Sour Brewing: Impact of *Lactobacillus amylovorus* FST2.11 on Technological and Quality Attributes of Acid Beers

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ABSTRACT

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This study was conducted to compare the effect of different acidification methods using *Lactobacillus amylovorus* FST2.11 as a starter culture on the microbiological, technological, and qualitative attributes of sour beers. Biological souring was performed in the mash after mashing, in the preboiling wort, or in the postboiling wort. This lactic acid bacterium strain was selected for its high sensitivity to hops, good growth at moderate levels of alcohol, and fast acidification in wort. Alcoholic fermentation was carried out using a commercial *Saccharomyces cerevisiae* (Safale US-05) strain. Desired acidification of the unhopped substrates (approximately 5–6 g/L of lactic acid) was achieved within 18 h of lactic fermentation. The lactic culture consumed maltose preferentially over monosaccharides, and uptake of free amino nitrogen was limited (8.0–12.6 mg/L). Yeast growth in soured substrates was delayed by 2–4 days compared with the unacidified control, but comparable end attenuations were achieved among all treatments. Among the soured beers, the lowest levels of off-flavors were found in preboil wort souring trials, whereas cofermented beers led to opalescent beers containing high levels of total diacetyl and acetoin. The low pH and the high level of staling compounds such as acetaldehyde suggest that sour beers suffered from low oxidative stability. Final beer qualities varied considerably depending on the souring method applied. Souring of wort by *L. amylovorus* FST2.11 before boiling was found to produce bright, tart beer with minor organoleptic failures while limiting the risk of bacterial cross-contamination within the brewing facility. The renewed interest that sour brewing has received among brewing communities worldwide calls for a better understanding of the advantages and disadvantages of different souring methods. The results from this study could help brewers to choose the most suitable approach according to equipment capabilities and sensorial likeness.

Sour beers are recognized as one of the oldest commercial beer styles, and recently they have been rediscovered by brewers worldwide as an additional way to diversify their product portfolio (12,65). Different approaches can be chosen to produce sour beers. Traditionally, they can be obtained after prolonged acidification and aging taking place over several months and involving different souring microorganisms, for example, wild yeasts (e.g., *Saccharomyces*, *Brettanomyces*, and *Kloeckera* spp.), acetic acid bacteria, and/or lactic acid bacteria (LAB) (1,59). The synergies between the microorganisms will ultimately determine the complex flavor profiles of these products. Most notable examples of sour beers produced in this way are the Belgian lambics and Flanders red ales and the German Berliner weisse. On the other hand, increasing demand for more rapid production of acidic beers has called for alternative approaches that avoid the long fermentation and maturation times. Commonly, this involves the acidification of mash and/or wort before or during alcoholic fermentation. In this regard, the addition of food-grade acid (e.g., lactic, phosphoric, or citric acids) represents a straightforward way to acidify with evident benefits with respect to ease of dosage and consistency. However, beers with added refined acids were also described as lacking flavor complexity, and their production is currently forbidden in facilities that follow the German beer purity law (Reinheitsgebot) (46). Under these circumstances, the use of pure or mixed cultures of LAB has been preferred. Species such as *Lactobacillus delbrueckii*, *L. amylovorus*, and *L. amylolyticus* are commonly used as commercial starter cultures. These strains are capable of homofermentative metabolism of sugars, releasing almost exclusively lactic acid as the major organic acid (17), although other secondary metabolites, such as diacetyl and acetoin, can be a determinant in the overall aroma and flavor profiles of cereal substrates (9). Biological acidification by LAB commonly involves a separate batch acidification of wort, with partial addition to the main mash and/or wort to regulate the pH level (32,36,37). During sour brewing, however, thorough acidification is needed to impart the characteristic sourness in the final beer. For this reason, acidification is often carried out using the whole brewing batch as a substrate and only after the mashing process is completed in order to preserve enzymatic activity.

Because LAB can cause spoilage of conventionally produced beers, souring before boiling is preferable to reduce cross-contamination risks in the cold side of the brewing process. This can be done by inoculating lactic cultures into the mash either after mash out or in the preboiling wort. In addition, this allows the brewer to use arbitrary hop regimes at levels that could otherwise inhibit certain LAB cultures (62). Nevertheless, souring wort after the boiling step could be favored to retain desirable volatiles released during lactic fermentation, as well as to contain negative effects caused by boiling of acid wort (e.g., weak hop isomerization and lower splitting rate of dimethyl sulfide precursors) (33). This latter method of acidification can be done before or as part of alcoholic fermentation.

To date, little research has been conducted to understand the impact that LAB acidification methods cause on the processing and product quality of sour beers. This study aims to compare the fermentative profiles as well as the technological and final quality attributes of beers brewed with different souring practices.

EXPERIMENTAL

Strains and Culture Conditions

The strain *L. amylovorus* FST2.11, originally isolated from a brewing environment, was stored as frozen stock in 40% glycerol at −80°C. The strain was routinely grown on malt extract (ME) agar plates (Fluka, Buchs, Switzerland) under microaerophilic conditions for 48 h at 40°C. Propagation prior to final inoculation was done overnight at 40°C in Congress wort (MEBAK I 3.1.4.9.1.2) (40) made with commercial pilsner malt (Weyermann, Bamberg, Germany). Alcoholic fermentation of acidified and nonacidified wort was carried out using a commercial, spray-dried *Saccharomyces cerevisiae* (Safale US-05, American Ale, Fermentis, Lesaffre, France) strain at a concentration of 0.1% (w/v) (approxi-

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mately 10^7 colony-forming units [CFU]/mL). All reagents used were analytical-grade from Sigma-Aldrich (Saint Louis, MO, U.S.A.), unless otherwise stated.

Characterization of LAB and Yeast Cultures

The amylolytic and proteolytic activity of *L. amylovorus* FST2.11 was tested qualitatively on starch and skim milk agar plates, respectively, according to the methods of Lowe et al. (38) and Axel et al. (3). Growth of *L. amylovorus* FST2.11 in hopped wort was assessed using a microtiter assay according to the method of Haakensen et al. (24) with modifications. Briefly, a range from 1 to 15 mg/L of isomerized hop extract (Isohop, 30% w/w iso-α-acids, Barth-Haas Group, Nürnberg, Germany) was prepared in Congress wort (8.5% w/w). International bitterness units (IBUs) were calculated as 1 IBU ≈ 1 mg/L of iso-α-acids. Similarly, alcohol sensitivity was assessed by diluting 96% v/v ethanol to a range between 1 and 10% (v/v). Growth under different extract levels was checked in a range between 2 and 20% (w/w) by producing a concentrated Congress wort using a higher initial grist-to-liquor ratio (50 g with 75 mL of liquor). For all trials, microtiter wells were filled with 200 µL of mixed substrate and inoculated with approximately 10^7 CFU/mL of an overnight FST2.11 culture. Absorbance was continuously recorded at 620 nm (A_{620}) over 72 h at 40°C. Likewise, acid sensitivity of yeast was assessed within a range of 1–15 g/L of D/L-lactic acid in Congress wort (pH range of 4.4 to 2.9). After inoculation of approximately 10^7 CFU/mL yeast cells in Congress wort, A_{620} values were recorded over 72 h at 20°C. All trials were performed as biological triplicates.

Brewing Trials

Commercial pilsner malt was used as base malt for all beers. A pilot-scale (60 L) brewhouse comprising a combined mash-boiling vessel, a lauter tun, and a whirlpool tank was used for mash and wort production. Hopping was not performed during this study. The souring and control trials were performed as outlined in Figure 1. The beers were brewed in duplicate, and each brew was, in turn, fermented (lactic and alcoholic fermentation) in duplicate.

Yeast control (YC). The malt was milled with a two-roller mill fitted with a 0.8 mm distance gap between the rollers. A grist-to-liquor ratio of 1:5 (6 kg of grist with 30 L of liquor) was chosen. A multistep infusion mashing regime was employed as specified in Figure 1. The heating rate was at 1°C/min between the temperature rests. The mash was pumped into the lauter tun, and two sparging steps of 5 L each were done. Lautering was performed at a wort retrieval rate of 0.5 L/min after collecting 2 L of turbid wort for recirculation. The wort was adjusted to an extract content of 10.5% (w/w) and boiled for 15 min. Hot trub precipitates were removed by means of a whirlpool, followed by cooling through a heat exchanger and in-line aeration using compressed clean air. The wort was filled in two Cornelius kegs (15 L each) and kept in a temperate room at 20°C for 2 weeks during alcoholic fermentation and maturation. Afterward, the brews were racked in clean kegs and stored chilled at 0°C for another 2 weeks before bottling.

Mash souring (MS). After mashing as described earlier, the mash was collected at mash-out temperature and filled in two kegs (11 L each). Carbon dioxide was flushed in the headspace to

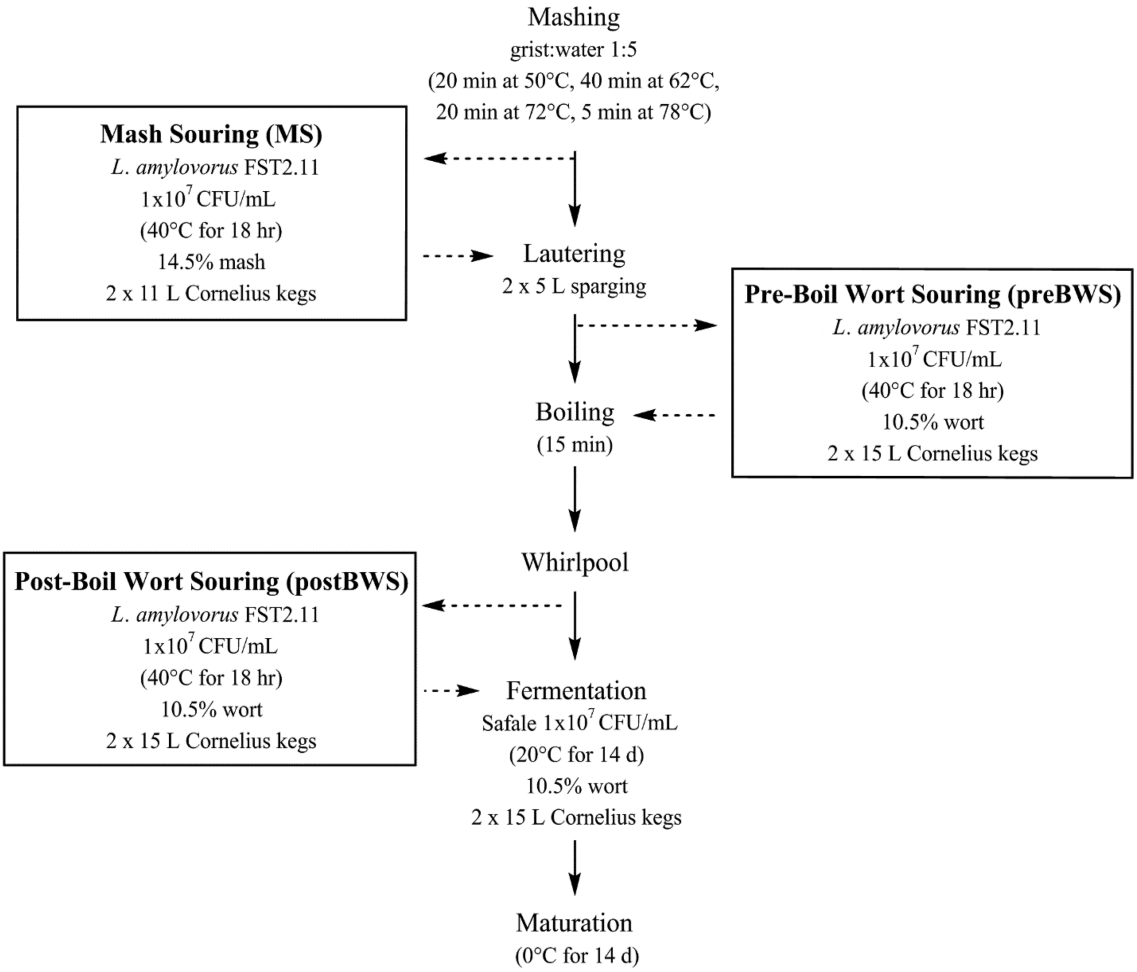

Fig. 1. Schematic flowchart of the souring trials.

provide microaerophilic conditions. The mash was cooled to a fermentation temperature of 40°C in around 2.5 h. Propagation of FST2.11 was done initially in Congress wort (24 h at 40°C) and subsequently in fresh control wort (16 h at 40°C). An aliquot corresponding to 5% v/v (approximately 10^7 CFU/mL) was inoculated in the mash followed by another CO_2 flushing. Fermentation was carried out for 18 h at 40°C until a pH of approximately 3.4 was reached. The two mashes were transferred back into the mash vessel, heated up to 78°C for 5 min, and pumped into the lauter tun for lautering. The acidified wort was boiled and brewed as done for the control trials.

Preboil wort souring (preBWS). After lautering and sparging, the collected wort was adjusted to an extract content of 10.5% (w/w) and held at 82°C for 10 min for sanitization purposes. Subsequently, the wort was filled in two kegs (15 L each), flushed with CO_2, and cooled to 40°C for FST2.11 fermentation (18 h) as described earlier. The acidified wort was boiled and further brewed as performed for the control trials.

Postboil wort souring (postBWS). Boiled wort was collected in two kegs (15 L each), and lactic fermentation was done as described for the preBWS trials. After 18 h of LAB fermentation, wort was cooled to yeast fermentation temperature (20°C) and aerated with clean air for 1 min at a flow rate of 2.4 L/min through a sterilized aeration stone (0.45 µm pore size). Yeast fermentation and beer maturation were done as described for the control trials.

Microbiological Analysis

Viable cell counts were determined throughout lactic fermentation (18 h) and alcoholic fermentation (14 days) and in final beers (28 days). The CFUs of FST2.11 were determined using ME agar supplemented with 0.001% (w/v) cycloheximide to prevent yeast overgrowth. The plates were incubated anaerobically at 40°C and assessed after 48 h. To follow yeast growth, ME agar plates with added 0.01% (w/v) chloramphenicol to suppress bacterial growth were counted after aerobic incubation at room temperature for 3 days.

Chemical and Technical Analysis

The mashes (250 mL each) were tested for the rate of filtration by gravimetric recording over 60 min through folded filter paper (Whatman 0860 1/2, GE Healthcare UK Limited, Chalfont, U.K.) according to the method of Lowe et al. (36). Viscosity of worts was assessed using a falling ball viscosimeter (MEBAK I 3.1.4.4.1) (40), and determination of β-glucan was done following the McCleary method (K-BGLU 05/15, Megazyme International Ireland, Bray, Ireland). Mashes and worts were analyzed for their buffering capacity according to the method of He et al. (28), extract (w/w) and alcohol content (v/v) (Alcolyzer Beer ME Analyzing System, Anton Paar, Graz, Austria), pH, and total titratable acidity (MEBAK II 3.2.3) (41). Free amino nitrogen (FAN) in worts was determined using the ninhydrin photometric method (MEBAK II 2.6.4.1.1) (41). Sugars (fructose, glucose, maltose, and maltotriose) and acids (lactic, acetic, and butyric acid) in mashes, worts, and beers were determined via HPLC (Agilent 1260 Infinity) using a refractive index detector or diode array detector for sugar or acid analysis, respectively. Sample preparation and HPLC running conditions were performed as described by Peyer et al. (50). The final beers were analyzed for the concentration of total soluble nitrogen (TSN) (MEBAK II 2.6.1.1) (41), L-lactic acid (K-LATE, Megazyme International), total polyphenol contents (MEBAK II 2.16.1) (41), and haze (beer haze meter, Haffman LTP-6B, Dr. Lange, Berlin, Germany). Color (MEBAK II 2.12.2) (41) was measured after centrifugation of beer samples at 4,500 × *g* for 5 min, and foam stability was determined according to the sparging method described by Lomolino et al. (35) with minor modifications. Briefly, 20 mL of decarbonated beer was placed at the bottom of a glass sintered tube (G-3 filter, pore size 16–40 µm, 3.2 × 20 cm), and all the liquid was brought to foam by sparging carbon dioxide at a constant flow rate (0.35 L/min) and pressure (30 kPa) for 15 s. Liquid and foam volumes were recorded throughout 15 min of observation, and foam stability was expressed as the half-time ($t_{1/2}$) of the foam height.

Fermentation By-products and Sensory Analysis

The following fermentation by-products were analyzed using gas chromatography–based methods: steam volatile aroma compounds (MEBAK II 2.23.6), highly volatile fermentation by-products (higher alcohols and esters) (MEBAK II 2.21.1), vicinal diketones (MEBAK II 2.21.5.1), acetoin (MEBAK II 2.21.5.4), bound sulfur dioxide (MEBAK II 2.21.8.2), and free dimethyl sulfide (MEBAK II 2.23.1.1) (41). Sensory analysis was carried out by 10 panelists (MEBAK Sensory Analysis Method 3.2.1) (42) and involved the descriptive analysis (smell, taste, and off-flavors) of the samples.

Statistical Analyses

Minitab software (version 17.0) was used for statistical calculations. One-way ANOVA was used to compare mean values between the samples. When F values were found to be significant, Tukey's multiple comparisons procedure was used to further determine any significant differences between the trials. The level of significance was determined at $P < 0.05$. Results are expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

Characterization of Bacterial and Yeast Cultures

When selecting the right lactic culture for acidification during brewing, different strain-specific attributes should be considered, such as adaptability to wort substrate, resistance to environmental stress conditions, and spoilage potential of the culture. Above all, the LAB strain should not become a biological hazard in premises that produce and handle beer with low hop dosages. For this reason, hop-sensitive strains are preferred over hop-resistant ones for souring purposes. The growth of FST2.11 was negatively affected by hop acids, and it was reduced by 22 and 66% when wort contained 1 and 2 IBUs, respectively, compared with an unhopped substrate (Table I). Iso-α-acids can cause sensitivity in LAB through dissipation of the transmembrane proton gradient, with subsequent decrease of the cytoplasmic pH and impairment of the metabolic activities of the cell (58). Inhibition was further increased when the substrate was corrected to the average beer pH of 4.5 (80 and 79% inhibition for 1 and 2 IBUs, respectively), and at IBU values of 5, corresponding to the starting IBU for commercial beers (e.g., lagers), no growth could be detected. As a comparison, hop-resistant LAB species (e.g., *Pediococcus* spp., *L. brevis*) have been found to grow in beer containing up to 29 IBUs after inoculation for 60 days (20). Nevertheless, the use of a hop-resistant LAB strain as an acidifying culture could be justified if souring is to be performed in an already-hopped substrate.

Growth of FST2.11 was found to be affected at a concentration of 8% (v/v) and higher of ethanol. This result was in agreement with a study by Gold et al. (21), who found that different *L. amylovorus* strains cultured on different carbohydrate sources showed good growth up to 8% ethanol (v/v), with some strains being able to grow even up to 16%. Overall, the authors found that the majority of the tested *Lactobacillus* strains were able to grow at 4% (v/v) ethanol. This moderate alcohol tolerance of lactobacilli is an important asset to ensure continued acidification during mixed fermentations.

The growth of FST2.11 increased linearly with the wort extract content (R^2 = 0.996) until a level of 14% (w/w) extract. High-

gravity wort can provide more nutrients as well as higher buffer capacity, which could promote LAB growth and delay self-inhibition owing to low pH. Bacterial growth slowed at extract values above 16% (w/w) and reached a growth plateau between 18 and 20%. Similar to brewing yeasts, industrial LAB strains used in food and feed fermentations are likely to be exposed to osmotic stress when a high amount of sugars is present. Strategies by LAB to counteract hyperosmotic conditions involve the uptake or synthesis of compatible solutes within the cell and their release or degradation under hypo-osmotic conditions (66).

Growth of yeast linearly decreased ($R^2 = 0.989$) with increasing lactic acid concentration. Narendranath et al. (45) reported that the minimal inhibitory concentration of lactic acid for *S. cerevisiae* was as high as 2.5% (w/v) when grown on a minimal medium (mineral salts, vitamins, and glucose), whereas a concentration of 0.2–0.8% (w/v) began to affect growth rates and ethanol production. Rogers et al. (54) reported that *S. cerevisiae* CBC-1 cultures failed to bottle carbonate sour beers with low pH (3.17) and total acidity of 2.5%. Although the yeast cells remained vital, a combination of low pH, high ethanol, lack of O_2, and other factors (e.g., limiting nutrients, acetic acid) was probably responsible for the metabolic alteration.

Microbiological Growth

Microbiological composition was followed throughout lactic and alcoholic fermentation (Fig. 2). As mentioned previously, the higher extract content of the mashes had a positive effect on the cell growth of FST2.11, reaching up to 3.2×10^8 CFU/mL, whereas values of up to 1.0×10^8 CFU/mL were found in wort. Nitrogen compounds (amino acids, peptides, and polypeptides) and phosphates are regarded as the main buffering compounds in barley malt substrates (16,63) and led to 28% higher buffer capacity in mash (2.36 ± 0.02) compared with wort (1.84 ± 0.02). The counterbalance of the pH drop during lactic fermentation has been

TABLE I
Relative Growth (%) of *Lactobacillus amylovorus* FST2.11 and Safale US-05 Yeast in Wort Substrates Under Different Hop, Alcohol, Extract, or Acidity Conditions[a]

Hop		Alcohol		Extract		Acidity	
Condition	Growth of FST2.11	Condition	Growth of FST2.11	Condition	Growth of FST2.11	Condition (pH)	Growth of US-05
IBU 1	78%	1% (v/v)	97%	2% (w/w)	12%	1 g/L (4.4)	97%
IBU 2	34%	2% (v/v)	91%	4% (w/w)	24%	2 g/L (3.94)	93%
IBU 4	17%	4% (v/v)	89%	6% (w/w)	39%	4 g/L (3.51)	82%
IBU 5	15%	6% (v/v)	80%	10% (w/w)	68%	6 g/L (3.29)	73%
IBU 10	9%	8% (v/v)	75%	14% (w/w)	88%	8 g/L (3.17)	53%
IBU 15	0%	10% (v/v)	20%	18% (w/w)	99%	10 g/L (3.05)	43%

[a] Absorbance values at post-exponential phase were considered, and percentages were calculated by comparing test and control values (set at 100%) corresponding to IBU 0 (pH 5.8), 0% v/v alcohol, 20% w/w extract, and 0 g/L of lactic acid (pH 5.88).

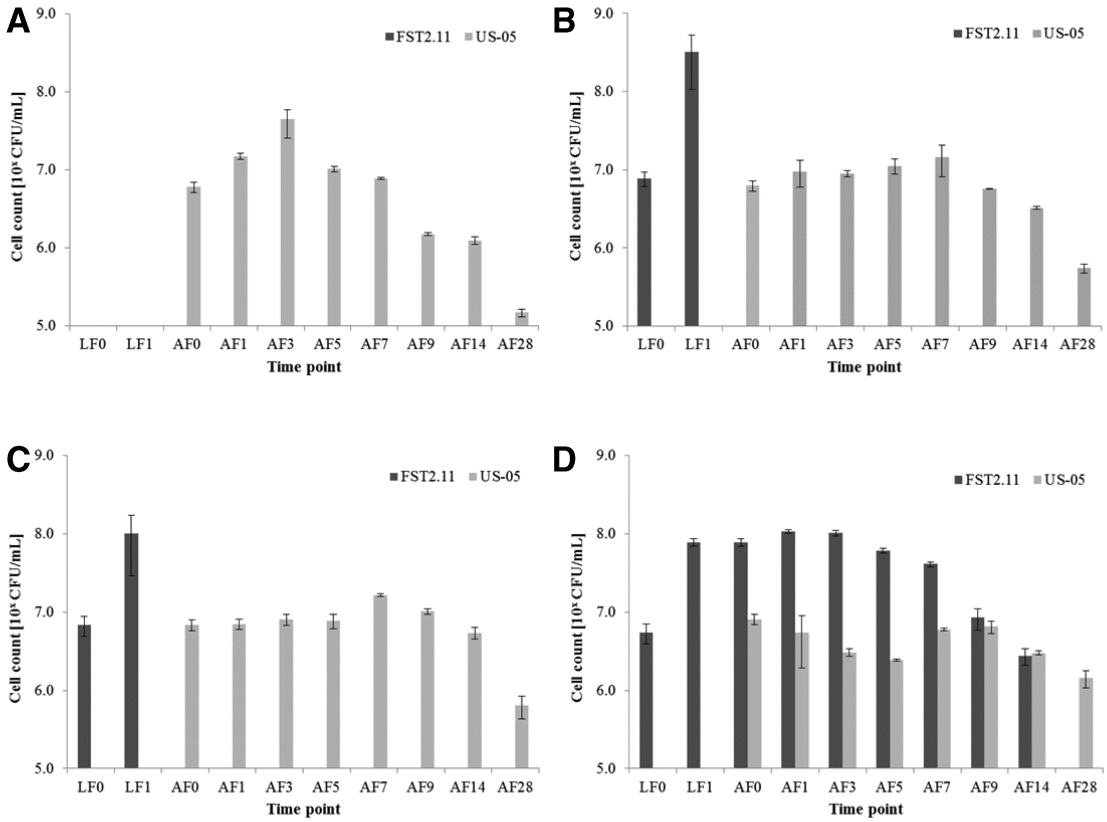

Fig. 2. Cell counts of lactic acid bacteria and yeast cells throughout lactic (LF) and alcoholic (AF) fermentations: **A,** yeast control; **B,** mash souring; **C,** preboil wort souring, and **D,** postboil wort souring.

reported to delay LAB self-inhibition and had a positive effect on the viability of probiotic lactic cultures (55). Finally, the presence of living FST2.11 during MS and preBWS trials could not be detected anymore after the boiling step.

Yeast growth was delayed by 2–4 days in the soured trials, and counts increased to a lesser extent (up to 1.7×10^7 CFU/mL) compared with the unacidified control, which in turn reached peak growth (4.5×10^7 CFU/mL) within 3 days of alcoholic fermentation. This trend confirmed the pretrial results (Table I), which showed decreased growth of US-05 with increased substrate acidification. Although no significant changes were detected in the yeast population between the MS and preBWS trials, yeast was negatively affected during postBWS fermentation. Here, the lactic culture continued to grow until the third day of alcoholic fermentation, whereas yeast showed an initial decrease but recovered after the seventh day. Bacterial population declined from the fifth day onward, most probably owing to increasing acidity, ethanol stress, and substrate depletion. The growth of yeast cells could have been encouraged by nutrients (e.g., nitrogen compounds) released during autolysis of LAB. In this regard, antagonistic and synergistic interactions between lactobacilli and yeasts and the exchange of their metabolites can induce significant differences in their growth and viability. Kedia et al. (31) showed that cofermentation of a 5% (w/v) malt suspension by yeast (isolate from Jiu Niang) and *L. reuteri* mixed at different inoculation ratios (2:1 and 1:2) could enhance LAB growth in both cases. On the other side, the yeast population was reduced in the 1:2 suspensions but reached the same levels at 2:1 after 30 h, compared with 1:1 inoculation ratio. The authors suggested that the yeast supplied the bacterium with essential nutrients for growth, while being inhibited at the same time by increasing ethanol contents. Instead, studies done by Schönfeld (56) showed that a yeast-to-LAB ratio of 4:1 (1×10^7 and 2.6×10^6 CFU/mL, respectively) during Berliner weisse fermentation favored yeast growth within the first 18 h of fermentation (7:1), decreasing to 1.7:1 after 64 h. Direct comparisons with this study, however, are limited, because the yeast-to-LAB cell ratio at the start of alcoholic fermentation was in clear favor of the LAB cells (1:7.8).

Fermentation Kinetics

Metabolite formation by LAB is a function of cell viability and vitality. The favorable growth condition during mash souring led to more extensive acidification (+56%) during MS compared with preBWS (Fig. 3A). However, as shown earlier, FST2.11 slowed down growth above an extract content of 14% w/w (Table I). For this reason, less acid was released proportionally to the extract content during mash fermentation (0.43 g/L of lactic acid per unit extract) compared with wort fermentation (up to 0.51 g/L of lactic acid per unit extract). Despite the increase in bacterial growth during the first three days of postBWS fermentation, additional acidification was contained, indicating limited LAB activity during alcoholic fermentation. Release of acid by-products (e.g., pyruvic, citric, and lactic) as well as the assimilation and removal of buffering compounds (6) during the fermentative metabolism of yeasts caused further acidification in all trials and a final pH of 4.13 in YC (Fig. 3B). The pH level did not drop for the soured trials, with both preBWS and postBWS beers reaching lower pH (3.44 and 3.45, respectively) compared with MS beer (3.75). In addition, YC showed a faster reduction in the extract content compared with the soured trials (Fig. 3C). This is in accordance with the higher yeast cell numbers recorded in this trial. Similarly, alcohol production was also faster in YC, reaching already after

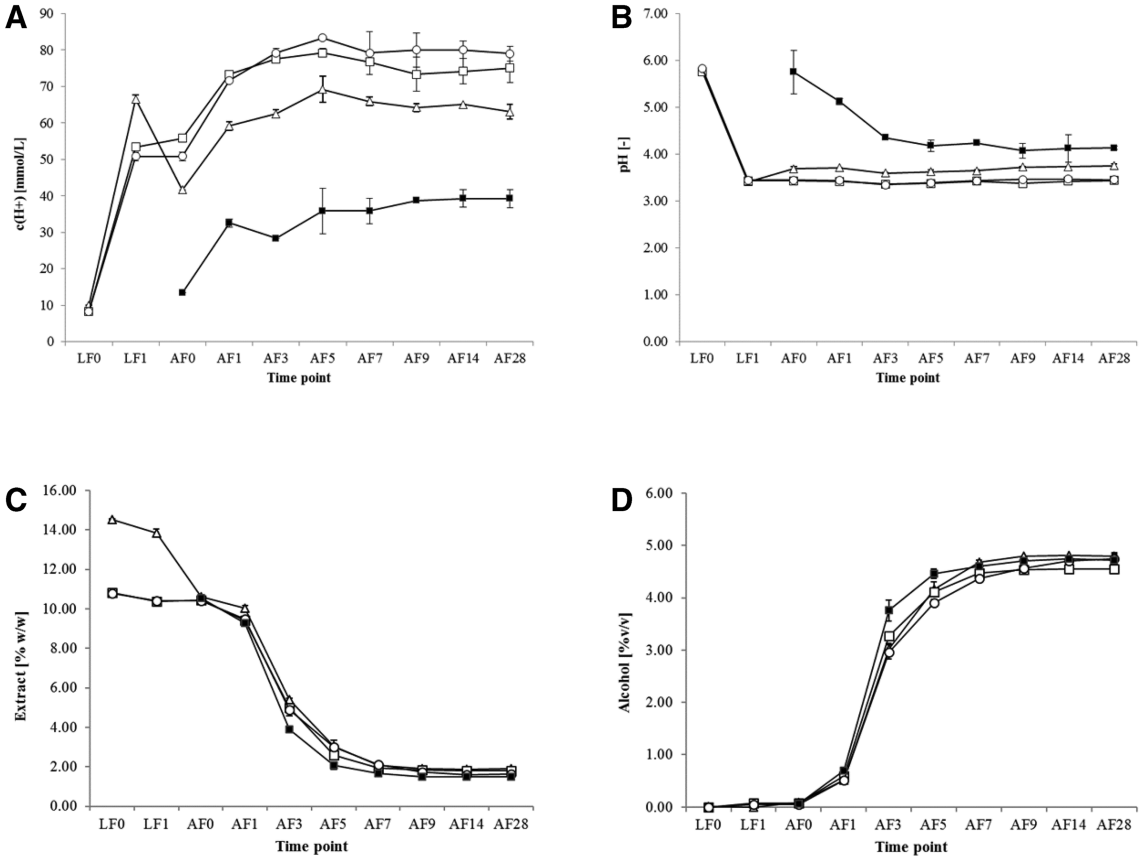

Fig. 3. Total titratable acidity (**A**), pH levels (**B**), extract (% w/w) (**C**), and alcohol (% v/v) (**D**) in mash and/or wort throughout lactic (LF) and alcoholic (AF) fermentation for yeast control (■), mash souring (△), preboil wort souring (□), and postboil wort souring (○).

the third day of alcoholic fermentation comparable levels of alcohol to that found only after the fifth day of fermentation of soured worts (Fig. 3D).

Metabolite Profile

In accordance with the obligate homofermentative metabolism of *L. amylovorus*, which converts glucose almost exclusively into lactic acid (>90% theoretical yield) (17), lactic acid was the main organic acid released by FST2.11 (Table II). This was present in equimolar quantities (50.2–55.4% of L-lactate) of both enantiomers, and no acetic acid could be detected. The lactic acid concentration rose throughout alcoholic fermentation in all trials because of the fermentative yeast metabolism as well as evaporation losses during further brewing operations. The highest final concentration of lactic acid was found in postBWS beers (5.82 ± 0.08 g/L). Maltose was quantitatively the preferred sugar consumed by FST2.11, followed by glucose and fructose, whereas maltotriose was not taken up. Sucrose could not be metabolized by this strain. The yields of lactic acid accounted for 165% during MS and 92 and 103% during preBWS and postBWS, respectively, related to the sugar consumed. The very high yields during MS suggest that the strain produced lactic acid from other sources not quantified in this study (e.g., dextrins). *L. amylovorus* strains can express extracellular amylases (69) that allow them to produce lactic acid directly from starch (52). *L. amylovorus* FST2.11 was found to possess amylolytic activities when tested on starch plates. Therefore, large polysaccharides, which are partially retained during lautering, could have served as a carbon source for the direct production of lactic acid. Nonetheless, malt amylases still active during lactic fermentation could have increased sugar concentration before inactivation owing to the low temperature and low pH.

Sugar consumption during alcoholic fermentation was faster in YC, with complete depletion of fermentable sugars after the fifth day. Yeast generally assimilates sugars in wort in the following order: sucrose, followed by the monosaccharides glucose and fructose, and finally maltose and maltotriose (25). In this regard, monosaccharides were quickly reduced after the first day of fermentation, maltotriose after the third day, and finally maltose after the fifth day. A similar priority of consumption was found also for the soured mash trials. Conversely, maltotriose and maltose were still present in both wort souring trials at the fifth day but disappeared after the seventh day of fermentation. At the end of alcoholic fermentation, sugars were entirely consumed in all trials, and no significant changes in final extract content were detected between the trials, thus confirming complete extract attenuation in all beers (Fig. 3C).

A limited consumption between 8.0 and 12.6 mg/L of FAN was found during lactic fermentations, leaving sufficient FAN (116.7–131.2 mg/L) for adequate yeast fermentation (100–150 mg/L) (15). Total consumption of FAN was higher during YC, pointing to a healthier fermentation during this trial, whereas lower consumption and higher end levels of FAN were registered for all the soured trials. A similar result was confirmed by Kedia et al. (31), who reported reduced consumption of FAN during cofermentation of *S. cerevisiae* and *L. reuteri* compared with the pure cultures. However, the proteolytic activities found for FST2.11 could have increased the amount of free nitrogen during lactic fermentation and cofermentation. This was suggested also by Rathore et al. (53), who related the increase in FAN concentration observed at the end of *L. acidophilus* fermentation of different malt substrates to the action of proteases secreted by the bacteria. However, it cannot be excluded that nitrogenous compounds could have also been released as a consequence of LAB autolysis (31,47).

Brewing-Relevant Analyses

Characteristics such as filtration performance of the mashes were compared with the viscosities and β-glucan content of the resulting worts. The rate of filtration for the MS proceeded faster in the first 30 min, compared with unacidified mashes (Table III). Various factors could have played a role here, such as the decrease in extract during lactic fermentation, as well as the enzymatic degradation of macromolecules (e.g. starch, dextrins, and proteins) that can positively affect the lautering rate (68). This was confirmed by measuring the viscosities of the clear worts obtained after filtration, which were lower for MS (1.61 ± 0.02 mPa·s) compared with the unacidified mashes (1.71 ± 0.02 mPa·s). Lowe et al. (36) ascribed improved lautering performances and lower viscosities of worts made from LAB-treated malt to the proteolytic activities of the starter cultures. However, a normalization of filtration rates between soured mash and normal mash occurred after 30 min of lautering. This, on the other side, could be attributed to a high number of suspended LAB cells (3.2×10^8 CFU/mL) present in the soured mash. The gradual accumulation of cells on the surface of the filter cake could increase the resistance to filtration (64) and ultimately slow down the wort runoff. The β-glucan levels in soured and unsoured mashes did not differ significantly, rejecting the degradation of β-glucans as a factor influencing faster initial filtrations (38).

Both MS and preBWS delivered clear beers after maturation (<1 EBC formazin unit). Slightly more turbid was YC, and clear opalescence was found in postBWS beers. Compared with the control, the high turbidity in postBWS beers could be caused by the higher number of suspended yeast cells, which can create a persistent haze if they are not filtered out. Because both settled and suspended cells were considered for total cell counts in this study, no conclusion on the influence of acidification on the flocculation extent of either microbial group can be drawn. However, differences in the flocculation ability of the yeast cultures can be expected, because this is strain-dependent and can highly depend on the environmental pH (60). The pH present during boiling can affect the extent of protein precipitation, thus final haze. This takes place most efficiently at the isoelectric point (pI) of the individual proteins, with most of them having a pI of around pH 5.2 (32). At lower and higher pH values, however, proteins gain positive and negative charge, respectively, and interaction is inhibited. The low pH values of MS and preBWS suggested that protein

TABLE II
Sugars (g/L) and Free Amino Nitrogen (mg/L) Consumption and Organic Acid (g/L) Production During Lactic and Alcoholic Fermentation in Yeast Control, Mash Souring, Preboil Wort Souring (PreBWS), and Postboil Wort Souring (PostBWS)

		Yeast control	Mash souring		PreBWS		PostBWS	
Analysis	**Unit**	**Alcoholic**	**Lactic**	**Alcoholic**	**Lactic**	**Alcoholic**	**Lactic**	**Alcoholic**
Glucose	g/L	–8.4 ± 1.0	–0.5 ± 1.0	–9.2 ± 1.1	–1.7 ± 0.6	–7.6 ± 1.0	–0.8 ± 0.6	–6.8 ± 1.8
Fructose	g/L	–1.1 ± 0.1	–0.2 ± 0.5	–1.8 ± 0.6	–0.5 ± 0.4	–1.2 ± 0.2	–0.3 ± 0.3	–0.9 ± 0.1
Maltose	g/L	–56.3 ± 5.9	–2.9 ± 1.1	–58.0 ± 2.4	–3.5 ± 1.3	–53.9 ± 5.3	–3.8 ± 1.5	–54.9 ± 4.1
Maltrotriose	g/L	–16.2 ± 1.9	+0.1 ± 0.6	–17.4 ± 0.3	+0.3 ± 1.1	–16.1 ± 1.7	–0.0 ± 1.2	–17.6 ± 1.3
Free amino nitrogen	mg/L	–68.8 ± 5.2	–8.1 ± 4.2	–69.0 ± 5.4	–12.6 ± 3.9	–53.0 ± 7.8	–8.0 ± 4.3	–59.2 ± 10.1
Lactic acid	g/L	+0.3 ± 0.6	+6.2 ± 0.7	+0.4 ± 0.5	+5.4 ± 0.2	+0.2 ± 0.4	+5.2 ± 0.7	+0.6 ± 1.1

interactions were not promoted during boiling. The values for total soluble nitrogen in the final beers showed no significant difference among the trials. Nevertheless, total polyphenols, also involved in haze formation, were lower in both MS and preBWS beers compared with postBWS and YC, suggesting that an unidentified removal mechanism occurred during boiling under acidic conditions.

During the brewing process, Maillard and browning reactions can lead to darker beers through the formation of color-bearing end products. These temperature-dependent, nonenzymatic reactions occur faster at higher pH values (57). The colors of all soured beers were accordingly brighter, whereas YC assumed darker colors. Pale beers were also reported in studies by Oliver-Daumen (48) and Pittner and Back (51) when biological acidification was applied during mash and wort compared with unacidified controls.

Together with turbidity, foam determines the first visual impression of beer quality to the consumer. Depending on the ability of the lactic cultures to degrade foam-active proteins, foam stability could be significantly influenced during sour beer production. However, this was seen in the past to not necessarily correlate with lower foam stabilities, because some of the proteins considered most important for foaming (LTP1 and protein Z) are resistant to enzymatic digestion (8). Lowe et al. (37) found that biological acidification by *L. amylovorus* FST 1.1 could improve the foam stability for a beer produced from 20% raw barley compared with that of 100% malt. The authors suggested that the proteolytic activity of the strain could have released more proteins to bind foam-destabilizing lipids. Even though *L. amylovorus* FST2.11 was found to possess proteolytic activities, foam analysis of soured beers showed that MS significantly promoted its stability compared with preBWS, but no differences were found compared with YC. A correlation between nitrogen content and foam stability was not apparent in this case. However, a prolonged contact time between spent grains and wort during MS could have led to the higher extraction of foam-stabilizing melanoidins from the malt husk (29). Although these compounds have been suggested as not being as powerful as proteins in stabilizing foam, they are less susceptible to the action of proteases (39). Melanoidins protect beers against the deleterious effects of lipid, as well as provide smaller bubbles (5). On the other side, the lysis of lactobacilli during boiling of preBWS could have released detrimental fatty acids (FAs), affecting foam qualities. It is reported that stressed and unhealthy yeast cells can release, other than proteases, also lipids that can damage foam (8). However, lysates of yeast cells have been seen to also contain mannoproteins, which in turn can act as foam stabilizers (10).

Flavor and Sensory Evaluation

Both yeast and lactic acid bacteria can add to the organoleptic profile by producing a variety of flavor compounds during fermentation (34). Whereas yeast release mainly esters, higher alcohols, FAs, aldehydes, and sulfur compounds during alcoholic fermentation, LAB excrete primarily organic acids, aldehydes, and a few esters during fermentation of liquid cereal-based substrates (49). Significantly higher levels of fermentation by-products, such as ethyl butyrate (tropical fruits), *n*-propanol (alcoholic), and isovaleric acid (sweaty, foot) (2) were found in postBWS beers compared with the other trials (Table IV). The formation of volatile compounds in mixed fermentation is strongly influenced by the activity and vitality of LAB and the yeast cultures and the release of metabolites that can act as flavor precursors. In this regard, amino acids likely released from the LAB proteolytic activity can be fed in the Ehrlich pathway of yeasts to form aldehydes or the corresponding alcohols (e.g., isovaleric acid) (13). Isovaleric acid is associated with the metabolism of branched amino acid and is a key odorant in different fermented foods, especially in cheese. It was reported by Guerzoni et al. (23) that this compound was overproduced as a response to acid stress from both pure cultures of *L. sanfranciscensis* and *S. cerevisiae* as well as in mixed fermentation of a liquid sourdough. The same author found that acid stress increased the levels of medium-chain FAs. FA levels were slightly raised for postBWS beers but not significantly different from the other beers. Considerable amounts of diacetyl and acetoin were found in postBWS samples. Diacetyl, a compound responsible for a butterscotch off-flavor, was above the sensory threshold for these beers; however, it was not detected by the sensorial panel. Diacetyl is formed through the nonenzymatic decarboxylation of α-acetolactate, a metabolite expelled by both LAB and yeast cultures during fermentation (19,61). This conversion is accelerated at lower pH values, as seen by Haukeli and Lie (27), who reported a fourfold increase when the pH in fermentations was lowered from 5.5 to 4.0. After primary fermentation by yeasts, the diacetyl formed is normally reabsorbed by the yeast cells and reduced to acetoin (cream, butter), which, in turn, is converted to 2,3-butanediol. The results suggested that this conversion was not completed in the postBWS soured beers. The substantial impairment of yeast metabolism during these trials could have caused a suboptimal reabsorption of diacetyl. Similarly, acetaldehyde (green apples, fruity) (44) was detected over its sensory threshold only in the soured beers, with the highest

TABLE III
Mash, Wort, and Beer Analyses of Yeast Control, Mash Souring, Preboil Wort Souring (PreBWS), and Postboil Wort Souring (PostBWS) Samples[a]

Analysis	Unit	Yeast control	Mash souring	PreBWS	PostBWS
Mash, filtration rate					
5 min	mL	93.1 ± 0.5b	114.4 ± 8.7a	94.6 ± 3.8b	97.2 ± 2.2ab
15 min	mL	150.5 ± 6.3ab	161.4 ± 1.2a	151.6 ± 3.9ab	150.7 ± 1.6b
30 min	mL	176.7 ± 2.8a	178.6 ± 4.7a	177.3 ± 0.8a	175.2 ± 0.7a
60 min	mL	192.5 ± 4.4a	186.9 ± 0.6a	191.4 ±1.6a	190.2 ± 2.2a
Wort (10.5 % w/w)					
β-Glucan	mg/L	373 ± 45a	378 ± 21a	391 ± 34a	364 ± 18a
Viscosity	mPa·s	1.74 ± 0.02a	1.58 ± 0.02b	1.64 ± 0.02b	1.74 ± 0.02a
Beer					
Haze	EBC formazin units	1.59 ± 0.24b	0.43 ± 0.15c	0.38 ± 0.04c	3.38 ± 0.58a
Total polyphenols	mg/L	106 ± 3a	87 ± 5c	90 ± 3bc	102 ± 1ab
Total soluble nitrogen	mg/L	648 ± 28a	611 ± 31a	615 ± 15a	650 ± 8a
Color	EBC units	6.4 ± 0.1a	5.2 ± 0.0c	4.9 ± 0.1c	5.8 ± 0.0b
Foam stability $t_{1/2}$	s	189 ± 10ab	224 ± 44a	161 ± 14b	206 ± 33ab

[a] Each value was expressed as mean ± standard deviation analyzed as duplicate from two independent brews. For each attribute, a different letter in each row denotes a significant difference at $P < 0.05$.

TABLE IV
Fermentation By-products (mg/L) Analyzed in Yeast Control, Mash Souring, Preboil Wort Souring (preBWS), and Postboil Wort Souring (PostBWS) Beer Samples After a Total of Four Weeks of Fermentation and Maturation[a]

Compound	Yeast control	Mash souring	PreBWS	PostBWS	Sensory threshold[b]
Esters					
Ethyl butyrate	0.22 ± 0.03b	0.15 ± 0.01b	0.18 ± 0.01b	0.33 ± 0.03a	0.4 (43)
Isobutyl acetate	0.05 ± 0.00b	0.09 ± 0.01a	0.07 ± 0.01a	0.06 ± 0.00b	1.6 (43)
Isoamyl acetate	0.50 ± 0.05a	0.80 ± 0.14a	0.65 ± 0.07a	0.60 ± 0.03a	1.0 (7)
Ethyl 2-phenylacetate	0.12 ± 0.00b	0.26 ± 0.02a	0.16 ± 0.12ab	0.17 ± 0.01ab	3.8 (7)
Ethyl acetate	11.30 ± 0.14a	14.20 ± 2.97a	11.75 ± 0.64a	14.05 ± 0.07a	30 (43)
Ethyl hexanoate	0.14 ± 0.01a	0.13 ± 0.01a	0.15 ± 0.01a	0.14 ± 0.00a	0.21 (43)
Ethyl octanoate	0.20 ± 0.01ab	0.11 ± 0.01c	0.15 ± 0.01bc	0.23 ± 0.02a	0.9 (7)
Ethyl decanoate	0.10 ± 0.00a	0.03 ± 0.01c	0.06 ± 0.01b	0.09 ± 0.01a	1.5 (43)
Fusel alcohols					
n-Propanol	19.5 ± 0.2b	20.1 ± 4.1ab	19.3 ± 1.3b	27.0 ± 0.6a	600 (7)
2-Phenylethanol	31.4 ± 1.7a	32.5 ± 0.4a	37.5 ± 1.7a	35.0 ± 2.2a	40–100 (7)
Isobutanol	28.9 ± 0.5b	43.9 ± 8.9a	32.9 ± 2.1ab	35.6 ± 0.0ab	100 (7)
Isoamyl alcohols	56.5 ± 0.2a	76.7 ± 15.8a	59.7 ± 3.8a	60.2 ± 0.7a	50 (7)
Fatty acids					
Isovaleric acids	0.83 ± 0.06b	0.72 ± 0.02b	0.50 ± 0.03c	1.15 ± 0.07a	1.5 (18)
Caproic acid	1.55 ± 0.07ab	1.10 ± 0.00c	1.40 ± 0.00b	1.75 ± 0.07a	8 (7)
Caprylic acid	3.60 ± 0.14a	2.40 ± 0.28b	3.35 ± 0.21a	3.75 ± 0.07a	15 (7)
Capric acid	1.14 ± 0.23ab	0.90 ± 0.28b	1.02 ± 0.11b	1.50 ± 0.00a	10 (18)
Ketones					
Diacetyl, total	0.10 ± 0.01b	0.07 ± 0.01bc	0.05 ± 0.00c	0.28 ± 0.00a	0.1–0.14 (26)
2,3-Pentanedione total	<0.01a	<0.01a	<0.01a	<0.01a	0.9 (7)
Acetoin	2.2 ± 0.2b	2.5 ± 0.1b	1.4 ± 0.3c	22.6 ± 0.2a	50 (43)
Polysulfide					
Dimethyl sulfide free	<0.01a	<0.01a	<0.01a	<0.01a	0.03–0.045 (43)
Linear aldehyde					
Acetaldehyde	5.35 ± 0.49c	40.25 ± 9.97a	14.25 ± 0.49b	14.05 ± 0.35b	10 (18)

[a] Each value was expressed as mean ± standard deviation analyzed as duplicate. For each compound, a different letter in each row denotes a significant difference at $P < 0.05$.
[b] Sensory threshold values retrieved from the literature referenced in parentheses.

level registered for the MS trial (40.25 mg/L), at four times higher than its sensory threshold in beer (10 mg/L) and eight times higher than YC. This compound is formed during alcoholic fermentation as an intermediate metabolite and reabsorbed by the yeasts after primary fermentation to be converted to carbon dioxide and ethanol (34). Numerous causes have been identified that enhance acetaldehyde levels (4). On one side, unhealthy yeast cells present at the end of fermentation can be impaired in their ability to reabsorb or finish the conversion from acetaldehyde to ethanol. Additionally, exposure to oxygen can lead to the oxidation of ethanol back to acetaldehyde (14). It is known that oxidative stability of beer is reduced at low pH values, contributing to the formation of staling compounds (22). The lower amount of polyphenols in the soured beers could have also led to higher oxidation events, which are triggered by the accelerated formation of reactive oxygen species (30). Acetaldehyde is particularly undesired, because it can be further involved in reactions leading to other staling compounds (67).

The soured beers were perceived as clearly sour with untypical ("not pure") smells identified for MS and postBWS, whereas a slight plum aroma was described for preBWS. Apart from postBWS samples, which had an astringent aftertaste, the sour beers lingered with a fading, sour aftertaste. Cell autolysis could have provoked the astringency, as well as influenced the overall flavor profile by releasing both substrates (lipids, proteins, and carbohydrates) and intracellular enzymes (11). An elevated astringency derived from the prolonged contact time with husk during MS was not detected. In this regard, a low pH during mashing was found in the past to limit their extraction (15).

CONCLUSIONS

Souring intended for sour beer production can be done at various time points along the brewing process, but the practice employed will ultimately influence attributes of both a technological and quality nature. The exposure of yeast cells to acidic conditions caused a delay in their growth and performance. Although this did not influence complete attenuation of sour beers, it might have impaired the reabsorption of off-flavors after primary fermentation, which primarily impacted the final quality of cofermented samples. High levels of acetaldehyde and decreasing lautering rates were detected when acidification was done in the mash. Further processing of soured mash also led to a dilution of their initial acidity level. Overall, the practice of souring wort before boiling emerged as the best way to obtain a sour beer with high acidity and minimal organoleptic failures.

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