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

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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 and the final beer was evaluated sensorically. XOS were utilized, metabolic compounds were produced (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 with reduced fermentation time (from 1-3 years to 4 weeks). The presented results demonstrate how hemicellulosic biomass can be valorised for beverage production, and to obtain sour beer with improved control.

Keywords:

Sour beer

Lactic acid bacteria

Secondary fermentation

Xylooligosaccharides

Hemicellulosic biomass

33 INTRODUCTION

34 Sour beer has an intentional acidic taste and is traditionally produced through spontaneous fermentation
35 involving lactic acid bacteria (LAB), acetic acid bacteria and yeasts such as *Saccharomyces* and
36 *Brettanomyces* [1-2]. This microbial consortium generates a wide range of metabolic compounds that
37 influence the organoleptic properties of the final product. Lactic acid and acetic acid are examples of
38 metabolic compounds which have sensory importance in beer. With reported sensory thresholds of 400
39 mg/L and 200 mg/L, respectively [3], both acids contribute to sourness and acidity [4]. Other metabolites
40 include esters such as ethylacetate, ethyl hexanoate and isoamyl acetate which contribute fruity flavours
41 [5], and alcohols such as 2-methyl 1-propanol and 3-methyl 1-butanol, both associated with alcohol
42 flavour [6]. A selection of metabolic compounds that can influence beer sensory properties is given in
43 table S1, along with sensory characteristics and reported sensory threshold in beer.

44 Fermentation of classic sour beers, including the Belgian styles Lambic, Geuze and Kriek [6], typically
45 takes several years [7]. However, difficulties with controlling complex mixed fermentations, in addition
46 to the time involved, can make production of commercial sour beer challenging. An increasing interest
47 in sour beers has emerged in recent decades and production strategies alternative to spontaneous
48 fermentations are being explored [8-9]. Fermentation with LAB prior to yeast fermentation is being
49 implemented in the brewing industry in order to produce sour beer rapidly and controllably [10]. Other
50 strategies include co-fermentation with LAB and yeast [11] and "primary souring", where an alternative
51 acid-producing yeast generates the sour taste [12]. Besides water and hops, malted barley is the main
52 ingredient in beer. Non-malt sources of extract, also known as adjuncts, are commonly added to modern
53 beers, providing additional substrates for the yeast fermentation [13]. Common adjuncts in beer
54 production include un-malted cereals such as barley, corn, rice, wheat and oats [14], but also non-cereal
55 substrates such as granulated sugar, sucrose-based syrups and malt extract [15]. Adjuncts allow the
56 manipulation of beer properties such as flavour, colour, drinkability and foaming properties, and may
57 increase brewery capacity through high gravity wort production [16]. As beer is consumed worldwide,

the replacement of a portion of barley malt with less expensive adjuncts is an efficient way to reduce production costs. The use of adjuncts can also reduce the carbon footprint from beer production, as the malting process (steeping, germination, kilning) entails considerable energy and water consumption [15]. Previous studies have investigated the use of alternative non-grain derived adjuncts such as banana [17], cocoa pulp [18] and sweet potatoes [19]. Emphasis is also being given to the use of regionally available adjuncts as this can reduce the need 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 for hardwood-derived xylans have been explored by the food industry [22-23]. Xylan has been shown to have antioxidant activity [25], it has been proposed as a nutritional fibre [26] and xylitol, derived from hydrolysis of xylan, 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 such as *Bifidobacterium bifidum* [30], *Roseburia* spp. and *Bacteroides* spp. [31-32] as well as *Bifidobacterium adolescentis* and *Lactobacillus brevis* [33].

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

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

88 MATERIAL AND METHODS

89 **Preparation of xylan**

90 Xylan was isolated from multiple batches of steam-exploded birch (*B. pubescens*) chips. Birch wood was
91 chosen due to its high abundance in Norway. Briefly, wood sawdust was pre-treated by steam explosion
92 (10 min residence time at 200ºC with a solid to liquid ratio of 1:1 w/w). The hemicellulose thus released
93 was extracted by hot water and the resulting molasses liquid was subjected to ultrafiltration using a
94 membrane with a cut off of 10 kilodalton (10 kDa) molecular weight (Alfa Laval). The retentate from the
95 ultrafiltration was collected and the permeate was further diafiltered and concentrated using a nanofiltration
96 membrane (TriSep). Ultrafiltration and nanofiltration were conducted on UF/NF model G (Gea filtration,
97 Denmark). Both the ultrafiltration and nanofiltration retentates were lyophilized using an ALPHA 2-4 LD
98 Plus freeze dryer (Christ, Germany) yielding two samples of xylan with a different degree of
99 polymerization. The nanofiltration retentate was used as a starting material in this study and was further
100 named low molecular weight (Mw) acetylated arabinoglucuronoxylan (AcAGX).

101 **Characterisation of low Mw AcAGX**

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

109 Neutral monosaccharides were analysed by High-Performance Anion-Exchange Chromatography
110 (HPAEC). Samples were autoclaved (121°C, 1 hour) with 4% H_2S0_4 to hydrolyse glyosidic linkages, cooled
111 and diluted to within an optimal concentration range (10 to 90 µg/mL). Arabinose, rhamnose, galactose,
112 glucose, xylose and mannose were detected and quantified using a Dionex™ ICS-3000 system (Thermo,

113 USA) with CarboPac™ PA1 columns – (guard 2 × 50 and analytical 2 × 250 mm) and an electrochemical
114 detector run in pulsed amperometric detector (PAD) mode. Analytes were eluted isocratically (1mM
115 NaOH) at 30 °C with a flow rate of 0.25 mL/min.

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

120 $$ASL\% = \frac{Abs\ at\ 240\ nm\ \times Volume\ hydrolysis \times Dilution}{\varepsilon \times weight\ DM(mg) \times Path\ length} \times 100$$

121 Acetic acid was quantified by HPLC. Deacetylation of xylan (10mg/mL) was carried out by alkaline
122 treatment (0.1 M NaOH, overnight at 4 °C). Acetic acid was quantified using an Ultimate 3000 HPLC
123 system (Thermo Scientific™, USA) with isocratic (5mM H_2SO_4) elution of a Rezex ROA-Organic Acid-
124 H+ column (300 × 7.8 mm) coupled to a security guard cartridge Carbo-H4 (4 × 3.0 mm). The released
125 acetic acid was detected at 210 nm, and quantification was based external calibration.

126 *Proteins*

127 Protein content was determined by micro-Kjeldahl. Samples (0.3 g) were hydrolysed in a Kjeldahl tube
128 with a digestion tablet (Kjeltabs Auto, Thompson and Capper Ltd., Runcorn WA7 1PH, UK) and 3 mL
129 H_2SO_4 (96 to 97%; Merck, Darmstadt, Germany) in an auto-digester (Foss Teactor, Foss analytical lab,
130 Hoganas, Sweden) for 60 minutes at 420 °C. Distillation and titration were carried out using a Foss Kjeltec
131 8400 analyser unit (Software version 1.5.18, Foss analytical lab, Hoganas, Sweden). The ratio of nitrogen
132 (% N) was converted to protein (% P) using a conversion coefficient of 6.25.

133 *Moisture and Ash*

134 The water content was determined by drying 0.2 g of sample at 105 °C for 20 h. Weight difference after
135 cooling in a dehydrator was considered water content. The remaining sample was incinerated at 600°C for
136 24 hours in an oven (Carbolite, Sheffield, England) to determine ash content. All measurements were
137 performed in triplicate.

138 **Preparation of xylooligosaccharides (XOS).**

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

153 **Production of base beers.**

154 Two base beers were produced according to the same recipe, but with different hopping. A 60L PRO pilot
155 scale brewery from CoEnCo (Oostkamp, Belgium, 2014) was used. Crushed malt (67 % Pilsner malt from
156 BestMalz, Germany, 33 % wheat malt from Weyermann, Germany) was mashed (1 kg malt: 4 L water) as
157 follows; 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 min, yielding wort with a 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 broth (De Man,
168 Rogosa and Sharpe, Oxoid Ltd., United Kingdom). The *L. brevis* strain was chosen because growth of this
169 microbe in beer had been proven previously, and for its ability to degrade XOS in pre-trials. For secondary
170 fermentation experiments in beer, *L. brevis* was first grown ON in MRS, before inoculation in a mixture of
171 85% MRS and 15 % beer (v/v). The mixture of 85/15 % MRS/beer was used to obtain high cell numbers
172 in the starter cultures while pre-conditioning the cells to beer related stress factors in a mild fashion. The
173 ON culture from MRS with beer was used to inoculate (1 %) the fermentation bottles in all experiments.
174 For the scaled-up fermentation, the starter culture in MRS with beer was centrifuged and the cell pellet was
175 re-suspended in a corresponding volume of beer and used as inoculum.

176 *Small scale fermentations*

177 XP2 (2 % w/v and 0.5 % w/v) was dissolved in beer LH, before the beer was centrifuged for 10 minutes at
178 4 °C (7000 × g, Heraeus Multifuge X3R, ThermoFisher, Germany, 2010) and sterile-filtered (0.22 µm,
179 Millipore Express™PLUS, Merck, Germany). The beer was then partitioned into four 50 mL bottles. Three
180 bottles were inoculated (1%) with *L. brevis*, and the fourth served as a negative, uninoculated control. A
181 corresponding experiment was prepared with xylose to serve as positive control, and also an experiment
182 with beer without substrate addition as negative control. The fermentation progressed at 25 °C for two

183 weeks. Two additional fully-corresponding experiments were prepared. One 2 % XP1 in beer LH, to
184 investigate the performance of XP1. The second one was prepared with 2 % XP2 in beer HH, and incubated
185 at 22 °C. This experiment was conducted to verify that the secondary fermentation still took place in beer
186 with higher hopping, and at regular ale fermentation temperature.

187 *Large scale XOS sour beer production by secondary fermentation*

188 Three 5 L fermentation flasks containing beer HH supplemented with 0.5 % XP2 were prepared, following
189 inoculation with *L. brevis*. Negative, non-inoculated, controls (2 × 5 mL tubes) were taken out from each
190 of the three 5 L flasks prior to fermentations. Positive xylose controls were prepared in 5 mL tubes without
191 centrifugation and sterile filtration. Beer with XP2 and positive xylose controls were inoculated (1%) with
192 *L. brevis* starter. Negative controls comprising beer without substrate additions were prepared in 5 mL tubes
193 without centrifugation and sterile filtration. The fermentation progressed at 25 °C for 4 weeks. After this
194 period the beer, referred to as "XOS sour beer", was stored at 4 °C for 4 weeks. Prior to sensory evaluation,
195 the beers were slightly carbonated using an Aqvia sodastreamer (AGA, Luleå, Sweeden), before
196 transferring to 0.33L bottles.

197 *Sampling.*

198 For the small-scale fermentations, fermentation bottles were swirled to obtain homogenized cell
199 suspensions, and samples of 0.4 mL were drawn at 0 h, and after 1 2, 3, 5, 7, 11, 14 and 28 days. Growth
200 of *L. brevis* was monitored by plate drop on MRS agar plates (15 % agar powder, VWR Chemicals, Leuven,
201 Belgium). pH was monitored throughout fermentation using a Sentron pH-meter with SI probe (Sentron,
202 Netherlands). Samples were then centrifuged (3 min, 16.1 × 1000 g) using a 5415 D centrifuge (Eppendorf,
203 Germany) to remove cells. 200 µL of the supernatant was frozen for further analysis. After the final
204 sampling, the remaining content in each fermentation flask was centrifuged at 7000 × *g*, 4 °C for 10 minutes
205 and the supernatants were frozen for further analysis. For the large-scale fermentation, samples of 50 mL
206 were drawn during the fermentation at 0 hours, and after 1 2, 3, 5, 7, 11, 14 and 28 days. A final sample
207 was taken at the time of the sensory evaluation. As beer fermentations are generally carried out without

208 stirring, samples were carefully drawn from the middle of unstirred fermentation vessels. MRS agar
209 supplemented with 25 mg/L cycloheximide (Sigma-Aldrich, St. Louis, USA) was used to monitor the
210 growth of *L. brevis*, while yeast was quantified using Rose-Bengal Chloramphenicol agar (RBC, Oxoid,
211 Basingstroke, UK). pH was monitored throughout fermentation, and samples were centrifuged as described
212 above and the supernatant was kept at -20°C for further analysis.

213 **Analyses.**

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

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

222

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

224 During fermentation by *L. brevis*, aliquots were removed at regular intervals. The amount of the mono- and
225 oligo- saccharides consumed was quantified by HPAEC using standard methodology. In brief, samples
226 were bound to a Dionex (Thermo Scientific) CarboPac PA1 analytical column (2 × 250 mm) in combination
227 with a CarboPac PA1 guard column (2 × 50 mm), equilibrated with 0.1 M NaOH. Carbohydrates were
228 detected by pulsed amperometric detection (PAD). The system was run at a flow rate of 0.25 mL/min. The
229 elution conditions during analysis were 0-10 min 0.1 M NaOH with a 0 to 0.1 M NaOAc gradient; 10-35
230 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
231 gradient; and 40-50 min 0.1 M NaOH. Commercial xylose and xylooligosaccharides (DP 2 to 6) from
232 Megazyme were used as standards.

233 *Headspace gas chromatography (HSGC).*

234 HSGC was used to analyse volatile compounds according to the method described by Grønnevik, et al. [38].
235 Samples were filtered through folding filters (pore size < 2 µm, Schleicher & Schuell, Dassel, Germany)
236 before 10 g were transferred to headspace vials (Machery Nagel, Dueren, Germany). The vials were sealed
237 with Teflon-coated septa with aluminium rings (PFTA/Si septa, Agilent Technologies, Wilmington, DE,
238 USA) and placed in a 7679A automatic headspace sampler connected to a 6890 GC system with flame
239 ionisation detector (Agilent Technologies). The headspace bath temperature was 50 °C and manifold
240 temperature 60 °C. The carrier gas was helium 6.0 (Aga, Norway) at a flow rate of 5.0 mL/min. Prior to
241 injection (0.5 min injection time, 10 PSI pressure), samples were mixed for 45 minutes (70 shakes/min) to
242 achieve equilibrium. A CP-SIL 5CB GC column (Varian, Middelburg, Netherlands) of 25 m × 0.53 mm
243 I.D. with film thickness 5 µm, was used to separate the compounds based on volatility and affinity for the
244 column matrix. Peak identification and quantification were done based on calibration with external
245 standards. The volatile compounds were tentatively identified. Used standards with corresponding retention
246 times and calibration ranges with corresponding R^2 can be reviewed in table S2 [39-40]. Open LAB EZChrom
247 software (version A.04.05, Agilent Technologies) was used to operate the system. The following
248 temperature scheme was applied during analysis: 35 °C for 5 min: increase of 10°C/min until 40°C for 2
249 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
250 10°C/min until 180 °C for 2 min; increase of 10°C/min until 200°C for 2 min.

251 *High performance liquid chromatography (HPLC).*

252 HPLC was used to analyse organic acids according to the method described by Grønnevik, et al. [38]. One
253 gram sample was mixed with 2.5 mL MilliQ water, 200 µL 0.5 M H_2SO_4 and 8 mL acetonitrile using a
254 MultiRS-60 BIOSAN rotator (Montebello Diagnostics A/S, Oslo, Norway) operated at 30 rpm for 30 min.
255 Samples were centrifuged for 15 min at 1470 × *g* using a Kubota 2010 centrifuge (Kubota Corporation,
256 Tokyo, Japan) and filtered through 0.2 µm PTFE membrane (Acrodisc CR 13 mm Syringe Filter, PALL,
257 Great Britain). The organic acids were separated at 30 °C on an Aminex HPX-87H column (Bio-Rad

258 Laboratories, Hercules, CA). The column was connected to a 1260 Infinity HPLC instrument (Agilent
259 Technologies, Singapore) with pump, autosampler, column oven, RI-detector (refractive index, used for
260 acetic acid) and DAD-UV detector (diode array detector- ultra violet, used for the other organic acids).
261 H_2SO_4 was used as mobile phase at a flow rate of 0.4 mL/min. Openlab CDS software (Agilent
262 Technologies) was used to operate the system. Detection and quantification were made using external
263 calibration.

264 *Beer characterisation.*

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

271 *Sensory evaluation by trained panel.*

272 Sensory evaluation of the beers was carried out by a professional sensory panel consisting of nine trained
273 assessors at the Norwegian Institute of Food, Fisheries, and Aquaculture Research (NOFIMA, Aas,
274 Norway). Panellists had been screened for sensory abilities (basic tastes, colour vision, odour detection,
275 tactile sensibility) and ability to communicate sensory descriptions of products recommended in ISO
276 (International organization for standardization) 8586 [41] in a sensory laboratory designed in accordance with
277 ISO 8589 [42]. EyeQuestion (v4.11.33, Logic8, Holland) was used for direct recording of data. Based on
278 previous experiments with beer, a list of relevant sensory attributes was generated. A training session was
279 carried out, in which the assessors were trained in the definition of 22 selected taste, texture, odour and
280 flavour attributes (Supplementary table S3). Sensory profiling according to Generic Descriptive Analysis
281 as described by Lawless and Heymann [43], was used to evaluate the two different beers in duplicate. Each
282 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 17± 1 °C in clear plastic cups, tagged with random three-digit codes. For each session, all samples 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) [44] 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

308 as two-dimensional score plots and loading plots. The former shows how the samples are grouped according
309 to the design, while the latter shows how the beer properties are affected by the design. In the ASCA score
310 plots one can display the uncertainty of the effect level means, similar to Tukey's test in ANOVA, using
311 confidence ellipsoids [45]. Uni-dimensional ANOVA for each compound was combined with Tukey's test for
312 honestly significant differences. This was done to obtain groups of effect levels, e.g. groups of beers, which
313 are not significantly different with respect to a chosen measured property. The ANOVA with Tukey's test
314 was carried out using R 3.6.1 (R Core Team 2019, Austria, Vienna), and statistical significance level was
315 set at $p < 0.01$.

316 RESULTS AND DISCUSSION

317 **Properties of low Mw AcAGX.**

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

323 **Preparation and characterization of the XOS samples.**

324 Enzymatic digestion and alkaline deacetylation of Low Mw AcAGX, were carried out in order to generate
325 shorter oligosaccharides and increase the fermentability by the strain of *L. brevis* used. Previous studies
326 have shown that XOS with a degree of polymerisation of 2-5 are favourable for microbial fermentation [46-
327 47] and unsubstituted oligos are more readily utilized by microbes [48]. Enzymatic digestion of low Mw
328 AcAGX, followed by alkaline deacetylation generated XP1. A second substrate was prepared to reduce the
329 pH observed in XP1 and increase the amount of substrate for fermentation by *L. brevis*. This was done by
330 using an initial deacetylation step with NaOH, followed by removal of alkali before enzymatic treatment.
331 This preparation scheme resulted in XP2, having some minor remaining xylanase activity (not observed for
332 prep 1). To determine the mass distribution and degree of acetylation, the two preparations were analysed
333 by HPAEC-PAD and MALDI-ToF MS. While low Mw AcAGX contained xylose and XOS ranging from
334 two to six units, XP1 contained very low, or no amounts of xylose and xylobiose (X_2), with predominant
335 XOS being xylotriose (X_3), xylotetraose (X_4), xylopentaose (X_5) and xylohexaose (X_6) (Fig. 1A). This was
336 partially caused by the acetyl substituents making the polymer less accessible to the enzyme [49-51], yielding
337 longer oligos, and partially by the loss of X_1 and X_2 in the cleaning step where NaOH and acetic acid was
338 removed. XP2 contains primarily X_2, but also considerable amounts of X_{3-6}. This agrees with previous
339 literature where Shearzyme® has been used [49]. As shown in Figure 1B, the low Mw AcAGX is heavily

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

342 **Small scale fermentations**.

343 *L. brevis* grew well in beer LH supplemented with XP1 (Fig. 2A), with an increase in CFU by 10^2 in 7 days,
344 reaching a maximum observed cell count of 2.1×10^8 CFU/mL. A simultaneous reduction of pH was
345 observed, from initial pH of 5 to 4.4 after 7 days (Fig. 2B). A reduction in CFU/mL and a very minor pH
346 drop was observed at the later sampling points. The final pH was higher than typical sour beer pH (below
347 pH 3.8) [8]. Comparing the HPAEC-PAD profiles at the initial and final sampling (Fig. 2C), X_1, X_2, X_3 and
348 X_4 were completely fermented after 28 days. The X_5 and X_6 were seemingly untouched. This suggests that
349 *L. brevis* can degrade XOS with xylose units up to four, but not higher. The high initial pH in this
350 fermentation is a result of a pH-elevating effect from XP1 in the beer. Despite successful acid production
351 from *L. brevis*, the final beer pH was too high for the beer to classify as a sour beer. Because of the high
352 pH contribution form XP1, all the subsequent growth experiments were conducted using XP2 as secondary
353 substrate in beer.

354 The initial growth of *L. brevis* was similar in regular LH beer, and the same beer supplemented with XP2
355 (Fig. 3A). In both beers supplemented with 0.5% and 2% XP2, the cell counts continued to increase during
356 the first week of fermentation reaching a maximum of 1.4×10^8 CFU/mL after 7 days for 0.5 %, and $2.3 \times$
357 10^8 CFU/mL after 5 days for 2 %. The growth curves for *L. brevis* in beer and beer supplemented with
358 xylose were similar, but with a lower maximal cell count, and an earlier decrease in cell numbers. The final
359 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
360 positive control and 1.4×10^6 for the beer negative control.

361 Corresponding reductions in pH were observed in *L. brevis* inoculated LH beer supplemented with 0.5%
362 xylose and 0.5% XP2 (Fig. 3B). The initial pH for both these beers was 4.1, and the final pH 3.4. The initial
363 pH of the beer with 2 % XP2 was slightly higher, at 4.3. This can be attributed to the pH-elevating effect
364 from the XP2 itself. Only a marginally lower final pH of 3.3 was obtained with the higher substrate dose.

365 It is noteworthy that the pH reduction was quicker with higher substrate dosage. In fact, after only 5 days
366 of secondary fermentation, the pH in the LH beer with 2% XP2 was 3.6, compared to 3.9 in beer with 0.5%
367 XP2 (Fig. 3B). This suggests that it is possible to carry out acidification of non-sour base beer in sour beer
368 production in less than a week with secondary fermentation, by using a high dose of secondary substrate.
369 No reduction of pH was detected in beer without secondary substrate addition. No pH-reduction was
370 observed in any of the uninoculated negative controls during the incubation period (Supplementary, Fig.
371 S1). As can be seen from the HPAEC-PAD profiles from the final sampling with *L. brevis* inoculated and
372 uninoculated beer (Fig. 3C), the secondary substrate in the 0.5% XP2 dosage is utilised to completion. The
373 peak representing X_2 is diminished in the *L. brevis* inoculated beer with 0.5% XP2 at the end of
374 fermentation, while X_2 is not fully consumed at the end of fermentation of beer with 2% XP2.

375 In the absence of a substrate for secondary fermentation, 560 mg/L lactic acid and 360 mg/L acetic acid
376 were present in the beer after 14 days of incubation with *L. brevis* (Fig. 3D). When the beer was
377 supplemented with 0.5% XOS, 2280 mg/L of lactic acid and 1740 mg/L acetic acid were detected at the
378 same time point. The corresponding values for beer with 2% XP2 were 3940 mg/L lactic acid and 2930
379 mg/L acetic acid. A similar trend, showing a higher concentration of compounds with increasing amount
380 of secondary substrate, was observed for acetaldehyde, ethyl acetate (Fig. 3E) and isoamyl acetate (Fig.
381 3F). The opposite was evident for acetoin (Fig. 3E) and diacetyl (Fig. 3F) where higher substrate
382 concentration yielded lower amounts.

383 These results demonstrate that the final pH of the beer after secondary fermentation, as well as the final
384 composition of metabolic compounds, can be adjusted by using different doses of secondary substrate.
385 Some organoleptically active compounds did not change during secondary fermentation or in response to
386 addition of a secondary substrate. Phenylethyl alcohol (Fig. 3E) is an example of this, showing that the
387 initial composition of beer prior to secondary fermentation, is important for the final composition of a sour
388 beer produced through this method. A similar experiment was also carried out at lower fermentation
389 temperature (22°C) in beer produced with approximately twice the amount of iso-α acids (Supplementary,

390 Fig. S2). *L. brevis* performed well under these conditions. Growth and pH-reduction were slower, but after
391 21 days incubation, a final pH of 3.7 was obtained in the beer. Thus, fermentation temperature and hopping
392 scheme can also be used to manipulate metabolic activity and final pH in sour beer produced through
393 secondary fermentation.

394 **XOS sour beer production through secondary fermentation.**

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

405 Lactic and acetic acid were produced throughout fermentation (Fig. 4D), reaching final concentrations of
406 1800 and 1200 mg/L after 28 days of secondary fermentation. At these concentrations, both acids were
407 well above their respective sensory detection limits and likely to influence the sensory properties of the
408 sour beer. Pyruvic and citric acid had opposite developments (Fig. 4E), being present in the base beer at
409 approximately 250 and 200 mg/L, respectively. After 5 days of *L. brevis* secondary fermentation, the
410 concentration of pyruvic acid was reduced to 20 mg/L, while citric acid was no longer detected. Acetoin
411 and diacetyl were not present in the base beer but were produced during the initial growth phase during
412 secondary fermentation (Fig. 4E). The concentration of both acetoin and diacetyl peaked after three days
413 (25 mg/L for acetoin and 8 mg/L for diacetyl) followed by a drop to below 5 mg/L for acetoin and below
414 the detection limit for diacetyl.

415 Citric acid is degraded to pyruvic acid and further by LAB metabolism, with diacetyl and acetoin being by-
416 products of this degradation [53-54]. This is evident from figures 4E and 4F, where diacetyl and acetoin were
417 produced while citric acid and pyruvic acid was degraded during the first three days of *L. brevis*
418 fermentation. Diacetyl has a very low detection limit in beer (0.1 mg/L) and is generally an unwanted
419 compound [55]. In the current secondary fermentation, the diacetyl concentration was diminished after 11
420 days. Emphasis should be made on allowing sufficient fermentation time during secondary fermentation.
421 This is not only important to achieve acid production, but also to allow the degradation of other unwanted
422 compounds produced in the initial phase of secondary fermentation.

423 *Characteristics and metabolic compounds in the final beer.*

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

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

465 base beer. The exceptions, which were higher in the base beer, were malty odour and flavour, and yeasty
466 odour and flavour. The high number of significant differences in sensory attributes between the XOS sour
467 beer and the base beer, point to a substantial effect from the XOS induced secondary fermentation on the
468 overall organoleptic perception. The beer increased in complexity from the secondary process as most of
469 the attributes, including total intensity odour and flavour, and perfumed odour and flavour, increased in
470 intensity. Some of the changes in these sensory properties might be brought about by the addition of the
471 secondary substrate itself. The contribution of the XOS on its own to the sensory properties has not been
472 investigated in the current study. It is however clear, looking at the metabolic data, that the XOS-induced
473 secondary fermentation by *L. brevis* causes multiple significant shifts in the concentration of metabolic
474 compounds known to have sensory relevance.

475 Succinic, lactic and acetic acids were not detected in the base beer but were produced in the secondary
476 fermentation and were present in the XOS sour beer. With succinic acid at 350 mg/L. lactic acid at 1750
477 mg/L and acetic acid at 1100 mg/L, all these organic acids were well above their respective sensory
478 thresholds (table S1). The level of organic acids in the XOS sour beer complies with this beer being
479 perceived as higher in sour odour, sour flavour and acidic taste. Acetaldehyde, which is associated with
480 fruit and green leaves (table S1) was higher in the XOS sour beer, at 5.6 mg/L compared to 1.6 mg/L in the
481 base beer. *L. brevis* strains have been shown to produce acetaldehyde from ethanol [56], and the increased
482 level in the XOS sour beer points to production during secondary fermentation. Ethyl acetate, ethyl
483 hexanoate, isoamyl acetate and ethyl octanoate, are all esters associated with fruity flavours (table S1).
484 Esters can be produced through lactic acid bacteria fermentation [57-58], as the slightly higher level in the XOS
485 sour beer suggests. No difference was found, however, between the base beer and the XOS sour beer
486 regarding fruity odour and fruity flavour, which agrees with the concentrations of fruity esters and
487 acetaldehyde, all being below their respective, reported flavour thresholds. The concentration of the volatile
488 alcohols 2-methyl-1-propanol, 3-methyl-1-butanol and 2-hexanol were very similar in the base beer and the
489 XOS sour beer, and well below their sensory thresholds (table S1).

490 The concentration of 2-butanol was also below the sensory threshold and was present at 0.13 mg/L in the
491 XOS sour beer but not in the base beer. This points to production during the *L. brevis* secondary
492 fermentation. 2-Butanol production by *L. brevis* strains has been reported [59]. The presence and combinations
493 of multiple compounds with sensory relevance, despite concentrations below detection thresholds, can
494 transform the sensory properties through synergistic effects [60]. The considerable number of significant
495 differences in sensory properties between the XOS sour beer and the base beer might be due to such effects.

496 The ANOVA with Tukey's test, which included the results from commercial sour beer reference as well as
497 the base beer and XOS sour beer, also revealed significant differences between all three beers. The full
498 table with attribute scores and ANOVA groups can be found in supplementary (Table S4). Attributes scored
499 significantly different between two or more beers are displayed in figure 5B. The XOS sour beer and the
500 commercial sour beer were perceived as similar to each other, but significantly different from the base beer
501 in the attributes dried fruit odour, total flavour intensity, acidic taste and astringency. Despite the vastly
502 higher content of lactic acid in the commercial sour beer, the XOS sour beer and the commercial sour beer
503 were perceived as having similar intensity in acidic taste. Lactic, acetic and succinic acids are all associated
504 with acidic taste. The higher concentrations of succinic and acetic acids in the XOS sour beer might
505 compensate for the lower lactic acid concentration, with respect to the acidic taste. The base beer, where
506 none of these acids were detected, was perceived as significantly less acidic taste, than both the sour beers.

507 It should be noted, that the reduction in carbonation level prior to sensorial evaluation carried out for the
508 commercial sour beer (by pouring the beer back and forth) might have caused loss of volatiles and reduced
509 intensity in flavour and odour attributes. It is, however, noteworthy, that the XOS sour beer was scored
510 between the base beer and the commercial sour beer, for all the sensory attributes where significant
511 differences were found in the ANOVA.

512 The commercial sour beer was different from the base beer and XOS sour beer in all characteristics
513 presented in table 2. The alcohol concentration in the commercial sour beer was 8 %, compared to
514 approximately 4 % in both other beers. The colour value was more than 20 EBC, compared to

515 approximately 10 in the base beer and XOS sour beer. The ADF in the commercial sour beer was above 90
516 %, compared to 80 % in the others, and original extract of 16 % compared to approximately 10.
517 Correspondingly, the final sugar concentration in the commercial sour beer was lower, at about 1 °Brix
518 compared to more than 2 °Brix in the base beer and XOS sour beer. Bitter taste and after taste were scored
519 differently between all three beers. Perfumed flavour and alcohol flavour were similar in the base beer and
520 the XOS sour beer, but different from the commercial sour beer. For all these attributes, the XOS sour beer
521 was scored between the base beer and the commercial sour beer in intensity. The alcohol flavour was scored
522 almost identically in the base beer and XOS sour beer, and lower than the commercial sour beer. This was
523 in expected, as the commercial sour beer contained twice the percentage of alcohol. These differences in
524 characteristics and sensory attributes were expected, as the commercial sour beer is produced through a
525 completely different process and based on a different recipe. While the XOS sour beer resulted from
526 secondary fermentation by a single *L. brevis* strain, the commercial sour beer reference was produced
527 through a traditional Lambic/Geuze fermentation by a complex microbial consortium. The active
528 fermentation time in the XOS sour beer production was less than 2 months, compared to several years for
529 the commercial sour beer reference. The objective of the current study was not to produce a beer with
530 identical properties and sensory qualities to a commercial sour beer, but to investigate an alternative
531 production process for sour beers. The commercial sour beer reference was included in the sensory analysis
532 to see if the result from this alternative process was at all comparable to a traditional sour beer product. The
533 descriptive sensory analysis showed that it is possible to significantly alter the sensory characteristics of
534 beer by implementing a substrate induced secondary fermentation with *L. brevis*. The results also showed
535 that the intensity of multiple sensory attributes was shifted in a way making the beer characteristics more
536 comparable to those of a traditionally produced commercial sour beer. If the purpose is to produce a sour
537 beer that is even closer in quality to a traditional sour beer, this could perhaps be achieved by altering the
538 base beer recipe or by including multiple bacterial strains in the secondary fermentation.

539 In summary, the current study demonstrates how XOS prepared from birchwood xylan can be used
540 as a specific substrate, directing secondary lactic acid bacteria fermentation in sour beer production. The
541 secondary fermentation in this study lasted for 4 weeks. Results from the small-scale fermentations point
542 to a potential for shortening the fermentation time even further, by adjusting substrate dosage and hopping
543 scheme. It should be noted that, while the small-scale fermentations were swirled prior to sampling, the
544 larger scale fermentations were not. Additional oxygen might have been introduced in the small-scale
545 fermentations, and this might be part of the explanation for the enhanced fermentation rate. This shows that
546 a secondary fermentation can be used in controlled sour beer production with shorter production time
547 compared to traditional spontaneous mixed fermentations. During the secondary fermentation, organic
548 acids and other metabolic compounds with sensory importance were produced. Actual sensory influence
549 was proven through descriptive analysis with a trained panel. The substrate induced secondary fermentation
550 caused multiple significant shifts in intensity of sensory attributes. Importantly, the resulting XOS sour beer
551 was scored as similar in intensity to a commercial sour beer reference in sensory attributes such as dried
552 fruit odour, total flavour intensity, acidic taste and astringency. *L. brevis* BSO 464*,* used in this study, was
553 well suited as it was able to degrade XOS and it was known to be able to sustain the harsh beer environment
554 [61]. Alternative bacteria and perhaps also yeast species could be used together with specific substrates to
555 generate beers with new sensory properties through secondary fermentations and potentially also co-
556 fermentations. Fermentation induced by hemicellulose derived substrates could pose an alternative for
557 improved utilization of cheap and renewable lignocellulosic feedstocks. Sour beer was used to prove this
558 concept in the current study. With further research, the approach could potentially be extended to products
559 such as non-alcoholic fermented beverages, sour dough bread and probiotic dairy products.

560 ABBREVIATIONS.

561 LAB – lactic acid bacteria

562 Mw – molecular weight

563 kDa – kilo Dalton

564 AcAGX – acetylated arabinoglucuronoxylan

565 RT – room temperature

566 HPLC – High Performance Liquid Chromatography

567 PAD – pulsed amperometric detector

568 ASL – acid soluble lignin

569 ON – over night

570 XOS – xylooligosaccharides

571 XP1 – xylooligosaccharide preparation 1

572 XP2 - xylooligosaccharide preparation 1

573 LH – lower hopping

574 HH – higher hopping

575 MRS – De Man, Rogosa and Sharpe

576 RBC – Rose Bengal Chloramphenicol

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

578 DHB – dihydroxybenzoic acid

579 HPAEC – High-performance anion-exchange chromatography

580 HSGC – Headspace gas chromatography

581 RI – refractive index

582 DAD-UV - diode array detector- ultra violet

583 ISO – International organization for standardization

584 ASCA – Analysis of variance simultaneous component analysis

585 ANOVA – Analysis of variance

586 PCA – Principal component analysis

587 Ara – arabinose

588 Rha – rhamnose

589 Gal - galactose

590 Glc – glucose

591 Xyl – xylose

592 Man – mannose

593 DA - degree of acetylation

594 CFU – colony forming units

595 ADF - Apparent degree of fermentation

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SUPPORTING INFORMATION DESCRIPTION.

Supporting information to the current paper contains four 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: Standards used for external calibration in HSGC analysis, with corresponding retention times.

Table S3: Attributes (odour, O; texture, taste, T and flavour, F) and descriptions used in descriptive sensory analysis of the beers.

Table S4: 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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764 # TABLES AND GRAPHICS

765 ## FIGURE CAPTIONS

766 **Figure 1**: Distribution of xylooligosaccharides in different preparations used in this study. A) HPAEC-PAD profile
767 of xylooligosaccharide standards (one to six xylose units), low Mw AcAGX, XP 1 and XP 2. MALDI-ToF MS
768 spectra of native low MW AcAGX (B), XP1 (C) and XP2 (D). "Xyl" = xylose, "Hex"= hexose, "Me"= methyl, "GlcA"
769 = Glucuronic acid, "Ac" = acetyl, all annotated peaks appear as sodium adducts except ζ = potassium adducts m/z
770 +16 compared to the corresponding sodium adduct, and * = sodium salts of the $MeGlcAXyl_n$.($[M+2Na]^+$) that are
771 commonly occurring in uronic acids [52]

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

775 **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
776 25°C for 14 days. A) Growth of *L. brevis* on different substrates. B) pH-development in inoculated samples. C)
777 HPAEC-PAD profile in beer LH with XOS (2% and 0.5%) at the end of fermentation with *L. brevis*, and profile in
778 non-inoculated negative control. D) Lactic acid and acetic acid final concentrations in beer and beer LH with XP2 (0.5
779 and 2%) of lactic and acetic acid (D), acetaldehyde, ethylacetate, acetoin and phenylethyl alcohol (E) and acetone,
780 diacetyl and isoamyl acetate (F). "N.d" = non-detected. Significantly different concentrations (according to Tukey's
781 test at significance level $p < 0.05$) are indicated with different letters.

782 **Figure 4**: Performance of *L. brevis* during incubation (25°C, 28 days) in 5 L HH beer supplemented with XP2 (0.5%).
783 All panels except C, are presented as averages of three replicates with standard deviation as error bars. A) Growth of
784 *L. brevis* during incubation. B) pH development in inoculated samples and non-inoculated negative controls. C)
785 HPAEC-PAD profiles of XOS standards and XOS profile at final sampling (28 days) from *L. brevis* inoculated
786 samples and non-inoculated negative controls. Development of lactic and acetic acid (D), pyruvic and citric acid (E)
787 and acetoin and diacetyl (F) during the fermentation.

788 **Figure 5**: Composition of metabolic compounds in the three beers described in the sensory analysis; base beer, XOS
789 sour beer and commercial sour beer. A) Variation in samples and replicate variation described by analysis of variance

790 simultaneous component analysis (ASCA) scores. The model explains 93% of the variation in metabolic compounds.
791 B) Loading weights for ASCA model in panel A. Dark grey bars show loadings for component 1 (70%) and light grey
792 bars show loadings for component 2 (30%). Concentration of succinic, lactic and acetic acid (C), acetaldehyde, ethyl
793 acetate, 2-methyl 1-propanol and 3-methyl 1-butanol (D), acetone, 2-butanol, 1-hexanol and ethyl hexanoate (E), and
794 isoamyl acetate and ethyl octanoate (F) in the different beers; base beer (light grey bars), XOS sour beer (dark grey
795 bars) and commercial sour beer (white bars). Significantly different concentrations in the compounds (according to
796 ANOVA at significance level $p < 0.05$) are indicated with different letters in the figure.

797 **Figure 6**: Descriptive sensory analysis of base beer, XOS sour beer and commercial sour beer. A) Base beer and XOS
798 sour beer: average scores for sensory attributes. Significantly different scores (according to t-test) between the two
799 beers are indicated with *. B) Base beer, XOS sour beer and commercial sour beer: significantly differently scored
800 sensory attributes in beers ($p < 0.05$), according to ANOVA with Tukey's test (Supplementary, Table S4).

801

802

TABLES

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

Table 2: Characteristics for commercial sour beer, base beer and XOS sour beer at the point of descriptive sensory analysis. Alcohol (%), colour value (EBC), apparent degree of fermentation (ADF, %), original extract (%) and sugar 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

FIGURES

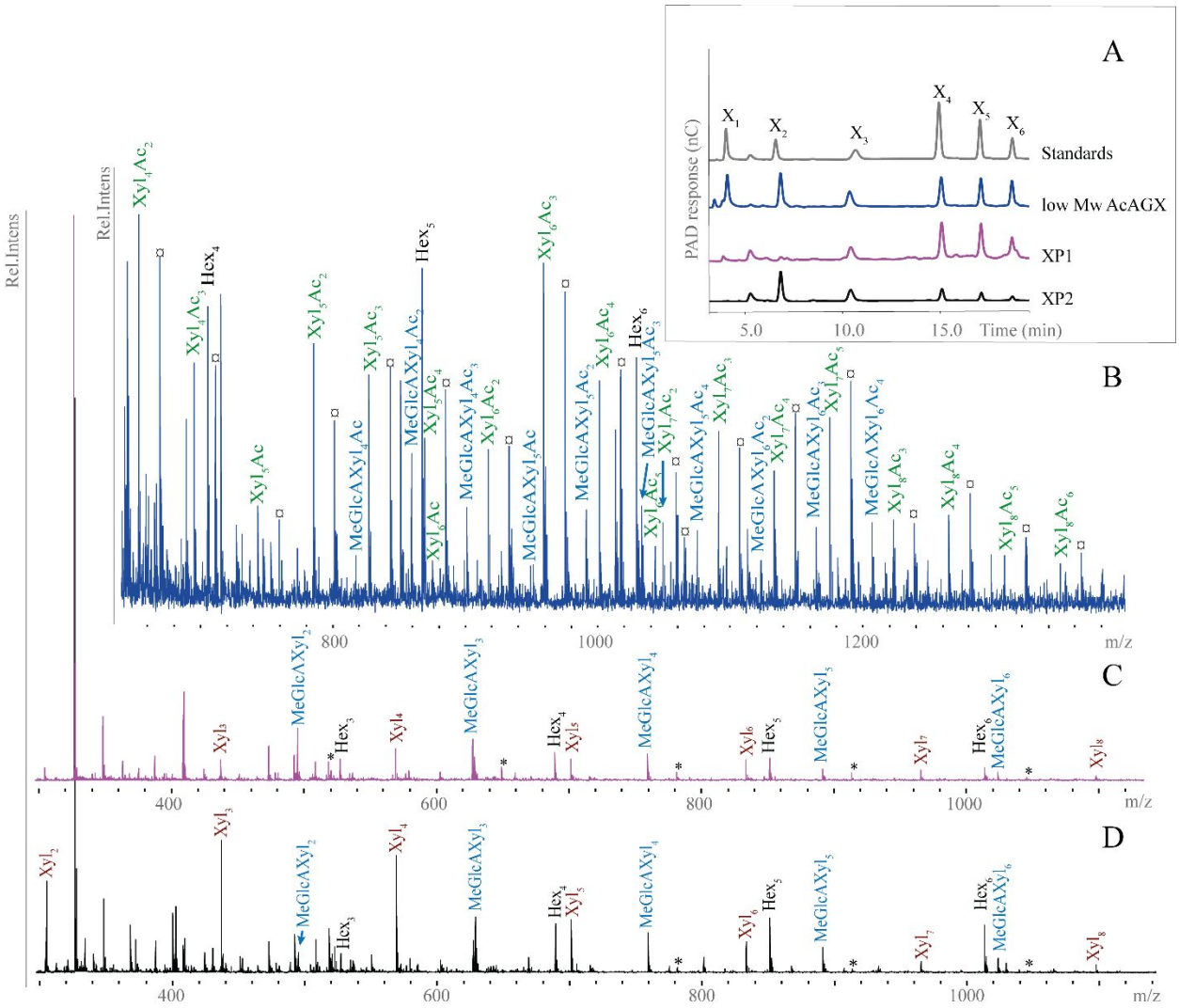


Figure 1

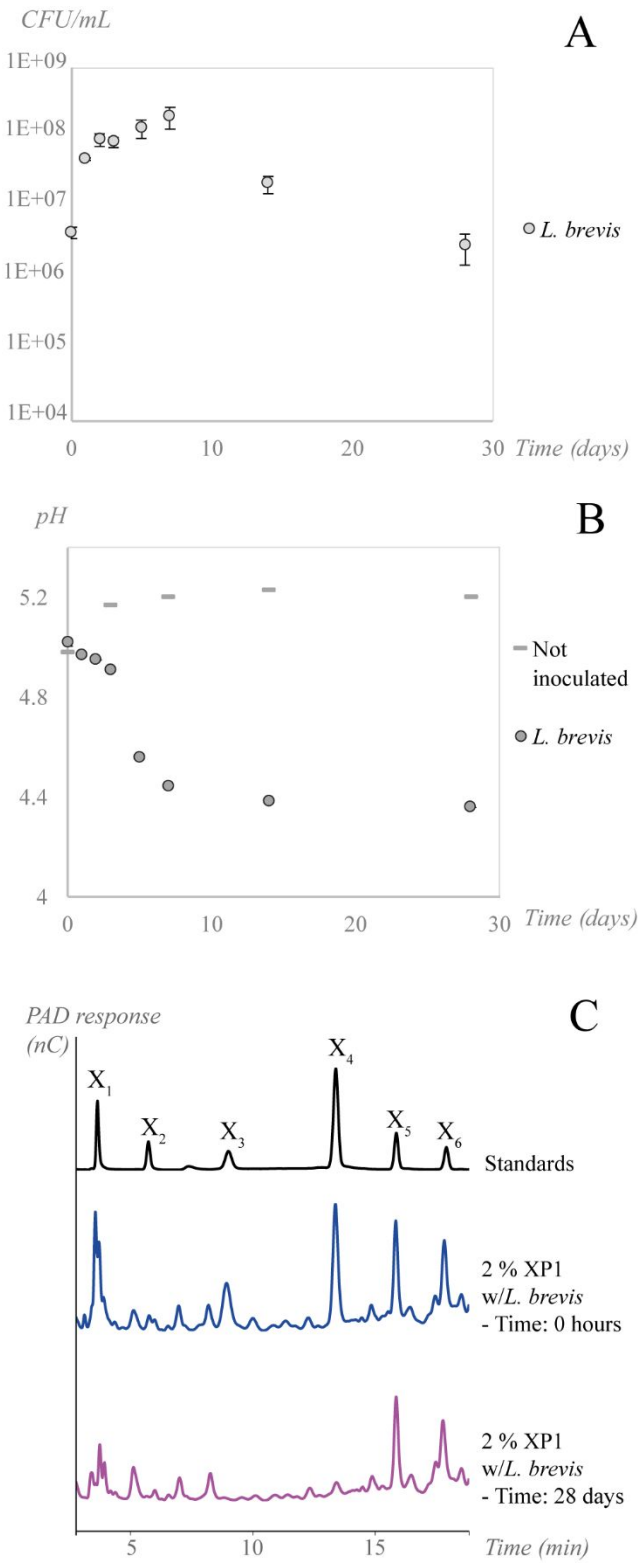

A
CFU/mL
1E+09
1E+08
1E+07
1E+06
1E+05
1E+04
0
10
20
30
Time (days)
L. brevis
B
pH
5.2
4.8
4.4
4
0
10
20
30
Time (days)
Not inoculated
L. brevis
C
PAD response (nC)
X1
X2
X3
X4
X5
X6
Standards
2 % XP1 w/L. brevis - Time: 0 hours
2 % XP1 w/L. brevis - Time: 28 days
5
10
15
Time (min)

Figure 2

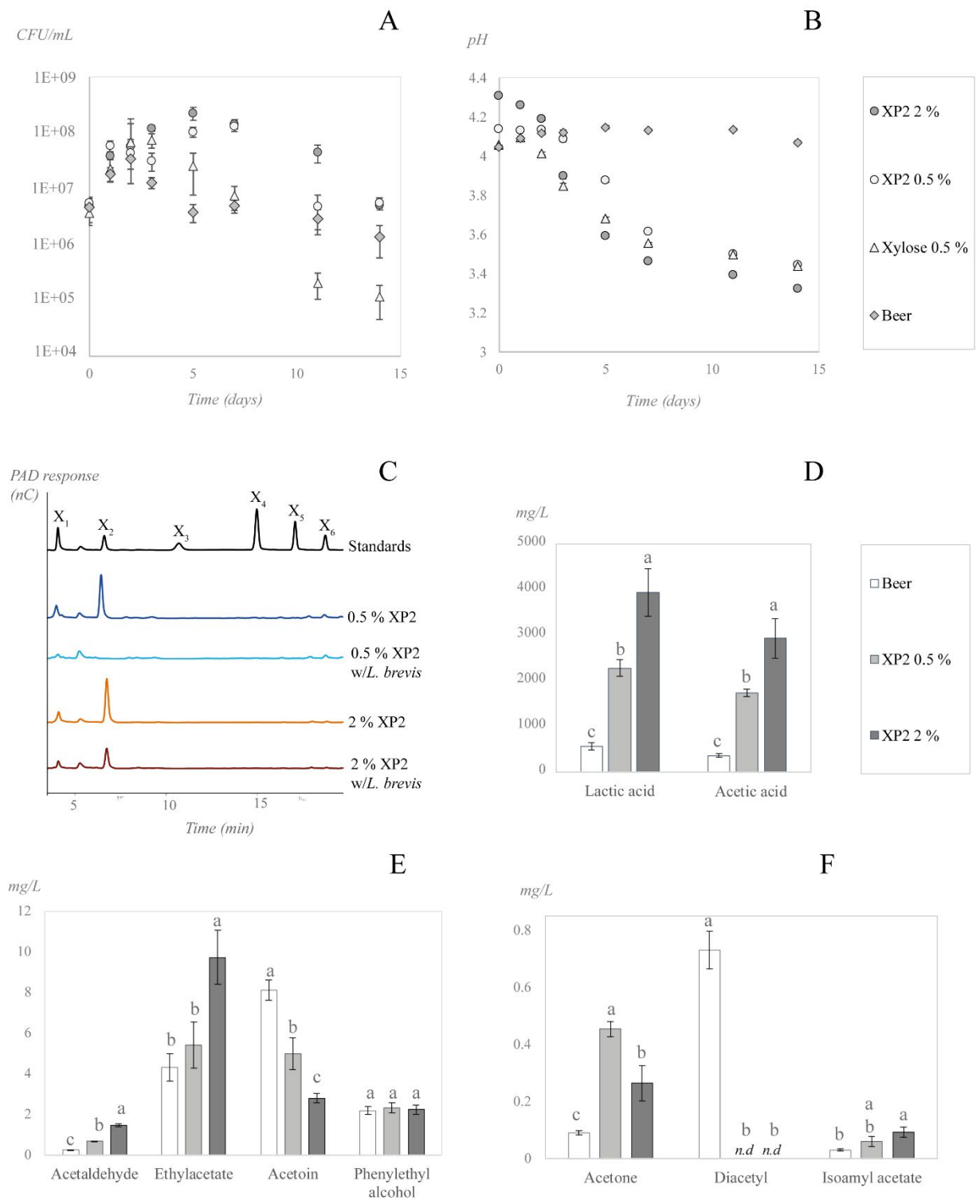

A
CFU/mL
1E+09
1E+08
1E+07
1E+06
1E+05
1E+04
0
5
10
15
Time (days)
B
pH
4.4
4.2
4
3.8
3.6
3.4
3.2
3
0
5
10
15
Time (days)
XP2 2 %
XP2 0.5 %
Xylose 0.5 %
Beer
C
PAD response (nC)
X1
X2
X3
X4
X5
X6
Standards
0.5 % XP2
0.5 % XP2 w/L. brevis
2 % XP2
2 % XP2 w/L. brevis
5
10
15
Time (min)
D
mg/L
5000
4000
3000
2000
1000
0
Lactic acid
Acetic acid
Beer
XP2 0.5 %
XP2 2 %
E
mg/L
12
10
8
6
4
2
0
Acetaldehyde
Ethylacetate
Acetoin
Phenylethyl alcohol
F
mg/L
0.8
0.6
0.4
0.2
0
n.d
n.d
Acetone
Diacetyl
Isoamyl acetate

Figure 3

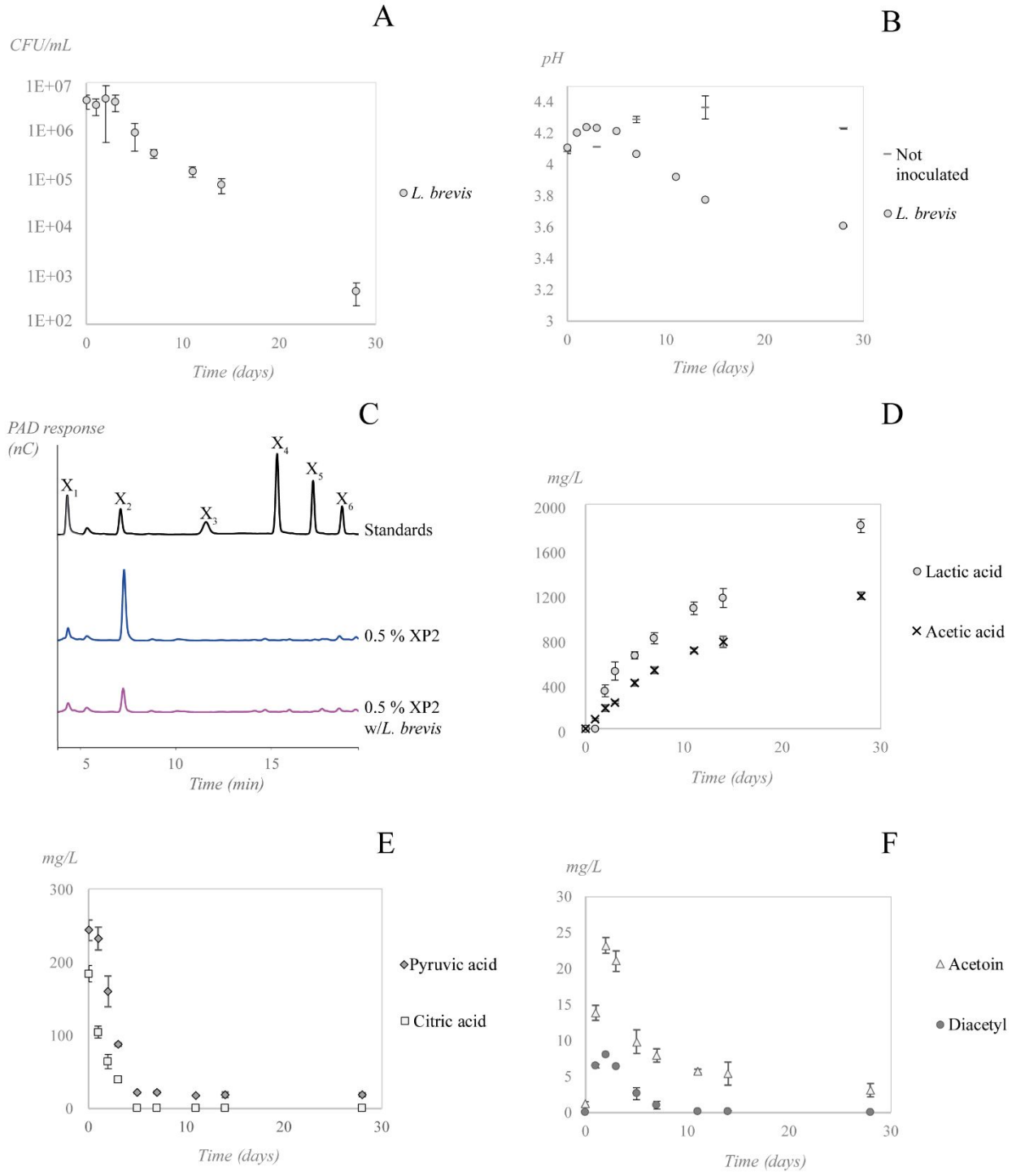

A
CFU/mL
1E+07
1E+06
1E+05
1E+04
1E+03
1E+02
0
10
20
30
Time (days)
L. brevis
B
pH
4.4
4.2
4
3.8
3.6
3.4
3.2
3
Not inoculated
L. brevis
C
PAD response (nC)
X1
X2
X3
X4
X5
X6
Standards
0.5 % XP2
0.5 % XP2 w/L. brevis
5
15
Time (min)
D
mg/L
2000
1600
1200
800
400
Lactic acid
Acetic acid
E
mg/L
300
200
100
Pyruvic acid
Citric acid
F
mg/L
30
25
20
15
10
5
Acetoin
Diacetyl

Figure 4

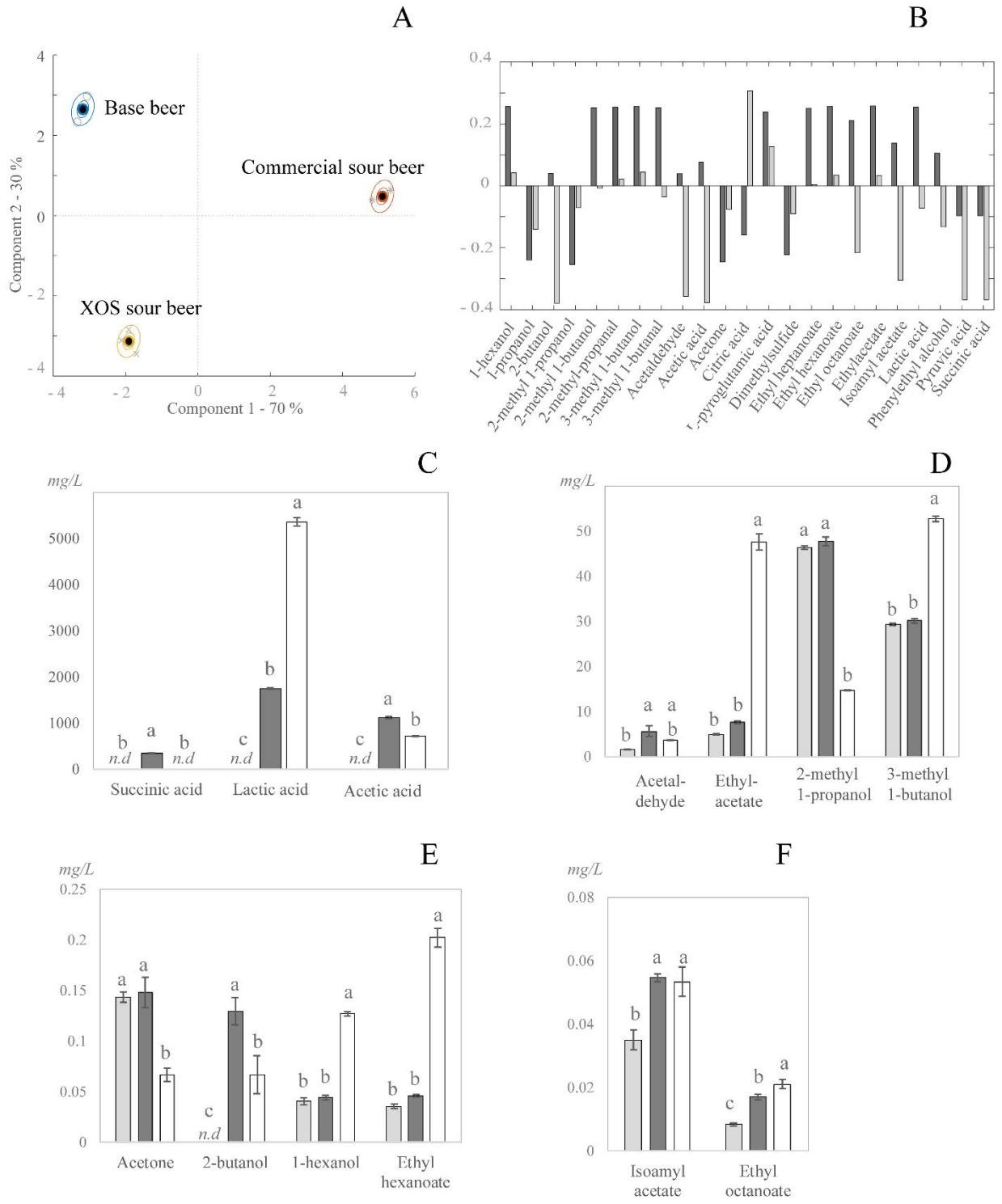

A
Base beer
Commercial sour beer
XOS sour beer
Component 2 - 30 %
Component 1 - 70 %
B
1-hexanol
1-propanol
2-butanol
2-methyl 1-propanol
2-methyl 1-butanol
2-methyl-propanal
3-methyl 1-butanol
3-methyl 1-butanal
Acetaldehyde
Acetic acid
Acetone
Citric acid
γ-pyroglutamic acid
Dimethylsulfide
Ethyl heptanoate
Ethyl hexanoate
Ethyl octanoate
Ethylacetate
Isoamyl acetate
Lactic acid
Phenylethyl alcohol
Pyruvic acid
Succinic acid
C
mg/L
Succinic acid
Lactic acid
Acetic acid
n.d
D
mg/L
Acetal-dehyde
Ethyl-acetate
2-methyl 1-propanol
3-methyl 1-butanol
E
mg/L
Acetone
2-butanol
1-hexanol
Ethyl hexanoate
F
mg/L
Isoamyl acetate
Ethyl octanoate

Figure 5

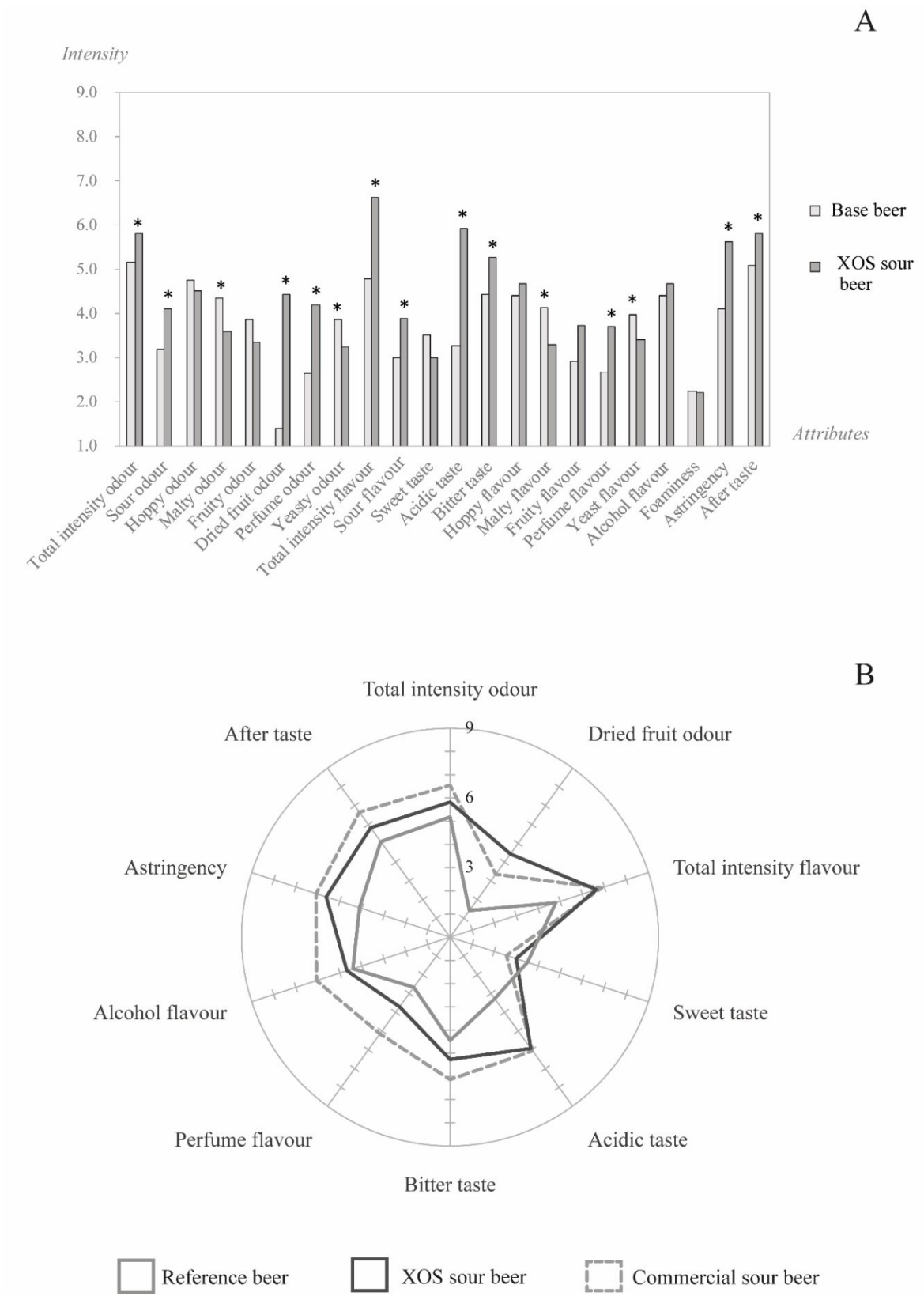
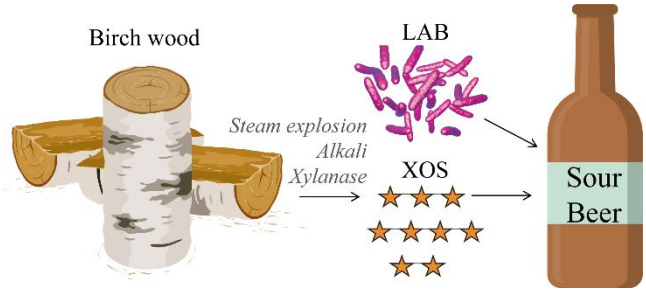

A
Intensity
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
Base beer
XOS sour beer
Attributes
Total intensity odour
Sour odour
Hoppy odour
Malty odour
Fruity odour
Dried fruit odour
Perfume odour
Yeasty odour
Total intensity flavour
Sour flavour
Sweet taste
Acidic taste
Bitter taste
Hoppy flavour
Malty flavour
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

Figure 6

TOC graphic


Birch wood
LAB
Steam explosion
Alkali
Xylanase
XOS
Sour
Beer