similar to the commercial sour beer in multiple sensory attributes, including dried fruit odour, total flavour intensity, acidic taste and astringency (Fig. 6B, table S3, paper III). It is also noteworthy, that for all the sensory attributes where significant differences were found between the three beers, the intensity for the XOS sour beer was scored closer than the base beer, to the commercial sour beer reference. The objective of the current study was not to produce a beer with identical properties and sensory

qualities to a commercial sour beer, but to investigate an alternative production process for sour beers. The descriptive sensory analysis showed that it is possible to significantly alter the sensory characteristics of beer by implementing a substrate-induced secondary fermentation with *L. brevis*. The results also showed that multiple sensory attributes were shifted in a direction making the beer characteristics more comparable to those of a traditionally produced commercial sour beer. The study represents a starting point, from which further work on complex sour beer production can originate.

Common discussion

All three methods investigated in the current PhD project represent feasible approaches for sour beer production. In paper I, a final pH in the range of 3.5 to 3.7 was obtained in beers produced through biological (A and B) or chemical (C and D) acidification and 4.0 for the reference (beer E) (Table 3, paper 1). This was below the pH limit to qualify as sour beer according to the suggested definition by Tonsmeire (2014) of pH > 3.9, but in the upper range of the pH values (3.2-3.6) found for commercial sour beers like Lambics (Thompson Witrick et al., 2017). The lactic acid quantities generated by *L. buchneri* pre-fermentation were substantially lower compared to the commercial Lambics, at ~1000 mg/L in beers A and B (Fig. 5A, paper I) compared to ~3700 mg/L to ~17 500 in commercial Lambics (Thompson Witrick et al., 2017). It is possible to obtain higher quantities through pre-fermentation with LAB in sour beer production. In a study by Peyer et al. (2017), more than 5000 mg/L lactic acid was obtained in sour beer produced through biological acidification of wort prior to yeast addition. In the study by Peyer et al. (2017), 10^7 CFU/mL *L. amylovorus* FST2.11 was incubated at 40°C, and the higher initial CFU/mL (10^6 CFU/mL in paper I), the higher fermentation temperature (18°C in paper I) and the different choice of *Lactobacillus* strain probably all contributed to the higher production of lactic acid.

The sour beers produced through co-fermentation in paper II were also in the upper range of those found by Thompson Witrick et al. (2017), at 3.6 for the *L. brevis* beer and 3.8 in the *L. plantarum* beer (Fig. 4C, paper II). Although, the lactic acid quantities generated during co-fermentation (~1800-2600 mg/L, Table S3, paper II) were higher compared to the pre-fermentation approach, these quantities were still lower compared to quantities in commercial Lambics. However, optimisation of the process may result in even higher lactic acid production, as can be seen below in paper III and in the study by Alcine Chan et al. (2019) in which they obtained more than 5000 mg/L lactic acid after 10 days co-fermentation with *L. paracasei* L26 and *S. cerevisiae* S-04. The co-fermentation was carried out in unhopped wort, and this might have contributed favourably to the lactic acid production. Higher temperature (30°C the first two days of fermentation) might also have played a role in this, along with strain selection.

The XOS induced secondary fermentation approach described in paper III, resulted in the highest quantities of lactic acid obtained in all experiments in papers I-III. In the small-scale experiment with high substrate dosage and in beer with low hopping, a final pH of 3.3 (Fig. 3B, paper III) and more than 3900 mg/L lactic acid was generated during 14 days of fermentation (Fig. 3D, paper III). Lactic acid production was lower with reduced XOS dosage in beer with higher hopping, 1750 mg/L in the larger scale fermentations (Fig 5C, paper III). Lower hopping and higher substrate dosage could result in higher lactic acid production also at larger scale, although prolonged fermentation time might also have an effect, as a bit of residual xylobiose was present at the end of secondary fermentation (Fig. 4C, paper III).

Concluding remarks and future perspectives

Pre-fermentation, co-fermentation and secondary fermentation with *Lactobacillus* and yeast, can all be used to produce sour beers within a short time frame (≤ 4 weeks of fermentation) compared to the traditional spontaneous fermentations used for Lambic production (typically 1-3 years). All three approaches resulted in beer products with reduced pH (3.3- 3.8), increased lactic acid concentrations (~1000-3900 mg/L) and increased intensity of the sensory attribute acidic taste.

The secondary fermentation approach is perhaps the superior method, if a sensory perception similar to that of a traditional Lambic product is wanted. Secondary fermentation with LAB, is also closer to what occurs in the spontaneous successive fermentation, where the main fermentation is followed by an acidification phase. In order to carry out this approach, specific strain properties are however required, as the bacterial strain needs to be robust towards beer related stress factors. Wood-derived XOS were used to promote *L. brevis* secondary fermentation of beer in paper III. Other types of non-food carbohydrate sources can also be used in similar approaches. Secondary fermentation induced by hemicellulose derived substrate could for instance pose an alternative for improved utilization of raw materials in the brewing industry. Brewer's spent grain is a by-product from the brewing industry that currently is simply disposed of or used as animal feed (Reis et al., 2015). Arabinoxylan constitutes 28 % of the dry weight of Brewer's spent grain (Mussatto and Roberto, 2005), and through proper pre-processing this can be used to generate oligos that are fermentable by lactic acid bacteria (Sajib et al., 2018). Further research within bioprocessing of hemicellulosic biomass for specific substrate preparation, and the use of these with suitable fermenting microorganisms, could offer utilization of non-food raw materials in food or beverage production and at the same time increase fermentation rates and improve process control. This approach could for instance be useful in water kefir, where high alcohol production by yeast can be problematic, and high CFU/mL of probiotic LAB is desired.

Great emphasis should be put on strain selection in conjunction with processing steps, when working with a limited number of microorganisms for sour beer production. Different microbial strains have different potential for fermentation performance and flavour generation, and they can respond differently to various fermentations conditions. The three studies constituting the current PhD project focus on one strain of *S. cerevisiae* in combinations with lactobacilli. The results represent a contribution to unveiling the potential for sour beer production, through controlled mixed fermentations with pure cultures. Further studies, including other and/or additional microorganisms (e.g. *Pediococcus* and/or *Brettanomyces*), could facilitate production of sour beer with flavour complexity even more comparable to that of traditional products. The studies included in the current PhD thesis represent a starting point, from which this further work can originate.

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Pre-fermentation with lactic acid bacteria in sour beer production

Anna Dysvik,[1]* Kristian Hovde Liland,[2] Kristine S. Myhrer,[3] Bjørge Westereng,[1] Elling-Olav Rukke,[1] Gert de Rouck[4] and Trude Wicklund[1]

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 the increasing interest in sour beer 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) was investigated as a timesaving and 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 performed to evaluate the sensory contribution from LAB. A significant effect was observed from LAB pre-fermentation, with respect to both volatile compounds (e.g. reduced 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 wort 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 Table 1. *Saccharomyces pastorianus* and *Saccharomyces cerevisiae,* 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 is accompanied by non-*Saccharomyces* yeast strains as well as various bacterial species *(13–15)*. Belgian brewing culture is famous for its long tradition of sour beer production. Most of the well known classic styles 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 airborne microorganisms come into contact with the wort, before it is transferred to wooden barrels used in previous fermentations. The remaining microbes 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 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 final beer character include *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 pure culture fermented ale or lager. A commercial ale or lager can be produced 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 rate, as does the progressively inhospitable growth environment. Ethanol, pH, carbon dioxide, substrate deprivation and inter-microbial competition for nutrients all contribute to reduced microbial growth and metabolism *(20)*. Commercial sour beer production can be challenging owing to the length of time required, as long term storage of barrels takes up space and reduces beer output. The 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.

* Correspondence to: Anna Dysvik, Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O.Box 5003, N-1432 Aas, Norway. E-mail: anna.dysvik@nmbu.no

[1] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, N-1433, Aas, Norway

[2] Faculty of Science and Technology, Norwegian University of Life Sciences, P.O. Box 5003, N-1433, Ås, Norway

[3] NOFIMA – Norwegian Institute of Food, Fisheries and Aquaculture Research, PB210, N-1431, Ås, Norway

[4] Faculty of Engineering Technology, KU Leuven Technology campus Gent, Gebroeders De Smetstraat 1, B9000, Ghent, Belgium

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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: position here horizontally
[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 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 is killed by boiling the wort before brewers' yeast is added and ethanol fermentation begins *(22)*. *Lactobacilli* are useful in pre-fermentation 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 fermentation, the ethanol and hop hurdles are circumvented, and sour beer can be produced in a 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 for sour beer production *(22)*, little research on this method can be found. The sensory contribution from *Lactobacillus* in beer produced through this two-step fermentation process seems to be partly unresolved. 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 that contribute 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). 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 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 work was originally isolated from silage grass *(28)* and 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

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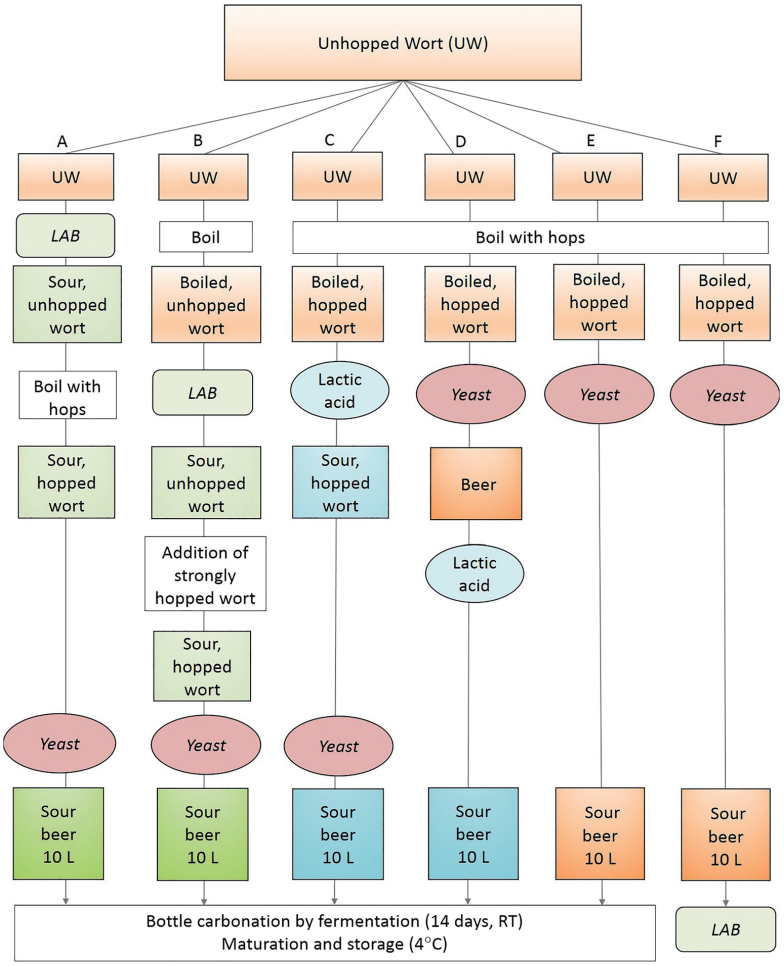

Figure 1. Experimental setup for production of beers A–F. LAB, lactic acid bacteria and RT, room temperature. [Colour figure can be viewed at wileyonlinelibrary.com]

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 sections.

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, pH fell from about 5.7 to 4.1. 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

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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. However, the LAB strain was unable to survive in the beer, and as no further development occurred, 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 stored at 4°C for maturation. The pH in all beers were monitored through the process using a PHM92 lab pH meter (Radiometer, Copenhagen, Denmark). Beer production was in triplicate. Samples (50 mL) were drawn from all beers throughout the production process and stored at −20°C until analysis of metabolic compounds. 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 - volatiles compounds Volatile compounds were analysed by headspace gas chromatography (HSGC) according to the method described by Grønnevik *et al. (29)*. The samples were filtered using grade 602h½ folding filters (pore size <2 μm, Schleicher & Schuell, Dassel, Germany) at 4°C to remove carbon dioxide. 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 10.0 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, on a 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

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

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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 refractive index detector (RI-detector). 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 (Anton Paar, Graz, Austria, 2014). Matured beer was sampled directly from bottles. Extract, ethanol, carbon 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, 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

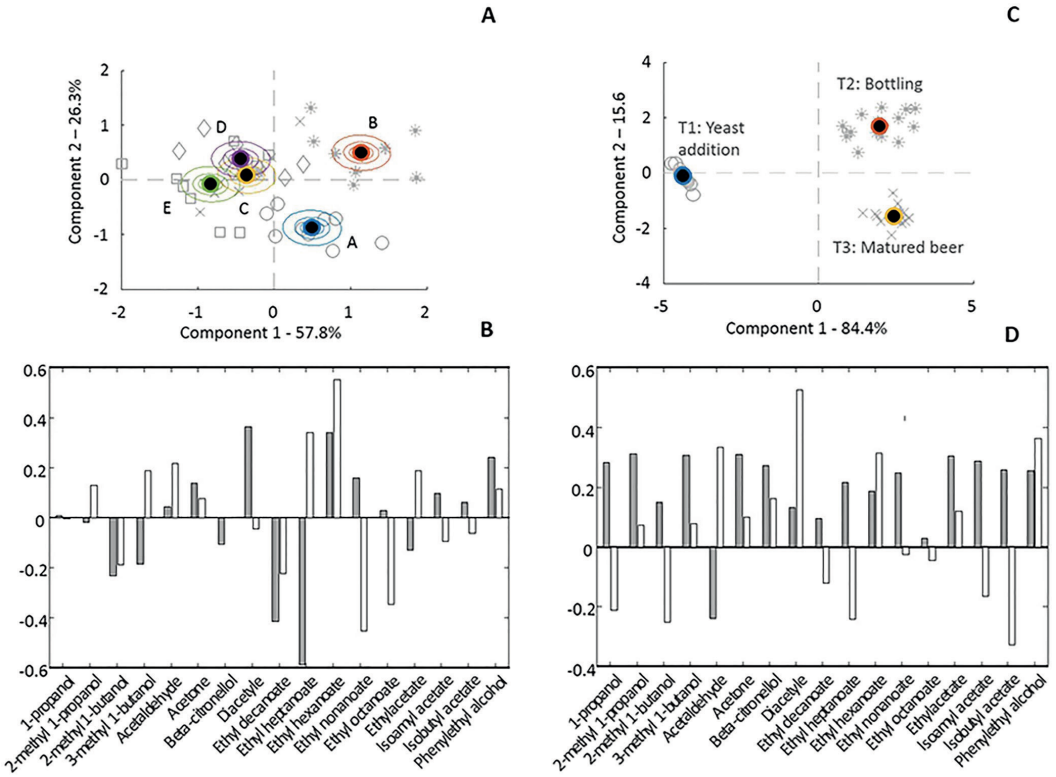

Figure 2. Variation in volatile compounds related to brewing method and time. (a) Variation in samples owing to brewing method (between the five beers A–E) and replicate variation, described by analysis of variance simultaneous component analysis (ASCA) scores. Brewing method explains 5.3% of the variation 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]

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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 were performed 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 taste, 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 brainstorming session where the assessors generated relevant words for the selected beer products. Before profiling, one session was used to train the assessors in the 21 selected odour, flavour, taste and texture attributes (Table 2), and agree on the consensus list for profiling and on the definition of each attribute.

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 any transfer of yeast sediment from the bottles. A 300 mL 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. Unsalted crackers were available together with warm or cold water for rinsing between samples. 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).

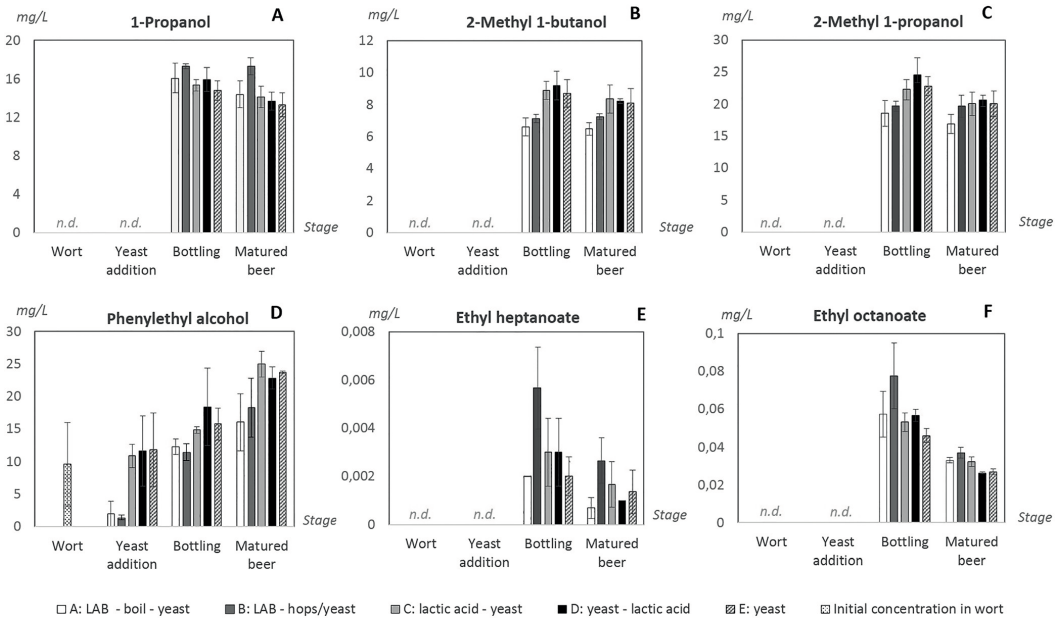

Figure 3. Volatile compounds with different development ($p < 0.01$) related to brewing method. The concentration at the sampling times (T0, wort; T1, yeast addition; T2, bottling; T3, matured beer) for each beer (A–E) is given with standard deviation as error bars. Non-detected values are indicated 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.

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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 (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. ASCA score plots 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 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 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 (HSD). 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 $p < 0.01$.

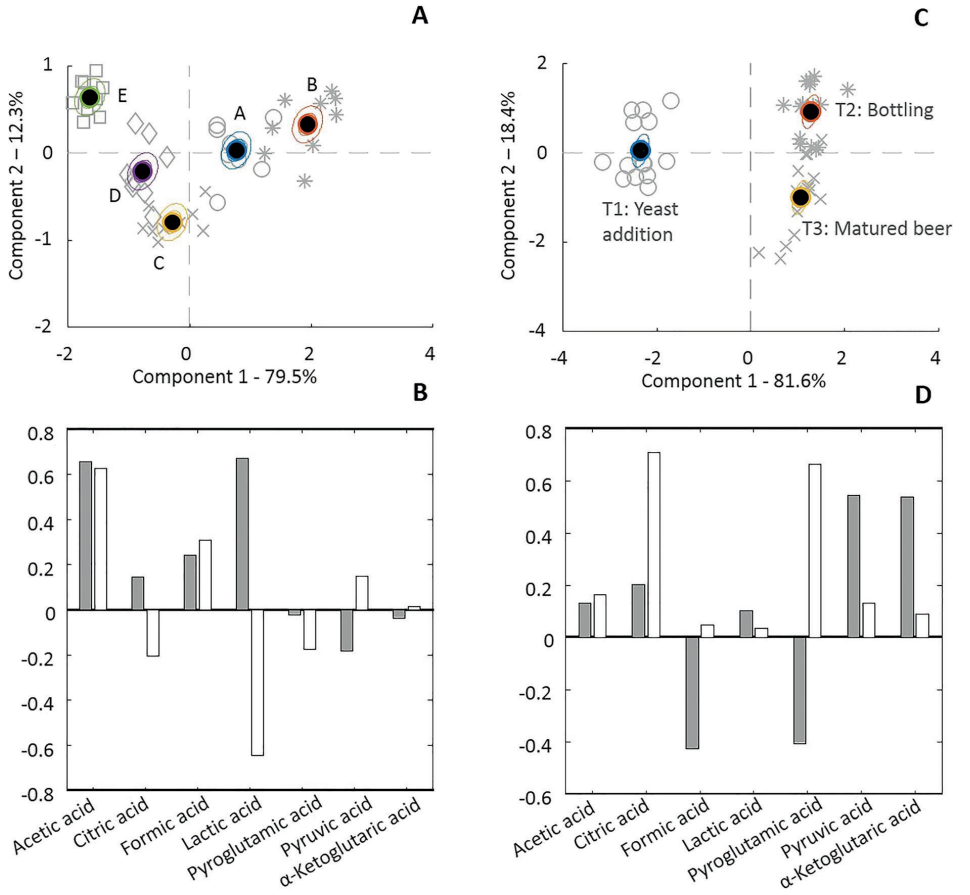

Figure 4. Variation in organic acids related to brewing method and time. (a) Variation in samples owing to brewing method (between the five beers A–E) and replicate variation, described by ASCA scores. Brewing method explains 28.4% of the variation in organic acids. (b) Loadings for ASCA model in (a). 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]

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Results and discussion

Metabolic compounds

The ASCA score plot in Fig. 2a displays variation in volatile compounds that can be attributed to the brewing method which explains 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. Of these, beers C–E are not significantly different from each other based on volatiles, suggesting no substantial influence from the presence of lactic acid 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 time. 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 and 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. However, the brewing method is, 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-methyl 1-butanol (Fig. 3c) and phenylethyl alcohol (Fig. 3d). Two were esters: ethyl heptanoate (Fig. 3e) and ethyl octanoate (Fig. 3f). The effect from LAB pre-fermentation was most pronounced with 2-methyl-1-butanol, 2-methyl-1-propanol and phenylethyl alcohol, which were lower in beers A and B, compared with beers C–E. The concentration 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 concentration 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

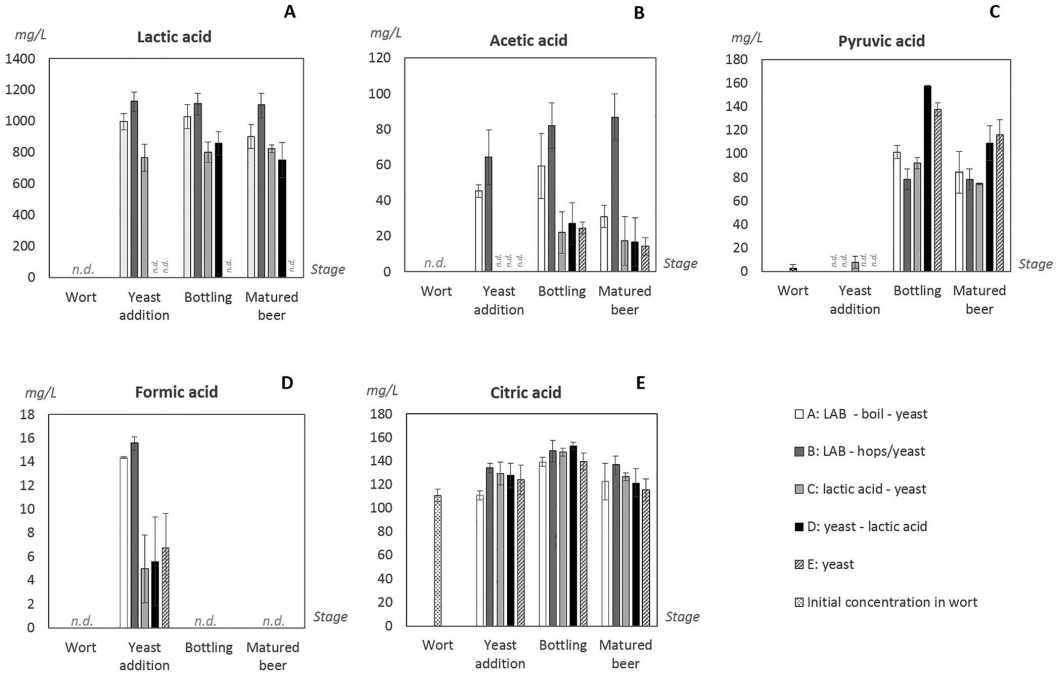

Figure 5. Organic acids with different development ($p < 0.01$) related to brewing method. The concentration at the sampling times (T0, wort; T1, yeast addition; T2, bottling; T3, matured beer) for each beer (A–E) is given with standard deviation as error bars. Non-detected values are indicated with '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.

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it difficult to pinpoint a sensory impact. Their presence could, however, influence overall sensory perception, as multiple compounds below threshold can influence flavour through synergistic effects *(32)*. Indeed, 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.

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 the 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. The time factor 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), 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 with yeast addition, bottling and matured beer stages compared with beers C–E. Lactic acid was 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 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.

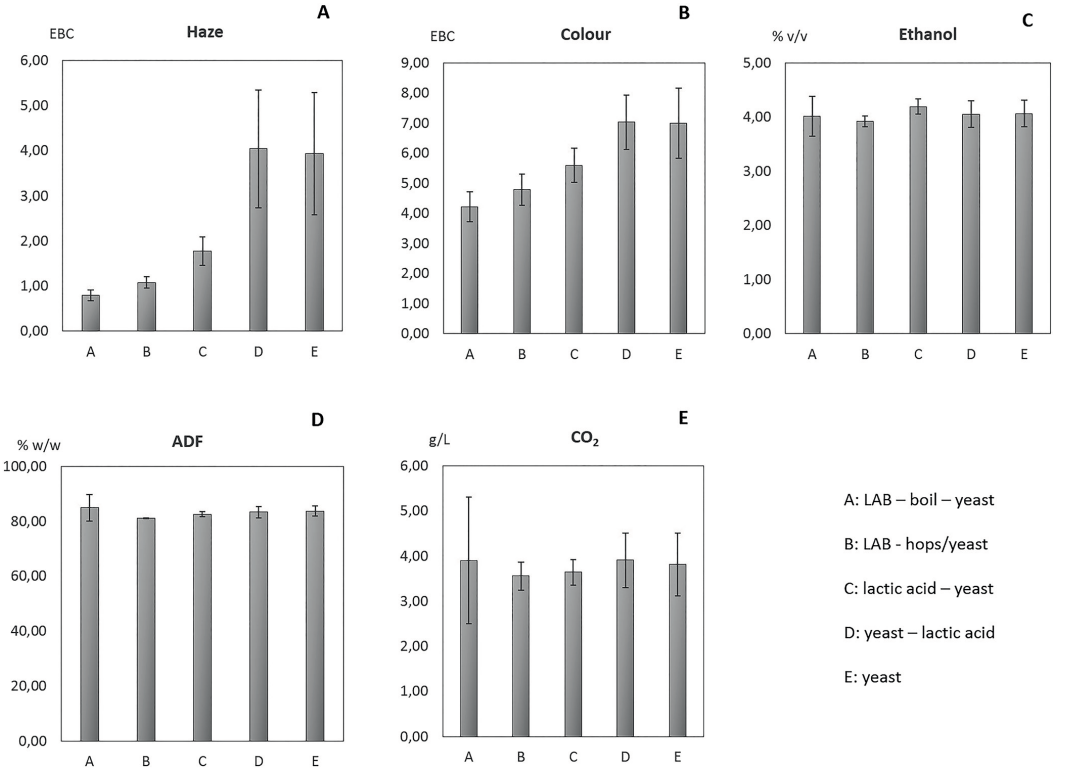

Figure 6. Properties for beers A–E. (a) Haze; (b) colour; (c) ethanol; (d) apparent degree of fermentation (ADF) and (e) carbon dioxide (CO_2).

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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.

Beer B was highest for all organic acids at the mature beer stage, except for pyruvic acid. A pronounced difference was observed for acetic acid, as a 3-4 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, which are both associated with fruity notes. The largest contribution from LAB was found with method B, where LAB is present longer. LAB is important for the flavour properties of other fermented alcoholic beverages, such as wine *(33)* and whiskey *(34)*. LAB is also important 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 in the beers for 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 (Fig. 6B), with lower levels for beers A–C 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 a lower pH. Yeast flocculation is important to obtain clear beer *(39)* and pH affects the flocculation behaviour of yeast *(40)*. Lower pH was associated with increasing flocculation in a study by Rogers *et al. (41)*.

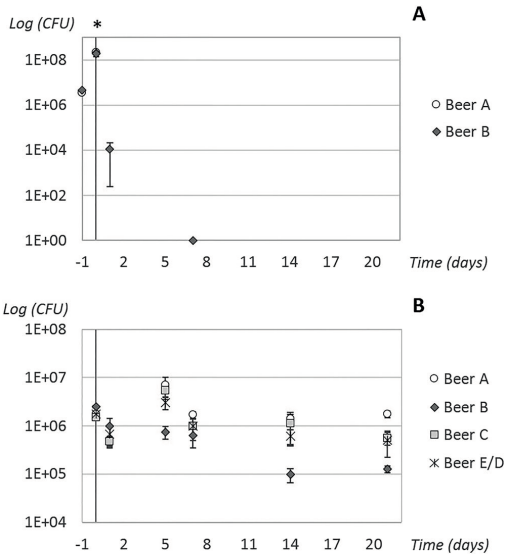

Figure 7. Microbial growth during three week fermentation of beers A–C and D/E. (a) LAB growth in beers A and B. (b) Yeast growth in beers A–C and D/E. Note that the range of the *y*-axis is $1–10^8$ in (a) and $10^4–10^8$ in (b). * Beer A, boiling with hops and yeast addition; beer B, addition of strongly hopped wort and yeast.

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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 affect.

No significant differences were detected between the beers with regard to ethanol, apparent degree of fermentation (ADF) or CO_2 concentration. The 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 influence the production 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 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 *(41)*.

Changes in pH 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 *ca.* 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 after secondary conditioning. The initial wort pH for beers D and E was 5.8. After three weeks of yeast re-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 in the small scale fermentations is shown 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 subsequent sampling points. LAB fermentation was 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 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, along with inhibitory effects from 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

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).

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the loss of LAB viability in beer B in the current study was caused by depletion of key amino acids, competition for nutrients, reduced pH, increasing ethanol or the introduction of hops. In all likelihood, all of these factors influence 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 is 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 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 robust with reduced pH and/or increased lactic acid concentrations. The growth pattern for beer B diverged from the others, as no increase in CFU/mL was observed, and about a 10-fold lower CFU/mL compared with beers A, C and D/E was observed two and three weeks into 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 monitored 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 given in Table 4, and spider plot profiles based on these are displayed in Fig. 8. Five of 21 assessed attributes scored

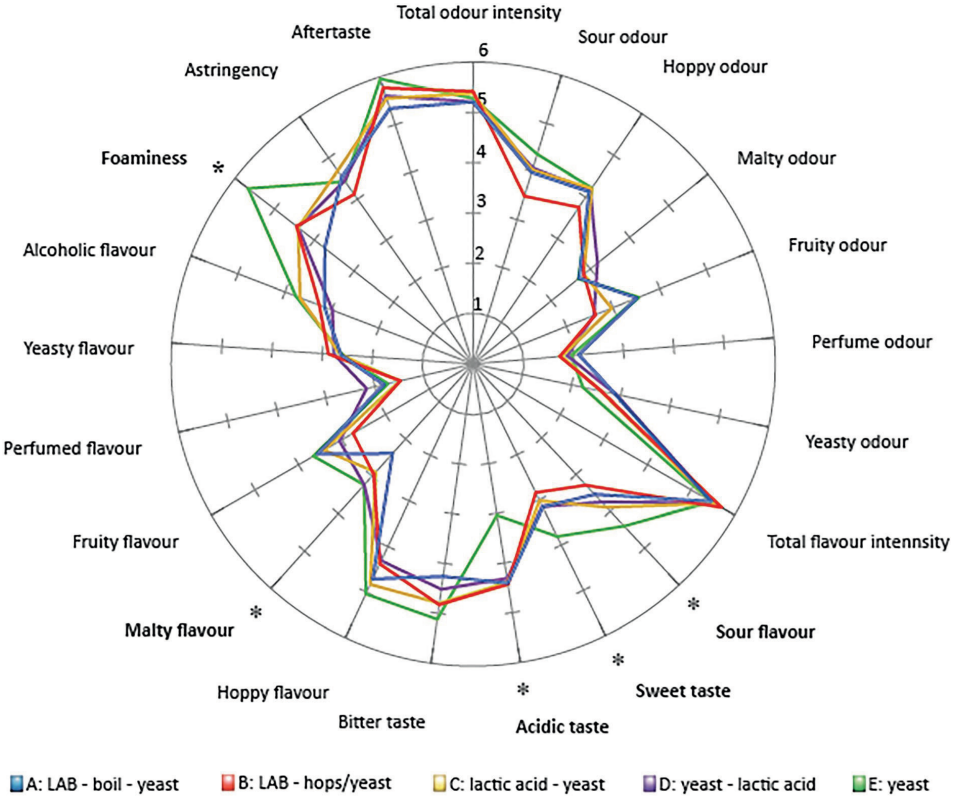

Figure 8. Average scores for sensory attributes in the descriptive analysis. Attributes scored significantly differently ($p > 0.05$) for two or more beers are marked by * and indicated in bold. The intensity of the attributes was scored on a non-structured continuous scale where the endpoints corresponded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

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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 work, 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 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 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. 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 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. 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, the sensory impact from this seems very slight. A potential drawback to the current study is the final compound analysis being carried out three months before the descriptive sensory analyses. However, this is considered to have only marginal effects

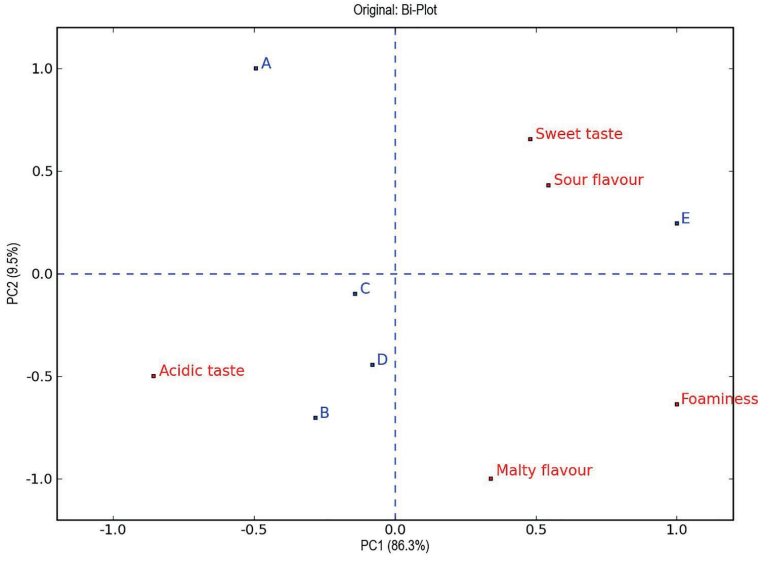

Figure 9. PCA bi-plot based on attributes with significantly different levels ($p < 0.05$) with beers as loadings and attributes as scores. PC1 explains 86.3% and PC2 explains 9.5% of the variation in the sample set [Colour figure can be viewed at wileyonlinelibrary.com]

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on the 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, as an alternative, non-spontaneous production techniques. Osburn *et al. (50)* reported 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 this *(50)*, there is to the best of our knowledge, no published work looking at alternative approaches to sour beer production. Extensive work has been reported 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 *(50)*, but to date no papers have been published where the contribution from LAB 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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Paper II

Co-fermentation of *Saccharomyces cerevisiae* with *Lactobacillus* strains tolerant to brewing-related stress factors for controlled and rapid production of sour beer

Anna Dysvik[1*], Sabina Leanti La Rosa[1], Kristian Hovde Liland[2], Kristine S. Myhrer[3], Hilde Marit Østlie[1], Gert de Rouck[4], Elling-Olav Rukke[1], Bjørge Westereng[1] and Trude Wicklund[1]

[1]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O.Box 5003, N-1432 Aas, Norway

[2]Faculty of Science and Technology, Norwegian University of Life Sciences, P.O. Box 5003, N-1433 Ås, Norway

[3]NOFIMA – Norwegian Institute of Food, Fisheries and Aquaculture Research, PB 210, N-1431 Ås, Norway

[4]Faculty of Engineering Technology, KU Leuven Technology campus Gent, Gebroeders De Smetstraat 1, B9000, Ghent, Belgium

* Corresponding authors, anna.dysvik@nmbu.no

Abstract

Increasing popularity of sour beer urges the development of novel solutions for controlled fermentations both for fast acidification and consistency in product flavour and quality. One possible approach is the use of *Saccharomyces cerevisiae* in co-fermentation with *Lactobacillus* strains, which produce lactic acid as a major end-product of carbohydrate catabolism. The ability of lactobacilli to ferment beer is determined by their capacity to sustain brewing-related stresses, including hop iso-α acids, low pH and ethanol. Here, we evaluated the tolerance of *Lactobacillus brevis* BSO464 and *Lactobacillus buchneri* CD034 to beer conditions and different fermentation strategies as well as their use in the brewing process in mixed fermentation with a brewer's yeast, *S. cerevisiae* US-05. Results were compared with those obtained with a commercial *Lactobacillus plantarum* (WildBrew™ Sour Pitch), a strain commonly used for kettle souring. In pure cultures, the three strains showed varying susceptibility to stresses, with *L. brevis* being the most resistant and *L. plantarum* displaying the lowest stress tolerance. During a 21-day co-fermentation with *S. cerevisiae*, *L. plantarum* and *L. brevis* were able to generate sour beer, and their presence positively influenced the composition of flavour-active compounds. Both sour beers were sensorially different from each other and from a reference beer fermented by *S. cerevisiae* alone. While the beer produced with *L. plantarum* had an increased intensity in fruity odour and dried fruit odour, the *L. brevis* beer had a higher total flavour intensity, acidic taste and astringency. Remarkably, the beer generated with *L. brevis* was perceived as comparable to a commercial sour beer in multiple sensory attributes. Taken together, this study demonstrates the feasibility of using *L. brevis* BSO464 and *L. plantarum* in co-fermentation with *S. cerevisiae* for controlled sour beer production with shortened production time.

Introduction.

Lactic acid bacteria (LAB) are gram-positive, non-sporulating bacteria with lactic acid as their main product of carbohydrate metabolism. LAB include homofermentative members, converting hexose sugars almost exclusively to lactic acid, and heterofermentative species fermenting hexose sugars to lactic acid, CO_2 and ethanol or acetic acid (von Wright and Axelsson, 2019). *Lactobacillus* is a genus within the LAB group with metabolism that is either obligate homofermentative (e.g. *L. acidophilus* and *L. delbrueckii*), obligate heterofermentative (*L. brevis* and *L. buchneri*) or facultative heterofermentative (*L. plantarum* and *L. sakei*) (Ibrahim and Ouwehand, 2019). Lactobacilli are frequently associated with food and beverages produced through mixed fermentations where both bacteria and yeast are involved. Examples of such products include wine (Wang et al., 2018, Mtshali et al., 2012), kefir (Guzel-Seydim et al., 2011), sake (Tsuji et al., 2018), sour dough bread (Ripari et al., 2016, Minervini et al., 2014) and beer (Vriesekoop et al., 2012).

Beer with intentional acidic taste, referred to as sour beer, is traditionally fermented as a spontaneous process where wort is inoculated by environmental exposure rather than active microbial inoculation (Van Oevelen et al., 1977, Verachtert and Derdelinckx, 2014). The traditional production methods for sour beer, such as lambic and geuze beers, originate from Belgium and are still in use today. The complex, multi-microbial fermentations that progress through these methods entail huge time investments, up to 3 years, and are challenging to control (Verachtert and Derdelinckx, 2014, Van Oevelen et al., 1977). Due to the difficulties associated with traditional sour beer production, and due to the increasing popularity of sour beer in recent decades, alternative production methods are being explored (Alcine Chan et al., 2019, Peyer et al., 2017, Osburn et al., 2018). During sour beer fermentation, yeasts generate ethanol and other metabolic products in the same manner they would in ale or lager fermentations. The presence of acid producing bacteria results in beer products with higher content of organic acids and reduced pH compared to other non-sour beers (Van Oevelen et al., 1977, Van Oevelen et al., 1976). Lactic and acetic acid constitute the most pronounced contribution from lactobacilli to the sensory properties of sour beer; in addition, lactobacilli have been proven to produce a wide range of other flavour-important metabolic products, including aldehydes, alcohols and esters (Dongmo et al., 2016, Salmerón et al., 2015, Stefanovic et al., 2017). The production of flavour-active, metabolites by *Lactobacillus* is strain dependent (Cui et al., 2019).

The environment where the microorganisms ferment is crucial for their metabolism and the resulting products. During the process of fermentation, the environmental conditions in which microbes reside is highly dynamic. Nutrients are depleted, metabolites such as organic acids and ethanol are produced, and cell densities increase. The ability of microorganisms to quickly adapt to these conditions are vital for their survival and continued metabolism. Previous studies have shown that exposure to environmental stresses results in changed gene expression in *Lactobacillus* (Guchte et al., 2002), ultimately shifting the

composition of the produced flavour-active metabolites and the organoleptic properties of the fermented food products (Serrazanetti et al., 2009). Stress induced shifts in production of metabolites from *Lactobacillus* sp. have been proven in different types of food, such as milk (Østlie et al 2005), fruit and vegetables (Wu et al., 2015) and kefir fermentation.

Beer during and after fermentation represents a stressful environment for multiple purposes. Low pH, presence of ethanol, low oxygen, nutrient depletion and presence of anti-microbial hop compounds all contribute to making beer relatively microbially stable towards microbial infection (Vaughan et al., 2005). Some bacteria are, however, able to sustain the harsh beer environment, which is unfortunate when their presence is unwanted (beer spoilers) but vital for production of sour beer. Lactobacilli are associated with both beer spoilage and wanted sour beer fermentations (Vriesekoop et al., 2012). Even though these lactobacilli can grow in beer, the environmental factors influence their metabolism. Lactobacilli are known as relatively tolerant towards ethanol compared to other bacteria (G-Alegría et al., 2004, Gold et al., 1992), and relatively low concentrations of ethanol have even proven to stimulate the metabolism of certain LABs (Mateo et al., 2010).

Literature is scarce on the impact of environmental stress factors on metabolite production by lactobacilli in the beer environment. The objective of the current study was to investigate the effect of beer-related stress factors on growth and metabolite production by three different lactobacilli in wort medium. The selected strains were *Lactobacillus brevis* BSO464, a strain previously proven as resistant to the harsh beer environment (Bergsveinson et al., 2016); *Lactobacillus plantarum* (WildBrew™ Sour Pitch), a commercial brewing strain commonly used for kettle souring (biologic acidification of wort prior to yeast fermentation); and *Lactobacillus buchneri* CD034, a strain previously used in research on kettle souring (Dysvik et al., 2019) but originally isolated from silage grass (Heinl et al., 2012). Furthermore, controlled co-fermentations with lactobacilli and *Saccharomyces cerevisiae* were evaluated as a time-saving method for sour beer production. The produced beers were assessed with respect to degradation of carbohydrates and amino acids, production of flavour-active metabolites as well as sensory properties.

Materials and methods.

Yeast, bacterial strains and growth conditions.

Three different *Lactobacillus* strains were used in the current study. *Lactobacillus brevis* BSO464 was purchased from Campden BRI (Gloucestershire, United Kingdom). *Lactobacillus plantarum* (WildBrew™ Sour Pitch) was purchased from Lallemand. *Lactobacillus buchneri* CD034 was kindly donated by the Department of Biotechnology at the University of Natural resources and Life Sciences, Vienna, Austria. Starter cultures for all three strains were prepared by propagating the bacteria twice in MRS medium (De Man, Rogosa and Sharpe, Merck, Darmstadt, Germany) and twice in wort medium at 30°C overnight. Cells were harvested by centrifugation (9000 × *g*, 10 min, 4°C), resuspended in wort medium (see below) supplemented with 15 % glycerol (v/v) to yield 1 % of the original culture volume and then stored at -80°C. The starter cultures were thawed at 4°C prior to use and inoculated directly. Viability after freezing and thawing was checked, and inoculations were made accordingly. The *Saccharomyces cerevisiae* US-05 was purchased from Fermentis, France. Dry yeast was rehydrated in sterile water at 1:10 (w/v) for 30 minutes at 22°C prior to inoculation in fermentation experiments. Unless otherwise stated, all fermentations were carried out in triplicate at 22°C under static conditions.

Wort production.

The wort used for the stress experiments and small-scale co-fermentations was prepared by dissolving concentrated brewer's wort (Pilsen Light, Pure malt extract, Briess Malt and Ingredient Co, Chilton, WI, US) in water at 120 g/L. The solution was then autoclaved. Solid material was separated from the solution after it had cooled down, the remaining clear solution is hereafter referred to as wort medium. The wort medium had a specific gravity of 1.033 (8.4°P). In the larger scale brewing experiment, wort was prepared using a 60L PRO pilot scale brewery vessel from CoEnCo (Oostkamp, Belgium, 2014). Crushed malt (33 % wheat malt from Weyermann, Bamberg, Germany and 67 % Pilsner malt from BestMalz, Heidelberg, Germany) was mashed in water at 0.25 kg/L according to the following scheme: 45 min at 65°C, 15 min at 72°C and 2 min at 78°C. The wort was separated from the spent grain and boiled for 60 minutes, yielding wort with specific gravity of 1.038 (9.6°P).

Stress experiments

Fermentation bottles (50 mL) were prepared with wort medium with various adjustments according to different stressors. The *reference* condition was wort medium, at 0 % (v/v) ethanol, 0 mg/L iso-α acids, pH 5, inoculated with *Lactobacillus* (10^6 CFU/mL), incubated at 22°C for 7 days. The conditions for the *high inoculation* trial differed from the reference regarding inoculation with 10^8 CFU/mL instead of 10^6 CFU/mL. The conditions for the *high temperature* trial were identical to the reference except incubation of the flasks at 30°C. Wort medium was supplemented with 5% (v/v) ethanol for the *Ethanol* trial, and 5 mg/L

of iso-α acids for the *Iso-α acid* trial. Two different mediums were prepared with reduced initial pH, one where the initial pH was reduced from pH 5 to 4 with lactic acid (*Low pH (lactic acid)*) and one where the initial pH was reduced from pH 5 to 4 with hydrochloric acid (*Low pH (HCl)).* Finally, a *multi-stressor* trial was conducted with wort medium containing 5 % (v/v) ethanol, 5 mg/L iso-α acids and reduced initial pH adjusted with lactic acid. Sampling was done at 0, 4, 8, 12, 24, 32, 48 and 72 hours and at 5 and 7 days. Growth was monitored by plate drop on MRS agar (VWR Chemicals, Leuven, Belgium) after serial dilutions in Ringers solution (Oxoid, Basingstoke, UK) and pH was monitored using a Sentron pH-meter with SI probe (Sentron, Netherlands). After the final sampling (7 days), remaining content in each fermentation flask was centrifuged at 7000 × *g*, 10 min, 4°C and the supernatants stored at -20°C prior to metabolite analysis.

Small scale co-fermentations with LAB and yeast.

The co-fermentation with lactobacilli and yeast was assessed in flasks containing 400 mL wort medium supplemented with 5 mg/L iso-α acids. The fermentation flasks were inoculated simultaneously with 10^7 cells/mL of lactobacilli and 10^6 cells/mL of yeast before incubation at 22°C for 21 days. Lactobacilli were inoculated at a higher ratio to give the bacteria an initial advantage and promote their contribution to the fermentation. The population dynamic was monitored during the fermentation process at established intervals (0, 14, 48, 72 hours and 4, 5, 7, 14 and 21 days); samples were plated both on MRS agar supplemented with 25 mg/L cycloheximide (Sigma-Aldrich, St. Louis, USA) and Rose-Bengal Chloramphenicol agar (RBC, Oxoid, Basingstoke, UK), to be able to differentiate lactobacilli populations from *Saccharomyces* populations, respectively. Samples were applied on plates after serial dilutions in Ringers solution, and CFU/mL for bacteria and yeast were obtained after 3 and 5 days incubation (aerobically, 30°C), respectively. pH was monitored as described above. After the final sampling, 50 mL from each fermentation flask was centrifuged (7000 × *g*, 10 min, 4°C) and the supernatant was stored at – 20°C prior to metabolite analysis. The remaining content from each flask was used for ethanol analysis.

Large scale co-fermentation experiment with LAB and yeast.

Fermentation tanks (10 L) were prepared with PRO pilot scale brewery wort supplemented with 5 mg/L iso-α acids. Inoculations, fermentation temperature and duration and monitoring of population dynamic was carried out as described above. Samples (80 mL) were withdrawn throughout fermentation, centrifuged (7000 × *g*, 10 min, 4°C) and the supernatant was kept at – 20°C for analysis of amino acids, carbohydrates, metabolites and ethanol. After the final sampling, tanks were kept at 4°C for 14 days before the beer was carbonated using an Aqvia sodastreamer (AGA, Luleå, Sweeden) and transferred to 0.33 L bottles for sensory analysis. Beer fermentations were carried out in triplicate with yeast alone, yeast in co-fermentation with *L. brevis* and yeast in co-fermentation with *L. plantarum*.

Chemical Analyses

Headspace gas chromatography (HSGC).

Volatile compounds were detected and quantified by HSGC according to the method by (Narvhus et al., 1998) with modifications. Samples (10,0 g) fermented by lactobacilli alone were directly transferred to headspace vials (Machery Nagel, Dueren, Germany), while samples (10,0 g) from co-fermentations by yeast and lactobacilli were first filtered through 602h ½ folding filters (pore size < 2 µm, Schleicher & Schuell, Dassel, Germany) to remove CO_2. Teflon-coated septa with aluminium rings (PFTA/Si septa, Agilent Technologies, Wilmington, DE, USA) were used to seal the vials before they were placed in a 7679A automatic headspace sampler. The sampler was connected to a 6890 GC system with flame ionisation detector (Agilent Technologies). The applied headspace bath temperature was 50°C and manifold temperature of 60°C. Helium 6.0 (Aga, Norway) at low rate 5.0 mL/min was used as carrier gas. Samples were mixed (45 min, 70 shakes/min) prior to injection (0.5 min injection time, 10 psi pressure). Analytes were separated on a CP-SIL 5CB GC column (Varian, Middelburg, Netherlands) of 25 m × 0.53 mm I.D. with film thickness 5 µm. The system was operated by Open LAB EZChrom software (version A.04.05, Agilent Technologies) and identification and quantification were carried out according to calibration with standards. The following temperature scheme was applied during analysis: 35°C for 5 min: increase of 10°C/min until 40°C and kept at 40°C for 2 min; increase of 30°C/min until 130°C and kept at 130°C for 4 min; increase of 30°C/min until 160°C and kept at 160°C for 4 min; increase of 10°C/min until 180°C and kept at 180°C for 2 min; increase of 10°C/min until 200°C and kept at 200°C for 2 min.

High performance liquid chromatography (HPLC).

Organic acids as well as fructose and maltotriose were detected and quantified by HPLC, according to the method described (Marsili et al., 1981) with modifications. Samples (1,0 g) were mixed with water (MilliQ), 0.5 M H_2SO_4 and acetonitrile in a MultiRS-60 BIOSAN turner (Montebello Diagnostics A/S, Oslo, Norway) operated at 30 rpm for 30 minutes. Samples were centrifuged for 15 min at 1470 × *g* using a Kubota 2010 centrifuge (Kubota Corporation, Tokyo, Japan) prior to filtration through 0.2 µm PTFE membrane (Acrodisc CR 13 mm Syringe Filter, PALL, Great Britain). Organic acids were separated on an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) with H_2SO_4 (0.05 M) as mobile phase (flow rate of 0.4 mL/min). The column, operated at 30°C, was connected to a 1260 Infinity HPLC instrument (Agilent Technologies, Singapore) with pump, autosampler, column oven, RI-detector (refractive index, used for acetic acid, fructose and maltotriose) and diode array detector-ultra violet (DAD-UV) detector, used for the other organic acids). Openlab CDS software (Agilent Technologies) was used to operate the system and detection and quantification were done according to calibration with standards. Maltose, sucrose and glucose were quantified by the K-MASUG enzymatic kit, (Megazyme, Wicklow, Ireland), used according to the instructions.

Statistical analysis of metabolic products.

Differences in metabolites from the stressor experiment were examined by Analysis of variance Simultaneous Component Analysis (ASCA) (Jansen et al., 2005). MATLAB (2019a, The Mathworks, Natick, MA) was used to fit the ASCA model, which split the variation in the dataset in three according to strain, stressor and strain-stressor interaction related variation. Confidence ellipsoids (Liland et al., 2018) were used to display uncertainty of the effect level means in the ASCA scores, similarly to Tukey's test in ANOVA. Uni-dimensional ANOVA for each compound was combined with Tukey's test for honestly significant differences. This was done to obtain groups of effect levels, e.g. groups of stressor conditions, which are not significantly different with respect to a chosen measured property. The ANOVA with Tukey's test was carried out using R 3.6.1 (R Core Team 2019, Austria, Vienna), and statistical significance level was set at $p < 0.05$. Variation in metabolites from the small- and larger scale co-fermentations were analysed by ASCA and ANOVA with Tukey's test.

Ethanol and apparent degree of fermentation (ADF).

Beer characterisation was carried out using a PBA-B instrument, consisting of a DMA 4500M density meter, an Alcolyzer Beer ME module with integrated colour measurement module, a CarboQC ME module and a PFD filling device. The entire instrumental setup was delivered by Anton Paar (Graz, Austria) and operated through Generation M instrument software version v2.42 (Anton Paar, Graz, Austria).

Free amino acids.

Free amino acids were identified and quantified using an HPLC method described by Bütikofer and Ardö (1999) and Moe et al. (2013) with the following modifications. Samples (5.00 g) were mixed with 5.00 mL internal standard solution (0.4 µmol/mL L-norvalin in 0.1 M HCl). The samples were mixed for 15 minutes (MultiRS-60 BIOSAN, Montebello Diagnostics AS, Oslo, Norway) before they were placed for 30 minutes in an ultrasonic water bath (Brandson 2510, Soest, Netherlands). The samples were then centrifuged for 40 minutes at 4°C at 2500×*g* (Thermo Scientific, Heraeus Multifuge X3R, Osterode, Germany) before the supernatant was mixed 1:1 with 4% trichloroacetic acid, kept on ice for 30 minutes and centrifuged for 5 minutes at 4°C at 15600×*g* (Eppendorf 5415D Microcentrifuge, Eppendorf, Hamburg, Germany). The supernatant was filtered through 0.2 µm cellulose acetate filters (VWR, USA) and stored at –20°C prior to further preparation. Borate buffer (350 µL, 0.4 M, pH 10.2) was mixed with samples (50 µL), and the samples were derivatised by allowing 5 µL to react for 0.15 min with 5 µL O-phthaldialdehyde (OPA) solution prior to injection. The samples were analysed using an Agilent 1200 HPLC system (Agilent Technologies, Singapore) consisting of a serial pump, auto injector, column oven, thermostat and fluorescence detector. The instrument was operated through Open LAB CDS software (Agilent Technologies). A sample volume of 10 µL was injected and analytes separated on an XTerra RP 18 column (150 x 4.6 mm; Waters, USA) operated at 42°C. Two mobile phases were used at 0.7 mL/min: eluent A (30

mmol/L sodium acetate trihydrate, 0.1 mmol/L triplex III, 0.25 % tetrahydrofuran, pH 7.2) and eluent B (100 mol/L sodium acetate trihydrate, 0.53 mol/L triplex III, 80 % acetonitrile, pH 7.2). The derivatized amino acids were separated by a stepwise linear gradient from 3.3 to 20.7 % eluent B over 13 min, from 20.7 to 30 % eluent B over 12 min and from 30 to 100 % eluent B over 4 min. Standard solutions for calibration were prepared according to the above description and free amino acids in the samples were identified and quantified according comparison with these standards.

Sensory evaluation of produced beers by trained panel.

A professional sensory panel of eight trained assessors at the Norwegian Institute of Food, Fisheries, and Aquaculture Research (NOFIMA), Aas, Norway was used for sensory evaluation of produced beers. All panellists were previously screened for sensory abilities (basic tastes, colour vision, odour detection, tactile sensibility) and communication skills regarding sensory descriptions of products recommended in ISO 8586 (ISO, 2012) in a sensory laboratory designed in accordance with ISO 8589 (ISO, 2007). A list of sensory attributes (Table S1) was generated and agreed upon by the panel, based on a brain storming session and previous experiments with beer. The assessors were trained in the definition of 22 selected taste, texture, odour and flavour attributes, prior to the actual experiment. The three different beers were evaluated in duplicate in a Sensory profiling according to Generic Descriptive Analysis as described by Lawless and Heymann (2010). The samples were evaluated by each assessor within each session in individual randomised order. The evaluation of eight samples in total was conducted in four sessions. A warm-up sample served in the beginning of the first serving, and a commercial sour beer reference (Geuze, Mariage Parfait, 2015, Boon Brewery, Belgium) was evaluated in duplicate at the end of the last session. One bottle from each of the three replicates of the three different beers, were mixed in a beaker before serving. Two bottles of the commercial sour beer reference were mixed in a beaker. A decarbonation procedure was carried out by pouring the beer back and forth between bakers 20 times and leaving the beer to rest for one hour prior to serving. The decarbonation of the commercial sour beer reference was done to obtain a similar carbonation level to the beers produced in this study. Clear plastic cups, tagged with random three-digit codes, were used to serve 30 mL of beer at 17± 1°C. All samples in one session were placed in the sensory evaluation booths at the same time and monadically evaluated at individual speed and registered continuously, using EyeQuestion (v4.11.33, Logic8, Holland). The assessors took a sip of the beer and rated all attributes by intensity on a non-structured continuous scale. The endpoints of this scale corresponded to 1 (lowest intensity) and 9 (highest intensity), and the scores were converted to a number between the endpoints by the Eye Question software. XLSTAT (v2019.1.3) 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 combined with Tukey's test and analysed further by Principal Component Analysis (PCA) using PanelCheck V1.4.2 (Norway).

Results and Discussion.

Stress experiment.

Initial screenings with *L. brevis L. plantarum* and *L. buchneri* were conducted to evaluate the effect of different beer-related stress factors on their growth and production of metabolites. The three investigated lactobacilli exhibited good fermentation performance in wort medium at reference conditions (Fig. 1A-C), with an increase of 2 $\log_{10}$ CFU/mL within the first 48 h. At the final sampling, L. *brevis* achieved 3.9 × 10^7 CFU/mL, *L. plantarum* reached 2.4. × 10^7 CFU/mL and *L. buchneri* reached 7.0 × 10^8 CFU/mL. These findings are in agreement with previous studies reporting that the nutrient sources present in malt-based media are favourable to lactobacilli growth (Charalampopoulos et al., 2002). A concurring pH drop was observed during fermentation at reference conditions for all LAB strains (Fig. 1A-C) from pH 5 to 3.2-3.7. The largest pH reduction was obtained with *L. plantarum*, where a final pH of 3.2 was obtained after 7 days in the reference trial. *Lactobacillus buchneri* reached pH 3.5 at the corresponding conditions, while *L. brevis* reached pH 3.7. Elevated growth rate and faster pH reduction was observed for all strains at higher temperature, but higher temperature had no effect on the final pH. This agrees with previous literature, where faster pH-drop, but equal final pH was associated with LAB fermentations at higher temperatures (Østlie et al., 2005, Narvhus et al., 1998).

Generally, the performance of *L. brevis* appeared to be more robust towards different stressor conditions, compared to the two other lactobacilli. The growth of *L. brevis* (Fig. 1A) seamed unaffected by iso-α acids alone, or in combination with ethanol and reduced pH (lactic acid) in the multi-stressor condition. The growth of *L. plantarum* (Fig. 1B) was severely affected by the presence of iso-α acids and no growth was observed under the multi-stressor condition. *L. buchneri* growth (Fig. 1C) was seemingly unaffected by iso-α acids alone, but severely hampered in the multi-stressor condition. The same trend was seen in pH development during fermentation, where a final pH of 3.7-3.8 was reached by *L. brevis* at all stressor conditions (Fig. 1A). For *L. plantarum* the pH remained unchanged during fermentation in the multi-stressor trial as it did not grow in these conditions, while a reduction from pH 5 to pH 4 was observed in the iso-α acid trial (Fig. 1B, lower). *L. buchneri* was able to generate a very slight reduction in pH in the multi-stressor trial, from pH 4.1 to pH 4.0, and a reduction from 5 to 3.6 in the presence of iso-α acids alone (Fig. 1C).

Based on the growth performance and the ability to reduce pH, the presence of iso-α acids in wort appear as the most stressful of the investigated environmental factors, especially when coinciding with other brewing related stress-factors, such as low pH and presence of ethanol. The antimicrobial action by iso-α acids, is due to their properties as ionophores that dissipate the transmembrane proton gradient in cells, and by this disrupt the proton motive force and impair cell metabolism (Simpson, 1993a, Ye et al., 1994,

Simpson and Smith, 1992, Simpson, 1993b). Iso-α acids affect microbial cells synergistically with low pH (Simpson and Hammond, 1991, Suzuki, 2011), and increased inhibition in the multi-stressor trial was thus expected. The ability to sustain in an environment with iso-α acids has been associated with the genes *hitA* (Hayashi et al., 2001), *horA* (Sami et al., 1997) and *horC* (Suzuki et al., 2005). The greater resistance towards iso-α acids by *L. brevis* was in accordance with expectations, as *hitA*, *horA* and *horC* have all been identified in *L. brevis* BSO 464. Overall, the higher robustness displayed by *L. brevis* was expected, as the ability of this strain to grow in beer has been demonstrated previously (Bergsveinson et al., 2016, Bergsveinson et al., 2015).

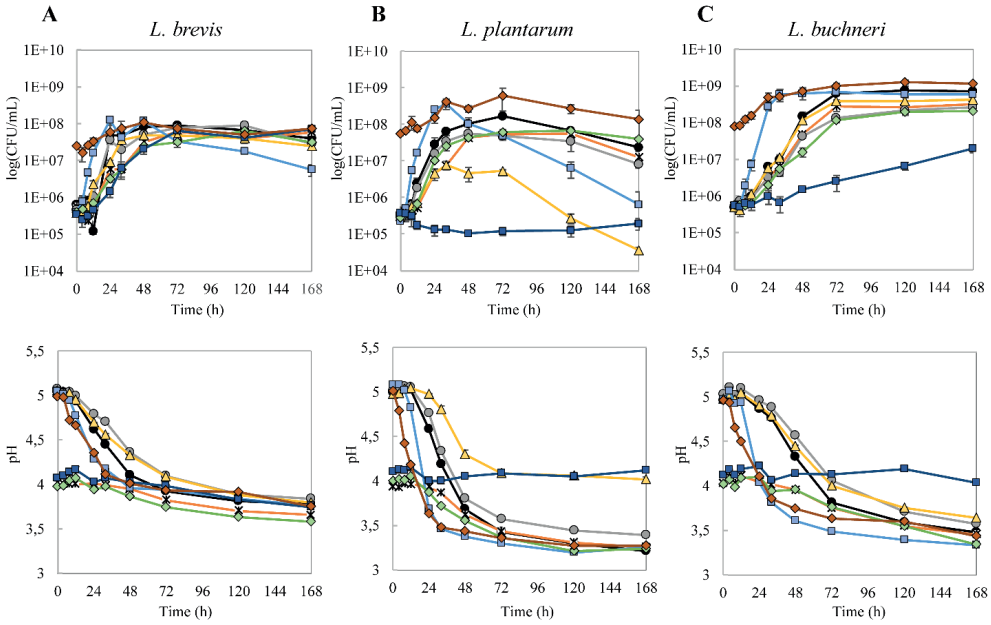

Figure 1. Growth kinetics (upper diagrams) and pH measurements (lower diagrams) during fermentation of wort medium with A) *L. brevis,* B) *L. plantarum* and C) *L. buchneri* during the reference trial (black line), ethanol trial (5 % ethanol, grey), low pH (initial pH 4) obtained with lactic acid trial (orange line) or HCl (green line), iso-α acids trial (5 mg/L, yellow line), high temperature trial (30°C, light blue line), multi-stress trial (5 % ethanol, initial pH 4 by lactic acid, 5 mg/L iso-α acids, dark blue line) and high inoculation trial (10^8 cells/mL, red line).

ASCA analyses of metabolites produced during fermentation in brewing-related stresses.

Metabolites generated during fermentation by the different lactobacilli in wort with different stressor conditions were analysed and visualised in ASCA plots (Fig. 2). The factor “strain” accounted for 38 % of the variation in the metabolic data and was the most important variable (Fig. 2A,). This was in accordance with expectations as production of metabolic products is known to be highly strain-dependent in lactobacilli (Cui et al., 2019). A clear separation of the metabolic profile of the three lactobacilli strains was observable in the ASCA score plot visualising strain-related variation (Fig. 2A). Component 1 explained 92 % of the strain variation in the data-set, while component 2 explained the remaining 8 %. The loading weights (Fig. 2A) showed that metabolites driving the strain-related variation both in component 1 and 2 included lactic acid, acetic acid and diacetyl. The production of these metabolites was significantly different between all three lactobacilli strains (Fig. S1A-C). *L. plantarum* generated the highest amount of lactic acid (4181 mg/L) and diacetyl (1.1 mg/L), but the lowest amount of acetic acid (208 mg/L) in the reference trial. At the same conditions *L. brevis* produced the lowest amount of lactic acid (1195 mg/L), no diacetyl and 382 mg/L acetic acid. *L. buchneri* did not produce diacetyl but generated 1822 mg/L lactic acid and the highest quantity of acetic acid (703 mg/L) in the reference trial. Higher relative production of acetic acid by *L. brevis* and *L. buchneri* was expected as these are both obligately heterofermentative, while *L. plantarum* is facultative heterofermentative and will produce primarily lactic acid as long as there is hexose available (von Wright and Axelsson, 2019).

The factor "environmental stress” explained 33.7 % of the variation in the metabolic dataset (Fig. 2B). All stressor conditions, except high inoculation, yielded metabolic compositions significantly different from the reference trial. This is evident from the separation of the various conditions in the ASCA score plot. The multi-stressor trial is furthest apart from the reference trial in component 1 (62% of the environmental stress variation in the model) and thus the most influential stress factor with respect to metabolic composition. The presence of ethanol alone was also influential. The most important driver of component 1 (62 % variation in the model) was 2-methyl 1-butanol and the most important driver of component 2 (17 % of the variation in the model) was ethylacetate (Fig. 2B). The production of 2-methyl 1-butanol was reduced in the multi-stressor trial for all three lactobacilli compared to the reference trial (from 0.61 to 0.24 mg/L for *L. brevis*, from 0.56 to 0 mg/L for *L. plantarum*, and from 0.4 to 0.10 mg/L for *L. buchneri*, Fig. S1I). This might be explained by the lower rate of metabolism in general for *L. plantarum* and *L. buchneri*, as the growth of both these was severely affected in the multi-stressor trial. Even though no pronounced effect on the CFU/mL of *L. brevis* was observed, the metabolic activity could still be influenced as observed for 2-methyl 1-butanol. The concentration of ethylacetate increased in the ethanol trial for lactobacilli compared to the reference trials (from 0 to 0.29 mg/L for *L. brevis*, from 0 to 0.23 mg/L for *L. plantarum*

and from 0.03 to 0.81 mg/L for *L. buchneri*, Fig. S1D). Ethanol is a constituent in ethylacetate, and ethylacetate synthesis is related to pathways connected to ethanol and acetic acid where various enzymes can be involved (Costello et al., 2013, Kallel-Mhiri and Miclo, 1993). Being a substrate for enzymatic ethylacetate synthesis, increased ethanol concentration is expected to facilitate synthesis of ethylacetate (Cristiani and Monnet, 2001, Liu et al., 2004).

The factor "interactions between strain and environmental stress" accounted for 20 % of the variation in the metabolic data. The separation of samples in the strain-stressor interaction ASCA score plot (Fig. 2C) suggests that the different lactobacilli were affected in different ways by the stressor conditions. The multi-stressor condition was most influential with respect to metabolite production, as the multi-stressor group for each strain is separated furthest away from its corresponding reference group in the score plot. The second most influential stressor was iso-α acids for *L. plantarum* and ethanol for *L. buchneri*. None of the conditions besides the multi-stressor affected *L. brevis* severely. Lactic acid, diacetyl and ethylacetate were all important contributors to component 1 and 2 in the strain-stressor interaction related ASCA (Fig. 2C). Lactic acid production was severely dependent on strain-stressor interactions (Fig. S1A). Indeed, while none of the stressor conditions yielded significant changes in lactic acid production for *L. brevis*, all stressor conditions caused a significant change in lactic acid production by *L. plantarum*. Reduced pH (by both lactic acid or HCl), presence of ethanol, iso-α acids and the multi-stressor trial reduced the lactic acid production significantly to between 215±62 and 3009±250 mg/L compared to 4181±326 mg/L in the reference trial for *L. plantarum*, while higher inoculation and temperature increased the lactic acid production for this strain to 4944±291 and 4926±227 mg/L, respectively. Higher lactic acid production was also obtained by higher inoculation level or temperature for *L. buchneri* (2342±86 and 2842±76 mg/L, respectively), while only the multi-stressor condition was able to significantly reduce the lactic acid production for *L. buchneri* (160±11 mg/L in the multi-stressor trial, 1822±73 mg/L in the reference trial). Lactic acid is the main metabolite generated by lactobacilli (Ibrahim and Ouwehand, 2019), and the strain-dependent difference in shifts in lactic acid yield mirrors the growth and pH-development results. The metabolic activity of *L. brevis* seems to be highly resistant towards environmental influence and produces similar quantities of lactic acid at all trials, while L. *buchneri* and *L. plantarum* are both more susceptible and produce higher or lower quantities in response to shifts in the environment. *L. brevis* did not produce diacetyl at any conditions, while *L. plantarum* did at all trials (Fig. S1C). Fermentation with *L. plantarum* at lower pH (HCl), in the multi-stressor trial, higher inoculation rate and high incubation temperature led to a decrease of diacetyl in comparison with the fermentation at reference conditions from 1.1±0.1 mg/L to 0.3-0.9±0.1 mg/L. The highest concentration of diacetyl across all strains and stressor conditions was generated by *L. buchneri* in the multi-stressor trial, yielding a final concentration of 2.1±0.1 mg/L. Diacetyl is associated with caramel and buttery flavours (Harrison, 1970). It is generally regarded as an off-flavour

in beer where the reported detection limit is as low as 0.1 mg/L (Vann and Sheppard, 2005). Even in the multi-stressor trial, *L. plantarum* which had very low metabolic activity, still produced 0.3 mg/L of diacetyl. The diacetyl produced by *L. buchneri* was well above the reported sensory threshold and would likely be sensorially influential if the fermented wort medium were to be tasted. Ethylacetate production was stimulated by ethanol for all strains (Fig. S1D). Ethylacetate is associated with fruity and solvent-like flavours (Meilgaard et al., 1979) and the reported sensory threshold in beer is 30 mg/L (Harrison, 1970). The generated quantities, of less than 1 mg/L for all lactobacilli (Fig. S1), were therefore below sensory thresholds. It should, however, be noted that the stimulating effect by ethanol on the generation of ethylacetate persisted in the multi-stressor trial for *L. brevis* (0.35±0.03 mg/L), but not for the two other lactobacilli. This can likely be attributed to the overall metabolic activity being hampered for *L. plantarum* and L. *buchneri* at these conditions, but not for *L. brevis*.

The results from the stressor trials illustrated that all three tested strains were capable of producing metabolites necessary for sour beer production when fermenting wort medium. The lactobacilli produced different metabolic compositions and displayed differences in tolerance towards beer related stress. *L. brevis* protruded as the more stress resistant strain, while *L. plantarum* displayed the lowest stress tolerance. *L. brevis*, *L. plantarum* and *L. buchneri* all generated substantial quantities of organic acids and reduced the pH of wort medium and are all candidates for sour beer production. However, the data suggest that attention should be paid to strain selection in conjunction with production method. Indeed, when using lactobacilli in controlled mixed fermentation for sour beer production, or other malt based fermented beverages, the properties of the selected strains could be detrimental to the final product properties. An example of this is the high quantities of diacetyl generated by *L. buchneri* in the multi-stressor trial, which represents the closest environment to that of beer.

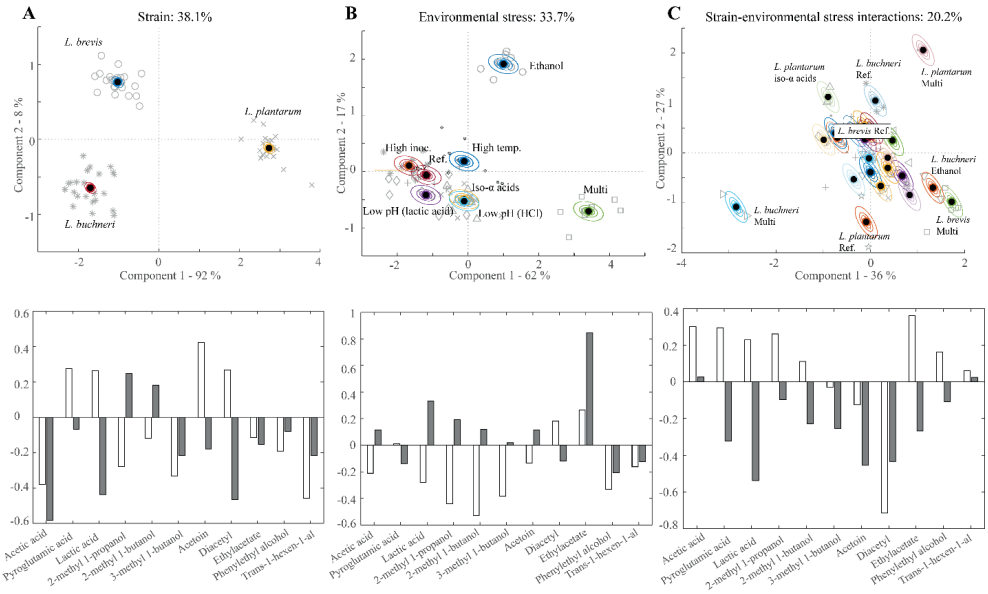

Figure 2. Metabolite variation in samples and replicate variation described by ASCA scores. The model is based on the metabolic composition at the end of fermentation (day 7) with the different lactobacilli strains grown under varying stressor conditions. A) Strain related variation, accounting for 38.1 % of the variation in the metabolic data displayed in a score plot (upper) with corresponding loading (lower). White bars show loadings for component 1 (92 %) and grey bars show loadings for component 2 (8 %). B) Environmental stressor related variation in the metabolic data, accounting for 33.7 % of the variation visualised in a score plot (upper) with corresponding loadings (lower). White bars show loadings for component 1 (62%) and grey bars show loading for component 2 (17 %). C) Strain-environmental stress interaction related variation, accounting for 20.2 % of the metabolic variation, visualised in a score plot (upper) with corresponding loading (lower). White bars show loadings for component 1 (36 %) and grey bars show loadings for component 2 (27 %).

Small scale co-fermentation experiment.

A lab-scale experiment (400 mL) where the three different lactobacilli were inoculated simultaneously with *S. cerevisiae* was conducted to investigate how the bacteria performed during co-fermentation for three weeks. The co-fermentation product by *S. cerevisiae* with *L. brevis, L. plantarum* and *L. buchneri* is hereafter referred to as the "*L. brevis* beer", "*L. plantarum* beer" and "*L. buchneri* beer". A reference fermentation with *S. cerevisiae* alone, referred to as "the reference beer", was also carried out. The growth medium was wort of the same composition as in the stressor experiments, supplemented with 5 mg/L iso-α acids. The presence of yeast did not affect lactobacilli viability as their cell counts were similar between single cultures (in the presence of iso-α acids) and co-cultures with *S. cerevisiae* (Fig. 3A). Vice versa, the acidic environment imposed by the lactobacilli was not detrimental to the yeast viability and no pronounced effect was observed between reference single strain beer culture and mixed strain culture beers (Fig. S2). For all four fermentations, a primary pH drop occurred within the five first days (Fig. 3B). The presence of lactobacilli was influential with respect to pH development, as the final pH was lower in all co-fermentations compared to the reference beer. The final pH in the reference beer was 4.0, compared to pH 3.4 for *L. brevis* beer, 3.8 for *L. plantarum* beer and 3.7 for *L. buchneri* beer. All lactobacilli were thus able to generate sour beers in the employed co-fermentation method, according to the definition suggested by Tonsmeire (2014) of beer with pH 3.1-3.9. *L. brevis* emerged as the more resistant strain with respect to beer fermentation, as it generated the lowest pH in co-fermentation with *S. cerevisiae*.

All lactobacilli were influential to the final metabolite composition after co-fermentation. As observed for pH development, *L. brevis* appeared as the most influential with regards to metabolite composition (Fig. 3C, Table S2). The ASCA model explained 82 % of the variation in the metabolites, where the *L. brevis* beer was separated from the others in component 1 (74.3% of the variation in the model) and the *L. buchneri* beer was separated from the others in component 2 (18.9 % of the variation in the model). Lactic and acetic acid were the most important drivers of component 1, while succinic acid and isoamyl acetate were important drivers of component 2 (Fig. 3D). The lowest quantities of organic acids were obtained in the reference beer (110±12 mg/L acetic acid) followed by the *L. plantarum beer* (124±3 mg/L acetic and 530±26 mg/L lactic acid) (Table S2). The second highest organic acid content was obtained in the *L. buchneri* beer (423±17 mg/L acetic, 878±38 mg/L lactic and 167±5 mg/L succinic acid) and the highest quantities were obtained in the *L. brevis* beer (951±25 mg/L acetic, 2300±55 mg/L lactic and 110±3 mg/L succinic acid). The greater influence exerted by *L. brevis* was not only due to its superior stress tolerance and ability to carry out its metabolism during co-fermentation, but also due to an inhibiting effect on *S. cerevisiae* metabolism. This effect was evident from the reduced ethanol production (Fig. 3E), reduced apparent degree of fermentation (ADF) (Fig. S3) and reduced production of a number of metabolites typical

for *S cerevisiae* (isoamyl acetate, 2- methyl 1-propanol, 3-methyl 1-butanol, 2-methyl 1-butanol, 1 propanol) in the *L. brevis* beer compared to the three other beers (Table S2).

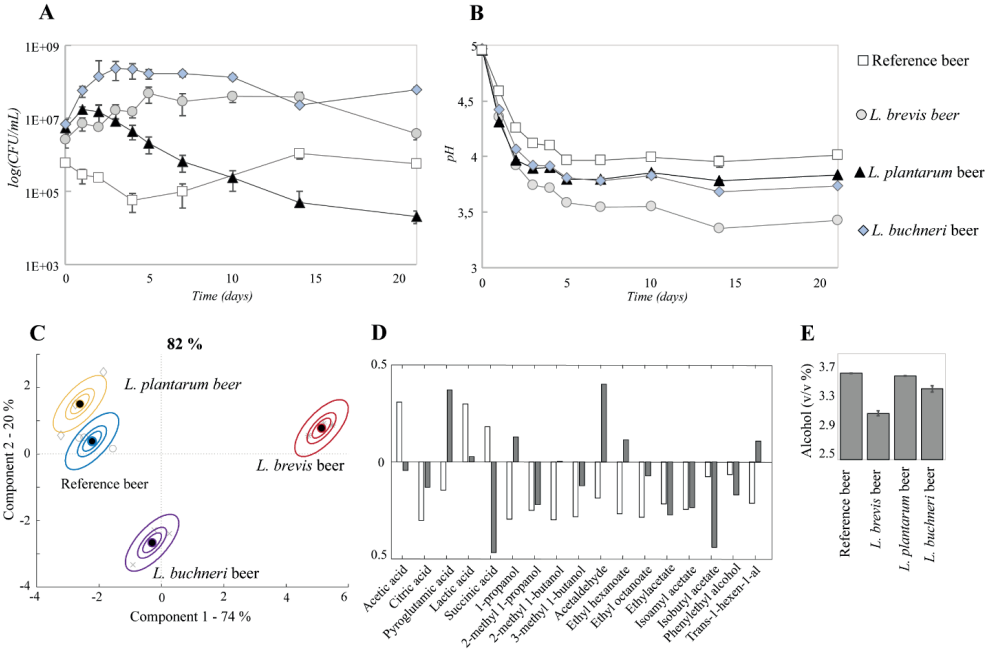

Figure 3. Small scale fermentations (400 mL) of reference*, *L. brevis*, *L. plantarum* and *L. buchneri* beers during 21 days of incubation at 22°C. A) Growth and B) pH development. C) Metabolite variation in samples and replicate variation described by ASCA scores. The model is based on the metabolite composition at the end of fermentation (day 21). The model explains 82.4 % of the variation in metabolites. D) Loading for ASCA model in panel C. White bars show loadings for component 1 (74.3%) and grey bars show loadings for component 2 (19.8 %). E) Final alcohol percentage (day 21).

* The four growth curves for *S. cerevisiae* in the reference, *L. brevis*, *L. plantarum* and *L. buchneri* beers are displayed in Fig. S2 in Supporting Information. Only the growth curve for yeast alone is displayed in the figure since the yeast growth curve was similar for yeast alone and in co-fermentation with LAB.

Beer production through co-fermentation.

Based on the results from the stress experiment, where undesirable accumulation of diacetyl was produced by *L. buchneri* in the multi-stressor trial (Fig. S1C) and based on *L. brevis* and *L. plantarum* protruding as most and least stress tolerant both in the stress trial and the small-scale co-fermentation, these strains were selected for upscaled beer production. In order to determine how the strains performed at larger scale in a brewery-like setting, 10 L fermentations at 22°C were conducted using wort. The growth patterns of the lactobacilli were comparable to the small-scale co-fermentations (Fig. 4B). For *L. brevis*, the maximum observed CFU/mL was observed after 48 hours (4.8×10^7) and dropped down to 1.6×10^5 CFU/mL after 21. The maximal observed CFU/mL for *L. plantarum* was 1.1×10^8 after 24 hours and was down to 3.3×10^3 CFU/mL after 21 days. *S. cerevisiae* growth was consistent with the small-scale co-fermentation in the respect that the CFU/mL were similar regardless of lactobacilli presence (Fig. 4A). The final sampling (21 days) was an exception, where the CFU/mL for *S. cerevisiae* was lower in co-fermentation with *L. plantarum* (3.3×10^2 CFU/mL) compared to *S. cerevisiae* alone (1.1×10^4 CFU/mL) or with *L. brevis* (0.7×10^4 CFU/mL).

The pH in all three fermentations dropped, with the majority of the pH reduction occurring during the five first days (Fig. 4C). As for the small-scale co-fermentation, *L. brevis* generated the largest pH-drop, with a final pH of 3.6, compared to 3.8 for *L. plantarum* and 4.1 for in the reference beer. Ethanol was produced all throughout the different fermentations (Fig. 4D), reaching a final concentration of 4.2% (v/v) in the reference, 4.1% in the *L. plantarum* beer and 3.8 % for *L. brevis* beer. As for the small scale-co-fermentation, a correspondingly lower ADF was observed in the *L. brevis* beer (Fig. S4). It could be argued that the minor reduction in ethanol production by yeast was merely a result of reduced fermentation pH, as the final pH was lower in the *L brevis* co-fermentation. However, in a study by Alcine Chan et al. (2019) looking at co-fermentation with *L. paracasei* L26 and *S. cerevisiae* S-04, no significant effect from bacterial co-fermentation on ethanol production was found. In a previous study we have tested for an eventual pH-effect by adding lactic acid to wort prior to yeast fermentation. No effect by reduced fermentation pH (final beer pH 3.7) was found on ethanol production or ADF for the same yeast strain (*S. cerevisiae* S-05) (Dysvik et al., 2019). These results suggest that the reduced performance by *S. cerevisiae* with respect to ethanol production and ADF in the *L. brevis* co-fermentation, was caused by some effect exerted by the presence of *L. brevis* itself. The results also illustrate how important choice of strain can be, in order to obtain wanted properties in the final product. In the current study, *L. brevis* generated higher quantities of organic acids (Table S3) and represented the better choice if low pH/high organic acid content in beer was wanted. However, *L. brevis* impaired the yeast metabolism in some way, and thereby generates a beer where fermentable sugars in the wort was exploited to a lower extent.

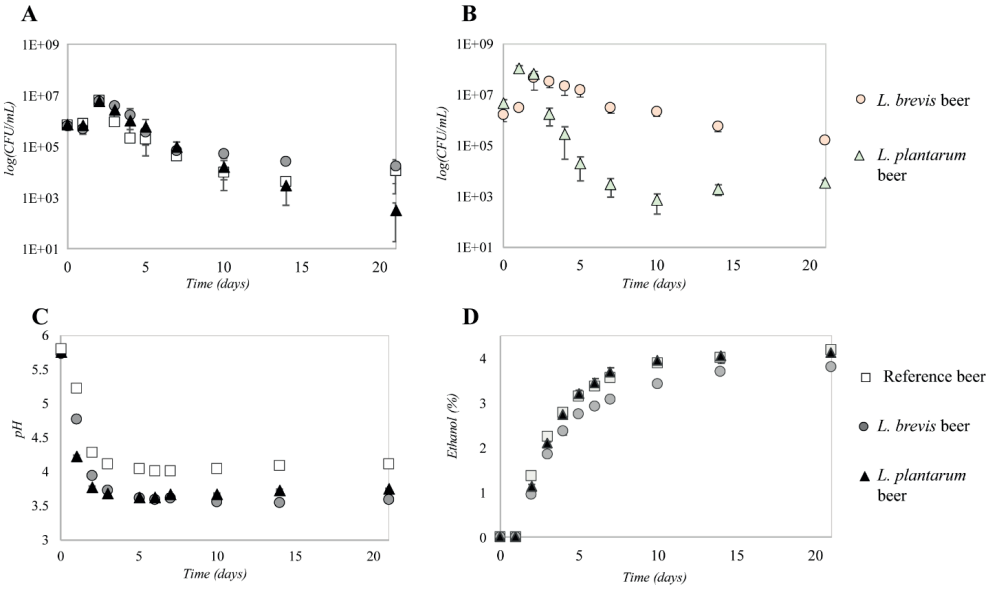

Figure 4. Fermentation (10L scale) in reference, *L. brevis* and *L. plantarum* beers during 21 days of fermentation at 22°C. A) *S. cerevisiae* growth. B) Lactobacilli growth in *L. brevis* and *L. plantarum* beers. C) pH development. D) Ethanol development.

Amino acid and carbohydrate throughout the fermentations were investigated in order study to if the reduced fermentation performance by *S. cerevisiae* in the *L. brevis* beer could be explained by altered degradation patterns. The presence of both lactobacilli was highly influential to the amino acid degradation pattern (Table 1). *L. plantarum* contributed to an elevated rate of depletion of all amino acids compared to the *L. brevis* beer and the reference beer, as the quantities of amino acids were lower after 24 hours in the *L. plantarum* beer. An exception was arginine, which was lower after 24 hours in the *L. brevis* beer (20 µmol/g) compared to the *L. plantarum* beer (26 µmol/g) and substantially lower compared to the reference (43 µmol/g). Despite the higher uptake rate, the concentration of most amino acids was similar after 21 days in the *L. plantarum* beer and the reference beer. *L. brevis* was, however more influential with respect to final amino acid composition, leaving higher quantities in the beer after 21 days of fermentation. Alanine was an example, at 0.57 µmol/g after 21 days in the *L. brevis* beer compared to 0.14 µmol/g in the reference beer. The same trend, with a lower degree of depletion in the *L. brevis* beer was observable for glycine, histidine, tyrosine and phenylalanine. Both lactobacilli seemingly produced γ-aminobutyric acid (GABA), as this amino acid accumulated in the co-fermented beers but decreased in the reference beer (Table 1). The increase was more pronounced in the *L. brevis* beer, where the concentration increased from 0.43 to 0.68 µmol/g in 21 days. GABA is produced from glutamic acid (Ueno, 2000) and production by lactobacilli has been proven previously (Choi et al., 2006, Li and Cao, 2010). GABA is a product of the glutamate

512 decarboxylase (GAD) system, which represents an acid stress response previously described in lactobacilli
513 (Higuchi et al., 1997). In the GAD system, decarboxylation of glutamic acid increases the intracellular pH
514 by consuming a proton. The arginine deaminase (ADI) pathway can also be part of the response towards
515 acid stress in lactobacilli (Champomier Verges et al., 1999). In the ADI pathway, ATP is generated as
516 arginine is converted to ornithine, carbon dioxide and ammonia. The elevated depletion of arginine, and the
517 production of ornithine (0.29 μmol/g after 21 days) in the *L. brevis* beer, suggests that the ADI pathway is
518 active in *L. brevis*. This coincide with previous literature where the ADI pathway is present in
519 heterofermentative LAB (e.g. *L. brevis*)(Liu et al., 1995). The amino acid results suggest higher activity in
520 the GAD and ADI systems in L. brevis compared to *L. plantarum*. This might be part of the explanation of
521 the increased influence during beer fermentation by *L. brevis*. Notably, ingestion of GABA has been
522 associated with a number of health benefits (Dhakal et al., 2012), and GABA is therefore classified as a
523 bioactive compound (Chou and Weimer, 1999) and may be a sought after compound in beer as a beneficial
524 feature.

525 The presence of both lactobacilli influenced the carbohydrate degradation (Table 1). *L. plantarum*
526 contributed to a quicker depletion of maltose, glucose, fructose and maltotriose (Table 1) compared to the
527 reference and *L. brevis* beer (lower concentrations after 1 day), but with similar final concentrations to the
528 reference beer (Table 1). In the *L. brevis* beer, the uptake was seemingly slower, as the concentrations of
529 all carbohydrates (except sucrose which is completely depleted in all beers after 24 hours) after 1 day was
530 slightly higher compared to the reference beer. Maltose and maltotriose were the most abundant
531 carbohydrate detected in the final beer. Concentrations of these two carbohydrates was higher at the end of
532 the fermentation in the *L. brevis* beer (3.12±0.07 g/L maltose and 2.85±0.13 g/L maltotriose) compared to
533 the reference beer (1.08±0.10 g/L maltose and 0.64±0.03 g/L maltotriose).

534 Overall, the reported data suggest that lactobacilli influenced the beer fermentation in different ways. While
535 *L. plantarum* contributes in the fermentation by depleting amino acids and carbohydrates quickly, its
536 presence did not disrupt the yeast fermentation extensively, as the final ethanol concentration and ADF are
537 highly similar to the reference beer. *L. brevis* did, however, affect the final ethanol concentration and ADF.
538 Part of this reducing effect on *S. cerevisiae* performance with respect to ethanol production and ADF, can
539 be explained by an interfering impact on the yeast ability to metabolise free amino acids and carbohydrates.

Table 1. Free amino acids and carbohydrates during fermentation (10 L, 22°C, 21 days) of the reference, *L. brevis* and *L. plantarum* beers. The initial sampling (0 h), 24 hours and the final sampling (21 days) are included in the table. All values are averages of triplicates. "n.d." = not detected

Amino acid	Amino acids (µmol/g)								
	Reference beer			*L. brevis* beer			*L. plantarum* beer		
	0 h	1 day	21 days	0 hours	1 day	21 days	0 hours	1 day	21 days
Alanine	0.85±0.08	0.91±0.09	0.14±0.02	0.90±0.01	0.89±0.03	0.57±0.02	0.88±0.03	0.51±0.03	0.33±0.01
Glycine	0.29±0.02	0.29±0.03	0.12±0.01	0.30±0.00	0.30±0.01	0.26±0.01	0.29±0.01	0.17±0.01	0.12±0.01
Valine	0.61±0.06	0.63±0.06	0.06±0.01	0.66±0.02	0.65±0.03	0.09±0.01	0.64±0.03	0.37±0.02	0.08±0.01
Arginine	0.47±0.05	0.43±0.04	0.04±0.01	0.47±0.00	0.20±0.01	n.d	0.48±0.02	0.26±0.02	0.09±0.01
GABA	0.41±0.04	0.42±0.04	0.29±0.01	0.43±0.01	0.48±0.02	0.68±0.04	0.42±0.02	0.25±0.02	0.44±0.01
Aspartic acid	0.40±0.04	0.38±0.03	0.04±0.01	0.42±0.00	0.39±0.02	0.09±0.00	0.40±0.01	0.21±0.01	0.06±0.01
Glutamic acid	0.52±0.05	0.50±0.04	0.05±0.01	0.55±0.01	0.47±0.02	0.06±0.00	0.54±0.02	0.31±0.02	0.05±0.00
Asparagine	0.51±0.06	0.42±0.04	0.02±0.01	0.55±0.01	0.44±0.02	0.02±0.00	0.52±0.02	0.24±0.02	0.03±0.00
Serine	0.43±0.04	0.35±0.03	0.05±0.01	0.46±0.00	0.35±0.01	0.05±0.00	0.45±0.02	0.20±0.01	0.04±0.00
Glutamine	0.28±0.03	0.24±0.02	0.04±0.00	0.30±0.00	0.21±0.01	0.04±0.00	0.29±0.01	0.13±0.01	0.04±0.00
Histidine	0.23±0.03	0.22±0.02	0.06±0.00	0.23±0.00	0.23±0.01	0.11±0.01	0.23±0.01	0.13±0.01	0.08±0.00
Threonine	0.27±0.03	0.20±0.02	0.03±0.01	0.28±0.00	0.21±0.01	0.03±0.00	0.27±0.01	0.12±0.01	0.04±0.00
Citrulline	n.d.	0.01±0.00	0.01±0.00	0.03±0.00	0.09±0.00	0.05±0.00	n.d	0.01±0.00	0.01±0.00
Tyrosine	0.33±0.03	0.33±0.03	0.04±0.01	0.35±0.00	0.33±0.01	0.10±0.01	0.34±0.01	0.19±0.01	0.05±0.00
Methionine	0.08±0.01	0.08±0.01	0.04±0.03	0.08±0.00	0.10±0.00	0.08±0.00	0.08±0.00	0.05±0.00	0.04±0.03
Isoleucine	0.33±0.03	0.33±0.03	0.04±0.01	0.36±0.01	0.34±0.01	0.03±0.00	0.35±0.01	0.19±0.01	0.05±0.01
Tryptophane	0.55±0.06	0.54±0.05	0.04±0.01	0.59±0.01	0.45±0.16	0.05±0.00	0.57±0.02	0.25±0.08	0.07±0.00
Phenylalanine	0.21±0.02	0.21±0.02	0.03±0.01	0.23±0.01	0.31±0.13	0.11±0.01	0.22±0.01	0.18±0.09	0.02±0.00
Leucine	0.65±0.07	0.57±0.05	0.07±0.01	0.70±0.01	0.60±0.02	0.07±0.00	0.68±0.03	0.34±0.02	0.11±0.01
Ornithine	0.14±0.08	0.13±0.08	0.12±0.07	0.20±0.00	0.17±0.01	0.29±0.02	0.20±0.01	0.11±0.00	0.04±0.01
Lysine	0.35±0.04	0.27±0.03	0.03±0.00	0.38±0.01	0.27±0.01	0.03±0.00	0.37±0.02	0.15±0.01	0.04±0.01
	Carbohydrates (g/L)								
	Reference beer			*L. brevis* beer			*L. plantarum* beer		
	0 hours	1 day	21 days	0 hours	1 day	21 days	0 hours	1 day	21 days
Glucose	6.97±0.17	4.95±0.15	0.01±0.00	6.83±0.10	5.11±0.24	0.01±0.00	6.98±0.36	3.08±0.09	0.02±0.01
Fructose	3.21±0.19	2.82±0.07	0.18±0.01	3.49±0.05	3.15±0.17	0.14±0.01	4.35±0.12	2.13±0.14	0.14±0.01
Sucrose	1.16±0.09	n.d.	n.d.	1.40±0.30	n.d.	n.d.	1.19±0.12	n.d.	n.d.
Maltose	52.87±0.87	46.40±3.46	1.08±0.10	52.88±0.91	47.84±1.35	3.12±0.07	54.84±1.02	33.14±0.82	1.20±0.02
Maltotriose	11.70±0.78	9.36±0.33	0.64±0.03	13.23±0.30	10.56±0.74	2.85±0.13	14.45±0.35	7.97±0.60	0.78±0.05

Metabolite composition and sensory analysis. The metabolite composition of the final beers was analysed, and the beers were evaluated sensorially. Correspondingly to the small-scale co-fermentation, *L brevis* seemingly exerted more influence with respect to metabolic composition than *L. plantarum* while co-fermenting with *S. cerevisiae*. This was visible from the ASCA score plot (Fig. 5A), where the *L. brevis* beer was separated from the reference beer in both component 1 and 2, while the *L. plantarum* beer only in component 2. Component 1 accounted for 68 % and component 2 accounted for 32 % of the variation in the ASCA, which explained 68.7 % of the variation in the metabolites. Lactic acid, acetic acid, ethyl hexanoate and ethyl octanoate were important drivers of the ASCA model, important in both component 1 and 2 (Fig. 5B). The highest concentration of organic acids was obtained in the *L. brevis* beer (2598±56 mg/L lactic, 942±11 mg/L acetic acid and 196±14 mg/l succinic acid) (Table S3). Significantly lower yet substantial quantities of organic acid were obtained in the *L. plantarum* beer (1791±94 mg/L of lactic and 89±26 mg/L acetic acid), while no lactic acid or succinic acid and only 31±4 mg/L acetic acid was generated in the reference beer. Lactic acid is associated with acidity and sourness (Van Oevelen et al., 1976) and has a reported taste threshold 400 mg/L (Engan, 1974). Acetic acid is associated with acidity, sour (Engan, 1974) and vinegary flavours (Van Oevelen et al., 1976) and has a reported sensory threshold of 200 mg/L (Engan, 1974). Both lactic and acetic acid were well above reported sensory thresholds in the *L. brevis* beer, which corresponds well with this beer being perceived as significantly higher than the *L. plantarum* and the reference beer in acidic taste in the sensory analysis (Fig. 5C, Table S4). The *L. brevis* beer was also scored as significantly higher in astringency compared to the other beers. This also corresponds well with the metabolic data, as astringency is partly related to organic acid content (Da Conceicao Neta et al., 2007), and higher perception of astringency is correlated with decreasing pH (Lawless et al., 1996).

The *L. plantarum* beer was perceived as highest in sour odour (Fig. 5C). The attribute “sour odour” is related to a fresh, balanced odour generally related to presence of organic acids (Table S1) (ISO, 2012). The *L. plantarum* and *L. brevis* beers were different in their organic acid content, not only in the total concentrations, but also in the relative ratios between the organic acids in the beer. The lactic:acetic acid ratio in the *L. brevis* beer was approximately 3:1, while the corresponding ratio in the *L. plantarum* beer was closer to 20:1. It could be speculated that this difference in the organic acid content and ratios somehow manifests as difference in perceived sour odour. The *L. plantarum* beer was also scored higher in fruity odour compared to the *L. brevis* and reference beer, and higher in dried fruit odour compared to the reference (Fig. 5C) (Table S3). This corresponds with the beer being higher in the fruity esters ethyl hexanoate and ethyl octanoate (Table S3). Ethyl hexanoate is associated with fruit, fennel and solvent flavours (Xu et al., 2017) and has a sensory threshold in beer of 0.3 mg/L (Harrison, 1970). Ethyl octanoate is associated with sweet and fruity flavours (Yonezawa and Fushiki, 2002) and a sensory threshold of 0.9-1.0 mg/L in beer (Pires and Bránayik, 2015). At 0.11±0.01 mg/L ethyl hexanoate and 0.03 mg/ ethyl

577 octanoate in the *L. plantarum* beer (Table S3), both esters were below the sensory threshold. Their presence
578 could, however, be influential to the sensory properties through synergistic, sub-threshold effects (Dalton
579 et al., 2000).

580 Further significant differences were found between all the three beers (excluding the commercial Geuze
581 beer from the statistical analysis) in the sensory analysis. Of 22 evaluated attributes (Table S1), 13 were
582 scored as significantly different between two or more of the beers (Fig. 5C, Table S4). Both beers produced
583 through co-fermentation with lactobacilli were perceived as sensorially different from the beer produced
584 through fermentation by *S. cerevisiae* alone. The *L. plantarum* beer was scored significantly higher
585 compared to the reference beer in sour odour, fruity odour, dried fruit odour, sour flavour and after taste
586 and significantly lower in sweet taste. The *L. brevis* beer was scored significantly higher compared to the
587 reference beer in total intensity flavour, acidic taste, bitter taste, after taste, foaminess and astringency and
588 significantly lower in sweet taste. The *L. brevis* and the *L. plantarum* beer were perceived as significantly
589 different from each other in sour odour, fruity odour, perfumed odour, yeasty odour, sweet taste, acidic
590 taste and astringency (Fig. 5C, Table S4).

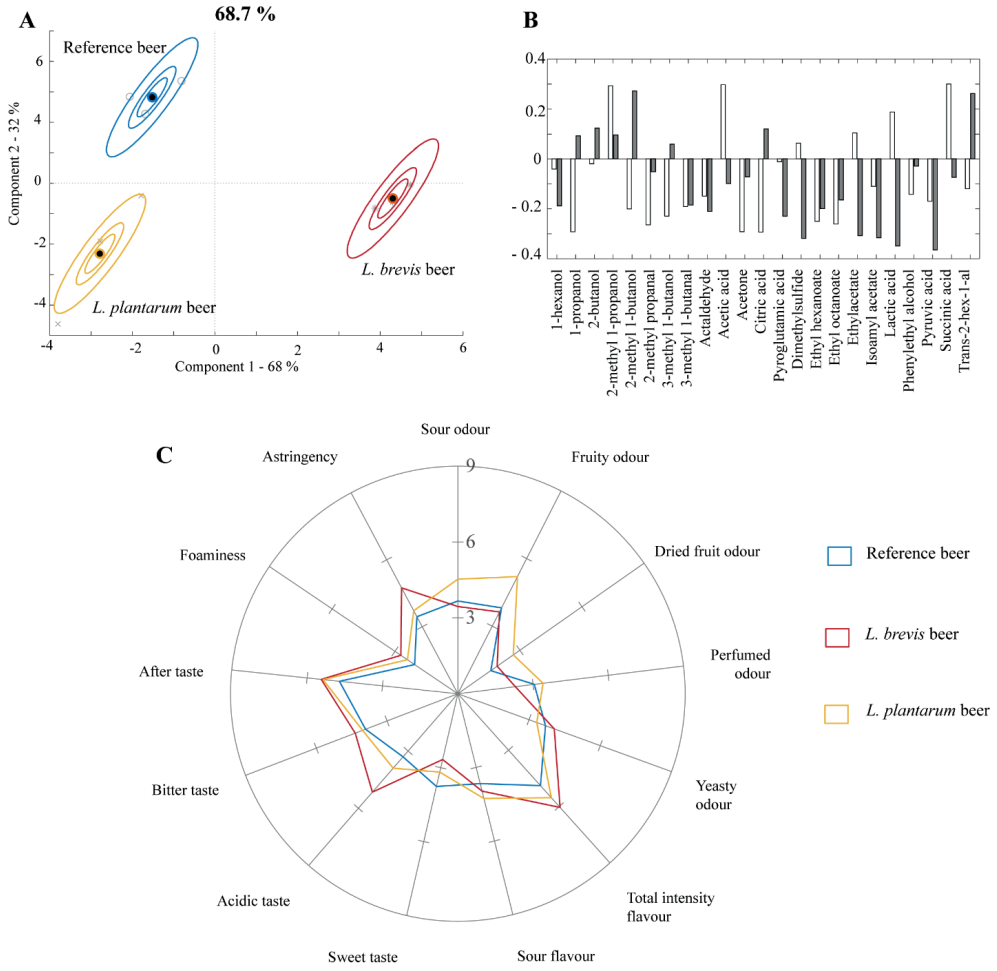

Figure 5. Properties of the final beer from 10 L fermentations at 22°C for 21 days in the reference, *L. brevis* and *L. plantarum* beers. A) Metabolite variation in samples and replicate variation described by ASCA scores. The model is based on the metabolic composition in the final beer products. The model explains 69 % of the variation in metabolites. B) Loading weights for ASCA model in panel A. White bars show loadings for component 1 (68 %) and grey bars show loadings for component 2 (32 %). C) Sensory properties for the three different beers. The graph only displays scores for sensory attributes assessed as significantly different between two or more of the beers.

A commercial sour beer reference (Boon Geuze) was evaluated at the end of the sensory analysis, in order to assess how the sour beers in the current study compared to a traditional sour beer. To visualize the difference in the flavour profile (Table S5) of the four beers, we generated a PCA bi-plot with beers as loadings and sensory attributes as scores (Fig. 6). The commercial Geuze beer is oriented oppositely to the three experimentally produced sour beers in component 1 in the PCA plot and was sensorially different. The Geuze sour beer was scored as significantly different from all other beers in total odour intensity, alcohol flavour, sour flavour and fruity flavour (Table S5). Being produced through a completely different method, the Geuze was expected to be sensorially different from the beers produced in the current experiment. The objective of the current study was not to replicate the sensory character of a geuze style beer, which originates through year-long spontaneous fermentation (Van Oevelen et al., 1977), but rather to get an idea of how beers produced through controlled co-fermentations compared to known commercial sour beers. It is noteworthy that both the *L. brevis* and *L. plantarum* beers were scored closer to the Geuze sour beer, significantly higher compared to the reference beer in total flavour intensity and after taste (Table S5). In addition, the *L. brevis* beer was perceived as similar to the Geuze sour beer and significantly different from the *L. plantarum* and reference beer in sweet taste, acidic taste and astringency.

613

614 **Figure 6.** PCA bi-plot with beers as loadings (blue) and attributes as scores (red), based on the sensory analysis of the
615 reference, *L. brevis* and *L. plantarum* beers. The PCA also includes sensory results from a commercial sour beer
616 reference included at the end of the sensory analysis. PC1 explains 73.3 % in the sample set, while PC2 explains
617 15.1%.

Concluding remarks.

This study shows that *L. brevis*, *L. plantarum* and *L. buchneri* displayed different responses to beer-related environmental stress factors. While *L. brevis* was robust towards stress, the metabolism of *L. plantarum* and *L. buchneri* was severely inhibited by multiple environmental stress-factors. The metabolic data revealed how a stressful environment can cause accumulation of unwanted, flavour active metabolic products during fermentation (i.e. accumulation of diacetyl in *L. buchneri* multi-stressor trial)... Remarkably, the current study demonstrates how controlled co-fermentation with *S. cerevisiae* and a stress-vulnerable *L. plantarum* can be used to produce sour beer within a 21-day fermentation period, resulting in a product with increased total flavour intensity, fruity odour and dried fruit odour. The study also demonstrates how a stress-tolerant *L. brevis* can be used to increase total flavour intensity and produce a sour beer, similar to commercial Geuze sour beer in acidic taste, sweet taste and astringency. Controlled mixed fermentations, and fermentations of wort with non-traditional microbes offers a great potential for creation of novel sour beer products with high production control and short production time. By extending the currently explored method to other mixed fermentations with multistrain yeasts and/or bacteria combinations, it might be possible to shift more sensory properties in the direction of traditional sour beer products and create beer beverages with novel organoleptic properties.

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Supporting information.

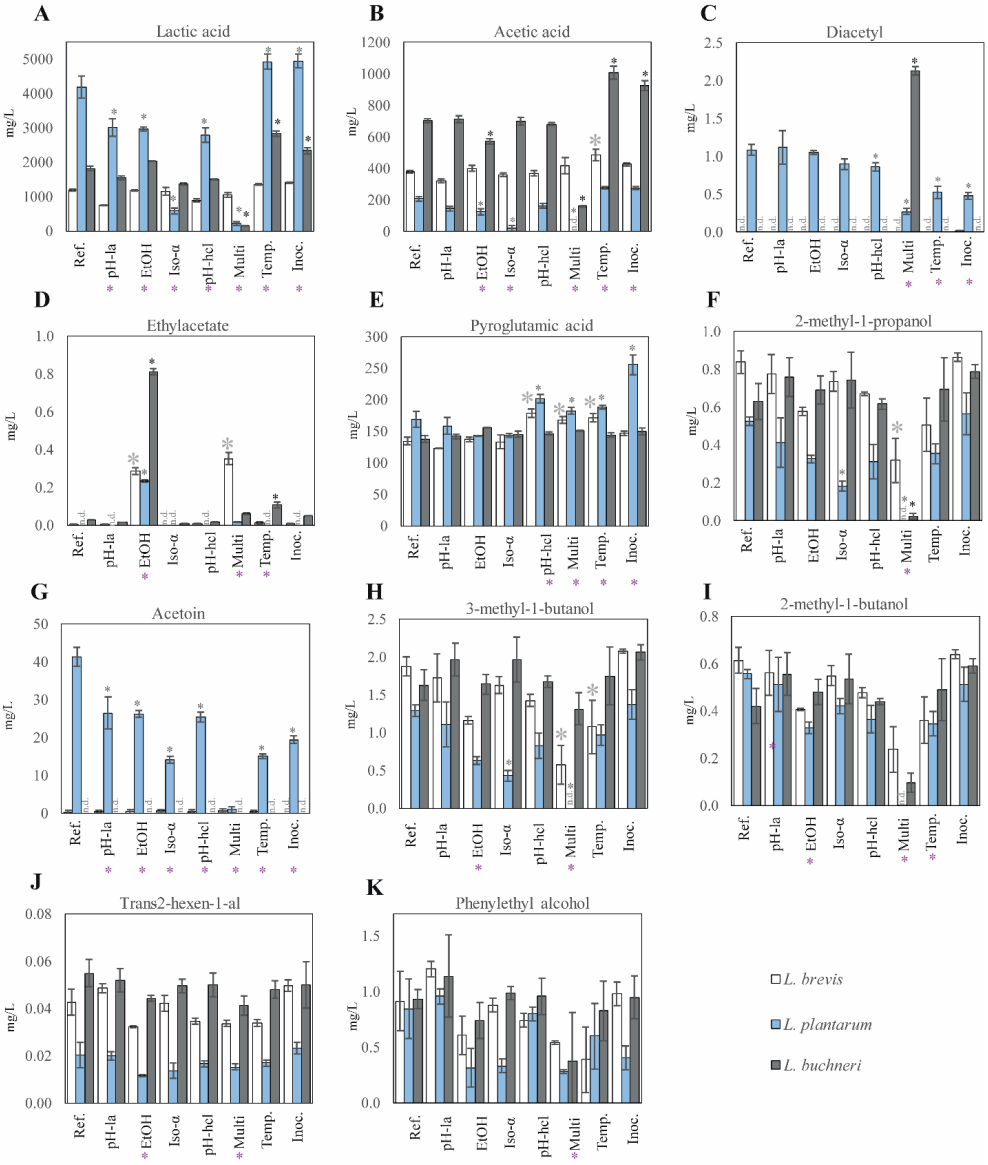

Figure S1: Metabolic compounds produced by different lactobacilli after 7 days of fermentation with varying brewing-related stresses. Average concentrations with standard deviation as error bars are presented for lactic acid (A), acetic acid (B), diacetyl (C), ethylacetate (D), pyroglytamic acid (E), 2-methyl 1-propanol (F), acetoin (G), 3-methyl 1-butanol (H), 2-methyl 1-butanol (I), trans-2-hexen-1-al (J) and phenylethyl alcohol (K). Ref = reference trial; pH-la,

= low initial pH (Lactic acid) trial; EtOH = ethanol trial; iso-α = iso alpha-acid trial; pH-HCl = low initial pH (HCl) trial; Multi = multi stressor trial with ethanol, iso-α acids and low initial pH (lactic acid); Temp. = high temperature trial; Inoc = high inoculum trial. Stressor dependent concentrations across all lactobacilli strains, significantly different to that obtained at the reference condition (significant stressor effect according to ANOVA at significance level $p < 0.05$) are indicated with a purple star at the stressor. Concentrations significantly different from that obtained by respective strains (significant strain-stressor interactions according to ANOVA at significance level $p < 0.05$) are indicated by with * for *L. brevis*, * for *L. plantarum* and * for *L. buchneri*. *n.d* = non detected values.

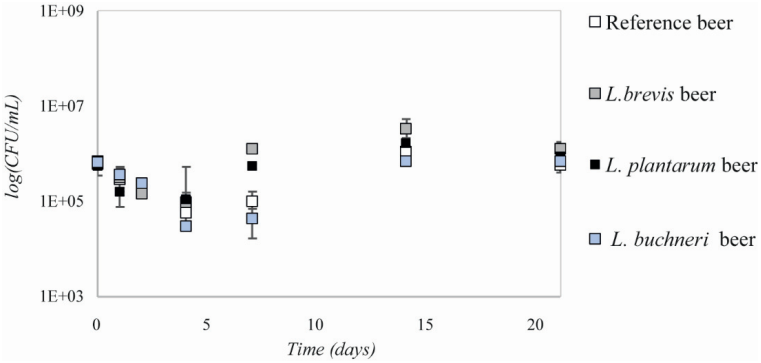

Figure S2: Growth of *S. cerevisiae* during 400 mL fermentation (21 days, 22°C) in reference (white squares), *L. brevis* (grey squares), *L. plantarum* (black squares) and *L. buchneri* beers (light blue squares).

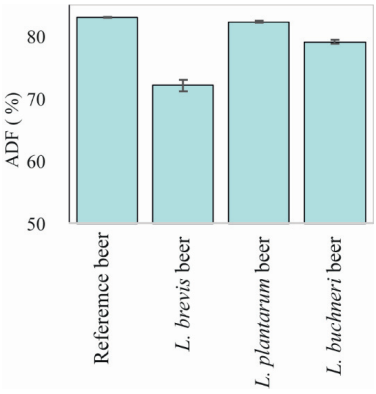

Figure S3: Apparent degree of fermentation (ADF) during 10L fermentations (22°C, 21 days) in reference, *L. brevis*, *L. plantarum* and *L. buchneri* beers.

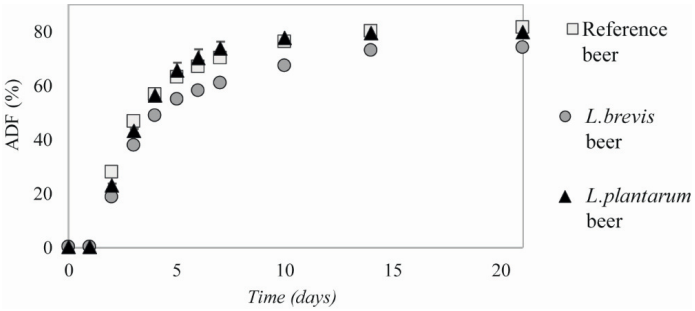

Figure S4: Development of apparent degree of fermentation (ADF) during 10 L fermentations (22 °C, 21 days) in reference (white squares), *L. brevis* (grey circles) and *L. plantarum* (black triangles) beers.

Table S1: 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 due to the presence of organic acids		*Sour-F*	Related to a fresh, balanced flavour due 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
Dried fruit-O	Odour of dried fruits (prunes, apricots, peaches)		*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 seconds after elimination of the product

Table S2. Metabolites after 400 mL fermentations (22 °C, 21 days) in reference, *L. brevis*, *L. plantarum* and *L. buchneri* beers. n.d. = non detected. Significant different concentrations according to ANOVA at $p < 0.05$ are indicated with different letters, where "a" gives the highest concentration group, "b" the second highest and "c" the lowest concentration group.

Metabolites (mg/L)	Reference		*L. brevis beer*		*L. plantarum* beer		*L. buchneri* beer	
Acetic acid	110.58 ± 11.62	c	951.41 ± 24.90	a	124.19 ± 2.64	c	422.59 ± 17.25	b
Citric acid	130.83 ± 0.74	a	n.d.	c	126.05 ± 2.41	a	118.19 ± 4.19	b
Pyroglutamic acid	194.09 ± 2.01	b	193.11 ± 1.73	b	238.42 ± 5.53	a	188.67 ± 1.22	b
Lactic acid	n.d.	d	2299.64 ± 55.08	a	531.00 ± 26.19	c	877.66 ± 38.3	b
Succinic acid	n.d.	b	111.76 ± 3.04	b	n.d.	c	166.50 ± 4.77	a
1-propanol	14.07 ± 0.70	a	7.30 ± 0.56	c	14.37 ± 0.67	a	10.77 ± 0.42	b
2-methyl 1-propanol	19.32 ± 0.65	a	16.26 ± 0.60	b	19.95 ± 1.07	a	20.32 ± 0.50	a
2-methyl 1-butanol	9.97 ± 0.30	a	7.70 ± 0.30	b	10.36 ± 0.24	a	9.52 ± 0.20	a
3-methyl 1-butanol	42.22 ± 1.21	a	35.20 ± 1.21	b	43.22 ± 1.11	a	42.40 ± 0.97	a
Acetaldehyde	24.74 ± 7.35	ab	8.50 ± 1.59	bc	32.13 ± 8.34	a	3.65 ± 0.26	c
Ethyl hexanoate	0.05 ± 0.00	a	0.02 ± 0.00	b	0.05 ± 0.01	a	0.04 ± 0.00	ab
Ethyl octanoate	0.03 ± 0.00	a	0.01 ± 0.00	b	0.03 ± 0.00	a	0.03 ± 0.00	a
Ethyl acetate	8.65 ± 0.24	a	6.15 ± 0.15	b	7.78 ± 1.16	ab	8.76 ± 0.45	a
Isoamyl acetate	0.32 ± 0.02	a	0.16 ± 0.01	b	0.29 ± 0.06	a	0.33 ± 0.03	a
Isobutyl acetate*	0.01 ± 0.00		0.01 ± 0.00		0.01 ± 0.00		0.02 ± 0.00	
Phenylethyl alcohol*	2.11 ± 1.03		2.16 ± 0.20		2.81 ± 0.48		3.00 ± 0.23	
Trans-1-hexen-1-al*	0.03 ± 0.00		0.03 ± 0.00		0.03 ± 0.00		0.03 ± 0.00	

* $p > 0.05$

Table S3. Metabolites after 10 L fermentations (22 °C, 21 days) in reference, *L. brevis* and *L. plantarum* beers. n.d. = non detected. Significant different concentrations according to ANOVA at $p < 0.05$ are indicated with different letters, where "a" gives the highest concentration group, "b" the second highest and "c" the lowest concentration group.

Metabolites (mg/L)	***S. cerevisiae***		***L. brevis* beer**		***L. plantarum* beer**	
Acetic acid	31.37 ± 3.81	c	942.06 ± 10.77	a	88.86 ± 25.61	b
Citric acid	199.25 ± 4.18	a	n.d.	c	176.17 ± 9.93	b
Pyroglutamic acid*	91.19 ± 3.47		95.80 ± 2.24		99.03 ± 8.19	
Pyruvic acid	9.02 ± 0.83	b	19.22 ± 0.80	b	105.45 ± 9.13	a
Lactic acid	n.d.	c	2598.02 ± 55.96	a	1791.61 ± 94.23	b
Succinic acid	n.d.	c	196.38 ± 13.95	a	n.d.	b
1-propanol	15.88 ± 0.62	a	10.11 ± 0.28	b	15.58 ± 0.71	a
2-methyl 1-propanol	21.66 ± 0.56	b	25.87 ± 0.49	a	19.06 ± 0.46	c
2-methyl-propanal	0.01 ± 0.00	ab	n.d.	b	0.01 ± 0.0	a
2-butanol*	0.02 ± 0.01		0.01 ± 0.01		0.01 ± 0.01	
2-methyl 1-butanol	8.71 ± 0.36	a	7.58 ± 0.13	b	8.09 ± 0.06	ab
3-methyl 1-butanol*	28.08 ± 1.02		25.77 ± 0.40		28.06 ± 1.16	
3-methyl-butanal*	0.01 ± 0.01		0.01 ± 0.01		0.01 ± 0.01	
Acetaldehyde*	10.23 ± 3.69		8.91 ± 4.34		20.18 ± 7.45	
1-hexanol*	0.02 ± 0.00		0.03 ± 0.01		0.03 ± 0.01	
Ethyl hexanoate	0.07 ± 0.01	b	0.05 ± 0.00	b	0.11 ± 0.01	a
Ethyl octanoate	0.02 ± 00.0	b	0.01 ± 0.00	b	0.03 ± 0.0	a
Ethyl acetate*	4.57 ± 0.40		6.87 ± 0.59		6.61 ± 1.28	
Isoamyl acetate	0.11 ± 0.01	b	0.14 ± 0.02	ab	0.21 ± 0.05	a
Phenylethyl alcohol*	2.08 ± 0.31		1.85 ± 0.21		2.19 ± 0.28	
Trans-1-hexen-1-al*	0.01 ± 0.00		n.d.		n.d.	
Acetone	0.08 ± 0.00	b	0.01 ± 0.01	c	0.11 ± 0.02	a
Dimethylsulfide*	n.d.		0.01 ± 0.0		0.01±0.00	

* $p > 0.05$

Table S4: Average sensory scores for all attributes evaluated in the descriptive analysis of the reference, *L. brevis* and *L. plantarum* beers. Beers receiving significantly different scores (p-value < 0.05, according to ANOVA analysis) are indicated with different letters, where group "a" has the highest score, group "b" second highest and "c" has the lowest score.

Attributes	*L. plantarum* beer	*L. brevis* beer	*S. cerevisiae*	p-value	Significant
Total intensity odour	5.44 a	5.30 a	5.30 a	0.897	No
Sour odour	4.53 a	3.45 b	3.67 b	0.007	Yes
Hoppy odour	3.76 a	4.11 a	4.08 a	0.574	No
Malty odour	3.59 a	3.37 a	3.40 a	0.699	No
Fruity odour	5.20 a	3.63 b	3.81 b	0.002	Yes
Dried fruit odour	2.68 a	1.90 ab	1.59 b	0.008	Yes
Perfumed odour	3.40 a	2.29 b	3.05 a	0.005	Yes
Yeasty odour	3.33 b	4.06 a	3.69 ab	0.027	Yes
Total intensity flavour	5.53 ab	6.05 a	4.89 b	0.001	Yes
Sour flavour	4.27 a	3.97 ab	3.66 b	0.014	Yes
Sweet taste	3.18 b	2.67 c	3.76 a	< 0.0001	Yes
Acidic taste	3.90 b	5.16 a	3.31 b	< 0.0001	Yes
Bitter taste	4.04 ab	4.35 a	3.91 b	0.033	Yes
Hoppy flavour	3.66 a	3.83 a	4.23 a	0.230	No
Malty flavour	3.40 a	3.29 a	3.67 a	0.161	No
Fruity flavour	5.26 a	4.89 ab	4.13 b	0.051	No
Perfume flavour	2.68 a	2.86 a	2.69 a	0.809	No
Yeast flavour	3.61 a	3.89 a	3.58 a	0.411	No
Alcohol flavour	3.89 a	3.74 a	3.80 a	0.842	No
After taste	5.37 a	5.44 a	4.71 b	0.005	Yes
Foaminess	2.41 ab	2.72 a	2.06 b	0.007	Yes
Astringency	3.72 b	4.74 a	3.45 b	< 0.0001	Yes

Table S5: Average sensory scores for all attributes evaluated in the descriptive analysis of the reference, *L. brevis* and *L. plantarum* beers. The table also includes sensory results from a commercial sour beer reference (Geuze). Beers receiving significantly different scores (p-value < 0.05, according to ANOVA analysis) are indicated with different letters, where group "a" has the highest score, group "b" second highest and "c" has the score concentration.

	Geuze	***L. plantarum* beer**	***L. brevis* beer**	***S. cerevisiae***	**p-value**	**Significant**
Total intensity odour	6.25 a	5.46 ab	5.30 b	5.30 b	0.012	Yes
Sour odour	3.29 b	4.41 a	3.45 b	3.67 ab	0.010	Yes
Hoppy odour	4.15 a	4.01 a	4.11 a	4.08 a	0.980	No
Malty odour	3.71 a	3.71 a	3.37 a	3.40 a	0.415	No
Fruity odour	3.25 b	5.03 a	3.63 b	3.81 ab	0.011	Yes
Drie fruit odour	3.34 a	2.64 ab	1.90 b	1.59 b	0.000	Yes
Perfumed odour	3.61 a	3.18 ab	2.29 b	3.05 ab	0.014	Yes
Yeasty odour	3.36 a	3.42 a	4.06 a	3.69 a	0.076	No
Total intensity flavour	6.91 a	5.61 b	6.05 b	4.89 c	< 0.0001	Yes
Sour flavour	2.84 b	4.19 a	3.97 a	3.66 a	< 0.0001	Yes
Sweet taste	2.67 c	3.23 b	2.67 c	3.76 a	< 0.0001	Yes
Acidic taste	5.22 a	3.92 b	5.16 a	3.31 b	< 0.0001	Yes
Bitter taste	6.17 a	3.96 b	4.35 b	3.91 b	< 0.0001	Yes
Hoppy flavour	4.46 a	3.69 a	3.83 a	4.23 a	0.050	Yes
Malty flavour	3.34 a	3.36 a	3.29 a	3.67 a	0.191	No
Fruity flavour	3.23 b	5.31 a	4.89 a	4.13 ab	0.001	Yes
Perfume flavour	3.58 a	2.54 b	2.86 ab	2.69 b	0.015	Yes
Yeast flavour	3.43 a	3.65 a	3.89 a	3.58 a	0.299	No
Alcohol flavour	5.58 a	3.88 b	3.74 b	3.80 b	< 0.0001	Yes
After taste	6.68 a	5.36 b	5.44 b	4.71 c	< 0.0001	Yes
Foaminess	2.61 a	2.31 ab	2.72 a	2.06 b	0.006	Yes
Astringency	5.40 a	3.61 b	4.74 a	3.45 b	< 0.0001	Yes

Paper III

Secondary lactic acid bacteria fermentation with wood-derived xylooligosaccharides as a tool to expedite sour beer production

Anna Dysvik[1*], Sabina Leanti La Rosa[1*], Fanny Buffetto[1], Kristian Hovde Liland[2], Kristine S. Myhrer[3], Elling-Olav Rukke[1], Trude Wicklund[1] and Bjørge Westereng[1]

[1]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O.Box 5003, N-1432 Aas, Norway

[2]Faculty of Science and Technology, Norwegian University of Life Sciences, P.O. Box 5003, N-1433 Ås, Norway

[3]NOFIMA – Norwegian Institute of Food, Fisheries and Aquaculture Research, PB 210, N-1431 Ås, Norway

* Corresponding authors, anna.dysvik@nmbu.no, sabina.leantilarosa@nmbu.no

ABSTRACT:

Xylooligosaccharides (XOS) from woody biomass were evaluated as substrate for secondary lactic acid bacteria (LAB) fermentation in sour beer production. XOS were extracted from birch (*Betula pubescens*) and added to beer to promote the growth of *Lactobacillus brevis* BSO 464. Growth, pH, XOS degradation and metabolic products were monitored throughout fermentations (2-4 weeks), and the final beer was evaluated sensorically. XOS were utilized, metabolic compounds were produced (up to 1800 mg/L lactic acid) and pH was reduced from 4.1 to 3.6. Secondary fermentation changed sensory properties significantly, and the resulting sour beer was assessed as similar to a commercial reference in multiple attributes, including acidic taste. Overall, secondary LAB fermentation induced by wood-derived XOS provided a new approach to successfully produce sour beer. The presented results both demonstrate how hemicellulosic biomass can be valorised for beverage production, and to obtain sour beer with improved control and reduced fermentation time.

Keywords:

Sour beer

Lactic acid bacteria

Secondary fermentation

Xylooligosaccharides

Hemicellulosic biomass

INTRODUCTION

Beer with intentional acidic taste, referred to as sour beer, is traditionally produced through spontaneous fermentations where lactic acid bacteria (LAB), acetic acid bacteria and yeasts such as Saccharomyces and Brettanomyces are involved [1-2]. This vast microbial consortium generates a wide range of metabolic compounds influential to the organoleptic properties of the final product. Lactic acid and acetic acid are examples of metabolic compounds with sensory importance in beer. With reported sensory thresholds of 400 mg/L and 200 mg/L, respectively [3], they both contribute to sourness and acidity [4]. Other compounds include esters such as ethylacetate, ethyl hexanoate and isoamyl acetate which contribute with fruity flavours [5], and alcohols such as 2-methyl 1-propanol and 3-methyl 1-butanol, associated with alcohol flavour [6]. A selection of metabolic compounds that can influence beer sensory properties is given in table S1, with sensory characteristics and reported sensory threshold in beer.

Fermentation of classic sour beers, including the Belgian styles lambic, gueze and kriek [6], typically takes multiple years [7]. Difficulties with controlling complex mixed fermentations, in addition to the time demand, can make production of commercial sour beer challenging. An increasing interest towards sour beers has emerged in recent decades, and alternative production strategies to the spontaneous fermentations are being explored [8-9]. Fermentation with LAB prior to yeast fermentation is being used in the brewing industry to produce sour beer rapidly and controllably [10]. Other explored strategies include co-fermentation with LAB and yeast [11] and "primary souring", where an alternative acid-producing yeast generates the sour taste [12]. Besides water and hops, malted barley is the main ingredient in beer. Non-malt sources of extract, also known as adjuncts, are commonly added to modern beer, providing additional substrates for the yeast fermentation [13]. Common adjuncts in beer production include unmalted cereals such as barley, corn, rice, wheat and oats [14], but also non-cereal substrates such as granulated sugar, sucrose-based syrups and malt extract [15]. Adjuncts allows the manipulation of the beer characteristics such as flavour, colour, drinkability and foaming properties, and may increase brewery capacity through high gravity wort production [16]. As beer is a worldwide consumed beverage,

the replacement of a portion of barley malt with adjuncts is an efficient way to reduce production costs, as most adjuncts are cheaper than malt. Use of adjuncts can also reduce the carbon footprint from beer production, as the malting process (steeping, germination, kilning) entail huge energy and water demands [15]. Previous studies have explored the use of alternative non-grain derived adjuncts including banana [17], cocoa pulp [18] and sweet potatoes [19]. Emphasis is being made on using regionally available adjuncts as their use can reduce the demand for expensive transportation.

The most abundant raw material on earth is lignocellulosic biomass, composed mainly of cellulose, hemicellulose and lignin [20]. Hemicelluloses constitute the second largest fraction of the dry weight biomass in hardwood and grasses, in which xylan is the prevalent polymer [21]. The common structural feature of xylan is the β-1,4-D-xylopyranose backbone, but the remaining structure varies with origin [22]. Glucuronoxylans found in hardwood can be decorated with acetyl substituents, as well as α-1,2-4-*O*-methyl-D-glucopyranosyl uronic acid [23-24]. Applications of hardwood-derived xylans have been explored within the food industry [22-23]. Xylan has been shown to have antioxidant activity [25], it has been proposed as a nutritional fibre [26] and xylan hydrolysate-derived xylitol can be used as sweetener [27]. Due to its water holding capacity, xylan has been suggested as bread ingredient [28]. It has also been tested as a hydrocolloid for texture improvement and dietary fibre enrichment in dairy products [29]. Hardwood derived xylan also has prebiotic potential. It has been shown to serve as carbon source in the fermentation of health-beneficial bacteria including *Bifidobacerium bifidum* [30], *Roseburia* sp. and *Bacteroides* sp. [31-32] as well as *Bifidobacterim adolescentis* and *Lactobacillus brevis* [33].

The xylan-degrading capabilities of microbes typically involved in food production, e.g. LAB, offer possibilities for utilizing hardwood derived xylan directly as a carbohydrate source in fermented foods and drinks. Beechwood xylan has been evaluated as a carbon source in fermentation of Pozol, a traditional, maize dough based, Mexican drink [34]. In this study, López-Hernández et al. showed that this substate promotes the growth of various *Weissella* spp. strains and other LAB, including sour beer-relevant strains such as *Lactobacilli*. In the current study, *xylan* was extracted from European white

83 birch (*B. pubescens*) wood chips. The potential of this product as a selective substrate for controlled
84 secondary fermentation in sour beer was investigated. Small- and large-scale fermentation experiments
85 were set up to determine the ability of this non-food carbohydrate source to enhance the growth of LAB
86 in beer, and the resulting beer was analysed with respect to metabolic compounds and sensory
87 properties.

MATERIAL AND METHODS

Preparation of xylan

Xylan was isolated from multiple batches of steam-exploded birch (*B. pubescens*) chips. Briefly, wood sawdust was pre-treated by steam explosion (10 min residence time at 200ºC with a solid to liquid ratio of 1:1 w/w). The released hemicellulose was extracted by hot water extraction and the resulting molasses liquid was subjected to ultrafiltration using 10 kilo Dalton (10 kDa) molecular weight cut-off (Alfa Laval) membrane. The retentate from the ultrafiltration was collected, the permeate was further diafiltered and concentrated on a nanofiltration membrane (TriSep). Ultrafiltration and nanofiltration were conducted on UF/NF model G (Gea filtration, Denmark). Both the ultrafiltration and the nanofiltration retentate were lyophilized on an ALPHA 2-4 LD Plus freeze dryer (Christ, Germany) yielding two xylan samples of different degree of polymerization. The nanofiltration retentate was used as a starting material in this study, further named low molecular weight (Mw) acetylated arabinoglucuronoxylan (AcAGX).

Characterisation of low Mw AcAGX

Uronic acids were quantified according to the method by Scott [35]. 0.3 mL of sample (1 mg/mL) were mixed with 0.3 mL sodium borate solution (2% boric acid and 3% NaCl dissolved in MilliQ water), 5 mL of concentrated sulfuric acid was added and samples were incubated for 40 minutes at 70°C. Hydrolysates were cooled down before 0.2 mL dimethylphenol (0,1% dissolved in glacial acetic acid) was added and samples were incubated at room temperature (RT) for 15 minutes to initiate a colorimetric reaction. Total uronic acid concentration was determined by comparing absorbance (λ = 400 and 450) to a glucuronic acid standard.

Neutral monosaccharides were analysed by High-performance Anion-exchange Chromatography (HPAEC). Samples were autoclaved (121°C, 1 hour) with 4% H_2S0_4 to hydrolyse glyosidic linkages, cooled down and diluted to optimal concentration range (10 to 90 µg/mL). Arabinose, rhamnose, galactose, glucose, xylose and mannose were detected and quantified using a Dionex™ ICS-3000 system (Thermo, USA) set up with CarboPac™ PA1 columns – (guard 2X50 and analytical 2X250 mm) and an

electrochemical detector run in pulsed amperometric detector (PAD) mode. Analytes were eluted isocratically (1mM NaOH) at 30°C with a flow rate of 0.25 mL/min.

Another part of the hydrolysate used for the neutral monosaccharide determination above was used to determine the ***acid soluble lignin*** (ASL). The lignin was determined per mg samples according the equation of NREL procedure [36]. Samples were analysed in triplicates (absorbance measured at 240nm). The extinction coefficient (Ɛ) and the pathlength used were 25 L/g/cm and 1cm, respectively.

$$ASL\% = \frac{Abs\ at\ 240\ nm\ \times Volume\ hydrolysis \times Dilution}{Ɛ \times weight\ DM(mg) \times Path\ length} \times 100$$

Acetic acid was quantified by HPLC. Deacetylation of xylan (10mg/mL) was carried out by alkaline treatment (0.1 M NaOH, overnight at 4°C). Acetic acid was quantified using an Ultimate 3000 HPLC system (Thermo Scientific™, USA) with isocratic (5mM H_2SO_4) elution on a Rezex ROA-Organic Acid-H+ column (300×7,8mm) coupled to a security guard cartridge Carbo-H4 (4×3.0mm). The released acetic acid was detected at 210 nm, and quantification was based on a standard curve of external standards of acetic acid.

Proteins

Protein content was determined by micro-Kjeldahl. Samples (0.3 g) were hydrolysed in a Kjeldahl tube with a digestion tablet (Kjeltabs Auto, Thompson and Capper Ltd., Runcorn WA7 1PH, UK) and 3 mL H_2SO_4 (96 to 97%; Merck, Darmstadt, Germany) on an auto-digester (Foss Teactor, Foss analytical lab, Hoganas, Sweden) for 60 minutes at 420°C. Distillation and titration were carried out on a Foss Kjeltec 8400 analyser unit (Software version 1.5.18, Foss analytical lab, Hoganas, Sweden). The ratio of nitrogen (%N) was converted to protein (%P) using a conversion coefficient of 6.25.

Moisture and Ash

The moisture weight was determined by drying 0.2g of sample at 105 °C for 20 hours. Weight difference after cooling down in a dehydrator was considered moisture. The remaining sample was burned at 600°C for 24 hours in an oven (Carbolite, Sheffield, England) to determine ash content. All measurements were performed in triplicates.

Preparation of xylooligosaccharides (XOS).

Low Mw AcAGX (50 mg/mL) was incubated with Shearzyme® (Novozymes, Denmark) overnight (ON) at 37 °C with shaking (100 rpm) to reduce the degree of polymerization. After the enzymatic degradation, the xylan was treated with 0.4 M NaOH ON at RT to achieve alkali deacetylation [37]. To remove NaOH and acetic acid from the product, the mixture was cleaned by filtration through a TriSep 2540-XN45-TSF membrane (Lenntech, The Netherlands) operated by a GEA model L filtration unit (GEA filtration, Denmark). The xylan was diafiltered with 300 L of water (permeate conductivity 40 uS/cm) before the retentate, hereafter referred to as XOS preparation 1 (XP1), was harvested. The pH of this product was 8.1. XP1 was freeze dried on an Alpha 2-4 LD Plus freeze-dryer (Christ, Germany) and used in secondary fermentation experiments in beer. In order to obtain an adequate substrate for secondary fermentation, low Mw AcAGX (17 mg/mL) was treated with 0.5 mM NaOH and the product cleaned by nanofiltration with approximately 350 L water. The retentate containing the product, was harvested when the permeate conductivity was below the detection limit in the operation unit, and the pH was 6.8. The product was then treated with Shearzyme® (37°C, 100 rpm, 5 hours) and freeze dried to obtain a dry powder, hereafter referred to as XOS preparation 2 (XP2).

Production of base beers.

Two base beers were produced according to the same recipe, but with different hopping. A 60L PRO pilot scale brewery from CoEnCo (Oostkamp, Belgium, 2014) was used. Crushed malt (67 % Pilsner malt from BestMalz, Germany, 33 % wheat malt from Weyermann, Germany) was mashed (1 kg malt: 4 L water) as follows; 1: 45 min at 65 °C, 15 min at 72 °C followed by 2 min at 78°C. The wort was separated from the

158 spent grain and boiled for 60 minutes, yielding wort with specific gravity of 1.040 (9°P). Hop pellets were
159 added at the beginning of the boiling step to yield an estimated final concentration of iso-α acids of 5 mg/L
160 in beer LH (Lower Hopping) and 10 mg/L in beer HH (Higher Hopping). Dry yeast (Safale US-05,
161 Fermentis, Gabriel Perl, France) was added to cooled wort (22°C) at 0.5 g/L. After primary fermentation
162 for 3 weeks, at RT, the base beers were kept at 4 °C for at least 2 weeks, to allow the yeast to sediment
163 prior to further experiments.

164 **Secondary fermentation experiments.**

165 *Bacterial strains and starter culture preparation.*

166 *Lactobacillus brevis* BSO 464 (hereafter referred as to *L. brevis*) was purchased from Campden BRI
167 (Gloucestershire, United Kingdom) and routinely grown at 30 °C without shaking in MRS medium (De
168 Man, Rogosa and Sharpe, Oxoid Ltd., United Kingdom). For secondary fermentation experiments in beer,
169 *L. brevis* was first grown ON in MRS, before inoculation in a mixture of 85% MRS and 15 % beer (v/v)
170 The ON culture from MRS with beer was used to inoculate the fermentation bottles in all experiments at 1
171 %. For the up-scaled fermentation, the starter culture in MRS with beer was centrifuged, the cell pellet was
172 re-suspended in a corresponding volume of beer and used as inoculum.

173 *Small scale fermentations*

174 XP2 (2 % w/v and 0.5 % w/v) was dissolved in beer LH, before the beer was centrifuged for 10 minutes at
175 4 °C (7000 × *g*, Heraeus Multifuge X3R, ThermoFisher, Germany, 2010) and sterile filtered (0.22 µm,
176 Millipore Express^{TM}PLUS, Merck, Germany). The beer was then partitioned into four 50 mL bottles. Three
177 bottles were inoculated (1%) with *L. brevis*, and one served as a negative, non-inoculated control. A
178 corresponding setup was prepared with xylose to serve as positive control, and a setup with beer without
179 substrate addition as negative control. The fermentation progressed at 25°C for two weeks. Two additional
180 full corresponding setups were prepared. One 2 % XP1 in beer LH, to investigate the performance of XP1.
181 The second one was prepared with 2 % XP2 in beer HH, and incubated at 22 °C. This experiment was

conducted to verify that the secondary fermentation still took place in beer with higher hopping, and at regular ale fermentation temperature.

Large scale XOS sour beer production by secondary fermentation

Three 5 L fermentation flasks containing beer HH supplemented with 0.5 % XP2 were prepared, following inoculation with *L. brevis*. Negative, non-inoculated, controls (2 × 5 mL tubes) were taken out from each of the three 5 L flasks prior to fermentations. Positive xylose controls were prepared in 5 mL tubes without centrifugation and sterile filtrations. Beer with XP2 and positive xylose controls were inoculated (1%) with *L. brevis* starter. Negative controls with beer without substrate additions were prepared in 5 mL tubes without centrifugation and sterile filtration. The fermentation progressed at 25° C for 4 weeks. After this period the beer, referred to as "XOS sour beer", was kept at 4°C for 4 weeks. Prior to sensory evaluation, the beer was carbonated using a Genesis sodastreamer (SodaStream International Ltd., Israel) to avoid the beer appearing completely flat, before transferring to 0.33L bottles.

Sampling.

For the small-scale fermentations, samples of 0.4 mL were drawn at 0 h, 1 2, 3, 5, 7, 11, 14 or 28 days. Growth of *L. brevis* was monitored by plate drop on MRS agar plates (15% agar powder, VWR Chemicals, Leuven, Belgium). pH was monitored throughout fermentation using a Sentron pH-meter with SI probe (Sentron, Netherlands). Samples were then centrifuged (3 min, 13.2 × 1000 rpm) using a 5415 D centrifuge (Eppendorf, Germany) to remove cells. 200 µL of the supernatant was frozen for further analysis. After the final sampling, remaining content in each fermentation flask was centrifuged at 7000 × *g*, 4°C for 10 minutes and the supernatants were frozen for further analysis. For the large-scale fermentation, samples of 50 mL were drawn during the fermentation at 0 hours, 1 2, 3, 5, 7, 11, 14 and 28 days. A final sample was drawn at the time of the sensory evaluation. MRS agar plates supplemented 25 mg/L cycloheximide (Sigma-Aldrich, St. Louis, USA) was used to monitor the growth of *L. brevis*, while yeast counts were followed using Rose-Bengal Chloramphenicol agar (RBC, Oxoid, Basingstroke, UK). pH was monitored

206 throughout fermentation, and samples were centrifuged as described above and the supernatant was kept at
207 -20°C for further analysis.

208 **Analyses.**

209 *Matrix assisted laser desorption–ionization time of flight (MALDI-ToF) mass spectrometry*
210 *(MS).*

211 MALDI-ToF analyses were performed with an Ultraflextreme MALDI-ToF/ToF MS instrument (Bruker
212 Daltonics, Germany) equipped with a 337-nm-wavelength nitrogen laser. All measurements were
213 performed in positive ion, reflector mode with 1000 shots taken per spectrum. For sample preparation, 1
214 µL of sample solution was mixed with 2 µL of matrix (0.9% w/v 2,5-dihydroxybenzoic acid [DHB] – 30%
215 acetonitrile [v/v]), directly applied on an MTP 384 target plate (Bruker Daltonics, Germany) and dried
216 under a stream of warm air.

217 *High-performance anion-exchange chromatography (HPAEC).*

218 During fermentation with *L. brevis*, aliquots were removed at regular intervals. The amount of the mono-
219 and oligo- saccharides consumed was quantified by HPAEC using standard methodology. In brief, samples
220 were bound to a Dionex (Thermo Scientific) CarboPac PA1 analytical column (2 × 250 mm) in combination
221 with a CarboPac PA1 guard column (2 × 50 mm), equilibrated with 0.1 M NaOH. Carbohydrates were
222 detected by pulsed amperometric detection (PAD). The system was run at a flow rate of 0.25 mL/min. The
223 elution conditions during analysis were 0-10 min 0.1 M NaOH with a 0 to 0.1 M NaOAc gradient; 10-35
224 min 0.1 M NaOH with a 0.1 to 0.3 M NaOAc gradient; 35-40 min 0.1 M NaOH with a 0.3 to 1 M NaOAc
225 gradient; and 40-50 min 0.1 M NaOH. Commercial xylose and xylooligosaccharides (DP 2 to 6) from
226 Megazyme were used as standards.

227 *Headspace gas chromatography (HSGC).*

228 HSGC according to the method described by Grønnevik, et al. [38], was used to analyse volatile compounds.
229 Samples were filtered through 602h ½ folding filters (pore size < 2 µm, Schleicher & Schuell, Dassel,
230 Germany) before 10 g were 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 applied headspace bath temperature was 50 °C and manifold temperature of 60 °C, and the carrier gas was helium 6.0 (Aga, Norway) at a flow rate of 5.0 mL/min. Prior to injection (0.5 min injection time, 10 PSI pressure), samples were mixed for 45 minutes (70 shakes/min) to achieve equilibrium. A CP-SIL 5CB GC column (Varian, Middelburg, Netherlands) of 25 m × 0.53 m I.D. with film thickness 5 µm, was used to separate the compounds based on volatility and affinity for the column. Identification and quantification were carried out according to calibration with standards. Open LAB EZChrom software (version A.04.05, Agilent Technologies) was used to operate the system. The following temperature scheme was applied during analysis: 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; increase of 10°C/min until 200°C for 2 min.

High performance liquid chromatography (HPLC).

HPLC, according to the method described by Grønnevik, et al. [38], was used to analyse organic acids. One g sample was mixed with 2.5 mL MilliQ water, 200 µL 0.5 M H_2SO_4 and 8 mL acetonitrile using a MultiRS-60 BIOSAN turner (Montebello Diagnostics A/S, Oslo, Norway) operated at 30 rpm for 30 minutes. Samples were centrifuged for 15 min at 1470 × *g* using a Kubota 2010 centrifuge (Kubota Corporation, Tokyo, Japan) and filtered through 0.2 µm PTFE membrane (Acrodisc CR 13 mm Syringe Filter, PALL, Great Britain). The organic acids were separated on an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA), operated at 30°C. The column was connected to a 1260 Infinity HPLC instrument (Agilent Technologies, Singapore) with pump, autosampler, column oven, RI-detector (refractive index, used for acetic acid) and DAD-UV detector (diode array detector- ultra violet, used for the other organic acids). H_2SO_4 at a flow rate of 0.4 mL/min was used as mobile phase. Openlab CDS software (Agilent

Technologies) was used to operate the system. Detection and quantification were done according to calibration with standards.

Beer characterisation.

Beer characterisation was carried out using a PBA-B instrument, consisting of a DMA 4500M density meter, an Alcolyzer Beer ME module with integrated colour measurement module, a CarboQC ME module and a PFD filling device. The instrumental setup was delivered by Anton Paar (Graz, Austria) and used to determine alcohol concentration, colour value, apparent degree of fermentation, original extract and sugar concentration. The equipment was all operated through Generation M instrument software version v2.42 (Anton Paar, Graz, Austria).

Sensory evaluation by trained panel.

Sensory evaluation of the beers was carried out by a professional sensory panel consisting of nine trained assessors at the Norwegian Institute of Food, Fisheries, and Aquaculture Research (NOFIMA, Aas, Norway). Panellists have been screened for sensory abilities (basic tastes, colour vision, odour detection, tactile sensibility) and ability to communicate sensory descriptions of products recommended in ISO (International organization for standardization) 8586 [39] in a sensory laboratory designed in accordance with ISO 8589 [40]. EyeQuestion (v4.11.33, Logic8, Holland) was used for direct recording of data. Based on previous experiments with beer, a list of relevant sensory attributes was generated. A training session was carried out, in which the assessors were trained in the definition of 22 selected taste, texture, odour and flavour attributes (Supplementary table S2). Sensory profiling according to Generic Descriptive Analysis as described by Lawless and Heymann [41], was used to evaluate the two different beers in duplicate. Each assessor evaluated samples within each session in individual randomised order. The evaluation of six samples in total was conducted in three sessions with a warm-up sample served in the beginning of the first serving. A commercial sour beer reference (Geuze, Mariage Parfait, 2015, Boon Brewery, Belgium) was evaluated in duplicate at the end of the last session.

Two bottles of the commercial sour beer reference were poured into one beaker, carefully avoiding the sediments in the bottles. Due to the high carbonation level in the commercial sour beer, this beer was poured back and forth between two beakers 20 times and left to rest for one hour prior to serving. Two bottles of the "Base beer" (prior to secondary fermentation) were mixed in one beaker. One bottle of each of the three replicates of "XOS sour beer" were mixed in one beaker. Beer (30 cL) was served at temperature 17± 1°C in clear plastic cups, tagged with random three-digit codes. All samples in one session were placed in the sensory evaluation booths at the same time and monadically evaluated at individual speed and registered continuously. The assessors were instructed to take a sip of the beer and rate the intensity of all attributes on a non-structured continuous scale with endpoints corresponding to 1 (lowest intensity) and 9 (highest intensity). Scores were converted to a number between 1 and 9 by the Eye Question software. All samples were expectorated, and unsalted crackers and warm and cold water was available for rinsing. EyeOpenR (v4.11.33, Logic8, Holland) was used to analyse the data using a paired t-test, for a base beer and XOS sour beer, and ANOVA (Analysis of variance) combined with Tukey's test for pairwise differences for all samples including the commercial. Significantly different attributes ($p < 0.05$) were selected based on the t-test and the ANOVA with Tukey's test.

Statistical analysis.

Analysis of variance Simultaneous Component Analysis (ASCA) [42] was used to examine differences in the metabolic compounds between the beers assessed in the sensory evaluation. The ASCA model was fitted using MATLAB (2018a, The Mathworks, Natick, MA). ASCA is a multivariate ANOVA combined with data compression. This means that variation due 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 Principal/Simultaneous Component Analysis (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

304 in ANOVA, using confidence ellipsoids [43]. Uni-dimensional ANOVA for each compound was combined
305 with Tukey's test for honestly significant differences. This was done to obtain groups of effect levels, e.g.
306 groups of beers, which are not significantly different with respect to a chosen measured property. The
307 ANOVA with Tukey's test was carried out using R 3.6.1 (R Core Team 2019, Austria, Vienna), and
308 statistical significance level was set at $p < 0.01$.

RESULTS AND DISCUSSION

Properties of low Mw AcAGX.

The composition of the Low Mw AcAGX used for further substrate preparation is presented in table 1 (the analyses accounts to 97.7% mass closure). As expected, xylose (83%) was the most abundant monosaccharide in the preparation. Arabinose (1.5%), uronic acids (3.3%), and rhamnose (1.2%) were also found in the sample. In addition, low levels of mannose (4.2%) and glucose (3.8%) and galactose (2.9%) were detected, indicating the presence of a minor amount of galactoglucomannan in birch wood.

Preparation and characterization of the XOS samples.

Enzymatic digestion and alkali deacetylation of Low Mw AcAGX, were carried out in order to generate shorter oligosaccharides and increase the fermentability by the used *L. brevis*. Previous studies have shown that XOS with a degree of polymerisation of 2-5 are favourable for microbial fermentation [44-45] and unsubstituted oligos are more readily utilized by microbes [46]. Enzymatic digestion of low Mw AcAGX, followed by alkali deacetylation generated XP1. A second substrate was prepared to reduce the pH observed in XP1 and increase the amount of *L. brevis* fermentable substrate. This was done by using an initial deacetylation step with NaOH, followed by removal of alkaline before enzymatic treatment. This preparation scheme resulted in XP2, having some minor remaining xylanase activity (not observed for prep 1). To determine the mass distribution and degree of acetylation, the two preparations were analysed by HPAEC-PAD and MALDI-ToF MS. While low Mw AcAGX contains xylose and XOS ranging from two to six units, XP1 contains very low, or no amounts of xylose and xylobiose (X_2), with predominant XOS being xylotriose (X_3), xylotetraose (X_4), xylopentaose (X_5) and xylohexaose (X_6) (Fig. 1A). This was partially caused by the acetyl substituents making the polymer less accessible to the enzyme [47-49], yielding longer oligos, and partially by the loss of X_1 and X_2 in the cleaning step where NaOH and acetic acid was removed. XP2 contains primarily X_2, but also considerable amounts of X_{3-6}. This agrees with previous literature where Shearzyme® has been used [47]. As shown in Figure 1B, the low Mw AcAGX is heavily

acetylated; the degree of acetylation (DA = 0.34) has been reduced in XP1 and XP2, making them more accessible for fermentation.

Small scale fermentations.

L. brevis grew well in beer LH supplemented with XP1 (Fig.2A), with an increase in CFU by two log units in 7 days, reaching a maximum observed cell count of 2.1×10^8 CFU/mL. A simultaneous reduction of pH was observed, from initial pH of 5 to 4.4 after 7 days (Fig.2B). A reduction in CFU/mL and a very minor pH drop was observed at the later sampling points. Comparing the HPAEC-PAD profiles at the initial and final sampling (Fig. 2C), X_1, X_2, X_3 and X_4 are completely fermented after 28 days. The X_5 and X_6 are seemingly untouched. This suggests that *L. brevis* can degrade XOS with xylose units up to four, but not higher. The high initial pH in this fermentation is a result of a pH elevating effect from XP1 to beer. Despite successful acid production from *L. brevis*, the final beer pH was too high for the beer to classify as a sour beer. Because of the high pH contribution form XP1, all the subsequent growth experiments were conducted using XP2 as secondary substrate in beer.

The initial growth of *L. brevis* was similar in regular LH beer, and the same beer supplemented with XP2 (Fig. 3A). In both beers supplemented with 0.5% and 2% XP2, the cell counts kept increasing during the first week of fermentation reaching a maximum of 1.4×10^8 CFU/mL after 7 days for 0.5 %, and 2.3×10^8 CFU/mL after 5 days for 2 %. The growth curves for *L. brevis* in beer and beer supplemented with xylose were similar, but with a lower maximal cell count, and earlier decrease in cell numbers. The final cell counts after 14 days were 5.2×10^6 for 2 % XP2, 5.8×10^6 for 0.5 % XP2, 1.2×10^5 for the xylose positive control, and 1.4×10^6 for the beer negative control.

Corresponding reductions in pH were observed in *L. brevis* inoculated LH beer supplemented with 0.5% xylose and 0.5% XP2 (Fig. 3B). The initial pH for both these beers were 4.1, and the final pH 3.4. The initial pH of the beer with 2 % XP2 was slightly higher at 4.3. This can be attributed to the pH-elevating effect from the XP2 itself. Only a marginally lower final pH of 3.3 was obtained with the higher substrate dose. It is noteworthy that the pH reduction was quicker with higher substrate dosage. In fact, after only 5

days of secondary fermentation, the pH in the LH beer with 2% XP2 was 3.6, compared to 3.9 in beer with 0.5% XP2 (Fig. 3B). This suggests that it is possible to carry out acidification of non-sour base beer in sour beer production in less than a week with secondary fermentation, by using a high dose of secondary substrate. No reduction of pH was detected in beer without secondary substrate addition. No pH-reduction was observed in any of the non-inoculated negative controls during the incubation period (Supplementary, Fig. S1). As can be seen from the HPAEC-PAD profiles from the final sampling with *L. brevis* inoculated and non-inoculated beer (Fig. 3C), the secondary substrate in the 0.5% XP2 dosage is utilised to completion. The peak representing X_2 is diminished in the *L. brevis* inoculated beer with 0.5% XP2 at the end of fermentation, while X_2 is not fully consumed at the end of fermentation of beer with 2% XP2.

In the absence of a substrate for secondary fermentation, 560 mg/L lactic acid and 360 mg/L acetic acid were present in the beer after 14 days of incubation with *L. brevis* (Fig. 3D). When the beer was supplemented with 0.5% XOS, 2280 mg/L of lactic acid and 1740 mg/L acetic acid were detected at the same time point. The corresponding values for beer with 2% XP2 were 3940 mg/L lactic acid and 2930 mg/L acetic acid. A similar trend, showing a higher concentration of compounds with increasing amount of secondary substrate, was observed for acetaldehyde, ethyl acetate (Fig. 3E) and isoamyl acetate (Fig. 3F). The opposite is evident for acetoin (Fig. 3E) and diacetyl (Fig. 3F) where higher substrate concentration yields lower amounts.

These results demonstrate that the final pH of the beer after secondary fermentation, as well as the final composition of metabolic compounds, can be adjusted by using different doses of secondary substrate. Some organoleptically active compounds did not change during secondary fermentation or in response to addition of a secondary substrate. Phenylethyl alcohol (Fig. 3E) is an example of this, showing that the initial composition of beer prior to secondary fermentation, is important for the final composition of a sour beer produced through this method. A similar experiment was also carried out at lower fermentation temperature (22°C) in beer produced with approximately twice the amount of iso-α acids (Supplementary, Fig. S2). *L. brevis* performed well under these conditions. Growth and pH-reduction were slower, but after

21 days incubation a final pH of 3.7 was obtained in the beer. Thus, fermentation temperature and hopping scheme can also be used to manipulate metabolic activity and final pH in sour beer produced through secondary fermentation.

XOS sour beer production through secondary fermentation.

A reduction in the concentration of dispersed bacterial cells was observed from day four, and all through the incubation period for the 5 L secondary fermented HH beer (Fig. 4A). Microbial growth likely took place, but was masked by inhomogeneous distribution of bacterial cells due to no mixing in the fermentation bottles. Metabolic activity by *L. brevis* was confirmed by pH-drop, X_2 depletion and development of organic acids and other metabolic compounds during incubation. During the fermentation, the pH dropped from 4.1 to 3.6 in the inoculated beers with XP2. No pH reduction was observed in the non-inoculated negative controls (Fig. 4B). The HPAEC-PAD profiles from the final sampling from *L. brevis* inoculated and non-inoculated beers shows a substantial reduction in available X_2 in the inoculated samples compared to the non-inoculated (Fig. 4C). This suggests that *L. brevis* utilizes the available X_2 from the XP2 preparation, with concomitant production of acids that causes the pH drop.

Lactic and acetic acid were produced throughout fermentation (Fig. 4D), reaching final concentrations of 1800 and 1200 mg/L after 28 days of secondary fermentation. At these concentrations, both acids were well above their respective sensory detection limits and likely to influence the sensory properties of the sour beer. Pyruvic and citric acid had opposite developments (Fig. 4E), being present in the base beer at approximately 250 and 200 mg/L, respectively. After 5 days of *L. brevis* secondary fermentation, the concentration of pyruvic acid was reduced to 20 mg/L, while citric acid was no longer detected. Acetoin and diacetyl were not present in the base beer but were produced during the initial growth phase during secondary fermentation (Fig. 4E). The concentration of both acetoin and diacetyl peaked after three days (25 mg/L for acetoin and 8 mg/L for diacetyl) followed by a drop to below 5 mg/L for acetoin and below the detection limit for diacetyl.

Citric acid is degraded to pyruvic acid and further by LAB metabolism, with diacetyl and acetoin being by-products of this degradation [51-52]. This is evident from figures 4E and 4F, where diacetyl and acetoin were produced while citric acid and pyruvic acid was degraded during the first three days of *L. brevis* fermentation. Diacetyl has a very low detection limit in beer (0.1 mg/L) and is generally an unwanted compound [53]. In the current secondary fermentation, the diacetyl concentration was diminished after 11 days. Emphasis should be made on allowing sufficient fermentation time during secondary fermentation. This is not only important to achieve acid production, but also to allow the degradation of other unwanted compounds produced in the initial phase of secondary fermentation.

Characteristics and metabolic compounds in the final beer.

Beer characterisation and metabolic compound analysis were carried out for both the base beer and the XOS sour beer prior to the sensory analysis. The same analyses were conducted for the commercial sour beer reference included in the sensory analysis. The two sour beers had similar pH, with 3.5 in the commercial and 3.6 in the XOS sour, while the base beer had pH 4.1. Apart from the difference in pH, the characteristics of the base beer and the XOS sour beer are comparable (table 2). A very minor increase in the sugar concentration, original extract and colour value, and a decrease in apparent degree of fermentation (ADF) was however observable in the XOS sour beer compared to the base beer. The decrease in ADF and increase in original extract and final sugar concentration can be attributed to the addition of fermentable carbohydrates in the form of xylooligosaccharides. The slight elevation in colour value can also be attributed to the substrate addition, as the powdered XP2 has a brownish tint.

The variation on the metabolic compounds from the analysis of the three beers is visualised in an ASCA score plot in figure 5A. Corresponding loading weights are presented in figure 5B. According to the model, which explains 93% of the variation in the metabolic compounds, all three beers are significantly different from each other. Component 1 in the ASCA model explains 70 % of the variation in the model, with 1-hexanol, 2-methyl 1-propanol, 3-methyl 1-butanol, acetone, ethyl hexanoate, ethyl acetate and lactic acid being important drivers. The XOS sour beer and base beer are similar in this component, but clearly

separated from the commercial sour beer. The XOS sour beer is clearly separated from the base beer in component 2, which explains 30 % of the variation in the model. Important drivers of component 2 includes 2-butanol, acetaldehyde, acetic acid, citric acid, ethyl octanoate, isoamyl acetate, pyruvic and succinic acid. Succinic and acetic acid, which are important drivers on component 2 in the ASCA, and lactic acid, driving component 1, are all different between the three beers (Fig. 5C). Succinic acid is only present in the XOS sour beer, at 350 mg/L. The highest concentration of lactic acid is found in the commercial sour beer, with more than 5000 mg/L compared to the XOS sour beer at 1750 mg/L. Acetic acid was highest in the XOS sour beer at 1100 mg/L, compared to 700 mg/L in the commercial sour beer. Neither lactic nor acetic acid were present in the base beer. Ethylacetate, 2-methyl 1-propanol and 3-methyl 1-butanol are all important drivers in component 1, where the commercial sour beer is separated from the two others. From figure 5D, these components are all similar between the base beer and XOS sour beer, but different in the commercial sour beer. Ethylacetate and 3-methyl 1-butanol were higher in the commercial sour beer, while 2-methyl 1-propanol was lower. Acetaldehyde, which drives component 2, was higher in the XOS sour beer compared to the two other beers. Acetone, 1-hexanol and ethyl hexanoate, all drivers of component 1, are also similar in the base beer and XOS sour beer, but clearly different in the commercial sour beer (Fig. 5E). Acetone is lower in the commercial sour beer compared to the two others, while 1-hexanol and ethyl acetate are higher. 2-butanol, which drives component 2, was not detected in the base beer but was highest in the XOS sour beer and significantly lower in the commercial sour beer. Isoamyl acetate and ethyl octanoate, both drivers of component 2, were similar in the XOS sour beer and commercial sour beer, but different from the base beer (Fig. 5F). Both ester concentrations were higher in the two sour beers, compared to the non-sour base beer.

Sensory analysis

Most of the sensory attributes assessed in the descriptive analysis, received significantly different scores between the base beer and the XOS sour beer, according to the t-test (15 out of 22 attributes, Fig. 6A). Most of the attributes were perceived as significantly higher in the XOS sour beer, compared to the base beer. The exceptions which were higher in the base beer were malty odour and flavour, and yeasty odour and flavour. The high number of significant differences in sensory attributes between the XOS sour beer and the base beer, point to a substantial effect from the XOS induced secondary fermentation on the overall organoleptic perception. The beer increases in complexity by going through the secondary process as most of the attributes, including total intensity odour and flavour, and perfumed odour and flavour, increase in intensity. Some of the changes in these sensory properties might be brought about by the addition of the secondary substrate itself. The contribution of the XOS on its own to the sensory properties has not been investigated in the current study. It is however clear, looking at the metabolic data, that the XOS-induced secondary fermentation by *L. brevis* causes multiple significant shifts in the concentration of metabolic compounds known to have sensory relevance.

Succinic, lactic and acetic acid were all non-detected in the base beer but have been produced in the secondary fermentation and are present in the XOS sour beer. With succinic acid at 350 mg/L. lactic acid at 1750 mg/L and acetic acid at 1750 mg/L, all these organic acids were well above their respective sensory thresholds (table S1). The level of organic acids in the XOS sour beer complies with this beer being perceived as higher in sour odour, sour flavour and acidic taste. Acetaldehyde, which is associated with fruit and green leaves (table S1) is higher in the XOS sour beer, at 5.6 mg/L compared to 1.6 mg/L in the base beer. *L. brevis* strains have been shown to produce acetaldehyde from ethanol [54], and the increased level in the XOS sour beer points to production during secondary fermentation. Ethyl acetate, ethyl hexanoate, isoamyl acetate and ethyl octanoate, are all esters associated with fruity flavours (table S1). Esters can be produced through lactic acid bacteria fermentation [55-56], as the slightly higher level in the XOS sour beer suggests. No difference was, however, found between the base beer and the XOS sour beer

in fruity odour and fruity flavour, which agrees with the concentrations of fruity esters and acetaldehyde, all being below their respective, reported flavour thresholds. The concentration of the volatile alcohols 2-methyl-1-propanol, 3-methyl-1-butanol and 2-hexanol were very similar in the base beer and the XOS sour beer, and well below their sensory thresholds (table S1). 2-Butanol was also below the sensory threshold, but present at 0.13 mg/L in the XOS sour beer, and non-detected in the base beer. This points to production through *L. brevis* secondary fermentation. 2-Butanol production by *L. brevis* strains has been reported [57]. The presence and combinations of multiple compounds with sensory relevance, despite concentrations below detection thresholds, can transform the sensory properties through synergistic effects [58]. The high number of significant differences in sensory properties between the XOS sour beer and the base beer might be induced by such effects.

The ANOVA with Tukey's test, which included the commercial sour beer reference as well as the base beer and XOS sour beer, also revealed significant differences between all three beers. The full table with attribute scores and ANOVA groups can be found in supplementary (Table S3). Attributes scored significantly different between two or more beers are displayed in figure 5B. The XOS sour beer and the commercial sour beer were perceived as similar to each other, but significantly different from the base beer in dried fruit odour, total flavour intensity, acidic taste and astringency. Despite the vastly higher content of lactic acid in the commercial sour beer, the XOS sour beer and the commercial sour beer were perceived with similar intensity in acidic taste. Lactic, acetic and succinic acid are all associated with acidic taste. The higher concentrations of succinic and acetic acid in the XOS sour beer might compensate for the lower lactic acid, with respect to the acidic taste. The base beer, where none of these acids were detected, was perceived as significantly lower in acidic taste, compared to both sour beers.

It is noteworthy, that for all the sensory attributes where significant differences were found in the ANOVA, the intensity for the XOS sour beer was scored closer than the base beer, to the commercial sour beer reference. The commercial sour beer reference was different from the base beer and XOS sour beer in all characteristics presented in table 2. The alcohol concentration in the commercial sour beer was 8 %,

compared to roughly 4 % in both other beers. The colour value was more than 20 EBC, compared to approximately 10 in the base and XOS sour beers. The ADF in the commercial sour beer was above 90 %, compared to 80 % in the others, and original extract of 16 % compared to roughly 10. Correspondingly, the final sugar concentration in the commercial sour beer was lower, at about 1 °Brix compared to more than 2 in the base beer and XOS sour beer. Bitter taste and after taste were scored different between all three beers. Perfumed flavour and alcohol flavour were similar in the base beer and the XOS sour beer, but different in the commercial sour beer. For all these attributes, the XOS sour beer was scored between the base beer and the commercial sour beer in intensity. The alcohol flavour was scored almost identical in the base beer and XOS sour beer, and lower than the commercial sour beer. This was in accordance with expectations, as the commercial sour beer contained twice the alcohol percentage. Differences in characteristics and sensory attributes were expected, as the commercial sour beer is produced through a completely different process and based on a different recipe. While the XOS sour beer resulted from secondary fermentation by a single *L. brevis* strain, the commercial sour beer reference was produced through a traditional lambic/geuze fermentation by a complex microbial consortium. The active fermentation time in the XOS sour beer production was less than 2 months, compared to multiple years for the commercial sour beer reference. The objective of the current study was not to produce a beer with identical properties and sensory qualities to a commercial sour beer, but to investigate an alternative production process for sour beers. The commercial sour beer reference was included in the sensory analysis to see if the result from this alternative process was at all comparable to a traditional sour beer product. The descriptive sensory analysis showed that it is possible to significantly alter the sensory characteristics of beer by implementing a substrate induced secondary fermentation with *L. brevis*. The results also showed that multiple sensory attributes were moved in a direction making the beer characteristics more comparable to those of a traditionally produced commercial sour beer. If the purpose is to produce a sour beer even closer in quality to a traditional sour beer, this could perhaps be achieved by altering the base beer recipe or by including multiple bacterial strains in the secondary fermentation.

In summary, the current study demonstrates how XOS prepared from birchwood xylan, can be used as specific substrate, directing secondary lactic acid bacteria fermentation in sour beer production. The secondary fermentation in this study lasted for 4 weeks. Results from the small-scale fermentations point to potential for shortening fermentation time even further, by adjusting substrate dosage and hopping scheme. This shows that a secondary fermentation can be used in controlled sour beer production with shorter production time compared to traditional spontaneous mixed fermentations. During the secondary fermentation, organic acids and other metabolic compounds with sensoric importance were produced. Actual sensoric influence was proven through descriptive analysis with a trained panel. The substrate induced secondary fermentation caused multiple significant shifts in intensity of sensoric attributes. Importantly, the resulting XOS sour beer was scored as similar in intensity to a commercial sour beer reference in attributes such as dried fruit odour, total flavour intensity, acidic taste and astringency. The currently used microbe, *L. brevis* BSO 464, was suited for the current study as it was able to degrade XOS and it was known to be able to sustain the harsh beer environment [59]. Alternative bacteria and perhaps also yeast species could be used together with specific substrates to generate beers with new sensory properties through secondary fermentations and potentially also co-fermentations. Fermentation induced by hemicellulose derived substrates could pose an alternative for improved utilization of cheap and renewable lignocellulosic feedstocks. Sour beer was used to prove this concept in the current study. With further research, the approach could potentially be extended to products such as non-alcoholic fermented beverages, sour dough bread and probiotic dairy products.

ABBREVIATIONS.

LAB – lactic acid bacteria

Mw – molecular weight

kDa – kilo Dalton

AcAGX – acetylated arabinoglucuronoxylan

RT – room temperature

HPLC – High Performance Liquid Chromatography

PAD – pulsed amperometric detector

ASL – acid soluble lignin

ON – over night

XOS – xylooligosaccharides

XP1 – xylooligosaccharide preparation 1

XP2 - xylooligosaccharide preparation 1

LH – lower hopping

HH – higher hopping

MRS – De Man, Rogosa and Sharpe

RBC – Rose Bengal Chloramphenicol

MALDI-ToF MS – matrix assisted laser desorption-ionization time of flight mass spectrometry

DHB – dihydroxybenzoic acid

HPAEC – High-performance anion-exchange chromatography

HSGC – Headspace gas chromatography

RI – refractive index

DAD-UV - diode array detector- ultra violet

ISO – International organization for standardization

ASCA – Analysis of variance simultaneous component analysis

ANOVA – Analysis of variance

PCA – Principal component analysis

Ara – arabinose

Rha – rhamnose

Gal - galactose

Glc – glucose

Xyl – xylose

Man – mannose

DA - degree of acetylation

CFU – colony forming units

ADF - Apparent degree of fermentation

ACKNOWLEDGEMENTS.

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SUPPORTING INFORMATION DESCRIPTION.

Supporting information to the current paper contains three tables and two figures.

Table S1: Metabolic compounds important for the organoleptic properties of beer, with reported sensory threshold in beer and flavour characteristics.

Table S2: Attributes (odour, O; texture, taste, T and flavour, F) and descriptions used in descriptive sensory analysis of the beers.

Table S3: Average sensory scores for all attributes evaluated in the descriptive analysis of base beer, XOS sour beer and commercial sour beer. Beers receiving significantly different scores (p-value > 0.05, according to ANOVA analysis) are indicated with different letters.

Figure S1: pH development in negative non-inoculated controls, during 14 days of incubation of beer LH with XP2 (2 or 0.5 %), xylose (0.5%) and beer without secondary substrate addition.

Figure S2: Growth (panel A) of *L. brevis* during fermentation (22°C, 28 days) in beer HH with XP2 2%, and pH development during incubation of inoculated samples (average of three replicates with standard deviation as error bars) and non-inoculated sample (one replicate).

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TABLES AND GRAPHICS

Table 1: Sugar composition of the low MW AcAGX. (*) Carbohydrates accounts for 88% of total dry mass of the sample, the monosaccharides are presented as relative %, estimated as anhydrosugars. (**) DA is the degree of acetylation. The ratio was evaluated on 1mg (acetyl groups or measured acetic acid (μmol) / Xyl (μmol) and quantified as bound acetate.*** Klason lignin was below the detection level (n.d.) to give an accurate value. Furthermore, all values in the table were corrected for the 6% moisture in the sample. Ara, arabinose; Rha, rhamnose; Gal, galactose; Glc, glucose; Xyl, xylose; Man, mannose; Uronic, uronic acids.

Component		**% (w/w)**
*Carbohydrate**	*Ara*	1.54 ± 0.27
	Rha	1.23 ± 0.42
	Gal	2.86 ± 0.13
	Glc	3.77 ± 0.43
	Xyl	83.04 ± 0.77
	Man	4.24 ± 0.42
	Uronic	3.33 ± 0.23
*Acetyl (DA: 0.34)***		7.45
*Klason lignin ****		n.d.
Acid soluble lignin		0.79 ± 0.16
Protein		0.45
Ash		1.04 ± 0.01

752 Table 2: Characteristics for commercial sour beer, base beer and XOS sour beer at the point of descriptive sensory
753 analysis. Alcohol (%), colour value (EBC), apparent degree of fermentation (ADF, %), original extract (%) and sugar
754 concentration (°Brix) are presented as averages of triplicates with standard deviations.

Beer	**Alcohol (% v/v)**	**Colour value (EBC)**	**ADF (% w/w)**	**Original extract (% Plato w/w)**	**Sugar concentration (°Brix)**	**pH**
Commercial sour beer	7.9 ± 0.01	23.1 ± 1.8	92.8 ± 0.3	15.8 ± 0.02	1.3 ± 0.00	3.5 ± 0.01
Base beer	4.3 ± 0	10.9 ± 0.1	81.0 ± 0.1	10.2 ± 0.01	2.1 ± 0.01	4.1 ± 0.01
XOS sour beer	4.4 ± 0.02	12.2 ± 0.08	78.5 ± 0.02	10.6 ± 0.03	2.4 ± 0.01	3.6 ± 0.01

755

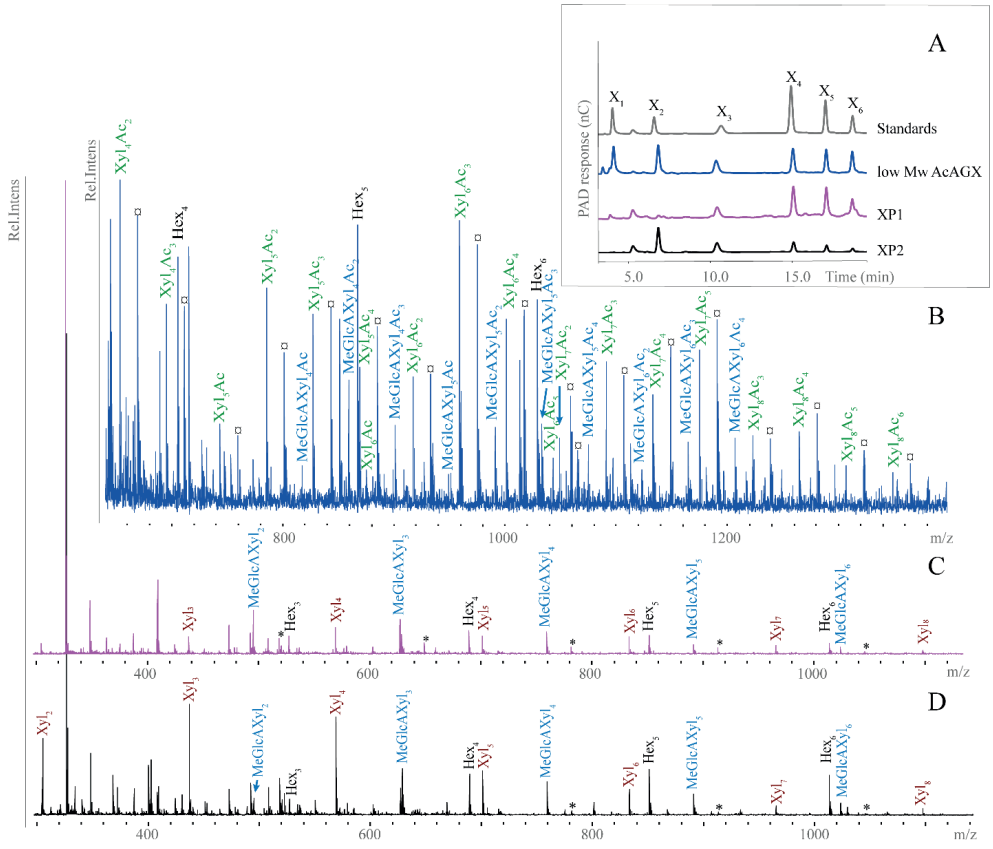
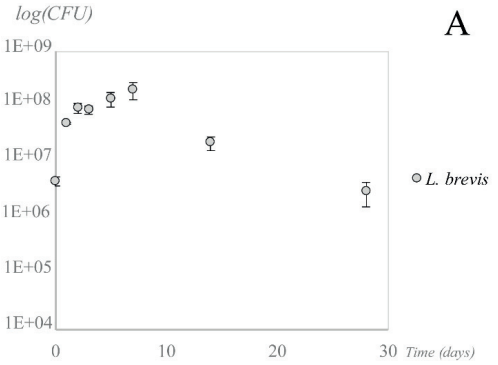
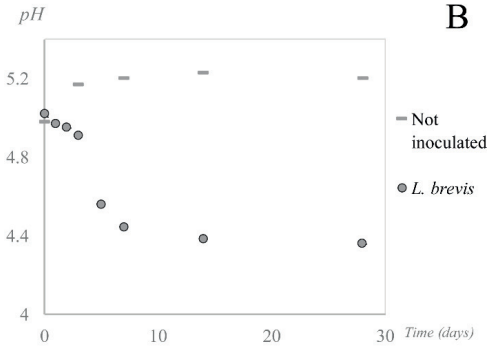
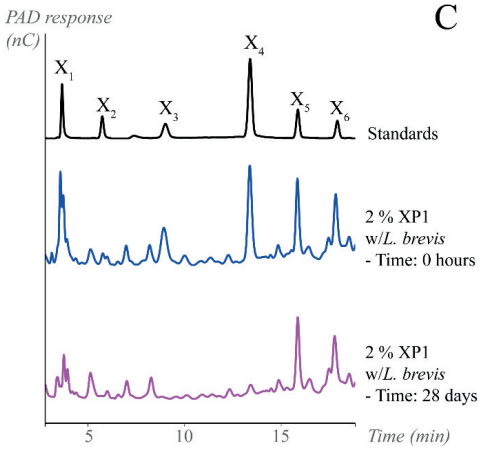


Figure 1: Distribution of xylooligosaccharides in different preparations used in this study. A) HPAEC-PAD profile of xylooligosaccharide standards (one to six xylose units), low Mw AcAGX, XP 1 and XP 2. MALDI-ToF MS spectra of native low MW AcAGX (B), XP1 (C) and XP2 (D). "Xyl" = xylose, "Hex"= hexose, "Me"= methyl, "GlcA" = Glucuronic acid, "Ac" = acetyl, all annotated peaks appear as sodium adducts except ¤ = potassium adducts m/z +16 compared to the corresponding sodium adduct, and * = sodium salts of the $MeGlcAXyl_n$.($[M+2Na]^+$) that are commonly occurring in uronic acids [50]

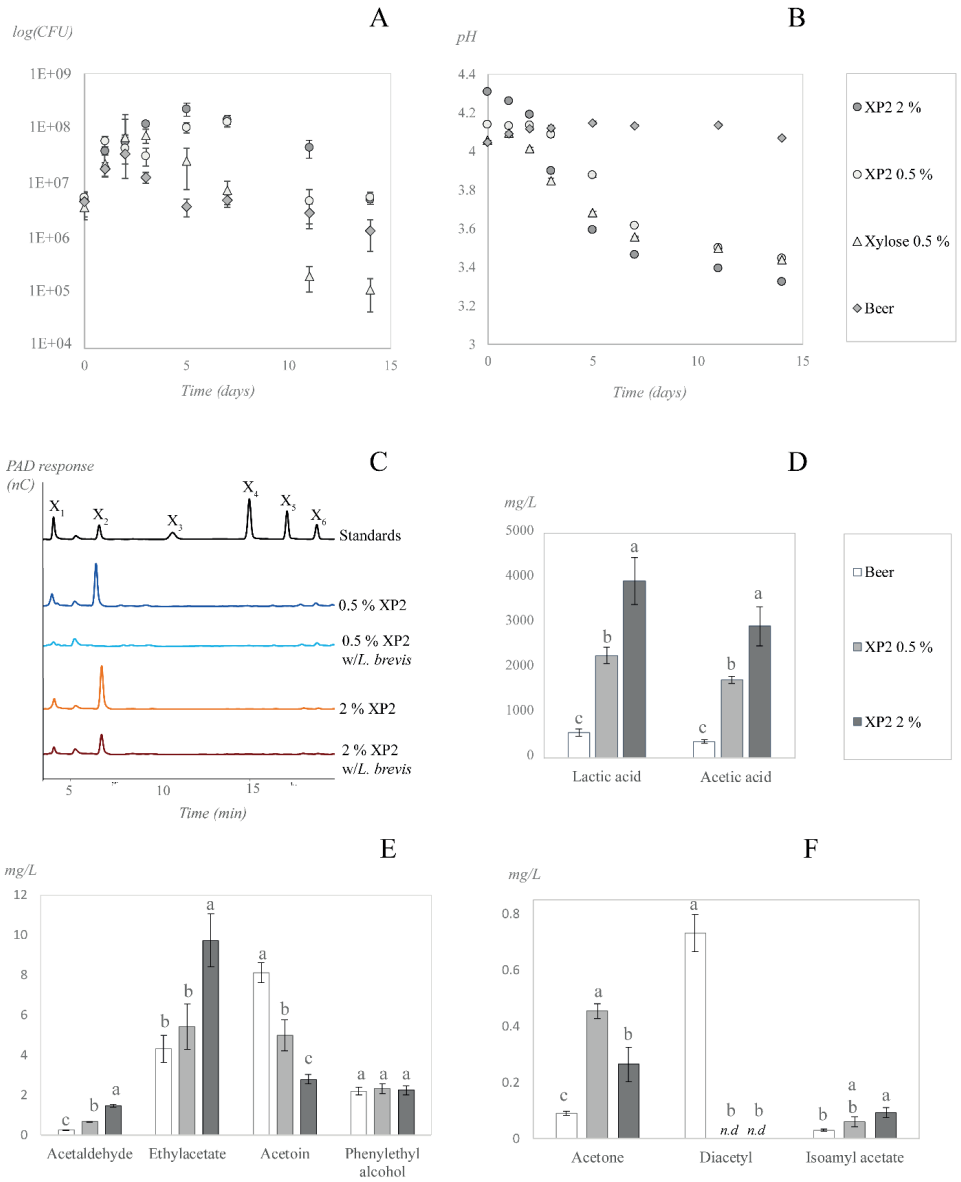

log(CFU)
A
1E+09
1E+08
1E+07
1E+06
1E+05
1E+04
0
10
20
30
Time (days)
L. brevis


pH
B
5.2
4.8
4.4
4
0
10
20
30
Time (days)
Not inoculated
L. brevis


PAD response (nC)
C
X1
X2
X3
X4
X5
X6
Standards
2 % XP1 w/L. brevis - Time: 0 hours
2 % XP1 w/L. brevis - Time: 28 days
5
10
15
Time (min)

763

764 Figure 2: Performance of *L. brevis* during fermentation in beer LH with XP1 at 2 % w/v. A) Growth of *L. brevis*
765 during incubation (25°C, 28 days). B) pH development. C) HPAEC-PAD profiles of XOS standards and XOS profile
766 at the initial and final sampling from the fermentation.



767

768 Figure 3: Fermentation of beer LH, beer LH with 0.5% xylose and beer LH with 0.5 and 2% XP2 in 50 mL bottles at
769 25°C for 14 days. A) Growth of *L. brevis* on different substrates. B) pH-development in inoculated samples. C)
770 HPAEC-PAD profile in beer LH with XOS (2% and 0.5%) at the end of fermentation with *L. brevis*, and profile in

771 non-inoculated negative control. D) Lactic acid and acetic acid, Final concentrations in beer and beer LH with XP2
772 (0.5 and 2%) of lactic and acetic acid (D), acetaldehyde, ethylacetate, acetoin and phenylethyl alcohol (E) and acetone,
773 diacetyl and isoamyl acetate (F). The scale is different for panel D, E and F. "N.d" = non-detected. Significantly
774 different concentrations (according to Tukey's test at significance level $p > 0.05$) are indicated with different letters.

775

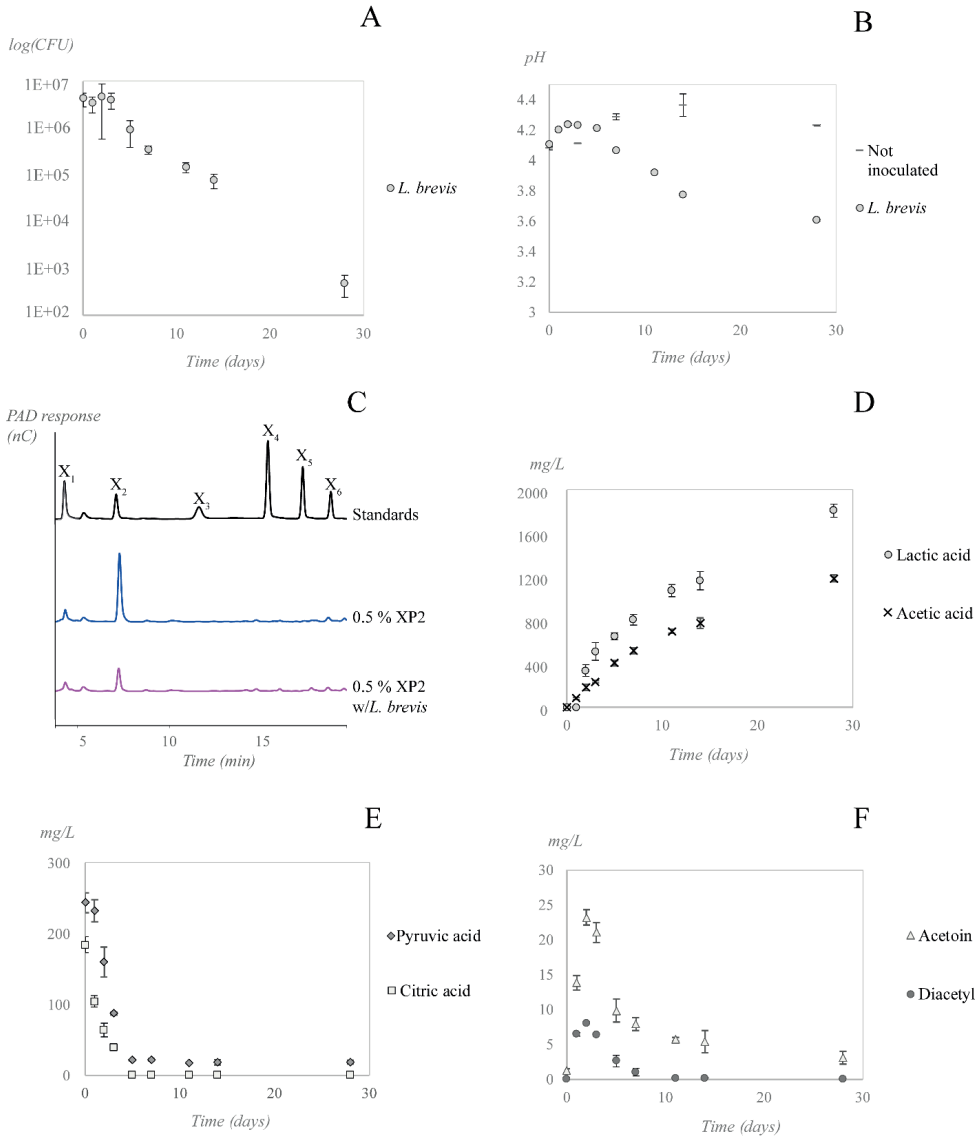
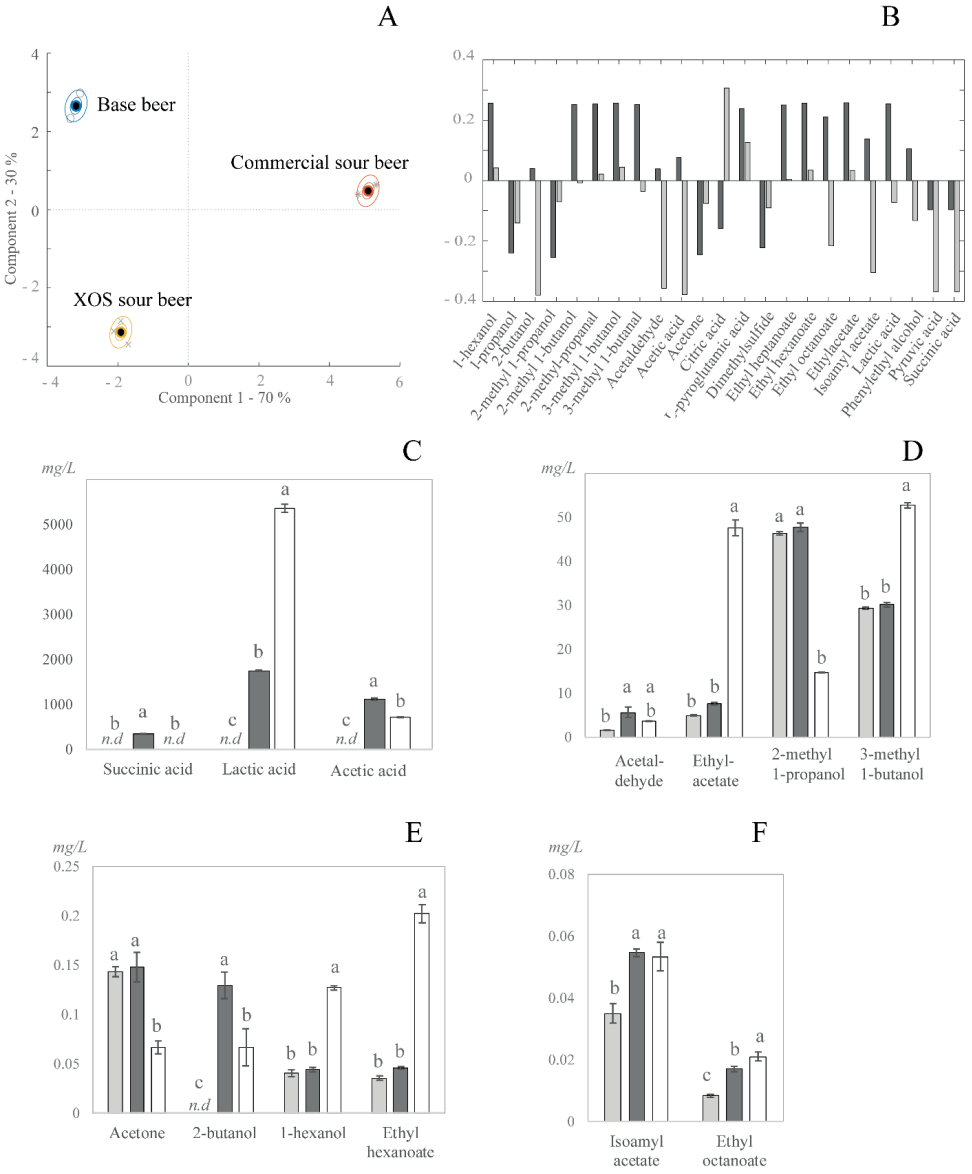


Figure 4: Performance of *L. brevis* during incubation (25°C, 28 days) in 5 L HH beer supplemented with XP2 (0.5%). All panels except C, are presented as averages of three replicates with standard deviation as error bars. A) Growth of *L. brevis* during incubation. B) pH development in inoculated samples and non-inoculated negative controls. C) HPAEC-PAD profiles of XOS standards and XOS profile at final sampling (28 days) from *L. brevis* inoculated

781 samples and non-inoculated negative controls. Development of lactic and acetic acid (D), pyruvic and citric acid (E)
782 and acetoin and diacetyl (F) during the fermentation. The scale is for panel D, E and F.

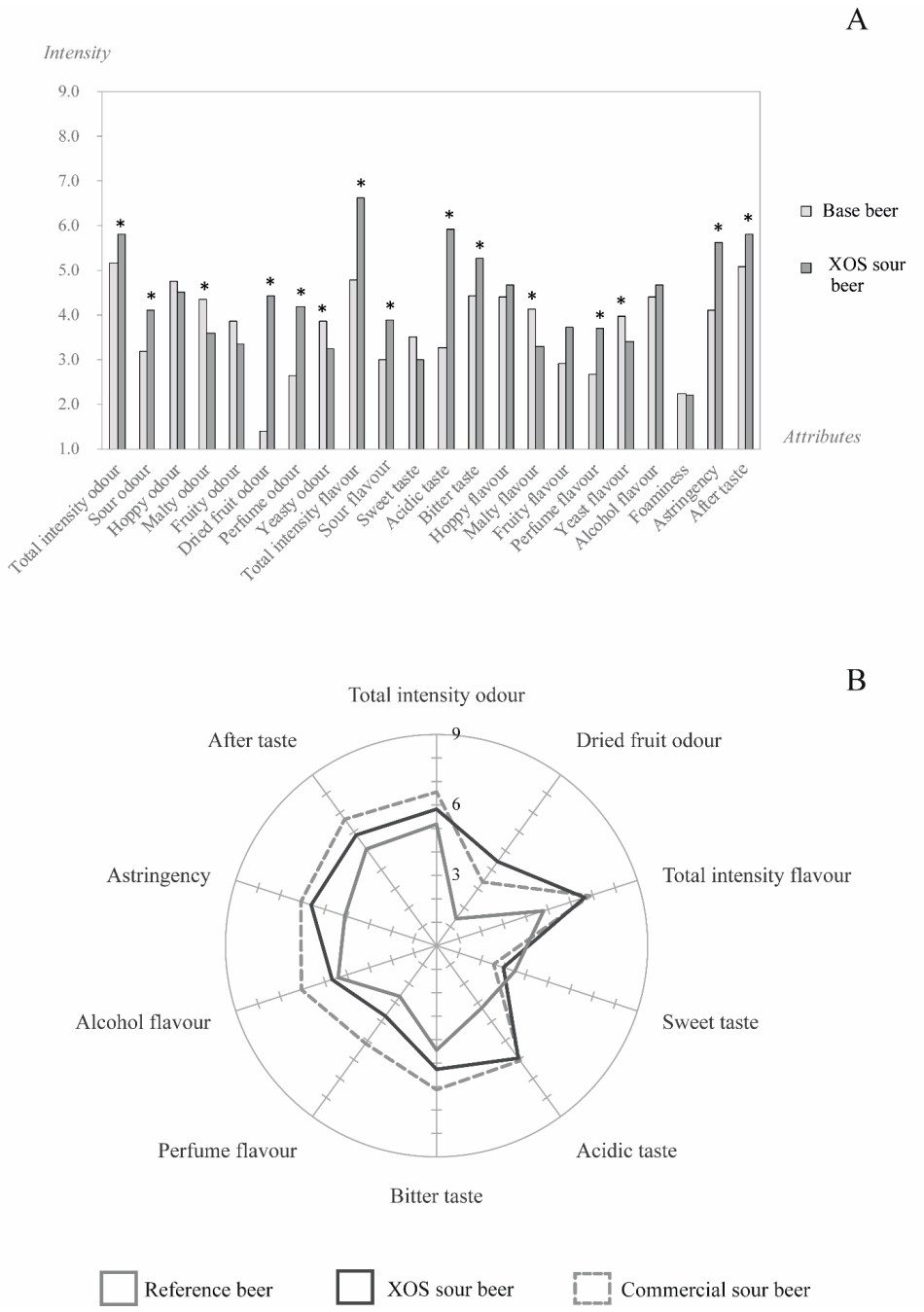
783



784

785 Figure 5: Composition of metabolic compounds in the three beers described in the sensory analysis; base beer, XOS
786 sour beer and commercial sour beer. A) Variation in samples and replicate variation described by analysis of variance
787 simultaneous component analysis (ASCA) scores. The model explains 93% of the variation in metabolic compounds.

B) Loading weights for ASCA model in panel A. Dark grey bars show loadings for component 1 (70%) and light grey bars show loadings for component 2 (30%). Concentration of succinic, lactic and acetic acid (C), acetaldehyde, ethyl acetate, 2-methyl 1-propanol and 3-methyl 1-butanol (D), acetone, 2-butanol, 1-hexanol and ethyl hexanoate (E), and isoamyl acetate and ethyl octanoate (F) in the different beers; base beer (light grey bars), XOS sour beer (dark grey bars) and commercial sour beer (white bars). Significantly different concentrations in the compounds (according to ANOVA at significance level $p > 0.001$) are indicated with different letters in the figure.


A
Intensity
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
Base beer
XOS sour beer
Attributes
Total intensity odour
Sour odour
Hoppy odour
Malty odour
Fruity odour
Dried fruit odour
Perfume odour
Yeasty odour
Total intensity flavour
Sour flavour
Sweet taste
Acidic taste
Bitter taste
Hoppy flavour
Malty flavour
Fruity flavour
Perfume flavour
Yeast flavour
Alcohol flavour
Foaminess
Astringency
After taste
B
Total intensity odour
Dried fruit odour
Total intensity flavour
Sweet taste
Acidic taste
Bitter taste
Perfume flavour
Alcohol flavour
Astringency
After taste
9
6
3
Reference beer
XOS sour beer
Commercial sour beer

795

Figure 6: Descriptive sensory analysis of base beer, XOS sour beer and commercial sour beer. A) Base beer and XOS sour beer: average scores for sensory attributes. Significantly different scores (according to t-test) between the two beers are indicated with *. B) Base beer, XOS sour beer and commercial sour beer: significantly differently scored sensory attributes in beers ($p > 0.05$), according to ANOVA with Tukey's test (Supplementary, Table S3).

SUPPORTING INFORMATION

Table S1: Metabolic compounds important for the organoleptic properties of beer, with reported sensory threshold in beer and flavour characteristics.

Compound	Sensory threshold in beer (mg/L)	Flavour characteristic
1-hexanol	4[1]	Resin, flower, green[2]
2-butanol	16[1]	Alcohol[3]
2-methyl 1-propanol	200[4]	Alcohol[5] Fruity[6], whiskey, winey[4]
3-methyl 1-butanol	70[7]	Alcohol[5] , Banana, nail polish remover[8]
Acetaldehyde	25[9]	Green leaves, fruity[5]
Acetic acid	200[10]	Tart, sour[10], vinegar[11]
Acetoin	>50[9]	Butter, creamy, green pepper[12]
Acetone	>100[9]	Pungent[13]
Citric acid	60[10]	Sour, lemon juice [10]
Diacetyl	0.1[9]	Caramel, butter[8]
Ethylacetate	30[9]	Fruity[14]
Ethyl hexanoate	0.3[9]	Fruit, fennel, solvent[14]
Ethyl octanoate	0.9-1.0[15]	Sweet, fruity[4]
Isoamyl acetate	2[9]	Banana, Solvent[14]
Lactic acid	400[10]	Sour[11], acrid[16]
Phenylethyl alcohol	125[4]/40[15] (lager beer)	Rosey[4]
Pyruvic acid	250[17]	Sour[17]
Succinic acid	200[10]	Sour, astringent, umami[18]
References: 1[1], 2[2], 3[3], 4[4], 5[5], 6[6], 7[7], 8[8], 9[9], 10[10], 11[11], 12[12], 13[13], 14[14], 15[15], 16[16], 17[17], 18[18]		

Table S2: 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 due to the presence of organic acids		*Sour-F*	Related to a fresh, balanced flavour due 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
Dried fruit-O	Odour of dried fruits (prunes, apricots, peaches)		*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 seconds after elimination of the product

7

8

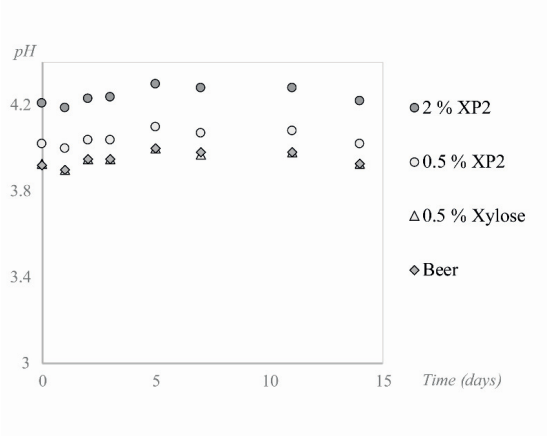


Figure S1: pH development in negative non-inoculated controls, during 14 days of incubation of beer LH with XP2 (2 or 0.5 %), xylose (0.5%) and beer without secondary substrate addition.

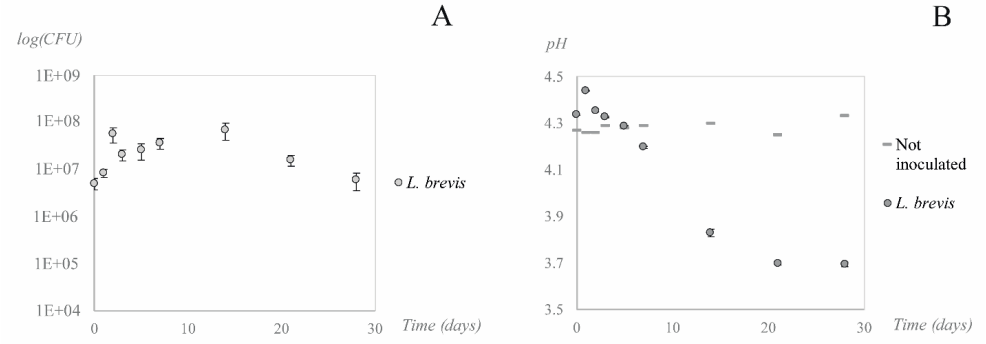


Figure S2: Growth (panel A) of *L. brevis* during fermentation (22°C, 28 days) in beer HH with XP2 2%, and pH development during incubation of inoculated samples (average of three replicates with standard deviation as error bars) and non-inoculated sample (one replicate).

Table S3: Average sensory scores for all attributes evaluated in the descriptive analysis of base beer, XOS sour beer and commercial sour beer. Beers receiving significantly different scores (p-value > 0.05, according to ANOVA analysis) are indicated with different letters.

Sensory attribute	**Base beer**	**XOS sour beer**	**Commercial sour beer**	**p-value**
Total intensity odour	5.18 B	5.82 AB	6.55 A	**0.003**
Sour odour	3.19 A	4.12 A	3.21 A	0.174
Hoppy odour	4.77 A	4.52 A	3.82 A	0.175
Malty odour	4.35 A	3.60 A	3.81 A	0.321
Fruity odour	3.87 A	3.36 A	3.34 A	0.625
Dried fruit odour	1.42 B	4.43 A	3.35 A	**0.003**
Perfume odour	2.65 A	4.20 A	4.44 A	0.053
Yeasty odour	3.86 A	3.24 A	3.47 A	0.194
Total intensity flavour	4.80 B	6.64 A	6.88 A	**<0.001**
Sour flavour	3.00 A	3.90 A	2.78 A	0.1
Sweet taste	3.51 A	3.00 AB	2.56 B	**0.042**
Acidic taste	3.28 B	5.93 A	6.06 A	**<0.001**
Bitter taste	4.45 C	5.27 B	6.14 A	**<0.001**
Hoppy flavour	4.41 A	4.67 A	3.85 A	0.293
Malty flavour	4.14 A	3.31 A	3.39 A	0.104
Fruity flavour	2.91 A	3.74 A	3.48 A	0.37
Perfume flavour	2.67 B	3.71 B	5.15 A	**0.001**
Yeast flavour	3.97 A	3.41 A	3.41 A	0.208
Alcohol flavour	4.41 B	4.68 B	6.06 A	**0.001**
Foaminess	2.25 A	2.22 A	2.35 A	0.829
Astringency	4.11 B	5.62 A	6.08 A	**<0.001**
After taste	5.09 C	5.83 B	6.66 A	**<0.001**

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Errata list

Page/section	Line	Changed from	Changed to
Table of content	15	*Format change*	additional space in front of "2.4 Challenges…" has been removed
Table of content	33	*addition*	"Errata list"
abstract	1	yeasts	yeast
abstract	2, 8, 21, 22	*Punctuation correction*	*, removed*
abstract	8	manuscript	manuscripts
abstract	15	was	were
abstract	28	high	a high
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samandrag	11	L. buchneri	*L. buchneri*
samandrag	13	*L. buchneri* fermentering	fermentering med *L. buchneri*
1	2, 3	*Punctuation correction*	*. removed*
1	7, 8, 20	*Punctuation correction*	*, inserted*
1	7	malt, and hops and yeast	malt, hops and yeast
1	8	law of (with…	law (with…
1	13	Brewers`	Brewer's
2	2 (figure text)	Brewers`	Brewer's
3	23	beers	beer's
3	25, 28, 31, 35	*Punctuation correction*	*, inserted*
3	28	*Punctuation correction*	*; removed*
3	31	probability	the probability
3	32	of	with
3	33	effect	effects
3	38	for	of
3	44	*Punctuation correction*	*, removed*
3	44	hurdle	hurdle that
3	45	range	ranges
3	45	pH	a pH
3	50	I	In
3	50	by	of
3	51	(Suzuki, 2011)	(Suzuki, 2011).
3	54	actions	action
4	59, 63, 80	*Punctuation correction*	*, inserted*
4	69	from CO_2	of CO_2
4	72	inhibit	inhibits
4	73	was	has been
4	80, 85	spoiler potential	spoilage potential
4	84	*Megasphaera* (Satokari et al., 1998) spp.	*Megasphaera* spp. (Satokari et al., 1998)
4-5	78 -91	*Format change*	*text justified*
7	92, 93	*Punctuation correction*	*. removed*
7	94	genera	genre
7	96	acid content	acid
7	96	... 3.0-3.9) compared	... 3.0-3.9) compared
7	98	of sour beer	in sour beer
7	98	originates	originate
7	99	strained	strain
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7	112	mean	means
7	117	on	in
7	120	months	month
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9	139, 146, 148	*Punctuation correction*	*, inserted*
9	139	where	in which
9	140	ferments	ferment

9	140	on	in
9	143	descries	described
9	144	beer	beers
9	145	where	in which
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10	154	geuze	Geuze
10	154	focus	focused
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10	161	report	reported
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10	163	were	had been
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10	166	mention	mentioned
10	168	mention	mentioned
10	169	where	in which
10	182	from	by
10	183	on at	in
10	183-184	laboratory of microbiology and biochemistry at the University of Leuven in Belgium	Laboratory of Microbiology and Biochemistry at the University of Leuven, Belgium
10	184	*Enterocateriaceae*	*Enterobacteriaceae*
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11	194	culture dependent	culture-dependent
11	198	on	in
11	199	by (Gorski, 2012)	(Gorski, 2012)
11	202	falsely	false
11	207	*Vibrio cholera*	*Vibrio cholerae*
11	216	for	in
12	229	fermentationS	fermentations
12	236	time-of-flight spectrometry	time-of-flight mass spectrometry
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12	241	*Enterobacteriacea*	*Enterobacteriaceae*
12	247	culture independent	culture-independent
12	247	have	has
12	250	culture dependent	culture-dependent
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13	269	on	at
13	271	force	forced
13	274	custom made	custom-made
13	282	*Enterobacteriae*	*Enterobacteriaceae*
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14	290	in present	present
14	295, 302	*Punctuation correction*	*, removed*
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14	301	culture independent	culture-independent
14	302	precent	present
14	304	bacteria	of bacteria
15	316	demonstrate	demonstrated
15	319	variation	variations
15	319	is	are
15	320	it	they
15	320	represents	represent

15	324	are	were
15	333	for	to
15	333	improved	improve
15	334	represent	represents
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17	341	common	a common
17	344	facultative	facultatively
17	347	fermentation	fermented
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18	380	aid	aids
18	380	hop sensitive	hop-sensitive
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18	389, 399	*Punctuation correction*	*, inserted*
18	389	other	others
18	390	exists	exist
18	397	cause	causes
18	399	increase	decrease
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19	408	though	through
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20	440	has	have
20	442	and	degrade
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23	453, 454	*Punctuation correction*	*. removed*
23	464	Experimentation and	Experimentation with and
23	468	description	descriptions
23	485	overattenuation	over-attenuation
24	493	*L. theremotolerans*	*L. thermotolerans*
24	505, 509	corresponds	corresponding
24	508	*S. cerevisae*	*S. cerevisiae*
24	510	an example	examples
24	510	is	are
25	527	*Punctuation correction*	*, removed*
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27	546	yeasts	yeast
27	557	brewers`	brewer’s
27	563	*Punctuation correction*	*, removed*
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27	567	attempts	attempt
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33	590	*Punctuation correction*	*, removed*
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33	599	LAB’s	LABs
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34	615, 620	*Punctuation correction*	*, inserted*
34	615	*L.buchneri*	*L. buchneri*
34	617	10L	10 L

34	625	were	was
34	629	been	has been
35	649	biological	biologically
35	658	different	differently
35	662	non	not
35	665	threshold. The	threshold. The
35	665	*Punctuation correction*	*, removed*
35	665	suggests	suggest
36	679	as	in
36	682	impair	impairing
36	687	affects	affect
36	693	strain	strains
36	695	for in	in
36	699	However, in	However, in
37	702	strain dependent	strain-dependent
37	705	point	points
37	708	*Punctuation correction*	*, inserted*
37	715	component	components
37	723	acid	acids
37	725	beer	beers
38	738	*Punctuation correction*	*, inserted*
39	748	*Punctuation correction*	*, removed*
39	748, 759, 762, 765, 768	*Punctuation correction*	*, inserted*
39	753	where	were
39	760	alkali	alkaline
39	765, 767, 774	acid	acids
40	780, 798	acid	acids
40	799	acids (Fig. 5C, paper III) acid	acids (Fig. 5C, paper III)
40	804	geuze	Geuze
40	806	L. brevis	*L. brevis*
40	809	found in the between	found between
41	814	substrate induced	substrate-induced
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42	833	*Punctuation correction*	*, removed*
42	834, 335, 838, 840	*Punctuation correction*	*, inserted*
42	837	optimizations	optimisation
42	837	of	to
42	839	who	in which they
42	844	paper	papers
43	871	rate	rates
43	873	are	is
43	879	*Punctuation correction*	*, inserted*

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Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

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