The power of sour – A review: Old traditions, new opportunities

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Short description: Because sour beers are becoming increasingly popular, this papers gives an overview of the most relevant souring microorganisms for sour beer production, as well as a review of the most common souring practices.

Abstract

Currently, there is a strong interest in sour beers, with more breweries producing sour styles and sales continuing to increase. There are many different ways for producing sour beer, offering breweries a variety of opportunities to pursue new beverages and diversify their portfolio. These methods range from modern kettle souring and short term mixed fermentation to traditional long term mixed fermentation and maturation in wooden barrels. While the first methods enable quick souring, these beers generally lack the flavour complexity that can be obtained through more traditional methods. Here, we discuss the microbiology of sour beers and review the most common production methods as well as the most important beer styles that are produced using these methods. Further, we report a number of novel methods that have the potential to produce the next generation of flavour-rich sour beers. Increased knowledge on the microbiology and flavour development in traditional sours will help developing such next generation of sour beers.

1. A brief history of sour beers

In the early days of brewing beer, about 9,000 years ago, the main two beer ingredients were grains and water [1,2]. Nevertheless, although unknown at that time, it is speculated that many microorganisms played a crucial role in the process, among which wild yeasts including *Saccharomyces* spp. were most probably responsible for alcoholic fermentation. Additionally, other microorganisms like lactic acid bacteria (LAB) were likely present, and influenced the brewing process. For example, acidification by LAB improves accessibility of starch, and it prevents growth of other microorganisms, thereby providing a favourable growth medium for *Saccharomyces* spp. [2]. Production of lactic acid also leads to a tart, sour taste, so it is very likely that in the early days of brewing most beers would sour. Nevertheless, microorganisms like *Lactobacillus* spp., *Micrococcus* spp., *Pediococcus* spp., and *Streptococcus* spp. can spoil beer and produce unpleasant aromatic compounds [3]. In the late 13th century, hops were increasingly added to prevent souring as it was observed that hops played a role in beer preservation [2,4]. Later, this property would be explained by the antimicrobial activity of its constituents in particular α-acids, iso-α-acids and β-acids, which are especially active against Gram-positive bacteria [5]. These acids act as ionophores which dissipate the pH gradient across the cytoplasmic membrane and reduce the proton motive force. Consequently, nutrient uptake driven by the proton motive force is inhibited, resulting in starvation and cell death [6-9]. In combination with the use of single strains (which became common since the 19th century) this resulted in a more clean, consistent beer with a longer shelf life [2,4].

Since then beer could be made in greater quantities and stored, as opposed to being drunk within a matter of days, and beer production shifted towards a highly controlled process. However, it was only after World War I, when grain prices dropped, that consumers switched from the traditional (sour) beers to the cheaper and consistent lager beers produced on an industrial scale. As a result of this trend, many (small) breweries disappeared, while the few surviving breweries expanded their production volume [4,10]. Since then, sour beers were seen as the beer for "peasants", causing the production of sour beers to decrease tremendously, and leaving only the most authentic sour beers (e.g. Belgian Lambic beers) on the market [4]. Nevertheless, near the end of the 20th century, the consumer's mindset changed again and the inverse trend was observed, i.e. an aversion towards mass-production and a desire for authenticity [4,10]. Consumers are now willing to spend more money for traditionally produced beers or beers with a more complex aroma than the typical industrial lager, leading to an increased demand for authentic beers and craft beers with an authentic taste**,** including sours [4,10,11]. The term 'sours' or 'sour beers' comprises many different beers and is not restricted to one explicit definition. However, there are a number of features that sour beers have in common, including (i) a sour taste, (ii) the occurrence of elevated concentrations of acids, particularly lactic acid and acetic acid, causing a low pH (sometimes as low as pH 3), and (iii) they are generally produced using both bacteria and yeasts [11].

Sour beers can be produced in many different ways, and encompass a wide variety of beer styles. The most common sour beer styles are Belgian, including Lambics, Flanders Red Ale and Old Brown, next to others like American Coolship Ale, Berliner Weisse and Gose. Additionally, a number of other barrel-aged and kettle-soured beers are available. In this review article, the microbiology of sour beers is briefly described. Additionally, the most common production methods as well as the most important beer styles that are produced with each of these methods are discussed. Finally, a number of novel methods are explained for producing the next generation of sour beers.

2. Sour beer microbiology

Most beers that are commercially available today only contain *Saccharomyces* spp., and the presence of any other microorganism suggests beer defects [12]. Important beer spoilage microorganisms include LAB, acetic acid bacteria (AAB), and *Brettanomyces* yeasts [3]. Such microorganisms spoil beer through acidification and haze formation, among other factors. However, these traits are desired in the brewing of sour beer. Knowledge of these microbial groups and their most important features is required to understand the process of beer souring. Some of the most important organisms responsible for beer souring are discussed below.

Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are the main source of lactic acid in fermented foods like yoghurt, sauerkraut, pickles, sourdough and sour beers. In addition to lactic acid, they can produce several compounds that contribute to taste, odour, colour, and texture of the foods [13,14]. Further, they can produce antimicrobial substances including bacteriocins that have the ability to inhibit pathogenic and food spoilage bacteria [15]. *Lactobacillus* and *Pediococcus* represent two LAB genera that are strongly associated with lactic acid production in sour beer, even though other LAB (*Oenococcus* spp., *Leuconostoc* spp., *Lactococcus* spp., …) have been isolated from sour beer as well [12].

Lactobacillus is a genus of rod-shaped, Gram-positive LAB, among which *Lactobacillus plantarum, Lactobacillus brevis* and *Lactobacillus delbrueckii* are the most important species in beer production [7]. *Lactobacillus* spp. can lower the pH of sour beer to approximately 3.3-3.4 [16]. *Lactobacillus* strains are generally sensitive to hops, although variation occurs [9]. Therefore, if growth of *Lactobacillus* spp. is desired, sensitivity of *Lactobacillus* spp. to hop bitter acids should be taken into account when deciding on the hop dosage. As a compromise, aged hops can be used instead of a fresh batch, as they generally contain less bittering agents and consequently less antimicrobial compounds [9]. A well-known example where this principle is applied, is the production of Belgian Lambic beers [17] (see further). *Lactobacillus brevis* is considered the most hop tolerant species of all lactobacilli, a property caused by a plasmid-encoded transporter protein HorA and a multi-drug transporter ORF5, which allow the transport of hop antimicrobial compounds out of the cytoplasm [18,19]. Therefore, *L. brevis* is the most common beer-spoilage bacterium found in breweries of non-sour beers. *Lactobacillus* species are generally desired for sour beer production, as they are capable to quickly produce large amounts of lactic acid, which causes a fast decrease in pH, without producing other flavour-active compounds, thus creating a 'clean' sour taste [9,20]. The underlying reason is that many lactobacilli operate using homofermentative metabolism, and only produce lactic acid from sugars [20]. The rapid decrease in pH is considered a defence mechanism towards other microorganisms that are not well adapted to growth at a lower pH [21,22]. If *Lactobacillus* species are used for beer souring in combination with other microorganisms (e.g. yeast species performing the primary fermentation), the decrease in pH should be monitored and controlled to avoid inhibition of other desired microorganisms. In addition, species from the *Lactobacillus* genus are aerotolerant, meaning that they do not require oxygen, but are also not harmed by it, and they grow best between 38 and 50 °C [20,23]. Importantly, some species, particularly *L. plantarum*, are able to produce 4-ethyl phenol and 4-vinyl phenol through decarboxylation of cinnamic acids [24,25]. In most beers these compounds are unwanted as they lend to medicinal, horses' sweat, or barnyard flavours [26], but in some beers they are highly appreciated (e.g. Lambic beers). Some strains of *L. brevis, L. acidophilus, L. johnsonii* and *L.*

plantarum also have α-glucosidase activity [27-30], which allows the breakdown of dextrins and, as a consequence, can lead to overattenuation of the beer [31]. Even though lactobacilli are desired souring agents in beer, they are generally used in combination with other souring microorganisms to increase flavour complexity [9,16], except for the production of Berliner Weisse, Gose or related beer styles [9]. In most cases, lactobacilli are pitched to the wort before boiling to avoid cross-contamination in the cold side of the brewing process. Nevertheless, souring wort after boiling can be preferred to retain desirable volatiles released during souring [16,32]. If the primary yeast and *Lactobacillus* species are pitched simultaneously, the pitching rate of *Lactobacillus* spp. should be high enough to allow lactic acid production. Another possibility is to pitch a mixed culture of a *Lactobacillus* strain and another strain breaking down dextrins into glucose molecules which, in turn, can be used by the *Lactobacillus* strain. One such organism is *Brettanomyces* (teleomorph *Dekkera*), which often excrete α- and β-glucosidases by which dextrins are degraded [31,33]

The genus *Pediococcus* represents another important rod-shaped, Gram-positive LAB in the production of sour beers and can lower the pH below 3.0 [34,35]. The main *Pediococcus* species found in sour beers, or used in brewing sour beer, is *Pediococcus damnosus* (previously listed as *Pediococcus cerevisiae*) [12,36]. The species is considered critical in the production of spontaneously fermented sour beers, like Lambic beers and American Coolship Ales [17]. *Pediococcus damnosus* is one of the most hop tolerant LAB, is homofermentative and grows best at 37 °C [9,37,38]. *Pediococcus* spp. are also known to withstand a lower pH than lactobacilli [39], which may be expressed by the fact that they can decrease the pH of beer even further than *Lactobacillus* sp. can. A main advantage of *Pediococcus* spp. to produce sour beer is that it takes time for it to begin lowering the pH dramatically. This gradual decrease in pH usually gives the primary yeast enough time to complete the wort fermentation [40]. Nevertheless, a major disadvantage of *Pediococcus* spp. is that they may also produce diacetyl, responsible for a rancid, buttery flavour [41]. To compensate, *Pediococcus* is often used in combination with *Brettanomyces*, which is able to convert diacetyl into compounds that are less flavour-active [42,43]. *Pediococcus* spp. may also cause 'ropiness' (also called 'sick beer') due to the production of exopolysaccharides [34]. 'Ropy' beer is more viscous and, in extreme circumstances, can form strands. Ropiness affects mostly the mouthfeel and appearance of beer, but may have no influence on the flavour. It is considered a temporary flaw in sour beer. However, some brewers claim that after the ropiness goes away (generally after a few weeks or months) it produces a deeper acidity and mouthfeel. In these cases, exopolysaccharide production is viewed as a positive process in the production of sour beer. When *Brettanomyces* species are present, their excreted glucosidases can speed up the breakdown of the exopolysaccharides and decrease the viscosity more rapidly [34].

Acetic acid bacteria (AAB)

Besides LAB, acetic acid bacteria (AAB) can also cause beer acidification, albeit with a different end product. Whereas lactic acid is considered soft and sweet-sour, acetic acid is often described as a harsher sourness, mostly resembling vinegar. However, if the beer contains lactic acid, the addition of acetic acid can, depending on the concentration, increase the flavour complexity of the beer and result in a more layered flavour profile. A well-balanced combination of lactic acid and acetic acid is one of the essential features that gives Lambic beers and other traditional Belgian sours the complex sourness for which these beer styles are known.

Acetic acid bacteria are strictly aerobic, Gram-negative bacteria and are valuable organisms for the production of vinegar, gluconic acid, cellulose, vitamin C, chocolate, kombucha, and sometimes also sour beers [44]. In general, they grow best between 25 and 30 °C and in the presence of 0.35 % acetic acid [45,46]. Acetic acid is produced through the oxidation of carbon sources into ethanol, followed by the oxidation of ethanol into acetaldehyde, and finally through the oxidation of acetaldehyde into acetic acid. When ethanol is depleted, AAB can also convert glycerol into cellulose, acetic acid and carbon dioxide [47,48]. In other fermentation environments, more specifically during chocolate fermentation, it was shown that AAB can convert lactic acid into acetoin, a compound with a pleasant, buttery flavour. Also in Lambic beers, an elevated concentration of acetoin is detected at times when AAB counts are higher [49,50]. AAB have also been shown to produce intracellular esterases that are responsible for catalysing the condensation reaction between acetic acid and ethanol, yielding ethyl acetate, i.e. a fruity-flavoured ester that is often found in sour beers [51]. At low concentrations, ethyl acetate is associated with pleasant fruity notes. However, at high levels it is described as having a solvent-like aroma, and therefore unwanted in beer and other beverages. The most common AAB in sour beers represent members of the genus *Acetobacter*, although *Gluconobacter* species have also been isolated [52,53].

Acetobacter species are commonly found in Lambic beers and American Coolship Ales [54], more specifically at the interface between the fermenting wort and air within the wooden barrels, where enough oxygen is available for their obligate aerobic growth [55]. The most important species found in sour beers are *Acetobacter lambici* [52], *Acetobacter orientalis* and *Acetobacter pasteurianus* [55]. In Lambic beers, it was observed that *A. orientalis* was only present for a few days at the beginning of the fermentation, while *A. pasteurianus* was found after seven weeks of fermentation and was still present until two years of maturation [55]. Competitive exclusion tests and a comparative genomic analysis revealed that this successive dominance might be related to adaptation towards high concentrations of ethanol and acetic acid [55]. Furthermore, *A. pastorianus* has been isolated from

Flanders Red Ales [56]. Acetic acid production by *Acetobacter* spp. is often undesired in sour beers, as they can rapidly produce too much acetic acid and ethyl acetate, which makes the beer taste like vinegar and solvent, respectively [55]. By contrast, acetic acid production by *Brettanomyces* spp. is more desired, as they usually produce more appropriate amounts of acetic acid [57].

Brettanomyces spp.

While unwanted in many beer styles and other beverages, *Brettanomyces* yeasts, particularly *Brettanomyces bruxellensis*, play a critical role in specific beer fermentations, such as the production of Belgian Lambic beers and American Coolship Ales, employing production methods similar to traditional Belgian Lambic [57]. In these fermentations, yeasts of the genus *Brettanomyces* live in perfect harmony with various other microbes, including LAB and AAB. *Brettanomyces* yeasts are responsible for many of the typical organoleptic characteristics of these beers, giving the beer its 'funky' character [57-59]. The unique aromatic properties of *B. bruxellensis* are increasingly recognised, with more and more brewers adding *B. bruxellensis* to their fermentations, either as a single culture or in combination with other cultures [58,59]. *Brettanomyces* species can use substrates that *Saccharomyces* spp. cannot use, such as dextrins, cellobiose, and nitrate [33,59,60]. Dextrins, such as maltotetraose and maltopentaose, are present as residual sugars after the main alcoholic fermentation in beer. *Brettanomyces* yeasts produce glucosidases, enabling them to hydrolyse these sugars into glucose [31,61], allowing them to reside in the fermentation for a long time, while yeasts belonging to *Saccharomyces* cannot. Consequently, *Brettanomyces* spp. can be used for the production of super-attenuated and low calorie beers [57]. However, such degree of attenuation can vary depending on the pitching rate and the strain [43]. *Brettanomyces* spp. produce little to no glycerol, and are able to produce considerable levels of acetic acid under aerobic conditions [62]. Ethyl esters such as ethyl acetate, ethyl hexanoate and ethyl octanoate are also produced in high quantities, contributing to tropical fruit and pineapple flavours [63]. The production of volatile phenols, including 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG), also contribute to the flavour profile characteristic for *Brettanomyces* sp. [57-59]. The detection threshold for these compounds is very low, with diverse flavour and aroma descriptors including horses' sweat, leathery, spicy, medicinal and smoky, among others [64]. Notably, in beer generally higher concentrations of 4-EG (clove-like or spicy aroma) are found than for 4-EP (medicinal, barnyard aroma) [65], resulting in a spiciness that is characteristic of beer fermented with *Brettanomyces* spp. [66]. Furthermore, *Brettanomyces* yeasts have a high β-glucosidase activity [57-59], which is particularly advantageous to survive in wooden barrels, especially when nutrient availability is low or barrels are emptied as it allows the breakdown of cellobiose present in wood. Additionally, this enzyme has the capacity to release flavour-active compounds from hops, resulting in an increase in volatile compounds such as linalool, which has a

lemon-woody flavour [67]. *Brettanomyces'* β-glucosidase has been characterised to be used as a potential natural bio-flavouring agent [68]. Two genes encoding β-glucosidases have been reported in *B. bruxellensis*. Curiously, most strains originating from beer seem to have only one of them, whereas *B. bruxellensis* isolates from wine have both genes [33].

3. Current souring methods

Currently, diverse methods are used for the production of sour beers, which range from spontaneous souring to inoculation-based methods, including modern kettle souring and more traditional mixed culture fermentation. An overview of the different production methods is shown in Fig. 1.

Spontaneous fermentation

In contrast to starter culture fermentations, spontaneous fermentations are not initiated through the inoculation of yeasts or bacteria as starter cultures. Instead, these beers are fermented by a mixture of wild, brewery-resident yeasts and bacteria that are inoculated in the wort and become dominant over the course of the fermentation process (Fig. 1). The microbiota that arises in spontaneously fermented beer is known to be complex, and yields sour beers with a unique flavour profile, that is difficult to mimic using starter cultures [57,69,70]. Acidity generally results from the production of lactic acid and acetic acid. Importantly, when presence of LAB is desired, it is recommended to take this into account when deciding on the hop dosage (see above).

The most prominent examples of such beers are Belgian Lambics, characterised by a spontaneous fermentation and maturation in wooden barrels that lasts for one to three years before bottling, or blending to produce Gueuze or fruit Lambic beers [69]. Gueuze is typically made by blending young (1-year-old) and old (2- to 3-year-old) Lambics, which is bottled for a second fermentation. Because the young Lambics are not fully fermented, the blended beer contains fermentable sugars, which allow a second fermentation to occur [17,69]. Lambic that undergoes a second fermentation in the presence of sour cherries before bottling results in Kriek Lambic, which is closely related to Gueuze. In Lambic beers, a mixture of malted barley, unmalted wheat and water is heated and then boiled with aged hops. The resulting wort is cooled down overnight in a large, shallow, open vessel known as the 'coolship', during which the wort becomes inoculated with environmental bacteria and yeasts [17,69]. After cooling, the wort is transferred into wooden barrels which are stored at cellar or ambient temperatures. Subsequently, the wort ferments and the Lambic beer matures in the same barrels leading to a cloudy, uncarbonated, bracing sour beverage (Fig. 1). A recent study has shown that the wooden barrels are an important microbial inoculation source for Lambic production, besides brewery air and brewery equipment, thereby helping to establish a stable

microbial community in the wort [71]. The specific flavour character of the beer originates from the metabolic activities of various yeasts, LAB, and AAB [72-74]. Additionally, since these beers are fermented and matured in the same barrel, their unique flavour profile is likely also affected by microbial autolysis, contributing both substrates (lipids, proteins, and carbohydrates) and intracellular enzymes to participate in diverse metabolic reactions [75-77]. Previous studies (both using culture-dependent and culture-independent techniques) of the Lambic beer fermentation process have identified a consistent microbial succession driven by changes in physicochemical parameters and substrate and metabolite compositions of the fermenting wort and the maturing Lambic [72-75,78]. An overview of the observed microbial succession during the fermentation and maturation process of Belgian Lambic beers is presented in Fig. 2. The first month is dominated by Enterobacteriaceae species like *Enterobacter* spp., *Klebsiella pneumoniae*, *Escherichia coli* and *Hafnia alvei*, together with non-*Saccharomyces* yeasts [78]. Enterobacteriaceae present during this stage produce several compounds responsible for the aroma of 1- to 2-month-old Lambic, including 2,3-butanediol, ethyl acetate, higher alcohols, and acetic acid, lactic acid, and succinic acid [79]. After 1 month, *Saccharomyces* spp. and LAB (primarily *P. damnosus*) dominate the fermentation, which lasts three to four months. Subsequently, *Brettanomyces* yeasts (mainly *B. bruxellensis*) replace *Saccharomyces* spp. and dominate the remainder of the fermentation and maturation, while producing a range of characteristic aroma compounds, including caprylic fatty acids and capric fatty acids and their ethyl esters [80,81]. *Brettanomyces* yeasts also hydrolyse the exopolysaccharides produced by *Pediococcus* spp. during the main fermentation, reducing the viscosity of the Lambic [34]. AAB are found throughout the whole fermentation period [72,74]. To avoid growth of Enterobacteriaceae, and associated production of toxic biogenic amines [82], Lambic brewers often acidify their wort prior to the spontaneous inoculation in the coolship, thereby killing off the Enterobacteriaceae [83]. The reason why the maturation phase can continue for such a long period is that the unmalted wheat, in combination with the traditionally applied turbid mashing, yields wort that contains elevated concentrations of dextrins. As the microorganisms occurring in the first stages of the process cannot consume these carbon sources, this enables *Brettanomyces* spp. to grow and develop, once other carbon sources are depleted. In addition, because of the exoglucosidase activity of *Brettanomyces* species, dextrin will be converted into glucose, which will then be available for *P. damnosus* to continue its growth [27,31]. A similar microbial succession was found for the American Coolship Ales [54]. However, the exact species composition seems to be different between both beers. More specifically, the enterobacterial species found in American Coolship Ales are different from the species found in Lambic beers, and *Saccharomyces cerevisiae* is already abundantly present from the start of the fermentation of the American Coolship Ales. It is speculated that these differences are caused by

the production environment [54]. American Coolship Ales are a seasonal product from craft breweries, which contrasts to traditional Belgian Lambic breweries that exclusively produce Lambic beers. It is thus likely that *Saccharomyces* spp., used for the brewing of other types of beers in the American craft-brewing sector, are enriched in these brewery environments. Another characteristic of American Coolship Ale fermentations is that the initial domination by Enterobacteriaceae is rapidly succeeded by LAB, where the latter can remain present at least until one year of fermentation. At this point, AAB become more prevalent [54].

Kettle souring

Kettle souring, also known as 'quick souring', is the more modern method for making sour beer based on the inoculation of a souring microbe (Fig. 1). In this method, wort with little or no hop is boiled, cooled down to a temperature of 40-50 °C and then pitched with a LAB (often *Lactobacillus* spp. and/or *Pediococcus* spp.), when the wort is still in the brew kettle. Subsequently, the wort is fermented by the LAB until the desired level of acidity is achieved, which typically takes 24-48 hours. Then, the wort is boiled again to stop the souring. When more bitterness is desired, additional hops can be added during this boiling step, simultaneously reinforcing that the LAB are not able to grow any longer. After the second boil and subsequent cooling, the wort is transferred to a fermenter and inoculated with brewing yeast to initiate primary fermentation [11,16,32]. The main advantage of this method is that it offers brewers a means to produce a sour beer in only a few days [32,84]. Further, when different microbes are sequentially pitched, conditions can be controlled for each culture individually (e.g. temperature, oxygenation, etc.) [16], leading to a consistent end quality. However, boiling the wort after fermentation by LAB drives off some flavour compounds produced during souring, making the beer a 'clean' sour with limited complexity [11,16,32]. More flavour complexity can be obtained by pitching non-conventional yeast cultures after the primary souring (possibly including a conventional fermenter), or by implementing a wood maturation step after primary fermentation [11]. Alternatively, one could opt for not performing the second boil. Nevertheless, this may prevent the fermenting yeast to develop as the acidification continues [16]. In this scenario, it may be an option to use *Pediococcus* spp. rather than *Lactobacillus* spp. Compared to *Lactobacillus* spp., *Pediococcus* spp. take more time to acidify the wort [40], which will give the fermenting yeast more time to start growing and fermenting. On the contrary, when using *Pediococcus* cultures for primary souring, higher concentrations of diacetyl may be produced in comparison to using *Lactobacillus* strains [41], and the viscosity of the beer may increase tremendously [34]. In turn, this may be solved by pitching *Brettanomyces* yeasts later on in the fermentation or maturation process [34]. Furthermore, kettle souring also has the disadvantage that during LAB fermentation, the brew kettle is occupied with the souring beer for around 48 hours, which prevents brewers from producing a next batch of beer [32].

Moreover, brewers who also produce non-sour beer styles often try to avoid the occurrence of bacteria in their brew environment to prevent them from contaminating a next batch of non-sour beer [16,32]. Despite being considered a 'one-trick-pony' (i.e. due to the lack in flavour complexity) kettle souring is used for a number well-known beer styles, including Berliner Weisse and Gose.

Berliner Weisse is a traditional German wheat beer style, dating back to at least the 16th century, that is typically described as a light alcoholic (around 3 % ABV), cloudy sour beer with a 'clean' sourness. Nowadays, many variations exist to the traditional production method. Originally, around 50-75 % wheat malt was used, supplemented with barley malt, with the stipulation that the malts are kilned at very low temperatures to minimise colour formation. In addition, the wort was not boiled, but rather cooled directly after lautering, with the relatively limited amounts of hop added during mashing [85,86]. When the wort is not boiled, malt aromatics are better preserved and a fresh bread dough aroma will be detected in the resulting beer. Furthermore, the starter culture consisted of a mixture between brewer's yeast and LAB in a ratio of 4:1 to 6:1 [85]. To promote LAB growth and acidification, the temperature of fermentation is raised to 35-45 °C [87]. Nevertheless, as the traditional method does not include a proper boiling step, a variety of microorganisms are able to survive through the brewing process. In the past, contaminations with slime-forming *Pediococcus* spp. were often reported [85,88]. Finally, a secondary fermentation takes place in the bottle through the addition of Kräusen, i.e. a yeast layer that is formed on top of the fermenting beer, to the green beer [85].

Nowadays, brewers generally only use around 30 % wheat malt [89] and after wort boiling, the wort is often divided into two parts [86]. Subsequently, one half is soured by pitching a homofermentative *Lactobacillus* strain, while the other half is fermented using a top-fermenting yeast, after which both parts are mixed again [86,90]. By splitting the souring and the fermentation, optimal conditions can be created for each microorganism. Furthermore, the amounts of wort and beer blended can be adjusted to achieve exactly the right amount of sourness, and the soured wort containing LAB can be boiled before blending to inhibit further LAB growth [86].

Another popular German-style kettle sour is Gose. The recipes for Gose beers are very divergent, and there is not much consistency and agreement on how to produce a traditional Gose. It is usually brewed with at least 50 % malted wheat. Dominant flavours in Gose include a lemon sourness, a herbal characteristic, and a strong saltiness. Alcohol content is usually between 4 and 5 % ABV. Gose beers typically do not have prominent hop bitterness, flavours, or aroma. The Gose characteristics that people generally do agree on, are the fact that *Lactobacillus* sp. is the only souring agent used during

kettle souring and that spices like coriander and salt are added to give the beers their typical sour and spicy flavour [11,91].

Mixed culture fermentation

Another method for sour beer production is a combination of the two methods discussed above, known as mixed culture fermentation (Fig. 1). In this method, a mixture of yeast (e.g. conventional brewer's yeast and/or one or more non-conventional yeast species) and bacteria (usually *Lactobacillus* spp. and/or *Pediococcus* spp.) is pitched to slightly hopped wort, and fermented in steel tanks for seven to eight weeks to create a fruity, refreshingly tart beer [92]. Some breweries bottle and sell this young beer as is, while others mature the beer for one to two years in large oak casks, where *Brettanomyces* spp. and wild yeasts that are resident in the wood referment the beer [92]. At high pitching rate, spoilage microbes generally do not stand a chance in the fermenting beer, and thus, potential production of off-flavours associated with these organisms is prevented [93]. In this respect, not only the pitching rate is important, but also the composition of the mixed culture plays a major role. For example, fast growing or fast fermenting *Lactobacillus* spp. and *Saccharomyces* spp. greatly affect the occurrence of other microorganisms, as they change the growth conditions in beer drastically, e.g. by producing lactic acid and ethanol and consuming the readily available fermentable carbon sources [20,57]. Depending on the microorganisms used, acidity can develop gradually or very rapidly. When the acidity is generated more gradually (e.g. by using *Pediococcus* spp. instead of *Lactobacillus* spp.), other species (e.g. *Saccharomyces* spp. and *Brettanomyces* spp.) also get the chance to develop and influence the flavour profile, thus creating more complex 'funky' beers [11,40,92]. Moreover, the aroma/flavour compounds produced by these species may be different from the compounds that they would produce when pitched as a single culture in not-acidified wort [94]. As mentioned above, one of the most promising microbial combinations is a combination of *Pediococcus* and *Brettanomyces* strains. Briefly, *Pediococcus* spp. acidify the beer quite slowly, giving *Brettanomyces* spp. enough time to develop, and add additional flavours to the beer. In addition, *Brettanomyces* spp. are able to 'clean' the beer, by consuming diacetyl and exopolysaccharides produced by *Pediococcus* species. In turn, *Brettanomyces* yeasts produce extracellular glucosidases that are able to convert complex carbohydrates like dextrins into sugars that can be fermented by *Pediococcus* spp.

Perhaps the most challenging aspect of utilising mixed culture fermentation is controlling the optimum balance between the microorganisms involved in order to achieve the desired flavour profile. Furthermore, it is not always easy to predict the outcome of the fermentation, and the added cultures may not always behave in the same manner [88,94]. In this regard, the viability of the pitched

organisms as well as the relative pitching rate of the strains within the mixed culture play an important role. Furthermore, in contrast to kettle souring, it is no longer possible to change the growth/fermentation conditions according to the pitched species. These factors lead to more variability in the quality of the end product [88,94]. To increase consistency, brewers sometimes add dregs from a previous, successfully brewed batch [11,92,95]. However, it should be noted that when dregs are preserved, the bacteria often grow faster than the yeasts in the dreg, leading to stuck fermentations. For this reason, it is often recommended to pitch fresh brewer's yeast together with the dreg from a previous batch [89,95].

Traditionally, many mixed-culture beer fermentations have been brewed in Belgium, with the most renowned groups being the Flanders Red Ales and the Flanders Brown Ales [56,74,92]. Flanders Red Ales are authentic sour beers from South-West Flanders, Belgium, with an alcohol percentage between 5 and 6 % ABV. Traditionally, the fermentation is initiated by re-pitching a mixed starter culture from a previous, successful batch of this beer, to the wort in open fermentation vessels. This starter culture typically contains the brewing yeast, together with several LAB species, among which both *Lactobacillus* spp. and *Pediococcus parvulus* have been identified [89,92]. After fermentation, the yeast is harvested and the beer is matured in vertical wooden barrels [74,92]. In a mature brew, molecular identification techniques have revealed the presence of *B. bruxellensis, P. damnosus, P. parvulus* and *A. pastorianus* [56,92]. The resulting beer is known to contain both lactic and acetic notes, as well as sweet, fruit and vanilla flavours [56,74,92]. Nowadays, according to the high council for authentic Flanders Red Ales (Hoge Raad voor Authentieke Vlaamse Roodbruine Bieren, HORARB; a group of brewers that unitedly strives for the European recognition of Flanders Red Ales as a regional product), Red Ales are produced through blending of young top-fermented beer with spontaneously fermented beer that has matured in vertical wooden barrels for at least 18 months [17].

Another traditional Flemish ale is Flanders Old Brown that is mainly produced in East Flanders, Belgium. Grists used for Flanders Brown Ales are similar to Flanders Red Ales, as they also contain a certain dose of darker base malts. The fraction of crystal malts used in Flanders Old Brown is smaller and is partially replaced by dehusked roasted malts that provide a slight cocoa flavour. To add a more complex, dark fruit aroma, additional specialty malts or dark sugar can be added [89]. After wort boiling, a mixed culture resembling the ones used for the production of Flanders Red Ales is pitched [89,92]. Besides the differences in malt composition, another important difference is that Old Brown ales are typically matured in stainless steel tanks instead of wooden foeders. Due to the minimal oxygen availability within the stainless steel tanks, growth of acetic acid bacteria is avoided and the vinegar undertones of Flanders Red Ales are thus not found in Flemish Old Browns. The Old Brown

Ales are therefore considered to have a softer sour character than the Flanders Red Ales, and instead contain notes of dark fruit, malt, sherry, raisins, plums and earth [11,89].

4. Novel souring methods

Whereas the souring methods discussed above already exist for a long time, novel souring techniques are being developed to meet the consumer's increasing demand for sours. One example is acidulation of the malt or mash (Fig. 1). Acidification during the malting process (sour malting) utilising LAB has several advantages, including increased malt yield and improved mash and wort filterability. In addition, LAB help with the malting process by contributing to barley germination and malt modification [19,88]. In sour mashing, *Lactobacillus* bacteria are added to the mash before any hops are added [11,16]. The grain is mashed as usual, then the temperature of the mash is lowered before inoculation with *Lactobacillus* sp. Next, the temperature is held at around 45-50 °C, and the mash is allowed to ferment until it reaches a pH of approximately 3.0-3.7. One disadvantage, however, is that holding the mash at a lowered temperature for an extended period increases the risk of infecting the mash with spoilers leading to off-flavours.

Another innovative souring technique was provided by Osburn *et al*. (2018) [32]. The authors present a primary souring technique without the use of LAB, but instead using a non-conventional yeast species that is able to produce both lactic acid and ethanol during wort fermentation (Fig. 1). Potential yeast species that can be used include *Hanseniaspora vineae*, *Lachancea fermentati*, *Lachancea thermotolerans, Schizosaccharomyces japonicus*, and *Wickerhamomyces anomalus*. Osburn and colleagues refer to this technique as 'primary souring' because the acidification occurs simultaneously with the primary fermentation and is executed by the same organism. This primary souring takes about two to four weeks and can be followed by maturation in wooden barrels or fruit addition. This souring process allows brewers to produce sour beers without the possibility of contaminating the brew environment by introducing LAB into the brew kettle or the fermentor. Furthermore, aroma compounds that would be driven off by boiling the wort after kettle souring are retained in this souring method [32]. In this regard, yeast suppliers are increasingly putting mixed cultures on the market for faster sour beer production.

Because brewers are continuously looking for ways to innovate and differentiate themselves, they have started a widespread experimentation within the sour beer sector. This experimentation has led to a strong interest in the production of innovative sour beers through barrel ageing of conventionally fermented beer, as a way to add additional flavours to the beer, including sourness and/or wood-derived flavours (Fig. 1). Such flavour characteristics may be derived from other beverages that were previously held in the barrel (e.g. various types of wine, sherry and whisky), or

from the wood itself [96]. This methodology gives brewers the opportunity to expand their portfolio with distinctive tasteful products of a noteworthy flavour complexity and nuance without having to change their brewing scheme, while keeping the risk of contaminating the brewing environment low [32]. Although wood fragments (e.g. wooden chips, cubes, spirals) may also be used to add woody notes to the beer, production of sour beer with the complexity and acidic characteristics of a traditional sour involves long term ageing in wooden barrels. The wooden walls of the barrels are slightly permeable, allowing slow ingress of oxygen, which in turn can be used by flavour producing microorganisms residing on the interior surfaces of the barrels [55,71]. However, until now wood maturation of craft beers remains largely a process of trial and error, which does not always lead to an end product with a pleasurable balance between sourness and wood characteristics, but instead it may give rise to unexpected, undesirable or even unpalatable results. For example, the beer style/type does not always match with the wood or the addition of acidity, or the maturation process may lead to unpleasant sourness or the production of off-flavours.

Several physical and chemical reactions occur between the wood and maturing beverage, including micro-oxygenation, extraction of volatile and phenolic compounds from the wood, evaporation or degradation of volatile compounds, and decomposition and esterification of wood (hemi)celluloses and lignins [97-98]. During the ageing process, the wood transfers a series of wood-related aromatic substances to the maturing beer, among which phenolic compounds like vanillin and vanillic acid are highly appreciated for their sensory qualities, imparting notes of sweetness or vanilla-like flavours to the beer, while guaiacol and its derivatives contribute to a smoky flavour [96]. The content and extraction of these compounds has been shown to vary substantially depending on a number of variables, including the wood species, its geographical region of origin, the type of wood seasoning (i.e. drying), the level of toasting, the history of the barrel, the number of times that the barrel has been used, the maturation temperature and period, and the intrinsic parameters of the beverage such as pH and alcohol content [99-103]. Additionally, microorganisms, especially LAB and yeasts, have been found to interact with the wood, which may lead to the (enhanced) production of volatile wood-derived compounds [104]. A profound knowledge of the interactions that occur between the maturing beer, the wood and microorganisms will be needed before this method can be implemented. When successful, it may be expected that improved process control measures can be developed, leading to a new generation of sour beers.

5. Conclusion

The sour beer trend continues to grow, with more breweries producing sour styles and sales continuing to increase. There are many different ways for producing sour beer, offering breweries the

opportunity to pursue new beverages and diversify their portfolio. However, these beers take more time to produce (except for kettle souring) and are more challenging to brew in comparison to regular 'clean' beer. Specific challenges for the sour beer sector include difficulty in reproducing the beers as well as increased risk of contaminating other beers that are brewed in the same environment, as their main microbial components are generally categorised as beer spoilers in non-sour beer styles. Other challenges include the production of new sours with a unique complex flavour profile. Increased knowledge on the microbiology and flavour development in traditional sours may help developing such next generation of sour beers. The advent of novel techniques to better monitor and understand the microbial ecology of these processes, including metabarcoding and functional metagenomics, will definitely aid in this regard. While techniques like metabarcoding are ideally suited to provide insight in the microbial composition and dynamics [73,75], functional metagenomics may help identifying active metabolic pathways and can reveal important functional changes during the process [105].

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7. References

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Figures

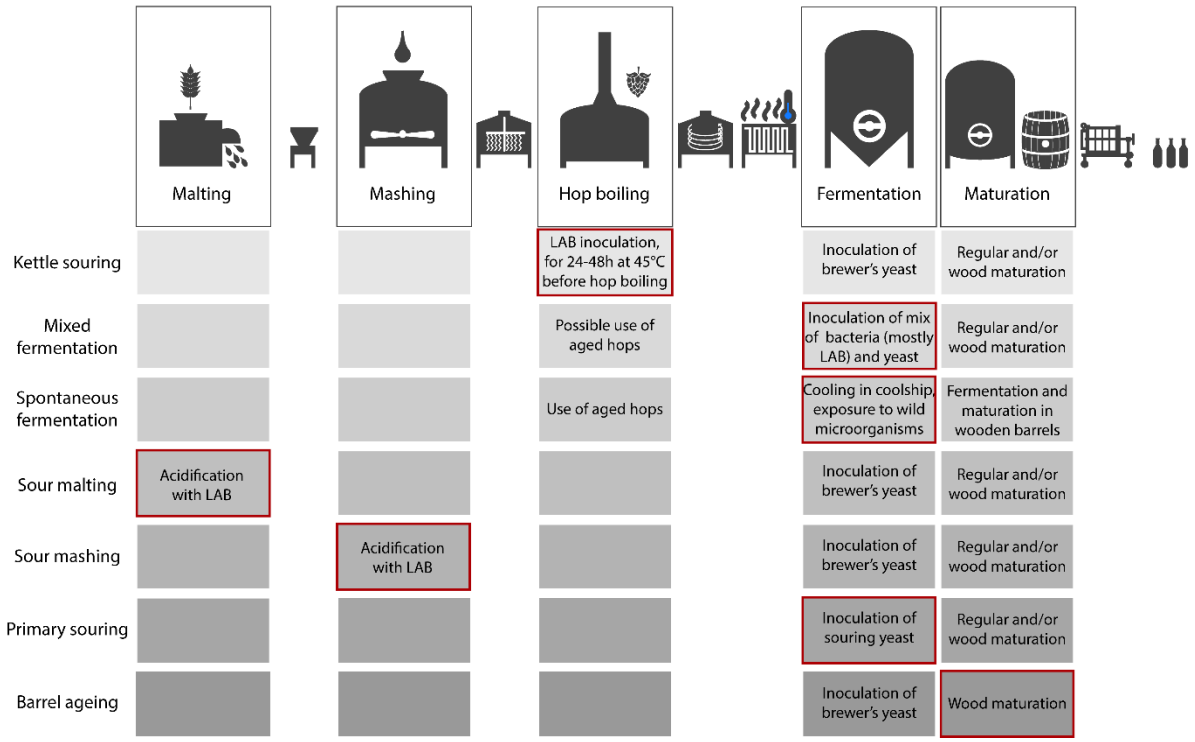

Fig. 1. Overview of different methods for producing sour beers, including (i) kettle souring, (ii) mixed fermentation, (iii) spontaneous fermentation, (iv) sour malting, (v) sour mashing, (vi) primary souring, and (vii) barrel ageing. At the top, a typical beer production process is shown from malting until bottling. The different steps that are the most crucial for sour beer production are indicated in framed boxes. The start of the souring is indicated with a red-framed box.

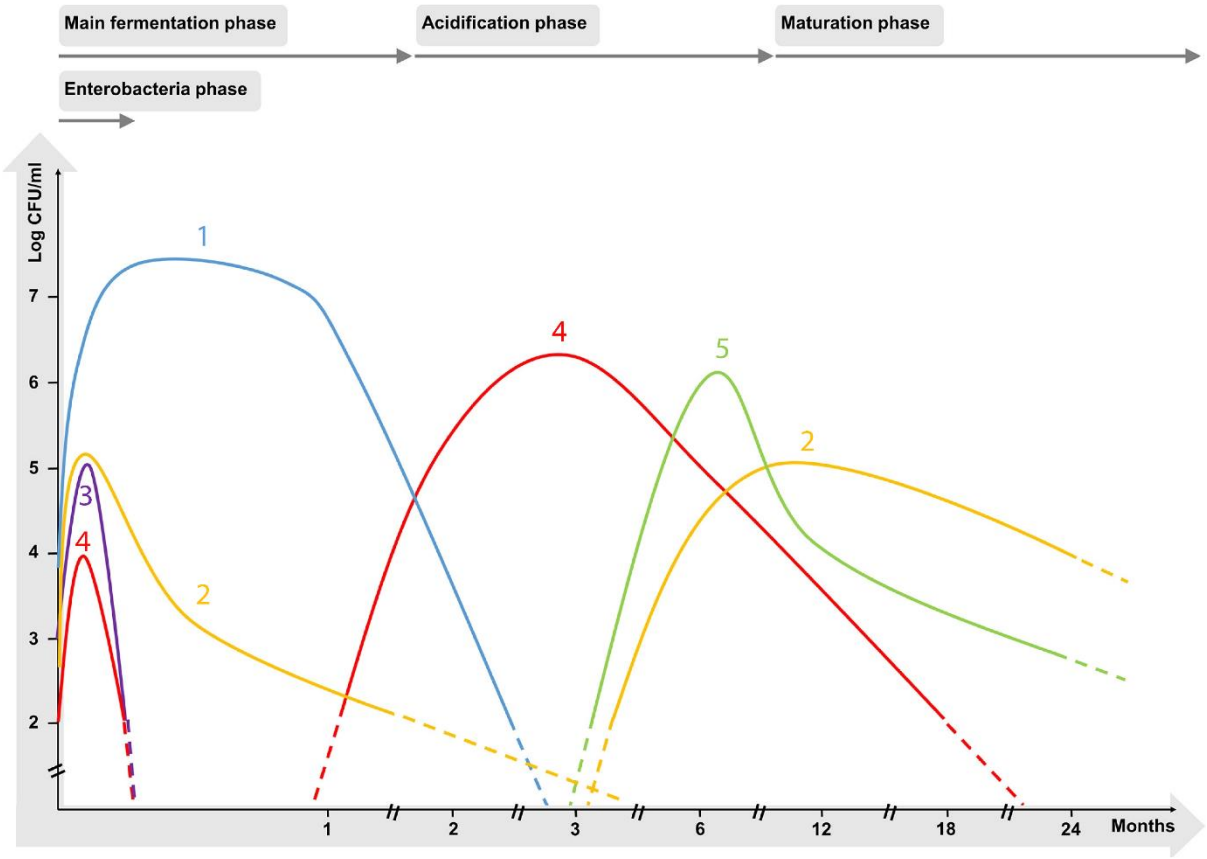

Fig 2. Microbial succession during a Lambic beer production process. Four phases can be distinguished, including (i) an enterobacterial and wild (oxidative) yeast phase, (ii) a main (alcoholic) fermentation phase, (iii) an acidification phase due to the growth of lactic acid bacteria (LAB) and acetic acid bacteria (AAB), and (iv) a maturation phase with the growth of *Brettanomyces* yeasts and LAB. Colours: blue (1): yeasts; orange (2), cycloheximide resistant yeasts; purple (3), Enterobacteriaceae; red (4), AAB; green (5), LAB [adapted from 17].