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Development of brewing science in (and since) the late 19th century: molecular profiles of 110-130 year old beers

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Abstract

The 19th century witnessed many advances in scientific enzymology and microbiology that laid the foundations for modern biotechnological industries. In the current study, we analyse the content of original lager beer samples from the 1880s, 1890s and 1900s with emphasis on the carbohydrate content and composition. The historic samples include the oldest samples brewed with pure *Saccharomyces carlsbergensis* yeast strains. While no detailed record of beer pasteurization at the time is available, historic samples indicate a gradual improvement of bottled beer handling from the 1880s to the 1900s, with decreasing contamination by enzymatic and microbial activities over this time span. Samples are sufficiently well preserved to allow comparisons to present-day references, thus yielding molecular signatures of the effects of 20th century science on beer production. Opposite to rather stable carbohydrate profiles, some aldehydes reach up to 40-fold higher levels in the historic samples as compared to present-day references.

1. Introduction

The emergence of human societies is closely linked to the onset of agriculture, use of controlled fireplaces, domestication of plants and craftsmanship for producing pottery or metal kettles. These factors have been prerequisites for the production of beer and other fermented beverages with analgesic, disinfectant and mind-altering effects (McGovern, Zhang, Tang, Zhang, Hall, Moreau, et al., 2004; Meussdoerffer, 2009; Walther, Hesselbart, & Wendland, 2014). In providing nutritive clean products, whose consumption was a social act, fermented beverages themselves have supposedly impacted strongly on the development of human societies (Libkind, Hittinger, Valerio, Goncalves, Dover, Johnston, et al., 2011). The emergence of a barley-based forerunner of modern beer dates at least to the time of 6000 BC. The molecular archaeology of beer samples has recently attracted interest in the recovery of a malt- and hop-containing beverage from a schooner that is believed to have sunk in the 1840s in the Åland archipelago southwest of Finland (Wilhelmson, Londesborouhg, & Juvonen, 2012). These samples with acidic pH and almost entirely degraded maltooligosaccharides due to bacterial infections date back to the time before the advent of pasteurization or pure brewing yeast cultures. Degraded samples permit only limited conclusions about what kind of beer originally was filled into the bottles.

Several advances in technology and knowledge mark the onset of scientific brewing towards the end of the 19th century. Industrial-scale brewing had rapidly emerged during the industrial revolution. In particular the popularity of cold-fermented lager beers from the middle of the 19th century posed new challenges in the cooling of brewing equipment and for yeast handling, however. Following the

development of scientific enzymology and microbiology, it became evident in 1860 that yeast fermentation converts sugar into ethanol and CO_2 (Sicard & Legras, 2011). The problem of contamination by wild spoilage yeasts was aggravated by the introduction of summertime lager beer fermentation. In 1883, Emil Christian Hansen isolated pure cultures of *Saccharomyces* strains at the Carlsberg Laboratory. One of these strains, Unterhefe No. 1, was chosen as production strain at the Carlsberg brewery due to its convincing brewing performance, donated to other breweries and entered the CBS strain collection as *Saccharomyces carlsbergensis* (Walther, Hesselbart, & Wendland, 2014).

Samples dating back to the development of scientific brewing towards the end of the 19th century could play a role in clarifying, how products and processes evolved during this time and how advances in the brewing process have altered the final product, lager beer, compared to its 19th century ancestors. In order to address these questions, three beer bottles filled with original lager beer from the late 19th century and early 20th century (Figure 1A) were obtained from the Carlsberg Museum bottle collection. More specifically, the three bottles can be dated to the 1880s or 1890s for bottle 1, the 1890s for bottle 2 and the 1900s for bottle 3. The extent of bottled beer pasteurization at the time has not been determined. Yeast cells were found in the cell slurries present in bottle 1, and Unterhefe No. 1 was isolated (Walther, Hesselbart, & Wendland, 2014).

Here, the content of the bottles was subjected to detailed analysis with a special emphasis on barley carbohydrates and aging markers. Complex changes in the chemical composition of beer during storage constitute a well-known problem even during normal shelf-life. Carbonyl compounds such as those deriving from Strecker degradation, Maillard reactions and fatty acid oxidation are of particular importance

in beer staling (Rodrigues, Barros, Carvalho, Brandão, & Gil, 2011; Saison, De Schutter, Delvaux, & Delvaux, 2008; Vanderhaegen, Neven, Verachtert, & Derdelinckx, 2006). Despite of their volatility, acyclic, aromatic and heterocyclic aldehyde markers formed by Strecker and Maillard reactions during beer aging were detected in historic samples at levels up to 40-fold of a present-day reference sample using gas chromatography.

Carbohydrates are the main carbon substrate of the fermentation process and polysaccharide fragments in the product are informative on production conditions and raw material usage (Marcone, Wang, Albabish, Nie, Somnarain, & Hill, 2013). Using high-resolution nuclear magnetic resonance (NMR) spectroscopy, all samples were found to contain significant quantities of carbohydrates, including fermentable sugars, even after up to 130 years. Various unconventional carbohydrate signals that are less abundant in present-day samples may be attributed to aging or to the use of 19th century cereal varieties. Despite a limited sample set of unique historical bottles, the carbohydrate profiles indicate a gradual improvement of beer handling from the 1880s to the 1900s and provide a reference to visualize the improved usage of raw materials due to 20th century biotechnology.

2. Materials and methods

2.1 Samples

Three dark brown glass bottles sealed with a cork and metal ring were obtained from the Carlsberg Museum bottle collection filled with original lager beer from the late 19th century and early 20th century. The bottles were present as unique copies, and were chosen as the oldest samples with entirely intact bottle glass as well as cork seals

and metal rings, thus indicating that the original material was contained in the bottles. The times where the fillers named on the bottle labels were licensed for bottling were used to date the bottles. All bottles were sprayed and washed extensively with ethanol (70% v/v), opened in a clean bench and the bottle content was removed by pipetting with sterile pipettes rather than by pouring. All three historic samples were characterized with a DMA35 handheld density meter, while limitations in sample amount allowed only beer sample 2 to be characterized with an Alcolyzer Beer Analyzing System (Anton Paar, Graz, Austria). The liquid of the bottles was directly frozen at -20 ºC. The bottom slurry was used for the re-isolation of cells. For comparison, a modern lager beer was used. Glucose, maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose reference compounds for HPLC were obtained from Sigma-Aldrich (St. Louis, MO, USA), Dextra Laboratories (Reading, UK) or Carbosynth (Compton, UK). Ethyl-glucoside was produced *in situ* as a reference compound for alcoholic glucosides using glucose (20 mg/ml), ethanol (20 mg/ml) and 100 units of *Bacillus stearothermophilis* α-glucosidase (E.C. 3.2.1.20, Megazyme, Bray, Ireland). The spectral positions of the ethyl-α-D-glucoside were determined without product purification using ^{1}H-^{13}C NMR spectroscopy.

2.2 Spectroscopy

NMR spectroscopy was applied as a robust, quantitative and non-destructive approach for the detection of various organic molecule classes without the need for sample derivatization or purification. Of the three historic and one reference (modern) lager beer samples, 500 µl were mixed 1:1 with D_2O containing 20% (v/v) d_3-acetic acid (Sigma-Aldrich, St. Louis, MO, USA) in order to acquire 1D ^{1}H NMR spectra including volatile compounds directly after the opening of the bottles (Nord, Vaag, &

Duus, 2004). The residual d_2-acetic acid signal of all samples was used for 1D ^{1}H NMR spectral normalization. The 1D proton spectra of Figure 1B, C were acquired by sampling 16384 complex data points during an acquisition time of 1.57 seconds and a recycle delay of 10 seconds using excitation sculpting for water suppression, while the 2D DQF-COSY spectrum was recorded with water suppression by presaturation as a data matrix of 4096×512 complex data points sampling 0.85 and 0.11 seconds in the direct and indirect dimensions, respectively. Ethanol content was measured by integration relative to an alcohol free reference sample spiked with 4 mg/ml ethanol and mixed 1:1 with D_2O containing 20% (v/v) d_3-acetic acid.

For ^{1}H-^{13}C NMR spectroscopy, 1.5 ml of the beer samples were lyophilized and redissolved in phosphate buffer (100 mM ,pH 6.5) in D_2O (99.9%; Cambridge Isotope Laboratories, Andover, MA, USA). The strong buffer was used to stabilize the pH and thus the signal positions of ionizable analytes. All NMR spectra were recorded at 298 K on an 800 MHz Bruker (Fällanden, Switzerland) DRX spectrometer equipped with a TCI CryoProbe and an 18.7 T magnet (Oxford Magnet Technology, Oxford, UK) using conventional linear data sampling. ^{1}H-^{13}C HSQC spectra were recorded with a spectral sweep width of 30 ppm (6027.86 Hz) around a ^{13}C offset of 95.0 ppm. All spectra employed hard excitation and refocusing pulses, only. Spectra were processed with extensive zero filling and shifted sine bell window function in both dimensions. Data were processed in Topspin 2.1 (Bruker, Fällanden, CH). All spectra were referenced relative to residual glucose with δ^1H_1=5.229 ppm and $\delta^{13}C_1$=92.99 ppm for α-glucopyranose (Petersen, Hindsgaul, & Meier, 2014).

2.3 Quantitation of unbranched starch fragments

159 Fluorescence labeling with 2-aminobenzamide prior to hydrophobic interaction liquid
160 chromatography (HILIC) analysis on a UPLC (Waters) was performed on reference
161 standards and beer as follows (Bøjstrup, Petersen, Beeren, Hindsgaul, & Meier, 2013;
162 Hughes & Johnson, 1982). Glucose, maltose, maltotriose, maltotetraose,
163 maltopentaose and maltohexaose were dissolved in water to concentrations of 1
164 mg/ml. 100 μl of these solutions were lyophilized. 100 μl of each beer sample were
165 lyophilized. To these lyophilized samples was added 100 μl of a 1 M solution of 2-
166 aminobenzamide in DMSO/AcOH (7:3), followed by adding 100 μl of a 1 M solution
167 of $NaBH_3CN$ in DMSO/AcOH (7:3). Samples were whirlmixed and incubated for 4
168 hours at 60 ºC, then cooled to room temperature and diluted (1:400) with a mixture of
169 10 mM ammonium formate buffer (pH 4.5) and acetonitrile (22:78). Samples were
170 centrifuged and directly analyzed. Of the labeled samples, 5 μl were injected into
171 Waters Acquity UPLC System equipped with Acquity glycan column, FLR detector
172 (excitation wavelength of 350 nm and emission wavelength of 420 nm) using an
173 Acquity UPLC BEH glycan 1.7 μm, 2.1×150 mm column with a VanGuard BEH
174 glycan 1.7 μm, 2.1×5 mm pre-column at room temperature.
175

176 **2.4 Solid-phase microextraction and chromatography**

177 Aldehydes and other volatile compounds were extracted using solid-phase
178 microextraction prior to detection and quantification by gas chromatographic
179 separation coupled to mass spectrometric analysis (GC-MS) with a Thermo Scientific
180 Quantum GC triple quadropole mass spectrometer. As an internal standard, 2-octanol
181 was added to each sample. Samples (2.5 ml) were prepared in 20 ml vials by adding
182 appropriate amounts of sodium chloride (final concentration 40 mg/ml), 50 μl NaN_3
183 (0.1 % w/v), 25 μl 2-octanol (final concentration of 200 μg/l) and ascorbic acid (to a

184 final concentration of 20 mg/ml). All samples were incubated for 10 min at 50 °C.
185 Solid-phase microextraction was performed using a
186 divinylbenzene/carboxen/polydimethylsiloxane fiber (DVB-CAR-PDMS) for an
187 extraction time of 40 min. A solgel-wax GC column (30 m/i.d. 0.25 mm/Film 0.25
188 μm) was used for all analyses. Helium gas was used as the carrier with a gas flow rate
189 of 1.2 ml/min. The thermal desorption time of analytes was 4 min. The MS detector
190 was operated in full scan mode at 70 eV with a scan range from 35 to 350 m/z. Data
191 were analysed using the ThermoXcalibur software (Version 2.2 SP1.48, Thermo
192 scientific) and compound identification based on the NIST version 2.0 mass spectral
193 database.
194
195 **3. Results**
196
197 **3.1 1D NMR spectra of old beer samples**
198 High-resolution nuclear magnetic resonance spectroscopy is an established method
199 for the compositional analysis of non-fractionated beer samples (Duarte, Barros,
200 Almeida, Spraul, & Gil, 2004; Duarte, Godejohann, Braumann, Spraul, & Gil, 2003;
201 D. Lachenmeier, Frank, Humpfer, Schäfer, Keller, Mörtter, et al., 2005). The complex
202 constitution of carbohydrates in beer has been addressed using NMR spectroscopy of
203 non-fractionated beer samples only rather recently (Beeren, Petersen, Bojstrup,
204 Hindsgaul, & Meier, 2013; Bøjstrup, Petersen, Beeren, Hindsgaul, & Meier, 2013;
205 Petersen, Hindsgaul, & Meier, 2014; Petersen, Motawie, Moller, Hindsgaul, & Meier,
206 2014; Petersen, Nilsson, Bojstrup, Hindsgaul, & Meier, 2014). Challenges in the
207 NMR analysis of carbohydrates relate to the narrow ^{1}H chemical shift range,
208 congestion with the water signal and the vast constitutional complexity (including

209 varying branching, ring sizes and anomeric configurations) of carbohydrates (Laine,
210 1994). Figure 1B displays ^{1}H NMR spectra of the historic beer samples designated
211 bottle 1, bottle 2 and bottle 3 (bottle 3 being the youngest sample at 110 years of age)
212 in comparison to a present-day lager beer. Carbohydrates are observed at considerable
213 amounts in all samples, whereas starch fragments (α-glucans) remain the main
214 constituents of samples above 100 years of age.

215 A noteworthy trend is observed for samples bottle 1, bottle 2 and bottle 3 in
216 the spectra of Figure 1B, where newer beers contain more α(1–4)-glucan signals (^{1}H
217 chemical shifts of 5.3-5.5 ppm), while older beers contain less α(1–4)-glucan signal
218 but more reducing end signals (^{1}H chemical shifts around 5.23 ppm). The spectral
219 signature of α(1–6)-branch points on the other hand is nearly identical in all historic
220 samples, but is different in present-day lager, which displays more extensive cleavage
221 of amylopectin towards the non-reducing end of branch points. While limit dextrins
222 appear to be stable in the historic samples, maltooligosaccharides apparently have
223 been degraded to smaller sugars, presumably after bottling, especially in the historic
224 beer bottle 1. Such activity in the bottled beer is consistent with the purification of
225 living Unterhefe yeast cells from the historic bottle 1. Specifically, the beer sample
226 from bottle 3 shows α-reducing end signals with an integral of 8% of the α(1-4)-
227 glucan signal and α(1-6) glucan signals with an integral of 14% of the α(1-4)-glucan
228 signals, as compared to 14% α-reducing end and 16% α(1-6) glucan signals for bottle
229 2, and 17% α-reducing end and 24% α(1-6) glucan signals for bottle 1 due to a loss of
230 intact α(1–4)-glycosidic bonds in the older bottles. As compared to a present day
231 reference with 12 % α-reducing end and 23% α(1-6) glucan signals relative to α(1-
232 4)-glucan signals, bottle 3 contains significantly more intact α(1–4)-glycosidic bonds.

233 Clues as to the differential stability of historic beer samples were sought in the
234 aliphatic and aromatic spectral regions of ^{1}H NMR spectra (Figures 1C, D). The
235 ethanol contents were 2.9%, 4.8% and 2.8% for samples of bottles 1, 2 and 3,
236 respectively. Historic samples showed classical amino acid and organic acid profiles
237 of beers. All historic samples had higher organic acid content than a present-day
238 reference, particularly more formate, lactate and acetate. The pH values of samples
239 from bottles 1, 2 and 3 were 4.95, 4.4 and 4.3, respectively, and thus comparable to
240 present-day beers. In comparison, values for bacterially degraded historic samples
241 from the 1840s were as low as pH 3 (Wilhelmson, Londesborouhg, & Juvonen,
242 2012). The higher pH of the beer from bottle 1, above the pKa of acetate, could
243 explain the survival of yeast cells, supposedly in a dormant state, for 130 years in this
244 particular bottle. Yeast cells clearly are not dormant due to the lack of a fermentable
245 carbon source in bottle 1 (Figure 1B), but presumably due to the lack of other
246 nutrients. The apparent extract was determined with a handheld density meter to 1.8,
247 2.3 and 4.9% for samples of bottles 1, 2 and 3, respectively. In addition, the sample of
248 bottle 2 was characterized on an Alcolyzer Beer Analyzing System to yield an EBC
249 color value of 54.8%, 3,59% real extract, 61,52 %real degree of fermentation and
250 2,3% apparent extract.
251
252 **3.2 Maltooligosaccharide quantitation**
253 The varying abundance and degradation of maltooligosaccharides in the beer samples
254 was probed by hydrophobic interaction liquid chromatography (HILIC) after the
255 fluorescence labeling of beer samples. Chromatograms of Figure 2 support the ^{1}H
256 NMR spectroscopic finding of maltooligosaccharide degradation to small fermentable
257 sugars, specifically glucose, especially in bottle 1, but lesser so in bottle 2 and

258 especially bottle 3.

259 Due to its stability, the beer in bottle 3 thus still gives a reasonable clue of
260 carbohydrate composition and content in lager beers from the beginning of the 20th
261 century. Maltotriose levels in bottle 3 (3.16 mg/ml) were more than 4-fold higher than
262 in a present-day reference (0.71 mg/ml), while maltotetraose was the most abundant
263 maltooligosaccharide in bottle 3 (3.9 mg/ml) and the present-day sample, albeit half
264 the concentration in the present-day sample (2.02 mg/ml) (Table 1). Not surprisingly
265 then, modern brewing methods have considerably improved the efficiency of raw
266 material usage. Notwithstanding, the HILIC traces of historic and present-day beers
267 show similar patterns of additional signals other than maltooligosaccharides. Thus,
268 variations in the composition of the most abundant sugars other than
269 maltooligosaccharides are limited, consistent with the similar profiles of limit dextrins
270 detected in ^{1}H NMR spectra.

271

272 **3.3 2D NMR-chemical detail of carbohydrate composition**

273 Highly resolved signatures of carbohydrate composition in the beer samples were
274 sought using homo- and heteronuclear 2D NMR spectroscopy. Figure 3A,B shows the
275 2D NMR detection of barley cell-wall carbohydrates, specifically arabinoxylan and β-
276 glucan in the historic beer samples. The beer of bottle 1 is largely devoid of
277 arabinoxylan and β-glucan, consistent with the action of microbial and/or enzymatic
278 activities in the bottle. Beers of bottles 2 and 3 contain arabinoxylan and β-glucan
279 signals that indicate a poor cleavage of these polysaccharides in beer production at the
280 turn of the last century, consistent with the fact that arabinoxylan and β-glucan pose
281 challenges for beer filterability throughout the 20th century and even today (Li, Lu,
282 Gu, Shi, & Mao, 2005).

283 Historic beer samples display various carbohydrate signals that are not found
284 in modern-style samples, or at considerably lower levels. Hence, the chemical
285 composition of carbohydrates and carbohydrate-adducts may be a possible aging
286 marker or may reflect the change in cereal varieties and malting practices since the
287 19th century (Ferrio, Alonso, Voltas, & Araus, 2006; D. W. Lachenmeier & Fügel,
288 2007; Linko, Haikara, Ritala, & Penttilä, 1998). Figures 3C and 4A indicate that
289 especially beer from bottle 1 contains glucans linked to primary alcohols that do not
290 derive from starch fragments (~4.93 ppm, at slightly lower frequency than the limit
291 dextrin α(1-6) branch points). Signal frequencies and sharp line widths indicate that
292 these signals arise from glucosides formed between α-anomeric glucose and low
293 molecular weight alcohols, for instance ethanol and glycerol. As the chemical
294 glycoside formation is acid catalyzed, while the beer from bottle 1 has the highest pH
295 of the historic beer samples, the formation of glucosides with low molecular weight
296 alcohols may result from enzymatic processes involving α-glucosidase instead. Such
297 an enzymatic, and hence specific mechanism, also seems plausible considering the
298 absence of corresponding β-glucosides in the spectral vicinity of gentiobiose (6-O-β-
299 D-glucopyranosyl-D-glucose, i.e. a glucopyranosyl β-linked to a primary alcohol
300 group at glucose C6, Figure 4B). Figure 4B indicates some further differences in
301 mono- and disaccharide composition of the beer samples and underlines the closest
302 resemblance of present-day lager beer by the beer of bottle 3, albeit with little
303 degradation of arabinoxylan to xylose and xylobiose fragments.
304
305 **3.4 Aging markers**
306 Beyond probing historical production conditions, the old beer samples were used to
307 validate and approximate limiting values of selected aging markers. The historic beer

samples were analysed using gas chromatographic separation coupled to mass spectrometric analysis (GC-MS). Several volatile compounds, for instance acetate esters (ethylacetate, isoamylacetate, phenylethyacetate), showed strongly reduced abundance, down to less than 1% of present-day reference values. The decline of these esters over more than a century could result from their high volatility and presumably does not allow simple conclusions about the flavor profile of beer at the onset of brewing science. In contrast, several aging markers (Vanderhaegen, Neven, Verachtert, & Derdelinckx, 2006) were analysed, including Strecker aldehydes (benzaldehyde, 3-methylbutanal) and Maillard aldehydes (furfural and 5-methylfurfural), showing 3-fold to 40-fold higher values than in the present-day reference (Table 2). Thus, these mentioned aldehydes in historic beer samples were well suited to observe chemical reactivity during beer storage and were validated as suitable aging markers. A comprehensive list of compounds analyzed by solid-phase microextraction and GC-MS is provided in the supplemental Table S1.

4. Discussion

The use of pure culture lager yeast strains by Emil Christian Hansen revolutionized lager beer production in the outgoing 19th century (Hansen, 1883). This first lager beer yeast strain is known as *Saccharomyces carlsbergensis*, originally termed *Unterhefe* No 1, and has been used in production since 1883 (Walther, Hesselbart, & Wendland, 2014). Here, we analyse the molecular profiles of historic lager beer samples intact beer bottles filled in the 1880s, 1890s and 1900s in comparison to present-day references in order (1) to retrace the biotechnological challenges and shortcomings in raw material handling at the onset of scientific brewing and (2) to

probe chemical, enzymatic and microbial reactivities during beer aging on historic timescales.

The enzymatic remodeling of carbohydrates in complex mixtures is a hallmark of many biotechnological production processes, including brewing. The carbohydrate composition in the historic beer samples indicates an improved stability of the bottled product from the 1880s to the 1900s. This finding is consistent with a growing awareness of the importance of keeping wine and beer germ free following the work of Pasteur in the 1860s and 1870s (Stewart & Russell, 1986). Two-dimensional NMR spectroscopy shows that enzymatic activities in the supposedly oldest beer bottle have led to the predominant degradation of maltooligosaccharides as well as barley arabinoxylan and mixed linkage (1,3-1,4)β-glucan. Such degradation decreases in the beer samples from the 1890s to the 1900s. The youngest historic beer sample from the early 1900s contains most of its maltooligosaccharides in form of maltotriose and maltotetraose and thus reflects the composition of present-day lager beers. The good preservation of starch fragments in historic samples uniquely allows the comparison with present-day samples and the identification of unconventional carbohydrates as possible products of historic cereal varieties. For instance, maltotriose and maltotetraose occur in the historic lager beer sample from the early 1900s at levels 2-4-fold higher than for typical present-day lager beers (see literature averages in Table 1), thus reflecting the push towards optimized processes in present-day brewing. The analysis of 10 recent lager beer samples of different years and sites from major breweries (Table 1) revealed that all the present day samples had lower levels of maltotriose and maltotetraose present in the historic lager beer sample from the early 1900s. This sample from the early 1900s has the lowest pH (4.2) and highest organic acid content (Figure 1C) of the historic samples, while containing the least degraded

oligosaccharides and lowest monosaccharide concentrations. Hence, the oligosaccharides can be considered chemically stable under weakly acidic conditions in aqueous medium for more than a century.

Opposite to linear oligosaccharides, branched limit dextrins are found in all historic beer samples at similar levels, and are evidently more resistant to amylase degradation than maltooligosaccharides (Beeren, Petersen, Bojstrup, Hindsgaul, & Meier, 2013). The limit dextrin structures in the historic samples are larger than those found in present-day lager beer, again consistent with the improved usage of raw materials in present-day lager beers, owing to the improved control over enzyme-dependent processes during the course of the 20th century. When not degraded in historic samples, the viscous cell wall polysaccharides arabinoxylan and mixed linkage (1,3-1,4)β-glucan occur as large fragments rather than oligosaccharides, in agreement with viscosity and filterability problems associated with these cell wall polysaccharides throughout the 20th century and until today.

The detection of living, dormant *Saccharomyces carlsbergensis* yeast cells and large amounts of alcoholic α-glycosides in the supposedly oldest sample is consistent with the presence of enzymatic activities after bottling. In addition, *Sporobolomyces roseus* (a beer spoilage yeast that is else not present in our lab) was detected in the same bottle 1. Determinations of the yeast species were based on rDNA sequencing. The presence of dormant yeast cells of 130 years of age in this sample may be favored by the only weakly acid sample pH (pH 4.9). Yeast dormancy in this sample occurs despite the presence of glucose above concentrations of 2 g/l, presumably as the consequence of cytostasis due to nitrogen starvation, ethanol or acidic pH. *Sporobolomyces* could have survived as spores, while *S. carlsbergensis* as a triploid yeast has no tendency to sporulate. The use of polymerase chain reaction

383 validates the presence of *S. carlsbergensis* DNA in the sample (Walther, Hesselbart,
384 & Wendland, 2014).

385 Various carbohydrate signals are observed in historic beer samples that are
386 absent from the present-day reference. Due to their low concentration and the absence
387 of suitable reference compounds, these additional carbohydrate signals remain
388 hitherto unassigned. Markers of beer aging were determined in historic and present-
389 day samples in order to estimate the changes in beer chemical composition beyond
390 carbohydrates on historic timescales. Using gas chromatographic analysis, we find
391 that several volatile compounds are reduced to less than 1% of present-day reference
392 values. In contrast, several aging markers including Strecker aldehydes and
393 heterocycles showed 3-40-fold higher values than in the present-day reference.

394 In concluding, we analyse lager beer samples bottled shortly after the advent
395 of scientific brewing, including some of the first brews using pure lager yeast culture.
396 Historic beer samples contain most of the carbohydrates that are found in present-day
397 beer. Historic samples reflect an improving control over enzymatic polysaccharide
398 degradation processes at the end of the 19th century, yielding samples that are stable to
399 chemical, enzymatic and microbial degradation for more than a century. In constrast,
400 severe changes in chemical composition are found for esters and aldehydes. These
401 finding underline the stability of carbohydrate profiles and their utility in molecular
402 archaeology.

403

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410 **Conflict of interest statement**

411 The authors declare no conflicts of interest.

412

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Figure Captions

Figure 1. (A) Historic bottles from the Carlsberg Museum bottle collection filled with original lager beer. Time ranges for their filling were deduced from the names of fillers given on the labels. (B) ^{1}H NMR spectrum of the spectral region containing α-anomeric sugar signals from starch fragments in the present-day reference and historic beer samples of the bottles shown in (A). The hump at 5.35 ppm results from α(1-4) signals near branch points. Horizontal lines designate α-(1 → 4)glycosidic bonds, vertical lines designate α-(1 → 6) glycosidic bonds and circles designate glucopyranosyl units, where the filled circle yields the signal in a structural motif indicated by open circles. (C) ^{1}H NMR spectrum of the aliphatic spectral region. Increased organic acid content, especially of lactate and acetate, is evident, while amino acids content does not appear significantly increased. Residual d_2-acetate signal derives from addition of d_3-acetate for stabilizing sample pH and internal referencing. (D) ^{1}H NMR spectrum of the aromatic spectral region, showing increasing formic acid content in historic samples 1-3 and the absence of broad protein background signals in the historic samples due to protein hydrolysis or precipitation. The ^{1}H NMR spectra are normalized relative to an internal residual d2-acetate standard signal.

Figure 2. HILIC traces of 2-aminobenzamide labeled beer samples for the quantitation of glucose, maltose and maltooligosaccharides in historic samples and a present-day reference. Absolute quantitations were derived using a mixture of standard compounds. Quantitations are tabulated in Table 1. G1-G6 represent α(1–4) linked glucans with dp 1 (glucose) to dp 6 (maltohexaose).

Figure 3. (A) ^{1}H-^{1}H- COSY and (B) ^{1}H-^{13}C HSQC spectra of barley cell wall polysaccharides in the historic beer samples. The spectra indicate nearly complete degradation in bottle 1, presumably after bottling, and intact β-glucopyranosyl (red) and β-xylopyranosyl (orange) polysaccharide chains with little chain cleavage in beers from bottles 2 and 3. In depictions of arabinoxylan fragments, squares designate arabinofuranosyl units, circles designate xylopyranosyl-units, horizontal lines indicate β-(1→4) glycosidic bonds, vertical lines indicate α-(1→2) glycosidic bonds, and diagonal lines indicate α-(1→3) glycosidic bonds. In depiction of β-glucan structures, circles designate glucopyranosyl units, horizontal lines indicate β-(1→4) glycosidic and diagonal lines indicate β-(1→3) glycosidic bonds. (C) Presence of alcoholic α-glycosides in beer from bottle 1 at ^{1}H-^{13}C HSQC spectral positions indicated by arrows. Ethyl-α-glucopyranoside was identified using an enzymatically synthesized reference compound.

Figure 4. (A) ^{1}H-^{13}C HSQC spectra of historic and present-day lager beer samples. Spectral regions corresponding to starch fragments are highlighted in bottle 1 and regions corresponding to β-glucan and β-xylan for sample bottle 2. Additional signals that are not present in present-day lager samples, or at lesser amounts, are highlighted by a grey area in sample bottle 3. (B) Spectral region highlighting differences in mono- and disaccharide compositions of historic and present-day lager beer samples.

Tables

Table 1. Glucose and maltooligosaccharide content of historic beer samples in g/l.

Sample	Glucose	Maltose	Maltotriose	Malto-tetraose	Malto-pentaose	Malto-hexaose
Bottle 1	2.54	0.13	0.04	0.05	nd	nd
Bottle 2	1.86	1.48	2.03	0.33	nd	0.04
Bottle 3	0.36	0.70	3.16	3.90	0.80	0.41
ref	0.02	0.39	0.71	2.02	0.33	nd
ref av[a]	0.07±0.03	0.15±0.26	0.46±0.36	1.51±0.60	0.39±0.20	0.19±0.08
lit av[b]	nd	0.05±0.10	1.25±0.71	3.45±1.31	nd	nd

[a]average determination by high-performance anion exchange chromatography with pulsed amperometric detection (as described in (Bøjstrup, Petersen, Beeren, Hindsgaul, & Meier, 2013)) of 10 contemporary commercial lager beer samples of major brewing companies. The analysis includes samples of the years 2011-2014, including sample variation from different sites.

[b]average determination by HPLC analyses of 18 lager beers reported in the literature (Ferreira, 2009).

561 **Table 2.** Strecker aldehydes and heterocyclic aldehydes produced during beer aging,
562 reported as parts per billion (w/v) as determined by GC-MS analysis.

Sample	Furfural	5-Methyl furfural	Benzaldehyde	3-Methyl butanal
Bottle 1	70.38	7.76	30.17	7.72
Bottle 2	143.91	10.74	138.91	9.83
Bottle 3	401.69	18.91	26.89	5.14
ref	14.44	0.56	10.87	nd

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