Microbial dynamics in traditional and modern sour beer production

Anna Dysvik[1*], Sabina Leanti La Rosa[1], Gert De Rouck[2], Elling-Olav Rukke[1], Bjørge Westereng[1] and Trude Wicklund[1]

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

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

* Corresponding author: anna.dysvik@nmbu.no

Abstract

Traditional sour beers are produced by spontaneous fermentations involving numerous yeast and bacterial species. One of the traits that separates sour beers from ales and lagers is the high concentration of organic acids such as lactic acid and acetic acid, which results in reduced pH and increased acidic taste. Several challenges complicate production of sour beers through traditional methods. These include poor process control, lack of consistency in product quality, and lengthy fermentation times. This review summarizes the methods for traditional sour beer production with a focus on the use of lactobacilli to generate this beverage. In addition, the review describes the use of selected pure-cultures of microorganisms with desirable properties, in conjunction with careful application of processing steps. Together, this facilitates production of sour beer with a higher level of process control and more rapid fermentation compared to traditional methods.

Introduction

Beer is a malt-based, alcoholic beverage consumed worldwide (1). The earliest written records of beer-consumption date to 2800 BC, but historians believe beer or beer-like beverages were consumed much earlier. Billions of litres are consumed each year, making beer among the most popular beverages today. According to the German Beer Purity Law from 1516, beer should only contain water, malt and hops. Yeast was later included on the ingredient list. This law, with some modifications, is still applied in countries such as Germany, but non-malt carbohydrate sources are extensively used in beer production worldwide (1).

Malt, usually wheat or barley, is milled and mixed with hot water in a mashing step. During the mashing, enzymes, including α- and β-amylases, degrade starch to fermentable carbohydrates. After mashing, the insoluble fraction, referred to as brewer's spent grain (BSG) is separated from the sugar-rich liquid, referred to as wort, in a process called lautering. The wort is then boiled with hops, before it is cooled and inoculated with yeast (Fig. 1A). The most commonly used yeast species for beer fermentation, also known as brewer's yeasts, are *Saccharomyces pastorianus*, used for fermentation of lager beer, and *S. cerevisiae*, used in ale production. During fermentation, the yeast, usually a single strain culture, utilizes the available carbohydrates, amino acids, and other nutrients in wort, to generate ethanol, carbon dioxide, higher alcohols, esters, and other metabolites (1).

Different processing steps reduce the beer's susceptibility to unwanted microbial growth during production. Examples of such processing steps include malt acidification, application of high temperatures during mashing, boiling, and pasteurisation, in addition to filtrations and application of low temperatures during storage (2). Furthermore, hops containing antimicrobial iso-α acids (typically 17-55 mg/L) also act as preservatives. By going through the fermentation

process, beer typically acquires a number of properties that make beer an inhospitable environment for microbial growth thus protecting against spoilage (2). These factors include ethanol, typically in the range of 3.5-5 % or higher, acidic pH, low oxygen and high carbon dioxide content as well as low quantities of available nutrients.

Ethanol in beer provides an important antimicrobial hurdle. In 1935, Shimwell showed that beers with higher ethanol content were more resistant to growth of *Lactobacillus brevis*, which was referred to as *Saccharobacillus pastorianus* at that time (3). The antimicrobial mode of action of ethanol is through inhibition of cell membrane function (4), and induction of cell membrane leakage (5). Ethanol-induced increase in membrane permeability causes a rise of protons influx into the cytoplasm, which makes it difficult for bacterial cells to maintain pH homeostasis (6). This is especially important in low pH environments, such as beer. Cell morphology and a variety of cellular functions can also be affected by ethanol (7).

Low pH represents an additional hurdle that microorganisms need to circumvent to grow in beer. Beer pH generally ranges between 3.4 and 4.7 depending on beer style, but most beers have a pH ranging between 4.0 and 4.5 (8). Acidic pH causes increased influx of organic acid, and acidification of the cytoplasm. This can damage various enzyme systems and hinder nutrient uptake and thereby interrupting cellular metabolism in general(9). Inability to maintain constant intracellular pH results in cell death (10). In addition to the direct effect of low pH, the acidic environment affects microbial cells survival synergistically with hop compounds (11).

When hops are added to beer, they introduce various antimicrobial compounds such as α-acids, iso-α acids, and β-acids. Iso-α acids are the most important antimicrobial compounds acting primarily as ionophores (12). Being week acids, undissociated iso-α acids can cross cell membranes and dissociate intracellularly where the pH is higher (13). The release of protons

causes a drop of the intracellular pH that demolishes the proton motive force ultimately affecting the whole cell metabolism (13). Other antimicrobial actions inherent to iso-α acids include induction of membrane leakage (14) and oxidative stress in the presence of manganese at low pH (15).

Carbon dioxide is formed during yeast fermentation of beer; CO_2 lowers beer pH and contributes to making it microbiologically stable. Further, the presence of CO_2 creates an anaerobic environment which inhibits growth of aerobic bacteria (2). CO_2 acts as a preservative through pH reduction and oxygen displacement, and through an inherent antimicrobial effect not yet fully elucidated (16). An inhibitory effect of CO_2 on a number of metabolic enzymes has been suggested as an important mode of action (17), as has disturbance of cell membrane function (18). Regardless of the mechanism, CO_2 exposure inhibits growth in both Gram-positive and Gram-negative bacteria (19), and higher levels of CO_2 in beer has been associated with reduced growth of beer spoilers (20).

During fermentation, yeast will consume the majority of nutrients. The available quantities of carbohydrates and amino acids in most beers are therefore low (21). Low nutrient content has been correlated with decreased susceptibility to bacterial growth (22).

Although the hurdles described above make the beer stable with respect to microbial growth, there are microorganisms capable of contaminating beer. The presence of microorganisms with beer spoilage potential can cause loss of colloidal stability, ropiness, aroma and taste defects among others (23). Lactic acid bacteria (LAB) (24), acetic acid bacteria (AAB) (25), *Enterobacteriaceae* (26), *Zymomonas*, *Pectinatus* spp. (27), *Megasphaera* spp. (28) are all bacteria associated with beer spoilage. Some yeasts, including *Brettanomyces*, *Candida*, *Hanseniaspora*, *Torulaspora*, *Pichia* and *Saccharomyces* also have beer spoilage potential (29).

92 It is a common belief that beer is resistant to food borne pathogens. Some studies have, however,
93 suggested that some foodborne pathogens, such as strains of *Escherichia coli* and *Bacillus*
94 *cereus*, are able to survive in beer (30). In the context of sour beers, microorganisms with beer
95 spoilage potential can be viewed in a different light, as the involvement of microorganisms
96 beyond conventional brewer's yeast is essential for the production of such beers.

97 **Sour beer.** Sour beer is a highly diverse genre of beer, not restricted to one specific definition
98 based on production process, raw material or geographic origin. A common denominator for sour
99 beer is higher concentrations of organic acids, causing reduced pH (pH 3.0-3.9) compared to
100 "regular beers". This leads to elevated intensity of corresponding sensory attributes such as
101 acidic taste (31). The elevated levels of organic acids in sour beer originate from the involvement
102 of acid producing bacteria in the fermentation process. While the fermentation of conventional
103 beer is usually limited to single strain yeast fermentations, sour beer originates through
104 fermentation by multiple microorganisms, including both yeasts and bacteria (32). Various
105 techniques for sour beer fermentations exist, including spontaneous fermentation, controlled
106 mixed fermentations and sour mashing and similar techniques, where the different
107 microorganisms are separated in time (33). Belgian brewing culture is famous for its sour beer
108 traditions, and classic sour beer styles of Belgian origin include Lambic and Lambic-derived
109 beers such as Geuze and Kriek, as well as Flanders red ale and old brown ale. Berliner Weisse
110 and Gose are sour beer styles of German origin (33). American coolship ale (ACA) is a product
111 from the American craft beer culture, with a production process heavily inspired by the classic
112 Belgian styles (34). The popularity of sour beer has increased in recent decades, and research is
113 being carried out on both traditional fermentations, as well as alternative production techniques.
114 The main focus of the current review, besides traditional sour beer products and challenges

115 associated with their production, is on lactic acid bacteria, their adaption strategies to beer
116 environments and their application in modern fermentation methods. Other microorganisms,
117 such as *Brettanomyces* and acetic acid bacteria (AAB), are also important in sour beer
118 fermentations; their role in sour beer production has been extensively reviewed in recent
119 publications (33, 35-38), and will not be covered in detail here.

120 *Brettanomyces* (also known as *Dekkera*) *bruxellensis* is the species most commonly associated
121 with beer fermentations, and the cause of "Brett character" that includes fruity, floral and tropical
122 taints, as well as medical, leathery, smoky and horsey aromas (39). Interest
123 in Brettanomyces within the brewing industry is due to its ability to generate a wide range of
124 flavour-active compounds including volatile phenolic compounds (40) and volatile esters
125 (41),(42). Further, the β-glucosidase enzymes, inherent to a number of Brettanomyces
126 strains (43),(44), facilitates liberation of volatile flavour compounds bound with glycoside bonds
127 in plant materials. Examples includes release of flavour-active compounds from cherries during
128 traditional Kriek production (45).

129 AAB are obligately aerobic bacteria that produce acetic acid as one of their main metabolic
130 products (46). AAB are recognized in production of vinegar, vitamin C and cellulose, but are
131 often considered problematic in the beverage industry due to their spoilage potential (47).
132 Despite this, they are vital contributors in the fermentation of a number of products including
133 cocoa and water kefir, and some AAB, such as *Acetobacter* and *Gluconobacter*, are also
134 important in spontaneous fermentations of sour beers (33, 47, 48). The produced acetic acid is
135 important to the pH and sensory acidity of sour beer, but AAB has also been associated with
136 other compounds important to sensory perception, such as ethyl acetate (49).

137

138 **Traditional sour beer products.** Lambic beers are produced through spontaneous fermentations
139 in which no active inoculation of microbial starter cultures is carried out (Fig. 1B). The boiled
140 wort is transferred hot to shallow, open vessels, known as coolship, and left to cool down,
141 completely open to the air, typically overnight (32). This exposure is assumed to facilitate
142 inoculation by environmental microorganisms present in air in the brewhouse (50-52). Microbial
143 inoculation may also occur from the barrels, which potentially host a large number of
144 microorganisms in a dormant stage in microcavities on the wood surface (53). To ensure that the
145 cooldown occurs within a reasonable amount of time, and as a means for some level of microbial
146 control, traditional Lambic brewing is only carried out during the winter months (32, 54). When
147 reaching the temperature of approximately 20°C, the wort is transferred to wooden barrels for
148 fermentation and maturation (32). According to the studies, carried out with classic culture
149 dependent techniques, a four-phase microbial succession takes place during fermentation into the
150 wooden barrels. The first phase first phase is often referred to as the enterobacteria phase, as
151 enterobacteria are dominating. Acetic acid bacteria and oxidative yeasts are also present during
152 this phase, which can prevail for a week (52) to a month (32, 55). Low concentrations of ethanol
153 and organic acids are produced during this first phase (52). The following phase is the main
154 fermentation phase, in which *Saccharomyces* spp. dominate for 3-4 months, followed by an
155 acidification phase dominated by LAB and AAB. Production of ethanol and carbon dioxide
156 dominates the main fermentation phase, and organic acids such as lactic acid and acetic acid are
157 produced during the acidification phase (52). The final phase is the maturation phase, where
158 *Brettanomyces* as well as *Lactobacillus*, *Pediococcus*, and acetic acid bacteria dominate, usually
159 from approximately 8 months onward (32). Production of esters such as ethyl acetate and ethyl
160 lactate are characteristic for the maturation phase (51, 52). More recent studies have been carried

out using culturing methods in conjunction with high throughput sequencing techniques to obtain higher quality information on the microbial species diversity. F. Spitaels et al. (55) showed that samples acquired throughout the fermentation process from two batches from a Lambic brewery had a similar microbial succession to that reported by D. Van Oevelen et al. (32), with an initial *Enterobacteriaceae* phase the first month, followed by a phase dominated by *Saccharomyces* spp. and *Pediococcus damnosus*, until *Dekkera bruxellensis* dominated after 6 months. This study, however, suggested that acidification and alcohol fermentation occurred simultaneously, rather than as an extended acidification phase as described previously(32, 51). These results corresponded well with those of N. A. Bokulich et al. (34), where samples obtained during a three-year fermentation period of spontaneously fermented American coolship ale were analysed. Another study on lambic beer has resulted in more than 2000 microbial isolates throughout the two-year fermentation, of which 400 were bacterial strains, and more than 1700 were yeast strains (52). The authors describe a distinct four-phase microbial succession, with an enterobacteria phase (first week), a main fermentation (24 h - 7 weeks), acidification (week 7 – 9 months) and maturation (6 months and onward). While the enterobacterial phase lasted for a month in traditional lambic production without wort acidification (55), J. De Roos et al. (52) showed that the enterobacterial phase did not occur when the wort was acidified by lactic acid addition . In a study focusing on Belgian red-brown acidic ales, the authors showed that the dominant operational taxonomic units (OTUs) are *Pediococcus*, *Acetobacteraceae*, *Lactobacillus*, *Dekkera*, and *Pichia*. Lactic acid and ethanol were the main metabolites, and ethyl acetate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate were identified as the main aromatic compounds (56).

183 Some industrial breweries produce lambic beers on a larger scale, in a process that diverges
184 somewhat from the traditional one. These breweries usually use modern processing methods,
185 such as pasteurisation, filtration, and forced carbonation for their lambic products (57). By using
186 modern equipment to chill wort, the production can be carried out year-round, not depending on
187 low winter temperatures for overnight cooling in shallow vessels. Industrial lambic breweries
188 also use wooden casks, but these are generally custom-made and far greater in size (170-200 hL)
189 compared to the retired wine casks used in traditional lambic breweries (57). Comparison of the
190 microbial succession during a one-year fermentation in an industrial lambic brewery and that
191 occurring during traditional production identified a core microbiota (57) . Microorganisms in this
192 community included *S. cerevisiae*, S. *pastorianus*, *D. bruxellensis*, and *P. damnosus*. Differences
193 between traditional and industrial fermentations included absence of the *Enterobacteriacea*
194 phase, explained by reduced initial pH due to lactic acid addition, and a larger variety of AAB in
195 industrial production.

196 The microbiota living on the inner surface of the wooden casks used in a traditional lambic
197 brewery has been shown to vary with barrel cleaning procedures and the general condition of the
198 casks with respect to age, wood thickness, and wood porosity. Based on 16s rRNA gene
199 sequencing, J. De Roos et al. (53) identified a variety of bacteria, including *Pediococcus*,
200 *Lactobacillus*, and *Acetobacter* and yeasts such as *Saccharomyces*, *Dekkera*, and *Pichia* possibly
201 acting as a source for microbial inoculation[45].

202 Lambic is the base beer for a variety of different beer styles. Geuze (also referred to as gueuze) is
203 a highly carbonated beer that is made by mixing young 1-year and old (2-years or more) lambic
204 following refermentation in bottles. Faro is made by mixing lambic with rock sugar (58). Kriek
205 is a fruit lambic, made by mixing sour cherries with a young lambic, allowing a second

206 fermentation on the fruit sugars (59). Raspberries can also be used in the same way in lambic
207 beer, resulting in a product referred to as framboise (58).

208 For ACA production, wort is cooled in open, shallow cooling vessels to favour spontaneous
209 inoculation by the environment before transfer to wooden barrels. The microbial succession is
210 similar to that of Belgian lambic, although some minor differences can be found (34).

211 Flanders red ale and old brown ales are originally products of spontaneous fermentation and
212 year-long maturation. Beside traditional methods, modern production of these beers is carried out
213 as controlled mixed fermentations in which inoculated yeast and bacteria ferment the wort,
214 before young beer is matured (60). Flanders red ale originates from West Flanders, is red
215 coloured, and is said to be “wine-like”. Flanders red ale is matured for up to two years in oak
216 barrels. Maturation in oak separates Flanders red ale from the old brown ales indigenous to
217 Eastern Flanders. The latter are described as more malt-driven, and less acidic (61).

218 Gose and Berliner Weisse are common German sour beer styles in which wheat malt makes up a
219 substantial fraction of the malt bill, and lactobacilli play important roles in fermentation. Both
220 beer styles, originating from Goslar and Berlin, respectively, represent products that are
221 produced both through traditional and with more modern methods. An important difference
222 between Berliner weisse and Gose is the spiciness of the latter, imposed by addition of salt and
223 coriander (33).

224 **Challenges associated with traditional sour beer production.** Production of sour beer through
225 spontaneous fermentation is associated with several challenges. These include inconsistent
226 product quality, wastage due to failed fermentations, and time consumption. A study by F.
227 Spitaels et al. (62) looking at microbiota and metabolites of aged Geuze clearly demonstrated

inconsistency in production, as the bottle-to-bottle metabolite variation made it impossible to generalize age effects on Geuze. The product variations that arise through the traditional process can be seen as a positive attribute, and are greatly appreciated by some consumers, as they represent a mark of authenticity and natural production. The product inconsistency can also be considered negative, especially if beer has to be discarded due to organoleptic failure after years if fermentation and maturation. The idea of using pure cultures in controlled mixed fermentations is appealing, not only because it can offer improved process control and product consistency, and potentially reduce production time for sour beers, but also because controlled mixed fermentations offer a tremendous potential for generation of novel products. Mixed fermentations of beer with pure cultures are utilised to an increasing extent in the craft brewing industry. The application of mixed cultures and non-conventional microbial strains to beer fermentation offers vast possibilities for flavour generation. In addition, the capacity of non-conventional brewing microorganisms for diverse carbohydrate utilization, allows the inclusion of non-conventional raw materials in beer production. This can be exploited as a tool to improve process control, besides being a method for direct conversion of non-food carbohydrate sources to food products through fermentation. *Lactobacillus* sp. are highly interesting in this regard. An example of this was recently presented, using xylooligosaccharides for controlled fermentation with *Lactobacillus* (63). Interestingly this study revealed an interesting ratio of acetic acid:lactic acid, that may favourably prevent extensive Acetobacter fermentation that is considered challenging in many products.

Lactobacilli and sour beer. Lactobacilli are Gram-positive rod-shaped bacteria that produce lactic acid as the main metabolic product of carbohydrate metabolism (64). Their metabolism is classified either as obligately homofermentative, meaning that they convert hexose sugars to

Applied and Environmental Microbiology

lactic acid almost exclusively, or as obligately or facultatively heterofermentative, converting hexose sugars to lactic acid as well as CO_2, and ethanol or acetic acid. Lactobacilli have a great safety record, and certain strains of some species are used as health-promoting probiotics, as well as starter cultures for fermentation of a vast variety of food products. They are associated with fermented dairy products such as yogurts (65) and cheeses (66), fermented vegetables (67), and fermented meat products (68, 69). Lactobacilli are also vital contributors to the production of a number of food products through mixed fermentations, where both bacteria and yeast participate, including kefir (70), water kefir (71), sour dough bread (72), and alcoholic beverages such as wine (73), sak e(74), and beer (2).

In beer, lactobacilli can be terrible spoilers or vital fermentation contributors, depending on the beer style and the strain properties. Lactobacilli are considered spoilers in ales and lagers, as these bacteria cause unwanted haze and sedimentation, off-flavours, acid formation, and ropiness (75). In sour beer, where production of acid is welcomed, lactobacilli can be appreciated contributors, vital to the wanted organoleptic characteristics developed through fermentation. Regardless of their presence as spoilers or as needed fermenters in beer, lactobacilli need to overcome the comprehensive sum of hurdles to be involved. A wide set of systems for detection and adaptation to stress are involved in this (21, 76).

Lactobacilli are generally inhibited from growing in beer by the presence of hop's iso-α acids. Some strains, however, are resistant to the antimicrobial actions of hops, and thus able to survive in beer (2, 75, 77). Genes associated with hops resistance in LAB include *horA*, *horC*, and *hitA* (78). The *horA* gene encodes an ABC transporter capable of expelling hops bitter acids from cells (79). The *horC* gene presumably encodes a proton motive force (PMF)-dependent multidrug effluence pump (80, 81). Products from *horA* and *horC* contribute to hops resistance

by lowering the net influx of hops bitter acids into the cell cytoplasm, thereby restricting their actions as antibacterial protonophores. The *hitA* gene is assumed to encode a divalent cation transporter that increase hop resistance by helping hop-sensitive bacteria transporting divalent cations, e.g. Mn^{2+}, into cells where the proton gradient has been dissipated (82). Other cellular adaptations are also involved in hop resistance in LAB, including modifications of the cell wall (83) and cell morphology (84). Presence of *horA* and/or *horC* is used as a genetic marker for ability to survive in beer (68).

Lactobacilli are generally tolerant to ethanol, which confers them competitive advantages in fermentative environments (85). They do, however, display huge variation in their resistance, as some (e.g. strains of *L. plantarum*) stop growing at 5-6% ethanol, while others can sustain environments with much higher concentrations (86, 87). While most LAB are inhibited above 13 % ethanol(88), reports exist of sake spoilers able to grow at 20 % ethanol[74]. U. Kleynmans et al. (89) reported lactobacilli able to resist 16 % ethanol, even at a pH as low as 3.3. Even though lactobacilli are generally able to sustain the ethanol levels in many beers, the role of ethanol tolerance on beer spoilage potential is not well characterised (90). Indeed, V. Pittet et al. (90) found no correlation between ethanol tolerance and ability to grow in beer.

Carbohydrate catabolism by lactobacilli causes accumulation of organic acids and reduction in pH in the environment in which they reside, making it inhospitable for many potential microbial competitors. Extracellular, undissociated acids can pass cell membranes, where they dissociate in response to the higher intracellular pH ultimately affecting enzyme activity and damaging DNA (91). Lactobacilli are not unaffected by acidic environments even though they inflict such an environment upon themselves. Strategies involved in their response to acidic stress include the glutamate decarboxylase (GAD) system. In the GAD system, extracellular glutamate is

297 internalised and decarboxylated to γ-aminobutyrate (GABA) in a reaction where a proton is
298 consumed before the decarboxylated product is exported to the extracellular environment. This
299 consumption of intracellular protons contributes to increased intracellular pH. In addition, the
300 decarboxylation can be coupled to an electrogenic transporter, which allows ATP generation
301 through the proton motive force (92, 93). The arginine deaminase pathway (ADI) (94) is another
302 system for maintaining pH homeostasis in lactobacilli (95) and other LAB (96). In the ADI
303 pathway, arginine is converted to ornithine, ammonia (NH_3), and carbon CO_2, and ATP is
304 generated. NH_3 is generated in the conversion, and reacts with intracellular protons, thus
305 contributing to alkalinisation of the cytoplasm. The F_0F_1-ATPase is a ubiquitous enzyme among
306 bacteria, which can facilitate the production of ATP in a reaction sustained by transmembrane
307 proton motive force, or expel protons from cells in an energy consuming process sustained by
308 ATP consumption (97). Active proton expulsion increases in acidic environments, and is vital for
309 maintaining pH homeostasis in lactobacilli (98) and other LAB (99). Several other systems are
310 known to be involved in the acid stress response of LAB comprehensively covered in the review
311 by M. van de Guchte et al. (92).

312 Lactic acid bacteria are known to be more resistant towards the presence of CO_2 than many other
313 bacteria (100). In addition they are able to sustain low oxygen levels, as lactobacilli are
314 anaerobic or aerotolerant (64).

315 During the fermentation of wort, conventional brewer's yeast utilizes sucrose, fructose, glucose
316 and maltose. Some strains can also utilize maltotriose. Poly- and oligosaccharides are also
317 present in wort, often referred to as dextrins (101). Dextrins can contribute to the sensory
318 perception, e.g. fullness, in ale or lager beer, but in mixed fermentations, these higher molecular
319 mass glycans can serve as substrate for microorganisms with carbohydrate degrading capabilities

320 exceeding those of conventional brewer's yeast. In traditional lambic production, a higher
321 content of such polysaccharides is promoted by inclusion of unmalted wheat in the grain bill (≤
322 30%), and the application of turbid mashing. Both of these factors contribute to reducing
323 enzymatic carbohydrate degradation during mashing, promoting a higher dextrin content in wort,
324 which is assumed to be important for sustaining the prolonged fermentation phases that occur
325 after the main fermentation in lambic production (37). Many lactobacilli have enzymes that
326 facilitate utilization of residual carbohydrates in wort, that are not degradable by conventional
327 brewer's yeast. Maltotriose, maltotetraose (102), maltopentaose, and more complex
328 maltodextrins can sustain growth of *Lactobacillus* (103), and genes encoding enzymes necessary
329 for cellular import and degradation of maltodextrins have been identified (104). Amylolytic
330 lactobacilli can also degrade starch (105), and some lactobacilli can also utilize cellobiose (106),
331 and xylooligosaccharides (63) (discussed in detail below). *Lactobacillus* involvement in super-
332 attenuation of lambic beer has been implicated. In super-attenuated or over-attenuated beer,
333 larger carbohydrate fraction has been fermented than the one that is degradable by brewer's yeast
334 alone (107). Although it is not the primary focus of the current review, it should be noted that
335 other microorganisms, including *Brettanomyces*, are able to degrade complex carbohydrates and
336 are equally important in super-attenuation of sour beer (39, 108).

337 As previously stated, lactobacilli must overcome the sum of hurdles in beer posed by ethanol,
338 low pH, the presence of iso-α acids (and other hops compounds), and nutrient depletion (Fig. 2),
339 to carry out metabolism in the beer environment. If *Lactobacillus* growth is required, e.g. in sour
340 beer production, this can perhaps be promoted by removing or reducing the stringency of one of
341 the hurdles discussed above, e.g. nutrient depletion. A specific substrate, known to promote
342 metabolism of a limited number of microorganisms, could for instance be added to beer, to

promote a rapid acidification phase in mixed or sequential fermentations. An example of such a substrate could for instance be lactose, which does not promote growth of *S. cerevisiae* but supports *Lactobacillus* metabolism (109).

Modern methods of sour beer production. Producing sour beers in controlled fermentations with pure cultures is by no means a new idea. In the late seventies, a study on the microbiology of spontaneous wort fermentation suggested the following question for future research: "*Can Lambic be made with pure cultures*?" (32). After four decades, there is still little evidence in the scientific literature of it having been pursued. Indeed, most of the scientific literature is focused on characterizing the microbiology and metabolite formation of spontaneous fermentation, rather than investigating alternative production methods that may offer improved process control and/or reduce fermentation times. Experimentation into, and development of alternative production methods have emerged in industry, and different modes of spontaneous, semi-spontaneous, and pure-culture fermentations are carried out for commercial production. An example of this is the "sour worting" method (Fig. 3B) where *Lactobacillus* fermentation for acid production is carried out prior to yeast fermentation, either by *Saccharomyces*, *Brettanomyces* or both, in oak barrels (31).

A strategy for simplifying and shortening the production process was explored by H. M. C. S. Kumara and H. Verachtert (108). They fermented wort from a lambic brewery for a short period (≤ 48 h) at high temperature (28°C) with *S. cerevisiae*, to obtain wort depleted of *S. cerevisiae* fermentable carbohydrates. The yeast cells were then removed, and the pre-fermented wort was pasteurised before inoculation with a mixed population from spontaneously fermenting, 1-year old lambic. In the same manner, a lambic at an earlier fermentation stage and higher carbohydrate content was pasteurised and reinoculated with the same mixed population from the

366 further progressed lambic fermentation. Using this process, the over-attenuation occurred in 30
367 days at 28°C, resulting in beers with more than 4000 mg/L lactic acid and 800 mg/L acetic acid
368 in both fermentations.

369 Single-strain fermentation with non-conventional, acid-producing yeast has also been attempted.
370 P. Domizio et al. (110) tested three different strains of *Lachancea thermotolerans* in three week-
371 long fermentations of wort at 14°C, in which they compared *L. thermotolerans* performance to
372 that of a conventional *S. cerevisiae* brewing strain. All the non-conventional strains were able to
373 degrade maltose, but not maltotriose. They were also able to produce comparable quantities of
374 ethanol (approximately 5% v/v) and higher quantities of lactic acid compared with *S. cerevisiae*.
375 A substantial increase in acidity was obtained with one of the tested strains (final pH 3.77
376 compared to 4.24 for *S. cerevisiae*). Even though the lactic acid content was higher for all *L.*
377 *thermotolerans* fermentations compared to *S. cerevisiae* fermentation, it only ranged from
378 approximately 100 to 300 mg/L, which is substantially lower than in most sour beers. K. Osburn
379 et al. (111) tested 284 (54 species, 26 genera) yeasts isolated in small scale beer fermentations
380 for their fermentation performance. Sensory testing of the resulting beers illustrated that many of
381 the strains generated beers described as tart or sour. The authors identified multiple yeast strains
382 capable of producing lactic acid and used four of these (strains of *Hanseniaspora vineae*,
383 *Lachancea fermentati*, *Schizosaccharomyces japonicus* and *Wickerhamomyces anomalus*) in
384 following brewing experiments where the wort was incubated at 21.7°C for 1 month.
385 Quantification of the lactic acid in the beers ranged from 900 to 4500 mg/L and the *W. anomalus*
386 fermented beer was perceived as very sour, with pear, apple and apricot aroma (K. Osburn et al.
387 (111)). This method, named “primary souring”, is as an alternative production route for sour

beer, solely relying on fermentation with yeasts that produce lactic acid as well as ethanol and CO_2.

The application of an initial biological wort acidification step (Fig. 3B) is another alternative production method for sour beer that has been explored both in industry (31) and in research (112). Biological acidification can be carried out in the mashing tun (sour mash), in the brewing kettle (kettle sour), or after the wort has been transferred to the fermentation vessel (sour wort). The concept is to carry out LAB fermentation in unhopped wort prior to yeast fermentation within a short time frame, typically 24-48 h. In this way the hurdle effects imposed by yeast fermentation (ethanol, nutrient depletion, low pH, etc.) and iso-α acids on LAB metabolism can be circumvented, and the ability of LAB to rapidly produce high quantities of lactic acid is exploited. When the desired level of lactic acid has been obtained, the wort is then boiled to stop bacterial fermentation followed by single strain fermentation with conventional brewer's yeast. An alternative to the inter-fermentation boiling step is addition of highly hopped wort upon yeast addition, to introduce antimicrobial iso-α acids after the wanted bacterial activity has transpired (113). In a study by L. C. Peyer et al. (112), *Lactobacillus amylovorus* was used for biological acidification of mash, pre-boil and post-boil worts. Acidified worts were subsequently inoculated with *S. cerevisiae* US-05. The authors showed how biological acidification at different time points in the pre-yeast fermentation process led to differences in the obtained beer product. Acidification of pre-boil wort emerged as an efficient method to ensure high acidity and minimal organoleptic failure (113). Pre-fermentation with *L. buchneri* prior to yeast fermentation was tested for production of sour beer (113). Sour beers (pH 3.5-3.7) with high lactic acid concentrations (~1000 mg/L) were produced in 3 weeks of fermentation. Although *L. buchneri*

410 made a significant contribution to the metabolite composition of the beer, the sensory influence
411 of this did not surpass the influence obtained with chemical acidification.

412 Two recent studies have explored novel strategies to expedite sour beer production and improve
413 the process control through co-fermentation of yeast and lactic acid bacteria tolerant to brewing-
414 related stresses (114) and through secondary fermentation using a woody-biomass derived
415 substrate (63) containing xylooligosaccharides that you also find in BSG. Two different
416 lactobacilli, *L. plantarum* WildBrew™ Sour Pitch and *L. brevis* BSO464 were selected based on
417 their ability to sustain various beer-related stress factors (ethanol, low pH, iso-α acids, etc.), and
418 used in separate co-fermentations with yeast (114). Sour beers (pH 3.6-3.8) with high lactic acid
419 concentrations (~1800-2600 mg/L) were successfully produced in as little as 3 weeks (Fig. 3C).
420 *L. plantarum* contributed to the sensory properties of beer by causing increased intensity in fruity
421 odour and dried fruit odour; while the *L. brevis* fermented beer had similar sensory properties to
422 a commercial sour beer in acidic taste and astringency. In another study, Dysvik at al. showed
423 that xylooligosaccharides (XOS) from birch wood can be used to selectively support
424 *Lactobacillus brevis* BSO 464 growth in the beer (63) (Fig. 3D). Sour beer with a pH of 3.3-3.6
425 and a lactic acid concentration of 1750-3900 mg/L was produced in only 2-4 weeks. XOS-driven
426 secondary fermentation shifted multiple sensory properties significantly, and sensory evaluation
427 of the produced XOS sour beer showed that the product was similar to that of a commercial sour
428 beer in dried fruit odour, total flavour intensity, astringency, and acidic taste.

429 Another approach has been investigated, in which co-fermentation with *Lactobacillus paracasei*
430 L26 and *S. cerevisiae* US-05 is used in sour beer production (115). A novel sour beer beverage
431 with sufficiently high lactobacilli count to represent a legitimate delivery vehicle for probiotics
432 was developed. Although the presence of ethanol in beer is problematic in a health-promoting,

probiotic context, the high viability of lactobacilli is noteworthy. The sour beer had a pH of 3.6, contained 10^9 CFU of probiotic lactobacilli per serving (100 mL) and more than 5000 mg/L of lactic acid.

Conclusions. Interest in sour beer has increased substantially in recent decades. Sour beer is traditionally produced through spontaneous fermentations in which complex microbial consortia are involved (Fig. 3). These can include different yeast (*Saccharomyces* spp. and *Brettanomyces* spp.) and bacterial species (*Lactobacillus* spp, *Pediococcus* spp, *Acetobacter* spp). A diverse range of metabolites are formed through the successive microbial progression of such fermentations, resulting in highly complex products, with respect to sensory properties. High quantities of organic acids, such as lactic acid and acetic acid, results in low pH and high intensity in sourness and acidic taste compared to ales and lagers fermented by pure, single cultures of *S. cerevisiae* and *S. pastorianus*, respectively. Several issues complicate production of sour beer through traditional methods. These include poor process control, lack of consistency in product quality, and lengthy fermentation times. Most of the sour beer research has been focused on understanding the complex spontaneous fermentation process, originating from traditional Belgian brewing culture. Pure-culture fermentations with strains of *Lactobacillus* and *S. cerevisiae*, in conjunction with careful application of processing steps, offer a valid alternative to facilitate production of sour beer with a higher level of process control and more rapid fermentation compared to traditional methods. Selection of strains based on their potential for substrate utilization and flavour generation could also open possibilities for using non-conventional sources of carbohydrates in beverages production through fermentation.

454 **REFERENCES**

455 1. Pires E, Brányik T. 2015. Biochemistry of beer fermentation. Springer International
456 Publishing AG Switzerland.
457 2. Vriesekoop F, Krahl M, Hucker B, Menz G. 2012. 125th Anniversary Review: Bacteria
458 in brewing: The good, the bad and the ugly. J Inst Brew 118:335-345.
459 3. Shimwell JL. 1935. The resistance of beer towards *Saccharobacillus pastorianus*. J Inst
460 Brew 41:245-258.
461 4. Casey GP, Ingledew WMM. 1986. Ethanol tolerance in yeasts. Crit Rev Microbiol
462 13:219-280.
463 5. Eaton LC, Tedder TF, Ingram LO. 1982. Effects of fatty acid composition on the
464 sensitivity of membrane functions to ethanol in *Escherichia coli*. Subst Alcohol Actions
465 Misuse 3:77-87.
466 6. Barker C, Park SF. 2001. Sensitization of *Listeria monocytogenes* to low pH, organic
467 acids, and osmotic stress by ethanol. Appl Environ Microbiol 67:1594-1600.
468 7. Kalathenos P, Russell NJ. 2003. Ethanol as a food preservative, p 196-217. *In* Russell NJ,
469 Gould GW (ed), Food Preservatives doi:10.1007/978-0-387-30042-9_10. Springer US,
470 Boston, MA.
471 8. van Leeuwen TA. 2006. A comparison of the chemical analysis of beers and judges'
472 scores from the 2004 Australian International Beer Awards (Honours Thesis). University
473 of Ballarat.
474 9. Neal AL, Weinstock JO, Lampen JO. 1965. Mechanisms of fatty acid toxicity for yeast. J
475 Bacteriol 90:126-31.

10. Booth IR, Stratford M. 2003. Acidulants and low pH, p 25-47. *In* Russell NJ, Gould GW (ed), Food Preservatives doi:10.1007/978-0-387-30042-9_3. Springer US, Boston, MA.

11. Simpson W, Hammond JRM. 1991. Antibacterial action of hop resin materials. European Brewing Convention, Proceedings of Congress:185-192.

12. Schurr BC, Hahne H, Kuster B, Behr J, Vogel RF. 2015. Molecular mechanisms behind the antimicrobial activity of hop iso-α-acids in *Lactobacillus brevis*. Food Microbiol 46:553-563.

13. Simpson WJ, Smith AR. 1992. Factors affecting antibacterial activity of hop compounds and their derivatives. J Appl Bacteriol 72:327-34.

14. Teuber M, Schmalreck AF. 1973. Membrane leakage in *Bacillus subtilis* 168 induced by the hop constituents lupulone, humulone, isohumulone and humulinic acid. Archiv für Mikrobiologie 94:159-171.

15. Behr J, Vogel RF. 2010. Mechanisms of hop inhibition include the transmembrane redox reaction. Appl Environ Microbiol 76:142.

16. King Jr AD, Nagel CW. 1967. Growth inhibition of a *Pseudomonas* by carbon dioxide. J Food Sci 32:575-579.

17. King Jr AD, Nagel CW. 1975. Influence of carbon dioxide upon the metabolism of *Pseudomonas aeruginosa*. J Food Sci 40:362-366.

18. Sears DF, Eisenberg RM. 1961. A model representing a physiological role of CO_2 at the cell membrane. J Gen Physiol 44:869-887.

19. Martin JD, Werner BG, Hotchkiss JH. 2003. Effects of carbon dioxide on bacterial growth parameters in milk as measured by conductivity. J Dairy Sci 86:1932-1940.

20. Hammond J, Brennan M, Price A. 1999. The control of microbial spoilage of beer. J Inst Brew 105:113-120.

21. Sakamoto K, Konings WN. 2003. Beer spoilage bacteria and hop resistance. Int J Food Microbiol 89:105-124.

22. Fernandez JL, Simpson WJ. 1995. Measurement and prediction of the susceptibility of lager beer to spoilage by lactic acid bacteria. J Appl Bacteriol 78:419-425.

23. Esmaeili S, Mogharrabi M, Safi F, Sohrabvandi S, Mortazavian AM, Bagheripoor-Fallah N. 2015. The common spoilage microorganisms of beer: Occurrence, defects, and determination-a review. J Food Sci Technol 7:68-73.

24. Garofalo C, Osimani A, Milanovic V, Taccari M, Aquilanti L, Clementi F. 2015. The Occurrence of beer spoilage lactic acid bacteria in craft beer production. J Food Sci 80:M2845-52.

25. Van vuuren HJJ, Loos MA, Louw HA, Meisel R. 1979. Distribution of bacterial contaminants in a south african lager brewery. J Appl Bacteriol 47:421-424.

26. Vuuren HJJv, Cosser K, Prior BA. 1980. The influence of *Enterobacter agglomerans* on beer flavour. J Inst Brew 86:31-33.

27. Lee SY, Madee MS, Jangaard NO, Horiuchi EK. 1980. *Pectinatus*, a new genus of bacteria capable of growth in hopped beer. J Inst Brew 86:28-30.

28. Satokari R, Juvonen R, Mallison K, von Wright A, Haikara A. 1998. Detection of beer spoilage bacteria *Megasphaera* and *Pectinatus* by polymerase chain reaction and colorimetric microplate hybridization. Int J Food Microbiol 45:119-127.

29. Jespersen L, Jakobsen M. 1996. Specific spoilage organisms in breweries and laboratory media for their detection. Int J Food Microbiol 33:139-155.

30. Kim SA, Kim NH, Lee SH, Hwang IG, Rhee MS. 2014. Survival of foodborne pathogenic bacteria (*Bacillus cereus*, *Escherichia coli* O157:H7, *Salmonella enterica serovar Typhimurium*, *Staphylococcus aureus*, and *Listeria monocytogenes)* and *Bacillus cereus* spores in fermented alcoholic beverages (beer and refined rice wine). J Food Prot 77:419-26.

31. Tonsmeire M. 2014. American Sour Beers: Innovative techniques for mixed fermentations. Brewer Publications, Boulder, Colorado.

32. Van Oevelen D, Spaepen M, Timmermans P, Verachtert H. 1977. Microbiological aspects of spontaneous wort fermentation in the production of lambic and gueuze. J Inst Brew 83:356-360.

33. Bossaert S, Crauwels S, De Rouck G, Lievens B. 2019. The Power of Sour - A Review: Old Traditions, New Opportunities. BrewingScience 72:78-88.

34. Bokulich NA, Bamforth CW, Mills DA. 2012. Brewhouse-resident microbiota are responsible for multi-stage fermentation of American Coolship Ale. PLoS ONE 7:e35507.

35. Spitaels F, Wieme AD, Snauwaert I, De Vuyst L, Vandamme P. 2017. Microbial Ecology of Traditional Beer Fermentations, p 179-196. *In* Bokulich NA, Bamforth CW (ed), Brewing Microbiology: Current Research, Omics and Microbial Ecology. Caister Academic Press, U.K.

36. Bokulich NA, Bamforth CW. 2013. The microbiology of malting and brewing. Microbiol Mol Biol Rev 77:157-72.

37. De Roos J, De Vuyst L. 2019. Microbial acidification, alcoholization, and aroma production during spontaneous lambic beer production. J Sci Food Agric 99:25-38.

544 38. Steensels J, Daenen L, Malcorps P, Derdelinckx G, Verachtert H, Verstrepen KJ. 2015.
545 *Brettanomyces* yeasts — From spoilage organisms to valuable contributors to industrial
546 fermentations. Int J Food Microbiol 206:24-38.
547 39. Crauwels S, Steensels J, Aerts G, Willems K, Verstrepen K, Lievens B. 2015.
548 *Brettanomyces bruxellensis*, essential contributor in spontaneous beer fermentations
549 providing novel opportunities for the brewing industry. BrewingScience 68:110-121.
550 40. Chatonnet P, Dubourdie D, Boidron J-n, Pons M. 1992. The origin of ethylphenols in
551 wines. J Sci Food Agric 60:165-178.
552 41. Spaepen M, Verachtert H. 1982. Esterase activity in the genus *Brettanomyces*. J Inst
553 Brew 88:11-17.
554 42. Spaepen M, Van Oevelen D, Verachtert H. 1978. Fatty acids and esters produced during
555 the spontaneous fermentation of lambic and gueuze. J Inst Brew 84:278-282.
556 43. Gondé P, Blondin B, Leclerc M, Ratomahenina R, Arnaud A, Galzy P. 1984.
557 Fermentation of cellodextrins by different yeast strains. Appl Environ Microbiol 48:265.
558 44. Daenen L, Saison D, Sterckx F, Delvaux FR, Verachtert H, Derdelinckx G. 2008.
559 Screening and evaluation of the glucoside hydrolase activity in *Saccharomyces* and
560 *Brettanomyces* brewing yeasts. J Appl Microbiol 104:478-88.
561 45. Daenen L, Sterckx F, Delvaux FR, Verachtert H, Derdelinckx G. 2008. Evaluation of the
562 glycoside hydrolase activity of a *Brettanomyces* strain on glycosides from sour cherry
563 (*Prunus cerasus* L.) used in the production of special fruit beers. FEMS Yeast Res
564 8:1103-1114.
565 46. Mamlouk D, Gullo M. 2013. Acetic Acid bacteria: physiology and carbon sources
566 oxidation. Indian J Microbiol 53:377-384.

47. De Roos J, De Vuyst L. 2018. Acetic acid bacteria in fermented foods and beverages. Curr Opin Biotechnol 49:115-119.

48. Spitaels F, Li L, Wieme A, Balzarini T, Cleenwerck I, Van Landschoot A, De Vuyst L, Vandamme P. 2014. *Acetobacter lambici sp. nov.*, isolated from fermenting lambic beer. Int J Syst Evol Micr 64:1083-1089.

49. Kashima Y, Iijima M, Okamoto A, Koizumi Y, Udaka S, Yanagida F. 1998. Purification and characterization of intracellular esterases related to ethylacetate formation in *Acetobacter pasteurianus*. J Ferment Bioeng 85:584-588.

50. Martens H, Dawoud E, Verachtert H. 1991. Wort enterobacteria and other microbial populations involved during the first month of lambic fermentation. J Inst Brew 97:435-439.

51. Verachtert H, Debourg A. 1995. Properties of Belgian acid beers and their microflora. The production of Gueuze and related refreshing acid beers. Cerevisia 20:37-41.

52. De Roos J, Vandamme P, De Vuyst L. 2018. Wort substrate consumption and metabolite production during lambic beer fermentation and maturation explain the successive growth of specific bacterial and yeast species. Front Microbiol 9:2763-2763.

53. De Roos J, Van der Veken D, De Vuyst L. 2019. The interior surfaces of wooden barrels are an additional microbial inoculation source for lambic beer production. Appl Environ Microbiol 85:e02226-18.

54. Verachtert H, Iserentant D. 1995. Properties of belgian acid beers and their microflora. Cerevisia 20:37-41.

588 55. Spitaels F, Wieme AD, Janssens M, Aerts M, Daniel H-M, Van Landschoot A, De Vuyst
589 L, Vandamme P. 2014. The microbial diversity of traditional spontaneously fermented
590 lambic beer. Plos One 9.
591 56. Snauwaert I, Roels SP, Van Nieuwerburg F, Van Landschoot A, De Vuyst L, Vandamme
592 P. 2016. Microbial diversity and metabolite composition of Belgian red-brown acidic
593 ales. Int J Food Microbiol 221:1-11.
594 57. Spitaels F, Wieme AD, Janssens M, Aerts M, Van Landschoot A, De Vuyst L,
595 Vandamme P. 2015. The microbial diversity of an industrially produced lambic beer
596 shares members of a traditionally produced one and reveals a core microbiota for lambic
597 beer fermentation. Food Microbiol 49:23-32.
598 58. Verachtert H, Derdelinckx G. 2014. Belgian acidic beers: Daily Reminiscences of the
599 Past. Cerevisia 38:121-128.
600 59. De Keersmaecker J. 1996. The Mystery of Lambic Beer. Scientific American 275:74-80.
601 60. Alworth J. 2015. The Beer Bible. Workman Publishing Company, New York.
602 61. Preedy VR. 2009. Beer in Health and Disease Prevention. Academic Press, Amsterdam.
603 62. Spitaels F, Van Kerrebroeck S, Wieme AD, Snauwaert I, Aerts M, Van Landschoot A,
604 De Vuyst L, Vandamme P. 2015. Microbiota and metabolites of aged bottled gueuze
605 beers converge to the same composition. Food Microbiol 47:1-11.
606 63. Dysvik A, La Rosa SL, Buffetto F, Liland KH, Myhrer KS, Rukke EO, Wicklund T,
607 Westereng B. 2019. Secondary lactic acid bacteria fermentation with wood-derived
608 xylooligosaccharides as a tool to expedite sour beer production. J Agric Food Chem
609 68:301-314 doi:10.1021/acs.jafc.9b05459.

610 64. Wright A, Axelsson L. 2019. Lactic acid bacteria: An introduction, p 1-16. *In* Gabriel
611 Vinderola, Arthur C. Ouwehand, Seppo Salminen, Atte von Wright (ed), Lactic Acid
612 Bacteria: Microbiological and Functional Aspects - Fifth Edition.
613 65. McFarland LV. 2015. From Yaks to Yogurt: The history, development, and current use
614 of probiotics. Clin Infect Dis 60:S85-S90.
615 66. Carafa I, Nardin T, Larcher R, Viola R, Tuohy K, Franciosi E. 2015. Identification and
616 characterization of wild lactobacilli and pediococci from spontaneously fermented
617 Mountain Cheese. Food Microbiol 48:123-132.
618 67. Petrović T, Dimitrijević S, Radulović Z, Mirković N, Rajić J, Obradović D, Nedović V.
619 2012. Comparative analysis of the potential probiotic abilities of lactobacilli of human
620 origin and from fermented vegetables. Arch Biol Sci 64:1473-1480.
621 68. Cocolin L, Dolci P, Rantsiou K, Urso R, Cantoni C, Comi G. 2009. Lactic acid bacteria
622 ecology of three traditional fermented sausages produced in the North of Italy as
623 determined by molecular methods. Meat Sci 82:125-132.
624 69. Fontana C, Bassi D, López C, Pisacane V, Otero MC, Puglisi E, Rebecchi A, Cocconcelli
625 PS, Vignolo G. 2016. Microbial ecology involved in the ripening of naturally fermented
626 llama meat sausages. A focus on lactobacilli diversity. Int J Food Microbiol 236:17-25.
627 70. Vardjan T, Mohar Lorbeg P, Rogelj I, Čanžek Majhenič A. 2013. Characterization and
628 stability of lactobacilli and yeast microbiota in kefir grains. J Dairy Sci 96:2729-2736.
629 71. Gulitz A, Stadie J, Wenning M, Ehrmann MA, Vogel RF. 2011. The microbial diversity
630 of water kefir. Int J Food Microbiol 151:284-288.

631 72. Minervini F, De Angelis M, Di Cagno R, Gobbetti M. 2014. Ecological parameters
632 influencing microbial diversity and stability of traditional sourdough. Int J Food
633 Microbiol 171:136-146.
634 73. Mtshali PS, Divol B, du Toit M. 2012. Identification and characterization of
635 *Lactobacillus florum* strains isolated from South African grape and wine samples. Int J
636 Food Microbiol 153:106-113.
637 74. Tsuji A, Kozawa M, Tokuda K, Enomoto T, Koyanagi T. 2018. Robust domination of
638 *Lactobacillus sakei* in microbiota during traditional japanese sake starter yamahai-moto
639 fermentation and the accompanying changes in metabolites. Current Microbiol 75:1498-
640 1505.
641 75. Suzuki K. 2011. 125th Anniversary Review: Microbiological instability of beer caused
642 by spoilage bacteria. J Inst Brew 117:131-155.
643 76. Geissler AJ, Behr J, von Kamp K, Vogel RF. 2016. Metabolic strategies of beer spoilage
644 lactic acid bacteria in beer. Int J Food Microbiol 216:60-68.
645 77. Bergsveinson J, Ziola BJBM. 2017. Investigation of beer spoilage lactic acid bacteria
646 using omic approaches. Brew Microbiol 245-274.
647 78. Bergsveinson J, Baecker N, Pittet V, Ziola B. 2015. Role of Plasmids in *Lactobacillus*
648 *brevis* BSO 464 Hop Tolerance and Beer Spoilage. Appl Environ Microbiol 81:1234.
649 79. Sakamoto K, Margolles A, van Veen HW, Konings WN. 2001. Hop resistance in the beer
650 spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette
651 multidrug transporter HorA. J Bacteriol 183:5371-5375.

80. Iijima K, Suzuki K, Asano S, Ogata T, Kitagawa Y. 2009. HorC, a hop-resistance related protein, presumably functions in homodimer form. Biosci Biotechnol Biochem 73:1880-2.

81. Suzuki K, Iijima K, Ozaki K, Yamashita H. 2005. Isolation of a hop-sensitive variant of *Lactobacillus lindneri* and identification of genetic markers for beer spoilage ability of lactic acid bacteria. Appl Environ Microbiol 71:5089.

82. Hayashi N, Ito M, Horiike S, Taguchi H. 2001. Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer. Appl Microbiol Biotechnol 55:596-603.

83. Behr J, Ganzle MG, Vogel RF. 2006. Characterization of a highly hop-resistant *Lactobacillus brevis* strain lacking hop transport. Appl Environ Microbiol 72:6483.

84. Asano S, Suzuki K, Iijima K, Motoyama Y, Kuriyama H, Kitagawa Y. 2007. Effects of morphological changes in beer-spoilage lactic acid bacteria on membrane filtration in breweries. J Biosci Bioeng 104:334-8.

85. Gold RS, Meagher MM, Hutkins R, Conway TJJoIM. 1992. Ethanol tolerance and carbohydrate metabolism in lactobacilli. J Ind Microbiol 10:45-54.

86. Wibowo D, Eschenbruch R, Davis CR, Fleet GH, Lee TH. 1985. Occurrence and growth of lactic acid bacteria in wine: A Review. Am J Enol Viticult 36:302-313.

87. Suzuki K, Asano S, Iijima K, Kitamoto K. 2008. Sake and beer spoilage lactic acid bacteria — A Review. J Inst Brew 114:209-223.

88. Nojiro K. 1984. Advances in sake brewing microbiology IV. J Soc Brew Jpn 79:229-235.

673 89. Kleynmans U, Heinzl H, Hammes WP. 1989. *Lactobacillus suebicus sp. nov.*, an
674 obligately heterofermentative *Lactobacillus* species isolated from fruit mashes. Syst Appl
675 Microbiol 11:267-271.
676 90. Pittet V, Morrow K, Ziola B. 2011. Ethanol tolerance of lactic acid bacteria, including
677 relevance of the exopolysaccharide gene gtf. J Am Soc Brew Chem 69:57-61.
678 91. Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich S, Maguin E. 2002. Stress
679 response in lactic acid bacteria. Antonie van Leeuwenhoek 82:187-216.
680 92. van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E. 2002.
681 Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek 82:187-216.
682 93. Higuchi T, Hayashi H, Abe K. 1997. Exchange of glutamate and gamma-aminobutyrate
683 in a *Lactobacillus* strain. J Bacteriol 179:3362-3364.
684 94. Cunin R, Glansdorff N, Piérard A, Stalon V. 1986. Biosynthesis and metabolism of
685 arginine in bacteria. Microbiol Rev 50:314-352.
686 95. Champomier Verges MC, Zuniga M, Morel-Deville F, Perez-Martinez G, Zagorec M,
687 Ehrlich SD. 1999. Relationships between arginine degradation, pH and survival in
688 *Lactobacillus sakei*. FEMS Microbiol Lett 180:297-304.
689 96. Arena ME, Saguir FM, Manca de Nadra MC. 1999. Arginine, citrulline and ornithine
690 metabolism by lactic acid bacteria from wine. Int J Food Microbiol 52:155-161.
691 97. Stock D, Leslie AGW, Walker JE. 1999. Molecular Architecture of the Rotary Motor in
692 ATP Synthase. Science 286:1700.
693 98. Corcoran BM, Stanton C, Fitzgerald GF, Ross RP. 2005. Survival of probiotic
694 lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars.
695 Appl Environ Microbiol 71:3060-3067.

99. Futai M, Noumi T, Maeda M. 1989. ATP synthase (H+-ATPase): results by combined biochemical and molecular biological approaches. Annu Rev Biochem 58:111-36.

100. Borch E, Kant-Muermans M-L, Blixt Y. 1996. Bacterial spoilage of meat and cured meat products. Int J Food Microbiol 33:103-120.

101. Boulton C, Quain D. 2001. The Biochemistry of Fermentation, p 69-142, Brewing Yeast and Fermentation doi:10.1002/9780470999417.ch3.

102. Møller MS, Goh YJ, Rasmussen KB, Cypryk W, Celebioglu HU, Klaenhammer TR, Svensson B, Abou Hachem M. 2017. An Extracellular Cell-Attached Pullulanase Confers Branched α-Glucan Utilization in Human Gut. Appl Environ Microbiol 83:e00402-17.

103. Spear GT, French AL, Gilbert D, Zariffard MR, Mirmonsef P, Sullivan TH, Spear WW, Landay A, Micci S, Lee BH, Hamaker BR. 2014. Human alpha-amylase present in lower-genital-tract mucosal fluid processes glycogen to support vaginal colonization by *Lactobacillus*. J Infect Dis 210:1019-28.

104. Nakai H, Baumann MJ, Petersen BO, Westphal Y, Schols H, Dilokpimol A, Hachem MA, Lahtinen SJ, Duus JO, Svensson B. 2009. The maltodextrin transport system and metabolism in *Lactobacillus acidophilus* NCFM and production of novel alpha-glucosides through reverse phosphorolysis by maltose phosphorylase. FEBS J 276:7353-65.

105. Reddy G, Altaf M, Naveena BJ, Venkateshwar M, Kumar EV. 2008. Amylolytic bacterial lactic acid fermentation — A review. Biotechnol Adv 26:22-34.

106. Gänzle MG, Follador R. 2012. Metabolism of oligosaccharides and starch in lactobacilli: a review. Front Microbiol 3:340-340.

718 107. De Cort S, Kumara HM, Verachtert H. 1994. Localization and characterization of alpha-
719 glucosidase activity in *Lactobacillus brevis*. Appl Environ Microbiol 60:3074-3078.
720 108. Kumara HMCS, Verachtert H. 1991. Identification of lambic superattenuating micro-
721 organisms by the use of selective antibiotics. J Inst Brew 97:181-185.
722 109. Domingues L, Guimarães PMR, Oliveira C. 2010. Metabolic engineering of
723 *Saccharomyces cerevisiae* for lactose/whey fermentation. Bioeng Bugs 1:164-171.
724 110. Domizio P, House JF, Joseph CML, Bisson LF, Bamforth CW. 2016. *Lachancea*
725 *thermotolerans* as an alternative yeast for the production of beer. J Inst Brew 122:599-
726 604.
727 111. Osburn K, Amaral J, Metcalf SR, Nickens DM, Rogers CM, Sausen C, Caputo R, Miller
728 J, Li H, Tennessen JM, Bochman ML. 2018. Primary souring: A novel bacteria-free
729 method for sour beer production. Food Microbiol 70:76-84.
730 112. Peyer LC, Zarnkow M, Jacob F, De Schutter DP, Arendt EK. 2017. Sour Brewing:
731 Impact of *Lactobacillus Amylovorus* FST2.11 on Technological and Quality Attributes of
732 Acid Beers. J Am Soc Brew Chem 75:207-216.
733 113. Dysvik A, Liland, K.L., Myhrer, K.S., Westereng, B., Rukke, E.O., de Rouck, G.,
734 Wicklund, T. 2019. Pre-fermentation with lactic acid bacteria in sour beer production. J
735 Inst Brew 125:342-356.
736 114. Dysvik A, La Rosa, SL, Liland, KH, Myhrer, KS, Østlie, HM, De Rouck, G, Rukke, EO,
737 Westereng, B, Wicklund, T. 2020. Co-fermentation involving *Saccharomyces cerevisiae*
738 and lactobacillus species tolerant to brewing-related stress factors for controlled and rapid
739 production of sour beer. Front Microbiol 11:279
740 doi:https://doi.org/10.3389/fmicb.2020.00279.

115. Alcine Chan MZ, Chua JY, Toh M, Liu S-Q. 2019. Survival of probiotic strain *Lactobacillus paracasei* L26 during co-fermentation with *S. cerevisiae* for the development of a novel beer beverage. Food Microbiol 82:541-550.

FIGURE LEGENDS

Figure 1. A. Schematic illustration of the beer production process. Grain is malted, milled and mashed, before wort is separated from Brewer's spent grain and boiled with hops. Yeast is added to chilled wort to ferment the sugary wort into ethanol-containing beer. **B**. Schematic illustration of the Lambic beer production process. Active inoculation of wort is not carried out. Boiled wort is cooled down in a shallow, open vessel (coolship), where it is spontaneously inoculated by exposure to the environment. The wort is transferred to wooden casks, where spontaneous fermentation by a variety of yeasts and bacteria can transpire.

Figure 2. Illustration of the hurdle effect in beer, where relatively low intensity hurdles such as iso-α acids, ethanol, low pH, high CO_2, low O_2 together pose a substantial antimicrobial effect.

Figure 3. Modern approaches to sour beer production. **A**, Traditional production process with spontaneous fermentation. **B**, Pre-fermentation with LAB, followed by yeast fermentation. **C**, Co-fermentation with yeast and LAB. **D**, Secondary fermentation with LAB, with wood-derived carbohydrates as substrate.