High-throughput dereplication and identification of bacterial and yeast communities involved in lambic beer fermentation processes

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Freek

22 mei 2014

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List of abbreviations

°P	Degree Plato
AAB	Acetic acid bacteria
ACA	American coolship ales
ACT1	Gene encoding actin
BAS	Barcoded amplicon sequencing
BLAST	Basic local alignment search tool
bp	Base pair
CFU	Colony forming units
CIS	Conductivity under ion suppression
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dnaJ	Gene encoding chaperone protein DnaJ
dnaK	Gene encoding chaperone protein DnaK
ELSD	Evaporative light-scattering detection
ESI	Electrospray ionization
FAME	Fatty acid methyl ester
FID	Flame ionization detector
GC	Gas chromatography
groEL	Gene encoding the 60-kDa chaperonin
HPAEC	High-performance anion-exchange chromatography
HPLC	High-performance liquid chromatography
ITS	Ribosomal internal transcribed spacer
LAB	Lactic acid bacteria
LC	Liquid chromatography
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
Mb	Megabases
MLSA	Multi-locus sequence analysis
m/z	Mass-to-charge ratio
ND	No data / Not determined
nt	Nucleotide
OD	Optical density
PAD	Pulsed amperometric detection
PCR	Polymerase chain reaction
pheS	Gene encoding phenylalanyl-tRNA synthase α-subunit
ppm	Parts per million
PPMCC	Pearson product moment correlation coefficient
qPCR	Quantitative or real-time PCR
RAPD	Random amplified polymorphic DNA
rep-PCR	Repetitive element sequence-based PCR
RNA	Ribonucleic acid
rpoB	Gene encoding DNA-directed RNA polymerase subunit beta
rRNA	Ribosomal RNA

SCFA	Short-chain fatty acid
SH	Static headspace
SIFT	Selected ion flow tube
SPME	Solid-phase microextraction
T-RFLP	Terminal restriction fragment length polymorphism
ULD	Under limit of detection
ULQ	Under limit of quantifitation
UPGMA	Unweighted pair group method with arithmetic averages
VBNC	Viable but non-culturable
VOC	Volatile organic compound

Part I
Introduction

Background and objectives

Spontaneously fermented acidic beers, such as lambic beers, are the product of a fermentation that can take up to three years and is initiated by a multitude of spontaneously inoculated microorganisms from the brewery environment at the time of brewing. These fermentations are poorly characterized from a microbiological point of view and management of the fermentation process relies on an organoleptic quality assessment by the brewer. Microbiological management in these breweries is minimal and aberrant fermentations are not uncommon. These so-called mixed fermentations are a challenging subject for biodiversity studies, since complex communities of yeasts and bacteria are present in a long-lasting process.

Biodiversity studies of fermented foods and beverages mostly focus on lactic acid bacteria and/or yeasts, but comprehensive studies of all microorganisms present during these fermentation processes are rare. Such studies are increasingly performed using culture-independent techniques because traditional culture-dependent approaches cannot cope with the identification of hundreds of isolates to fully characterize the microbiota present. Culture-dependent results are also regarded as less informative, since some microorganisms can be present in a viable but non-culturable (VBNC) state, and as biased, because isolation media favor the cultivation of specific microorganisms only. Nevertheless, the isolation of the microorganisms that dominate the fermentation can give a wealth of information about the genetic and metabolic characteristics that enable these microorganisms to adapt to the fermentation matrix and allows their exploitation as starter cultures in industrial fermentations.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a method routinely used for the identification of bacteria and yeasts in medical microbiology. This technique enables a fast identification based on the comparison of cell protein profiles, provided that an identification database is available. Several commercial systems exist but their databases focus on human pathogens, and therefore MALDI-TOF MS has not been widely adopted in food

microbiology. Yet, the method has an impressive throughput capacity, which has already been applied in a few biodiversity studies for the dereplication of large numbers of isolates (*i.e.*, for the recognition and elimination of isolates that represent the same species from complex communities).

The present study aimed to characterize the bacterial and yeast communities in the lambic beer fermentation process, by exploiting the potential of MALDI-TOF MS as a high-throughput dereplication tool. The main goal of the present study was to characterize the microbial communities at several time points during the lambic beer fermentation process and in several fermentation batches, using both culture-dependent and culture-independent techniques. In this effort, MALDI-TOF MS was used as a high-throughput dereplication tool of the cultivable communities and state-of-the-art taxonomic methods were used to accurately identify their various members. This effort also implied the development of MALDI-TOF MS as a key technology for the high-throughput characterization of all cultivable microbiota. This included optimization of the sample preparation for the generation of high-quality mass spectra of various groups of bacteria and yeasts. A second goal was to compare the microbiota of the lambic beer fermentation process in a traditional lambic brewery located in the Senne river valley with that of an industrial lambic brewery not located in the Senne river valley. Gueuze beers are the product of a spontaneous bottle refermentation of mixtures of young and old lambic beers, mature further after bottling. A final goal was to examine, the effect of aging on the microbiota and metabolites of bottled gueuze beers.

Thesis outline

PART I comprises the background, objectives and outline of this thesis.

PART II presents a comprehensive overview of the literature on beer and spontaneous fermented beverages (**Chapter 1**). In particular, the characteristics of the spontaneous fermentation process for the production of lambic beers is highlighted (**Chapter 2**). Furthermore, an introduction to MALDI-TOF mass spectrometry and its current applications for the identification and dereplication of bacteria and yeasts is discussed (**Chapter 3**).

PART III presents the experimental work performed in the present study. In **Chapter 4**, the spontaneous fermentation processes of traditionally (**Chapter 4.1**) and industrially (**Chapter 4.2**) produced lambic beers are examined. In the frame of these biodiversity studies, two new acetic acid bacteria were isolated and described, namely *Acetobacter lambici* sp. nov. (**Chapter 5.1**) and *Gluconobacter cerevisiae* sp. nov. (**Chapter 5.2**). The last chapter of this part (**Chapter 6**) addresses microbiota and metabolite changes during the aging of bottled gueuze beers.

In **PART IV**, the general conclusions of the results obtained are discussed and an outlook on future applications is given.

The summary of the thesis is given in **PART V**.

Part II
Literature overview

Chapter 1. Beer and spontaneous fermentations

1.1 Malting and beer production

Beer is among the oldest fermented beverages and is the product of a fermented sugar extract from grains. The grain extract used is mostly malted barley, although other grains whether or not malted are used as well (Briggs *et al.*, 2004). Grains such as maize, wheat, sorghum, oat and rice were used in the past and are still used in some beer types (Taylor *et al.*, 2013), but generally malted barley is regarded as the most suitable grain for beer production (Briggs *et al.*, 2004). In the malting process the grains are steeped in water, by which the grains swell and the conditions for their germination are optimized. Due to the germination, the seedling produces and activates several enzymes to enable its growth (Briggs *et al.*, 2004; Palmer, 2006). Of all the enzymes, the amylases are of the highest interest for the brewer. These enzymes convert the endosperm of the grain (mostly starch) to more simple carbohydrates, *i.e.*, glucose, maltose, and maltotriose, that are used by the seedling to support its growth (Palmer, 2006). After five days of germination and growth, the grains are dried and the seedlings are removed, but the enzymes remain in the dried grains (Palmer, 2006). The temperature and duration of drying determines the color of the malt (from light to dark) and of the color and flavor of the resulting beer. The endogenous enzymes that are produced during malting are exploited by the brewers to degrade the starch in the malt during the mashing (lautering) step at the start of the brewing process. During mashing several rests at the optimal temperatures of the enzymes enable proteolysis by proteases, β-glycan breakdown by β-glucanases and starch conversion to maltose by the α- and β-amylases (Briggs *et al.*, 2004). After the mashing process, the mash is filtered and the wort is boiled. After boiling, the wort is cooled and ready for fermentation. The density of wort and beer is generally expressed in degrees Plato (°P).

Currently, several beer types are produced worldwide and they are mainly classified by the type of fermentation (lager *versus* ale beers) and the type of malt used for their production. Two well-known exceptions to the exclusive use of barley malt in the brewing process are wheat beers, which are brewed using wheat malt next to

barley malt, and the so-called Happo-shu or low-malt beers, which contain between zero and 66% barley malt, in addition to several adjuncts (rice, corn, sorghum, potatoes, starches, sugar syrups) (Kawasaki & Sakuma, 2009).

Lager beers are the most commonly produced type of beers (Howard, 2014). A strain of a lager yeast such as *Saccharomyces bayanus* or *Saccharomyces pastorianus* is used for its production (Bokulich & Bamforth, 2013). These yeasts are also known as *Saccharomyces carlsbergensis*, but although still widely used in the literature, this name is trivial and has no scientific meaning (Bokulich & Bamforth, 2013). These yeasts are cryotolerant and fermentation is performed at low temperature (4 to 10°C) for 10 to 14 days (Briggs *et al.*, 2004). At the end of the fermentation process, the yeast cells clump together in a process called flocculation (Bokulich & Bamforth, 2013) and sink to the bottom of the fermentor, mostly a cylindroconical fermentor, used for beer production (Russell, 2006).

Ale beers are mostly produced using strains of *Saccharomyces cerevisiae*, the fermentation temperature is higher compared to the fermentation temperature of lager beers (about 20 to 25°C) and the fermentation requires about 5 to 7 days (Briggs *et al.*, 2004). Most lager brewing companies also produce ales. Also smaller craft breweries produce this type of beer, as it requires less brewing equipment. Fermentation of ale beers can take place at room temperature and does not require a cooling capacity. Additionally, in contrast to lager beers, which are bitter and less diversified in taste, ale beers have a more diversified taste (Howard, 2014).

Both ale and lager beers are pitched with a yeast starter culture after the wort is boiled, cooled and aerated. These starter cultures are generally axenic yeast cultures, but occasionally a mixture of multiple yeasts is used for primary fermentation or secondary bottle fermentation (Vanderhaegen *et al.*, 2003). Primary fermentation refers to the main alcoholic fermentation during the brewing process, whereas secondary bottle fermentation refers to refermentation or reconditioning in bottles. Although these beers are pitched with a starter culture to produce stable products in every fermentation batch, spoilage by growth of wild yeasts or bacteria in the starter cultures, during fermentation or in finished beers, is detrimental for the quality of the beer (Suzuki, 2011).

Fermentations that are performed using a mixture of yeasts and bacteria are generally referred to as mixed fermentations. These wort fermentations can be initiated spontaneously or by a starter culture of yeast and bacteria (Bokulich & Bamforth, 2013). All mixed fermentation beverages are acidic and a little tart and therefore they are refreshing and well appreciated by the consumers (De Keersmaecker, 1996). Below an overview is presented of the main mixed fermentation beverages and their fermentation characteristics.

1.2 Spontaneous mixed fermentation beers

1.2.1 Lambic beers

Acidic lambic beers, obtained by a spontaneous fermentation, are probably the oldest known beers (De Keersmaecker, 1996). These beers are the products of a mixed fermentation that can proceed up to three years and traditionally ferment in wooden casks (Verachtert & Iserentant, 1995). Most knowledge about these beers originates from studies performed in the 1970s through 1990s, in particular by the research group of Prof. em. Hubert Verachtert (Faculty of Bioscience Engineering, KU Leuven, Leuven, Belgium). These studies focus on the microbiota and metabolites in several phases of the fermentation process of lambic beers and on some specific characteristics of the microbiota involved. However, only culture-dependent analyses were performed (Martens *et al.*, 1991, 1992; Shanta Kumara & Verachtert, 1991; Spaepen *et al.*, 1978, 1979; Van Oevelen *et al.*, 1976, 1977; Verachtert, 1983; Verachtert & Dawoud, 1984; Verachtert & Iserentant, 1995; Verachtert *et al.*, 1989).

Lambic beer is traditionally brewed only during the cold winter months, from October until March, because the lambic wort has to be cooled to approximately 20°C within the timeframe of one night. Lambic beer is traditionally produced using about 66% malted barley and 33% unmalted wheat. The use of at least 30% unmalted wheat is regulated by law (Belgisch Ministerie van Economische Zaken, 1993). Traditionally, the lambic wort production starts with a turbid mash method which is a combination of the English infusion and decoction process (Figure 1.2.1). Hot water is added during the English infusion process to increase the temperature

of the mash. During decoction, the brewer boils a part of the mash separately to rupture the starch granules and subsequently reintroduces it into the mash tun to increase the total mash temperature, ensuring the rests at the enzymes' optimal temperatures (Briggs *et al.*, 2004). During turbid mashing, the brewer does not reintroduce the separately boiled wort (called slime) into the mash tun, so that not all of the wort passes through all temperature rests (Figure 1.2.1). The use of unmalted wheat and the turbid mashing step with separate slime cooking results in a wort that is rich in malto-oligosaccharides or dextrins. These dextrins are non-fermentable by conventional *Saccharomyces* brewing yeasts (Shanta Kumara & Verachtert, 1991), but they can be fermented by *Dekkera* (the asexual form of this yeast is named *Brettanomyces*) yeasts that are also present during the maturation of red(brown) acidic ales of South-West-Flanders (Martens *et al.*, 1997). The wort is boiled for 3 h, which is a long period compared to other beer types, and a high amount of aged hops is added to enhance the microbiological stability of the beer without resulting in a bitter hop flavor (Verachtert & Derdelinckx, 2005; Vriesekoop *et al.*, 2012). After wort cooking, the wort is cooled in an open vessel, called the cooling tun or coolship, which is mostly located in the attic of the brewery (Figure 1.2.1). After overnight cooling, the wort is inoculated by the microbiota of the environment. As lambic beers were originally only produced in the Senne river valley (southwest of Brussels) and in the southeast of Brussels, it was believed that the responsible microbiota were present in the air of this region (Verachtert & Iserentant, 1995). To which extent this claim is genuine is not really known.

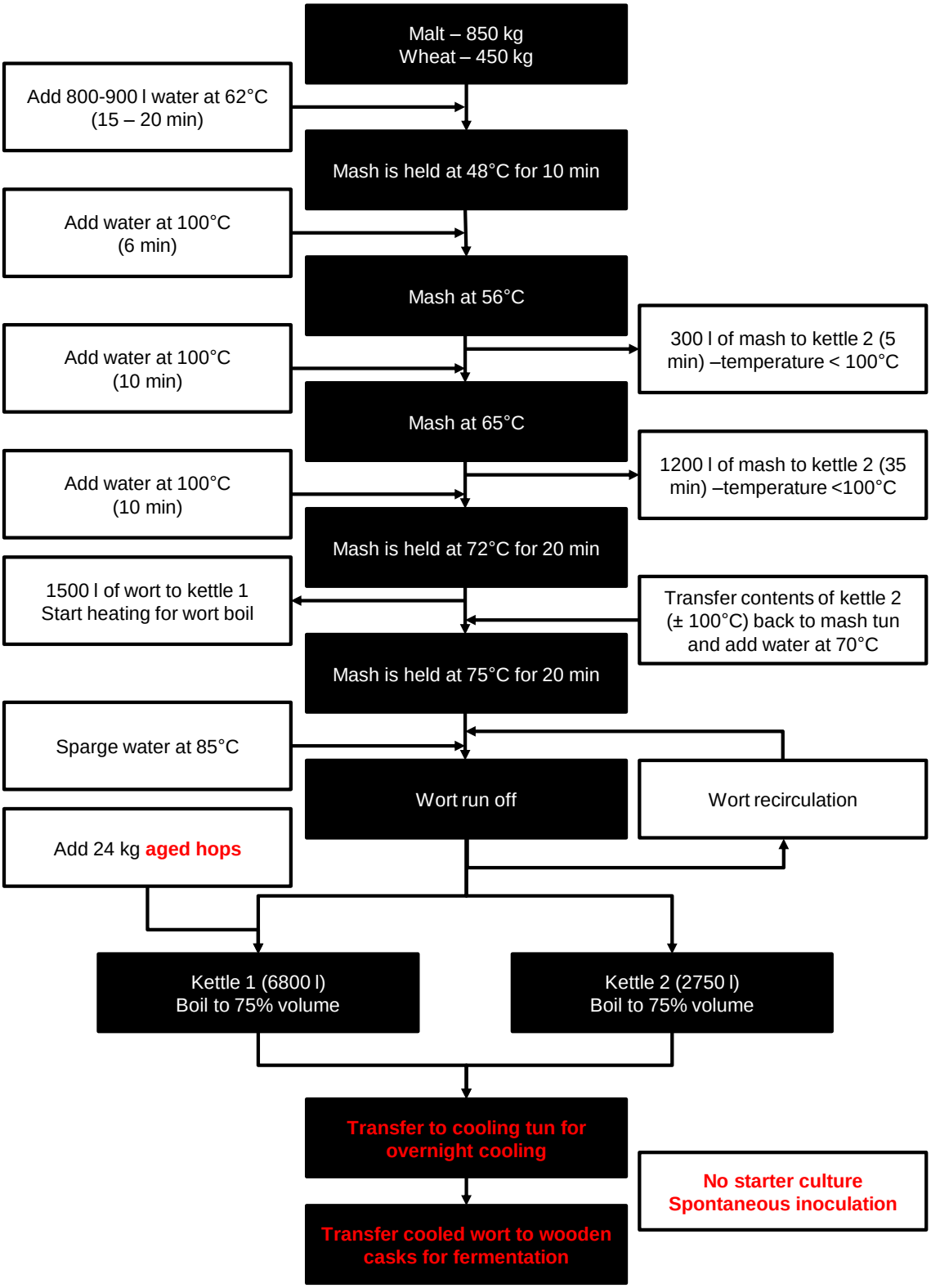


Figure 1.2.1 Example of a brewing scheme in a traditional lambic beer brewery, making use of turbid mashing and two boiling kettles. The typical lambic beer fermentation process characteristics, next to the unusual mashing scheme, are indicated in red font.

The lambic beer fermentation process has been reported to consist of four phases (Figure 1.2.2) (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). The fermentation starts with an enterobacterial phase, which is initiated by bacteria of the *Enterobacteriaceae* family that are introduced in the wort during the overnight cooling in the cooling tun (Martens *et al.*, 1991; Van Oevelen *et al.*, 1977). This enterobacterial phase is dominated by several species and ends after about one month (Martens *et al.*, 1991). The taxonomy of several of these species has been modified since their description in previous studies of the lambic beer fermentation process. Below, the up-to-date species names are reported but the corresponding names that were used in the original papers are shown between square brackets. The dominant *Enterobacteriaceae* include *Klebsiella pneumoniae* [*Klebsiella aerogenes*] (Brisse *et al.*, 2006), *Enterobacter cloacae*, *Enterobacter aerogenes*, *Escherichia coli*, *Citrobacter freundii*, *Shigella sonnei* and *Hafnia alvei* (Martens *et al.*, 1991). The number of *Enterobacteriaceae* cells present in brewery air is however low, so it has been hypothesized that wort inoculation during cooling is not homogeneous and bacteria are probably adsorbed to particles present in the air (Martens *et al.*, 1991). The disappearance of the *Enterobacteriaceae* after about one month of fermentation is explained by the depletion of glucose, the increase in ethanol concentration and the decreased pH of the wort (Martens *et al.*, 1991). Oxidative yeasts, such as *Hanseniaspora uvarum*, are the main yeast species present during the enterobacterial phase of the lambic beer fermentation process (Van Oevelen *et al.*, 1977). *Hanseniaspora uvarum* has a low fermentative capacity and is commonly found during the spontaneous fermentation of wines and ciders where its contribution to flavor complexity is increasingly appreciated (Bezerra-Bussoli *et al.*, 2013).

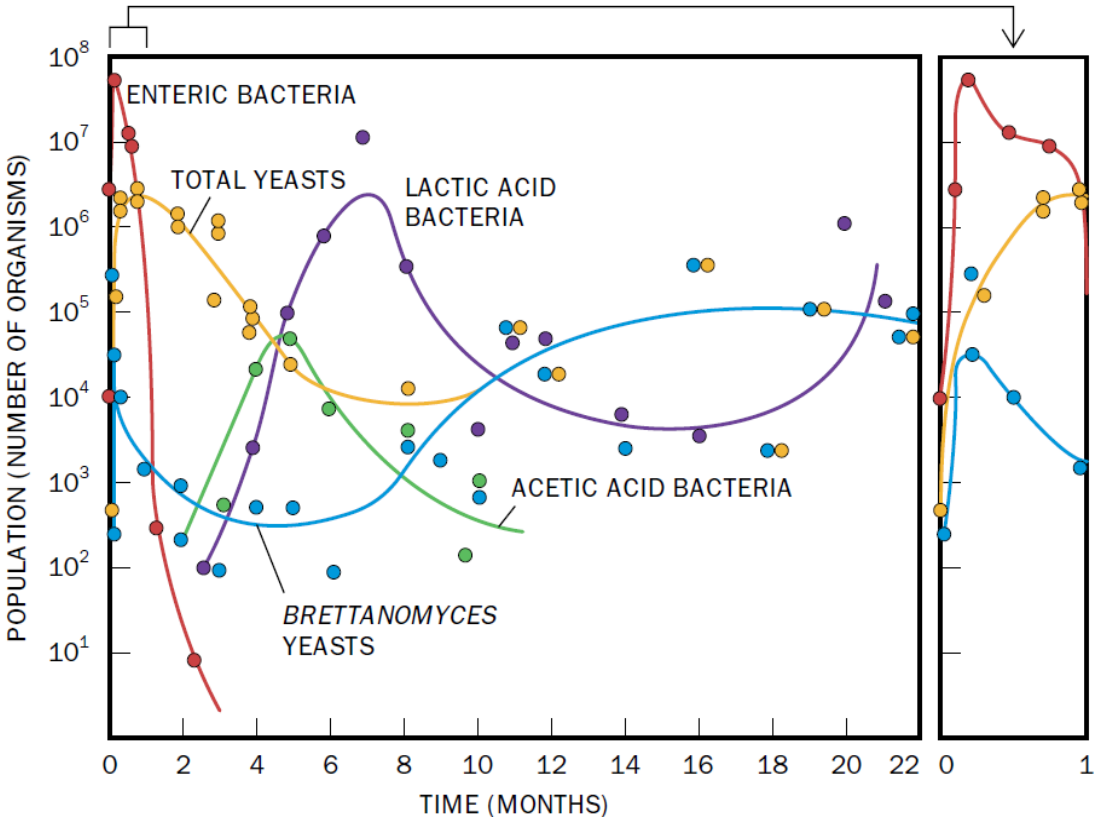


Figure 1.2.2 The lambic beer fermentation phases as described by Verachtert and Iserentant (1995). The figure was adapted from De Keersmaecker (1996).

The second phase of the lambic beer fermentation process is referred to as the main or ethanol fermentation phase. *Saccharomyces* spp. dominate the fermentation process from month 1 until month 4 (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). *Saccharomyces cerevisiae* and *S. bayanus/pastorianus* are identified as the main actors during this stage (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). After the main fermentation phase, oxidative yeasts, *i.e.*, *Cryptococcus* spp., *Candida* spp., *Pichia* spp. and *Torulopsis* spp. form a pellicle at the top of the liquid and serve as an oxygen barrier (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995).

After the depletion of the carbon sources that can be fermented by *Saccharomyces* spp. (simple sugars up to maltotriose), the fermentation enters a next phase referred to as the acidification phase. During this phase, mainly lactic acid bacteria (LAB) (*Pediococcus damnosus* [*Pediococcus cerevisiae*] and *Lactobacillus brevis*) produce

large amounts of lactic acid, which is a typical metabolite and flavor of lambic beers (Van Oevelen *et al.*, 1976).

After ten months, the bacterial counts decrease and a new phase in the lambic beer fermentation process is initiated by the increase of *Dekkera* spp. In this context, *Dekkera bruxellensis* is worth mentioning, as its name refers to the environment of Brussels where lambic beer production originates from. During this final phase, which is called the maturation phase, several specific metabolites and flavor compounds are produced by a synergistic action of LAB and *Dekkera* yeasts (Shanta Kumara & Verachtert, 1991; Van Oevelen *et al.*, 1976, 1977; Verachtert & Iserentant, 1995). Such components include the esters ethyl acetate and ethyl lactate, but also the long-chain fatty acids and their esters such as ethyl caprylate and ethyl caprate (Spaepen *et al.*, 1978). Only minimal concentrations of ethyl caprate are present in most other beers and this can thus be considered as a typical aroma component of lambic beers (Spaepen *et al.*, 1978). However, a beer produced by the mixed fermentation of a LAB-harboring pitching yeast with a secondary cask fermentation (see Chapter 1.3.1) contains comparable concentrations of these long-chain fatty acids and their esters (Spaepen *et al.*, 1979).

Dekkera spp. in combination with LAB degrade the residual dextrins that are not fermented by *Saccharomyces* spp. (Shanta Kumara & Verachtert, 1991). Lambic beers reach a high attenuation during the maturation phase, resulting in a residual density that may be below 1°P (Shanta Kumara & Verachtert, 1991; Verachtert & Iserentant, 1995). Super-attenuation or overattenuation was already described by Andrews and Gilliland (1952). In the latter paper, the authors pointed out that a primary attenuation limit, typical for an axenic *S. cerevisiae* culture, and a secondary attenuation limit, typical for an axenic *D. bruxellensis* culture, can still be overcome by the use of a mixed culture of yeasts and bacteria (Andrews & Gilliland, 1952). Hence, there is a synergistic effect of the yeast and bacterial cultures during the degradation of dextrins and starch (Andrews & Gilliland, 1952). A similar finding was made by Shanta Kumara and Verachtert (1991), who demonstrated that *Dekkera* is the main contributor to the super-attenuation of lambic beers, but its effect is more pronounced in a mixed culture with *Pediococcus* (Shanta Kumara & Verachtert, 1991). *Dekkera* produces α-glucosidase, an enzyme capable of dextrin

degradation (Shanta Kumara & Verachtert, 1991). This α-glucosidase shows intracellular as well as extracellular activities and acts by removing a single glucose molecule from the dextrin polymer (De Cort *et al.*, 1994; Shanta Kumara *et al.*, 1993). The enzyme is fast acting, as under optimal conditions malto-oligosaccharides shorter than maltotetraose are not found in the presence of the enzyme (Shanta Kumara *et al.*, 1993). The low pH of lambic beers, however, may explain the slow process of overattenuation *in situ* in lambic beers (Shanta Kumara *et al.*, 1993).

Besides lambic beers, lambic brewers produce gueuze and fruit lambic beers, while gueuze blenders ('geuzestekers' in Dutch) buy lambic beers from lambic beer brewers to produce their own beers. Gueuze beers are produced by the refermentation of a mixture of young lambic beer that contains a lot of dextrins and old lambic beer that contains dextrin-hydrolyzing microorganisms (Verachtert & Iserentant, 1995). The pellicle yeasts survive in the initial stages of the refermentation process, although they do not multiply (Verachtert & Iserentant, 1995). Their presence can be explained by the breaking of the pellicle during the emptying of the casks.

1.2.2 American coolship ales

Acidic beers are currently attracting interest worldwide, especially in the USA (Bokulich *et al.*, 2012). In the American craft brewing industry, which is the collective name for small- to mid-scale breweries, the production of lambic beers is mimicked and the resulting beers are called American coolship ales (ACA) (Bokulich *et al.*, 2012). Breweries adopt the open cooling vessels and fermentations are performed in wooden casks or stainless steel fermentation tanks (Bokulich *et al.*, 2012). Recently, the microbiota of an ACA fermentation process has been studied using primarily culture-independent techniques (Bokulich *et al.*, 2012). Culture-dependent techniques were limited to the use of two aerobically incubated bacterial isolation media, the collection of two bacterial isolates per colony morphotype and their identification using 16S rRNA gene sequence analysis (Bokulich *et al.*, 2012). The latter technique is not sufficiently discriminatory for accurate species level identification (Cleenwerck *et al.*, 2010; De Bruyne *et al.*, 2007, 2008; Mollet *et al.*, 1997; Naser *et al.*, 2007). The community diversity of multiple barrels of multiple

fermentation batches was studied through bar-coded 16S rRNA gene amplicon sequencing (BAS) and terminal restriction fragment length polymorphism (T-RFLP), a technique with a sensitivity similar to that of denaturing gradient gel electrophoresis (DGGE) but with a higher automation capacity (Bokulich & Mills, 2012). Cell numbers were studied using quantitative polymerase chain reaction (PCR) assays (Bokulich *et al.*, 2012).

Bokulich *et al.* (2012) have reported fermentation phases in the production of ACA similar to those of lambic beer fermentation processes. *Enterobacteriaceae* and some oxidative yeasts dominate the first phase of fermentation, but *S. cerevisiae* is from the start of the fermentation the most dominant yeast (Bokulich *et al.*, 2012). Members of the *Enterobacteriaceae* family are dominant up to 1 month, but some species can be isolated up to 12 weeks in the fermentation process (Bokulich *et al.*, 2012). ACA are seasonal products of these breweries and it is likely that *S. cerevisiae* is enriched in the brewery environment by its use in other types of beers produced in these breweries, probably explaining their early dominance during ACA fermentation (Bokulich *et al.*, 2012). From week 4 onwards, LAB are the most dominant bacteria; *Lb. brevis* is the only bacterial species isolated during the whole fermentation process, but *Pediococcus* is the most dominant LAB from week 4 onwards based on T-RFLP and BAS analyses (Bokulich *et al.*, 2012). *Dekkera* is detected from week 11 onwards in minor numbers, but represents a dominant yeast after 1 year. Minor numbers of acetic acid bacteria (AAB) are found during the whole fermentation process (Bokulich *et al.*, 2012). Interestingly, fermentation profiles are very similar between batches and between barrels, even when barrels have a different origin, are new or are reused (Bokulich *et al.*, 2012).

1.3 Other mixed fermentation beers

1.3.1 Acidic ales of South-West and -East-Flanders

West of the Scheldt, non-spontaneous mixed acid beer fermentation was originally applied for beer production based on “Gruyt” (basically herbs) in the absence of hops. Non-spontaneous mixed fermentation is used in two types of Belgian acidic ales, namely the red acidic ales of South-West-Flanders (Roeselare) and the redbrown acidic ales that are produced in South-West- and South-East-Flanders.

The red acidic ales of South-West-Flanders were traditionally produced using an in-house starter culture that contains yeasts and LAB by the reuse of the starter in every fermentation batch (Martens *et al.*, 1997). The number of bacteria is kept low by acid (mainly phosphoric acid) washing of the yeast suspension (Martens *et al.*, 1997). These ales have a vinous acidic character and their production starts with mashing of malted barley and cooked unmalted maize (Martens *et al.*, 1997). The main ethanol fermentation phase proceeds for about seven days and is followed by a secondary lactic acid fermentation phase that proceeds for another four to five weeks (Martens *et al.*, 1997). Finally a long maturation phase of 20 to 24 months occurs in large oak wooden casks and *P. damnosus* and *Pediococcus parvulus*, together with *Dekkera* spp. and AAB (due to natural micro-oxygenation of the wood) are an active part of the microbiota during this phase (Martens *et al.*, 1997). Remarkably, this 'tandem' fermentation process (the main fermentation followed by cask maturation) is imported in Belgium around 1860 from Northern England, where it was used for the production of old English Porter beer (Claussen, 1904; Martens *et al.*, 1997). *Dekkera* spp. were present in old English Porter beer until the production process was altered and stainless steel fermentation vessels were used instead of wooden casks, indicating a need of these yeast species to be in contact with the wood (Martens *et al.*, 1997). The production of redbrown acidic ales is very similar, with the fermentation being initiated by repitching of LAB-harboring yeast starter cultures and the use of open fermentation vessels from which the yeast is harvested at the end of the fermentation, followed by maturation in oak vessels (Martens, 1996; Martens *et al.*, 1997). Redbrown acidic ales differ from old brown ales in that the latter beers are not oak-aged, but they are also produced in South-East-Flanders.

1.3.2 Berliner Weisse

Berliner Weisse is a trademarked beer, only allowed to be brewed in Berlin (Burberg & Zarnkow, 2009). The mash is made with a 2:1 to 3:1 ratio of wheat malt and barley malt, has a low initial density of around 7 to 9°P and the level of carbonation in the finished product is high (Burberg & Zarnkow, 2009; Verachtert & Derdelinckx, 2005). Traditionally, the wort is not boiled, but rather cooled directly after lautering, with the hops being added during the mashing, although in modern Berliner Weisse

production a heating step is incorporated (Burberg & Zarnkow, 2009; Verachtert & Derdelinckx, 2005). The fermentation is traditionally carried out in an open fermentor by the reuse of a yeast culture that harbors LAB and generally has a 4:1 to 6:1 yeast:LAB ratio (Burberg & Zarnkow, 2009). The secondary fermentation is carried out in bottles by the addition of Kräusen (a foam that is formed on top of the fermenting beer) to the green beer in the bottles, after which the bottles are stored for a duration of three weeks to three years (Burberg & Zarnkow, 2009). The resulting beer is 95% attenuated and has a pH of 3.0 (Burberg & Zarnkow, 2009). More recently, these beers are also produced by dividing the wort into two parts, after which one half is fermented with a homofermentative *Lactobacillus* and the other half with an ale yeast (Verachtert & Derdelinckx, 2005).

1.3.3 Sorghum beers

On the African continent, tropical cereals such as maize and sorghum are used for the production of beers, since barley (a cool-season, temperate cereal) cultivation is not viable (Taylor, 2003). Moreover, sorghum is the only viable food grain in regions with semi-arid and sub-tropical climatic conditions (Taylor, 2003). Sorghum beers are widely produced in sub-Saharan Africa and are well-known under their local names, such as burukutu (Nigeria), tchapalo (Ivory Coast), dolo (Burkina Faso), pito (Ghana), munkoyo (Zambia) and bili bili (Chad) (Abegaz, 2007; Faparusi *et al.*, 1973; Lyumugabe *et al.*, 2010, 2013; Marcellin *et al.*, 2009; N'Guessan *et al.*, 2011; Nanadoum & Pourquie, 2009; Sawadogo-Lingani *et al.*, 2007; Schoustra *et al.*, 2013; Taylor, 2003; van der Aa Kühle *et al.*, 2001; Zulu *et al.*, 1997). Sorghum beers are traditionally opaque, but some commercial clear versions exist as well (Hibbett & Taylor, 2013; Nanadoum & Pourquie, 2009). These beers are mostly produced by the women of agropastoral families (which perform agriculture by growing crops and keeping livestock) on a weekly basis and are often sold (Dancause *et al.*, 2010). Production methods differ between countries and recipes are often household-specific (Taylor, 2003).

As an example, the production of bili bili starts with a malting of the sorghum grains; steeping, germination and drying of the grains takes about one week (Nanadoum & Pourquie, 2009). After the milling of the sorghum malt, the flour is steeped for at

least 2 h, after which the supernatant is removed from the residue (Nanadoum & Pourquie, 2009). The residue is cooked for an average of 2 h to ensure gelatinization of the starch (Nanadoum & Pourquie, 2009). The thick mash of the residue is mixed with the supernatant at a temperature of 65 to 70°C. Subsequently, this mixture is left to cool overnight in open air (Nanadoum & Pourquie, 2009). During overnight cooling, the wort acidifies by the activity of LAB, which are spontaneously inoculated from either the sorghum malt or from the surrounding air (Nanadoum & Pourquie, 2009). Alternatively, in some sorghum beers, the LAB are introduced by backslopping (Taylor, 2003). In dolo and pito beer, *Lactobacillus fermentum* is reported as the predominant LAB (Sawadogo-Lingani *et al.*, 2007), whereas in burukutu beer the predominant LAB are identified as *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lb. brevis* and *Lactobacillus delbrueckii* (Faparusi *et al.*, 1973). After boiling the acidified wort, either a dried yeast obtained from a previous fermentation or a part of the previous beer is added to start the fermentation, in which *S. cerevisiae* dominates (Nanadoum & Pourquie, 2009). This yeast species is dominant in all sorghum beer main fermentation phases, which take place overnight (Faparusi *et al.*, 1973; N'Guessan *et al.*, 2011; Nanadoum & Pourquie, 2009; van der Aa Kühle *et al.*, 2001). The next morning, the beer is ready to be sold and has a shelf-life of about 1 day (Nanadoum & Pourquie, 2009). In burukutu beer, also a high number of AAB is found, which are now all classified as *Gluconobacter oxydans* (Faparusi *et al.*, 1973).

1.3.4 Other cereal-based beverages

Chica

Chica is a traditional beverage produced in South America and was already produced by the Incas (Vallejo *et al.*, 2013). The production starts by steeping and germination of maize grains to get a sort of maize malt (Vallejo *et al.*, 2013). Alternatively, the maize is chewed to convert the starch into fermentable sugars by the action of the amylase in saliva (Gomes *et al.*, 2009). Besides maize, also cassava and cane sugar can be used in the production of chica (Gomes *et al.*, 2009). After cooking, the mixture is poured into clay pots, which are buried, and the liquid is left to ferment for one up to six days (Gomes *et al.*, 2009; Vallejo *et al.*, 2013). The end-

product of the fermentation primarily contains *S. cerevisiae* yeasts (Vallejo *et al.*, 2013). It is unknown if bacteria are involved in the fermentation of chica. Since the clay fermentation pots are reused for every fermentation and no bacteria or yeasts are pitched to start the fermentation, the microbiota involved in the fermentation of chica probably penetrates into the clay surface; a new yeast species, *Candida theae,* was recently isolated from chica clay fermentation pots found in a tomb (Chang *et al.*, 2012).

Boza

Boza is a fermented beverage that is produced in Turkey and other Balkan countries (Kabak & Dobson, 2011). Its production starts with the boiling of a mixed flour of millet, rice and wheat, and water (Kabak & Dobson, 2011). After filtering, the supernatant is inoculated with a part of a previous fermentation batch of boza, sourdough or yoghurt (Altay *et al.*, 2013; Kabak & Dobson, 2011). The mixture ferments at 30°C for 24 h (Altay *et al.*, 2013; Botes *et al.*, 2007; Kabak & Dobson, 2011). The microbiota present during the fermentation can vary significantly, depending on the inoculum and region of production. Generally, a variety of LAB is found during these fermentations, including *Lactobacillus* and *Leuconostoc* spp. (Altay *et al.*, 2013; Botes *et al.*, 2007; Kabak & Dobson, 2011). Several yeast species are found, but in contrast to other fermented cereal-based beverages, *Saccharomyces* spp. are not always found (Botes *et al.*, 2007). Instead, *Candida* spp. and *Pichia* spp. can be the dominant yeasts in boza fermentation (Altay *et al.*, 2013; Botes *et al.*, 2007; Kabak & Dobson, 2011). Opportunistic pathogenic yeasts have been isolated from Bulgarian boza, highlighting the need for starter cultures (Botes *et al.*, 2007). The shelf-life of boza is about 15 days and it is acceptable for consumption until the pH drops below 3.5 (Altay *et al.*, 2013).

1.4 Other mixed fermentation beverages

1.4.1 Natural cider

Ciders are fermented beverages produced from apple juice. The apples are crushed in mills and the juice is extracted by batch mechanical presses (Morrissey *et al.*, 2004). For traditional fermentation, must is not inoculated with yeasts, but rather a spontaneous fermentation process occurs that is initiated by the indigenous yeasts

of the apples (primarily *H. uvarum* and *D. bruxellensis*) and the press house (primarily *S. cerevisiae* and *D. bruxellensis*) (Morrissey *et al.*, 2004). Irish cider fermentation requires about 25 days in wooden casks and the fermentation process is divided into a fruit yeast fermentation phase (*H. uvarum*; up to 5 days), followed by a fermentation phase with *S. cerevisiae* from day 5 to 12 (Morrissey *et al.*, 2004), and finally, a maturation phase that is dominated by *D. bruxellensis* from day 12 and is detectable up to 9 months (Morrissey *et al.*, 2004). In parallel, a malolactic fermentation takes place (see below). Dependent on the apples used for must production, the main and malolactic fermentations together can proceed up to 105 days in the case of French cider (Salih *et al.*, 1988). In other cider fermentations, the main fermentation phase can be initially dominated by *S. bayanus*, after which *S. cerevisiae* becomes dominant at the final stage of the main fermentation phase (Suárez Valles *et al.*, 2007). *Dekkera anomala* has been reported in bottled French cider (Coton *et al.*, 2006), but *Dekkera* spp. are not always present in French cider fermentations (Laplace *et al.*, 2001; Salih *et al.*, 1988).

The maturation of the ciders can be performed in stainless steel vats, since the use of wooden presses and fermentation casks sometimes results in uncontrolled fermentations and variations in quality (Morrissey *et al.*, 2004). However, when using a new stainless steel pneumatic press and stainless steel fermentors, the fermentation is sometimes incomplete and with inconsistent flavor profiles, highlighting the importance of the indigenous yeasts from the manufacturer's traditional press house (Morrissey *et al.*, 2004). The pressing method of the apples influences the fermentation. Traditional pressing using a wooden press lasts for three days and allows *Saccharomyces* yeasts, present in low numbers on the apples, to enrich in the initial must (Suárez Valles *et al.*, 2007). In contrast, pneumatically pressed apple juice (8 h) is initially rich in *Hanseniaspora* spp., after which *Saccharomyces* spp. become dominant (Suárez Valles *et al.*, 2007). It should be mentioned, however, that the latter study only focused on the pace of pressing and did not take into account the material composition of the presses, nor the fact that the traditionally pressed apple juice was fermented in a wooden cask in contrast to the pneumatically pressed must that was fermented in a stainless steel fermentor (Suárez Valles *et al.*, 2007). Indeed, wooden fermentation casks can introduce a

microbiota that has penetrated the wood during a previous fermentation (Swaffield & Scott, 1995). For instance, apple musts obtained by traditional and pneumatic pressing and fermented in wooden and stainless steel fermentors have been compared (del Campo *et al.*, 2003). Traditional presses showed the most profound influence on the rate of fermentation and malolactic fermentation, irrespective of the type of fermentor used (del Campo *et al.*, 2003). When the must was produced with a pneumatic press, the pace of the fermentation in the wooden cask was still higher compared to the fermentation in the stainless steel fermentor (del Campo *et al.*, 2003).

During the malolactic fermentation the tart L-malic acid, which occurs naturally in the apple must, is converted into the softer-tasting L-lactic acid (Fugelsang & Edwards, 2007). *Acetobacter aceti*, *Lb. plantarum* and to a lesser extent *Oenococcus oeni* are present during the malolactic fermentation of Irish ciders (Swaffield & Scott, 1995). In contrast, in French cider, *O. oeni* is the only LAB found throughout the fermentation (Salih *et al.*, 1988). *Oenococcus oeni* is currently also used as starter culture for the production of Brazilian ciders (Dierings *et al.*, 2013).

1.4.2 Natural wine fermentation

Similar to natural cider fermentation, natural wine fermentation is not initiated by the addition of a yeast starter culture to the must. Instead, the fermentation is started by the indigenous yeasts present on the grapes or introduced by the equipment used in the vinery (Bezerra-Bussoli *et al.*, 2013; Diaz *et al.*, 2013; González-Arenzana *et al.*, 2012a; Ocón *et al.*, 2010b). Typically, fermentation proceeds about 9 days and should not last longer than 20 days (Diaz *et al.*, 2013; Ocón *et al.*, 2010b). As is the case with cider, non-*Saccharomyces* yeasts (mostly less ethanol-tolerant) dominate the first phase of the fermentation process and *S. cerevisiae* or *S. bayanus* become dominant towards the end of the fermentation process (Bezerra-Bussoli *et al.*, 2013; Diaz *et al.*, 2013; Ocón *et al.*, 2010b; Wang & Liu, 2013). The non-*Saccharomyces* yeasts mostly belong to the genus *Hanseniaspora*, with *H. uvarum* as one of the most commonly present yeast species on the surface of grapes (Ocón *et al.*, 2010a; Wang & Liu, 2013). Since this latter species has a high ethanol tolerance, it can also be detected at the end of the

alcoholic fermentation phase (Ocón *et al.*, 2010b; Wang & Liu, 2013). The grape temperature at the time of pressing plays a key role in the success of the spontaneous fermentation of natural wines, since a higher temperature favors a rapid proliferation of *S. cerevisiae* (Diaz *et al.*, 2013).

As for natural cider fermentations, less attention has been drawn to the bacterial microbiota present during the fermentation of natural wines. Producers of spontaneously fermented wines increasingly realize the importance of a well-performed malolactic fermentation on the wine quality and therefore natural malolactic fermentations are studied to obtain starter cultures (González-Arenzana *et al.*, 2012a, 2012b). *Oenococcus oeni* is the major bacterial species present during all phases and the only species at the final phase of natural malolactic fermentation in Spanish Tempranillo wines (González-Arenzana *et al.*, 2012a, 2012b). This species is already detected during the alcoholic fermentation phase, although also other LAB are initially present, including primarily *Lb. plantarum*, *Lactobacillus mali* and *Leuc. mesenteroides* (González-Arenzana *et al.*, 2012a, 2012b, 2013a, 2013b). Several *O. oeni* strains can be present during the malolactic fermentation, but in most cases only one strain is dominant (González-Arenzana *et al.*, 2013a, 2013b). The wine temperature at the end of the alcoholic fermentation determines the length of the subsequent malolactic fermentation, which ranges from 11 to 239 days (González-Arenzana *et al.*, 2012a, 2012b, 2013a, 2013b).

Chapter 2. Characteristics of lambic beer brewing and the microbiota involved

2.1 Use of old hops and wooden casks during lambic beer brewing

Lambic beer brewing has some unique characteristics compared to other beers. These include the use of malted barley and unmalted wheat, the unusual mashing scheme, the prolonged wort boiling, the overnight cooling of the wort in the cooling tun and its spontaneous inoculation, and the use of old hops. Furthermore, these beers are traditionally left to ferment and mature in wooden casks that were used for wine or cognac production previously.

Hops are widely used in virtually all beers; it enhances the microbiological stability and gives an appreciated bitterness to the beer palate (Briggs *et al.*, 2004). Hops can also generate fruity flavors through the presence of hop oils (Bamforth, 2000). Hop resins, subdivided into α-acids (*e.g.*, humulones) and β-acids (*e.g.*, lupulones) are the main hop compounds with antimicrobial activities (Sakamoto & Konings, 2003). These compounds also enhance the foam stability of beer (Bamforth, 2000). During boiling, the α-acids isomerize to *iso*-α-acids which are more bitter and more easily dissolved in beer than the corresponding non-isomerized forms (Sakamoto & Konings, 2003). In contrast, β-acids are poorly soluble in beer and have no important role in the bittering properties of hops under normal brewing conditions (Sakamoto & Konings, 2003).

In lambic beer brewing, the bitter hop flavor could interfere with the acidic tart flavor. For that reason, traditional lambic beer brewers use only aged hops. These hops lost most of their bittering capacities due to the oxidation of the humulones to humulinic acids but keep their antibacterial properties (Mikyška & Krofta, 2012). However, also fresh hops can be used for lambic beer brewing (Verachtert & Derdelinckx, 2005).

Next to the use of old hops, the use of old wine barrels is typical for the production of traditional lambic beers. Wine is stored in casks to mature, during which time

polyphenols and tannins extracted from the wood give the wine an appreciated flavor and high quality (De Rosso *et al.*, 2008; Garde-Cerdán & Ancín-Azpilicueta, 2006; Guzzon *et al.*, 2011). The flavor of the wood is mainly determined by lactones, volatile phenols and phenolic aldehydes (Sterckx *et al.*, 2012). In addition, wines undergo a natural clarification and a micro-oxygenation, which improves the aging process (Garde-Cerdán & Ancín-Azpilicueta, 2006). Once a certain amount of compounds have been extracted from the wood, these barrels are of no further use for the winemakers and are even considered a hazard, since they are considered to be a major factor in the conservation of *Dekkera* infections in wineries (Licker *et al.*, 1998). For the production of lambic beer (and cider), these wood compounds are of no interest (del Campo *et al.*, 2003), and the used barrels can be purchased at a reduced price compared to new barrels. Additionally, some of the wine flavor compounds, which penetrated the wood, can diffuse into the beer.

The beer fermentation process is less controlled when wooden casks are used instead of stainless steel fermentors, since wooden casks are very difficult to clean (Barata *et al.*, 2013; Guzzon *et al.*, 2011; Puig *et al.*, 2011; Suárez *et al.*, 2007). Therefore, wooden casks are only used for the production of mixed fermentation beers, where the brewer acknowledges and exploits the microbiota harbored in the cask wood. Indeed, microorganisms are capable of penetrating wooden surfaces up to 1.2 cm within a period of 2 weeks (Swaffield & Scott, 1995; Swaffield *et al.*, 1997). Likewise, cells of *D. bruxellensis* have been detected at depths up to 8 mm into the wood, which corresponds with the maximum level of wine penetration (Barata *et al.*, 2013; Wedral *et al.*, 2010). Because of the depth of microorganism penetration, cask cleaning does not remove all microorganisms present (Barata *et al.*, 2013) and biofilms can be formed within the wood that protects the microorganisms from cleaning procedures and other stresses (Guzzon *et al.*, 2011). Additionally, a natural micro-oxygenation occurs in wooden casks and it is estimated that, depending on its porosity, 10 to 45 mg O_2/L per year can diffuse through the wood (De Rosso *et al.*, 2008). This allows the survival and growth of aerobic microorganisms for a prolonged time (Hidalgo *et al.*, 2010; Torija *et al.*, 2009).

Because of the increasing demand for spontaneously fermented beers and to decrease the area needed for the storage of the casks, the applicability of stainless

steel fermentors in the production of lambic beers has been considered (Verachtert & Derdelinckx, 2005). Stainless steel fermentors would not only decrease the production area needed but would also facilitate a better microbiological control over the process, since stainless steel fermentors can be cleaned thoroughly, which would avoid aberrant fermentations (del Campo *et al.*, 2003; Licker *et al.*, 1998; Oelofse *et al.*, 2008; Verachtert & Derdelinckx, 2005). Verachtert and Derdelinckx (2005) reported that lambic beer fermentations in stainless steel tanks are very similar to those in wooden casks, although the amount of acetic acid and its ester ethyl acetate are higher in the stainless steel fermentors.

2.2 *Enterobacteriaceae*, *Saccharomyces bayanus*/*pastorianus* and *Saccharomyces cerevisiae*, microbiota dominating the early steps of the lambic beer fermentation

The *Enterobacteriaceae* family belongs to the class of *Gammaproteobacteria*. Many species enclosed in the genera of this family are well known plant and human pathogens. There are however several unresolved taxonomical problems, mainly due to the lack of straightforward differential biochemical tests and the limited taxonomic resolution of the 16S rRNA gene sequence (Dauga, 2002). These outstanding problems are increasingly solved through the use of multi-locus sequence analysis of housekeeping genes and polyphasic taxonomy studies (Brady *et al.*, 2013). Members of the *Enterobacteriaceae* family are fast-growing, are mostly linked to poor hygienic practices in food microbiology and may cause food poisoning (Baylis *et al.*, 2011). Yet, members of the *Enterobacteriaceae* family have been found in the initial phases of a multitude of spontaneously fermented food products and beverages, such as lambic beers, American coolship ales, cocoa beans, and cheese (Bokulich *et al.*, 2012; Chaves-López *et al.*, 2006; Papalexandratou *et al.*, 2011; Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995).

Saccharomyces bayanus/*pastorianus* and *S. cerevisiae* are the two main species found during the main alcoholic fermentation phase of lambic beer (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). *Saccharomyces bayanus*/*pastorianus* yeasts are cryotolerant and cannot be separated on the basis of 26S rRNA gene sequence analysis (Josepa *et al.*, 2000). Technically, *S. bayanus* and *S. pastorianus* cannot be

regarded as distinct species in the ecological and evolutionary sense, since they are hybrids and are thus the product of the brewing environment and do not occur in nature (Libkind *et al.*, 2011). *Saccharomyces pastorianus* is the product of domestication and hybridization of *S. cerevisiae* (which is thermotolerant) and *Saccharomyces eubayanus* (which is cryotolerant). The latter was isolated from *Nothofagus* trees in Patagonia (Libkind *et al.*, 2011). In addition, it has been demonstrated that *S. bayanus* is a complex hybrid of *S. eubayanus*, *S. pastorianus* and *S. uvarum* (which is also cryotolerant) (Libkind *et al.*, 2011). Hybrids are commonly used in fermentation processes and combine the properties of their ancestor species (Peris *et al.*, 2012). Similarly, the yeast strains used in the production of several Belgian trappist beers are hybrids of the species *S. cerevisiae* and *Saccharomyces kudriavzevii* (González *et al.*, 2008). Since these strains evolved separately after the hybridization events, these hybrids can differ significantly from each other and from their ancestral species in their biochemical and physiological properties (González *et al.*, 2008).

2.3 Lactic acid bacteria, the microbiota responsible for the acidification of lambic beer

[*Pediococcus cerevisiae*] and *Lactobacillus* spp. are reported in lambic beer and other mixed fermentation beers (Bokulich *et al.*, 2012; Martens *et al.*, 1997; Van Oevelen *et al.*, 1977). The name [*P. cerevisiae*] was used in the past for at least two species that are known today, *i.e.*, *P. damnosus* and *Pediococcus pentosaceus* (Garvie, 1974). *Pediococcus damnosus* is present in American coolship ale fermentations and lambic beer isolates previously identified as [*P. cerevisiae*] also belong to this species (Bokulich *et al.*, 2012; Martens *et al.*, 1997; Van Oevelen *et al.*, 1977). It is therefore the dominant LAB species in lambic beer fermentation. *Pediococcus damnosus* is a homofermentative LAB species, which implies that it only produces lactic acid during fermentation (Verachtert & Iserentant, 1995). This is in contrast to *Lactobacillus* spp., isolated from several mixed fermentation beers, which can be heterofermentative and not only produce lactic acid but also other acids, CO_2 and ethanol (Martens *et al.*, 1997).

LAB are isolated from a wide range of fermented foods and their acidification effect mostly has a beneficial effect on the fermentation and shelf-life of the fermented end-products by preventing the prevalence of pathogens and food spoilage bacteria (Ross *et al.*, 2002). During ale and lager beer fermentations, however, these bacteria are considered detrimental, because they can cause off-flavors by the production of metabolites such as lactic acid (Suzuki, 2011). Besides off-flavors, LAB cause turbidity and alter the viscosity of beer, rendering it unsellable because of decreased consumer appreciation (Menz *et al.*, 2010). Several beer spoilage LAB are resistant to the antimicrobial compounds of hops, making them a real hazard in beer production (Suzuki, 2011). Nevertheless, these bacteria are indispensable during the mixed fermentation of beers, such as lambic beers and redbrown acidic beers, as they provide the tart flavor, which is a key characteristic of such beers (Van Oevelen *et al.*, 1976; Verachtert & Iserentant, 1995).

2.4 *Dekkera*, the main yeast responsible for the maturation of lambic beer

Of all microorganisms present in the lambic beer fermentation, *Dekkera* yeasts, which appear during the maturation phase of the brewing process, are probably the most intriguing ones. *Dekkera* cells succeed in growing when other microorganisms are declining under the harsh conditions of the low nutrient and acidic wort environment (Blomqvist *et al.*, 2010; Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995) and are found in low numbers already during the first three months of the lambic beer fermentation process (Van Nedervelde & Debourg, 1995). *Dekkera* spp. are responsible for the super-attenuation of the beer, together with *P. damnosus* (Shanta Kumara & Verachtert, 1991) and may originate from the old wine barrels (Licker *et al.*, 1998). Additionally, they are indispensable for the typical lambic beer flavor and produce several metabolites that are characteristic for lambic beers in high amounts (Shanta Kumara & Verachtert, 1991; Spaepen & Verachtert, 1982; Spaepen *et al.*, 1978, 1979; Van Oevelen *et al.*, 1976; Verachtert & Iserentant, 1995).

2.4.1 Taxonomy and occurrence of *Dekkera*

Dekkera is the sexual (teleomorphic) form of the asexual (anamorphic) genus *Brettanomyces*. Although both names are widely used in the literature, lambic beer brewers know the genus best by its asexual name *Brettanomyces*. In the early 1900s, most European breweries adopted the use of single-strain starter cultures for brewing as described by Hansen (Claussen, 1904). It was noted, however, that especially English breweries did not adopt the use of single yeast strains for the production of their beers (Claussen, 1904). These beers were comparable to modern stout beers (Martens *et al.*, 1997). The use of a single *Saccharomyces* culture for the primary fermentation did not enable the development of the so-called English aroma of beer (Claussen, 1904). This aroma was typically developed during a spontaneous secondary fermentation during beer maturation in a wooden cask in traditionally produced beer (Claussen, 1904). Claussen hypothesized and proved that the presence of another yeast in the production of English stock beers was responsible for this secondary fermentation (Claussen, 1904). The yeast was subsequently isolated and named "*Brettanomyces*", highlighting its origin (Great Britain) and referring to the so-called English aroma the beer obtains in its presence (Oelofse *et al.*, 2008). Claussen however did not propose a formal taxonomic name and it was only during a study of lambic beer in 1921 that Kufferath and Van Laer (Licker *et al.*, 1998) identified yeasts with the same characteristics as those described by Claussen (1904). They named these yeasts *Brettanomyces bruxellensis* and [*Brettanomyces lambicus*], and provided a formal description of the genus *Brettanomyces* (Licker *et al.*, 1998). The genus *Dekkera* was proposed and described by Van der Walt (1964), when ascospore formation in *B. bruxellensis* and [*Brettanomyces intermedius*] was found. Both genera contain a lot of synonymous species (Smith *et al.*, 1990). The asexual genus name *Brettanomyces* has been used in early reports of the lambic beer microbiota (Shanta Kumara & Verachtert, 1991; Shanta Kumara *et al.*, 1993; Spaepen & Verachtert, 1982; Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995).

This dual nomenclatural system in mycology has been the subject of many debates, but recently, the "One Fungus = One Name" consortium made progress in the unification of the nomenclature of yeasts (Taylor, 2011). Changes in the

International Code of Nomenclature for algae, fungi, and plants to enforce this unification were made under influence of this consortium (McNeill *et al.*, 2012; Taylor, 2011) and according to Art. 57 of the International Code of Nomenclature for algae, fungi, and plants (Melbourne Code), the sexual name *Dekkera* should be used until it is rejected by the General Committee (McNeill *et al.*, 2012; Norvell, 2011). Therefore, the name *Dekkera* will be used throughout the present study, whenever appropriate. The same rule will be applied to other yeast names, but the asexual name by which a particular yeast might be best known will be given with its first appearance in the text.

Next to its reported occurrence in old Porter beers (Martens *et al.*, 1997), Berliner Weisse (Verachtert & Derdelinckx, 2005) and lambic beers (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995), *Dekkera* yeasts are also known as spoilage microorganisms in the wine and soft drinks industries (Oelofse *et al.*, 2008; Smith & Grinsven, 1984) and in beer (Suzuki *et al.*, 2008). Additionally, these yeasts occur in sourdough (Meroth *et al.*, 2003), cheese and fermented milk, fruit flies and bees (Oelofse *et al.*, 2008).

2.4.2 Economical relevance of *Dekkera*

Dekkera spp. are well known wine spoilage yeasts, especially of high quality wines that are fermented and aged 'on lees' in wooden barrels, where they mature on the deposits of *Saccharomyces* yeasts of the main alcoholic fermentation phase (Renouf *et al.*, 2008). *Dekkera* yeasts cause phenolic and mousy off-flavors and odors, which are described as resembling horse sweat, clove, burnt plastic or barnyard-like, making the wine unsuitable for the market (Conterno *et al.*, 2006; Grbin & Henschke, 2000; Licker *et al.*, 1998). These off-flavors are primarily caused by the conversion of *p*-coumaric acid and ferulic acid to 4-ethylphenol and 4-ethylguaiacol, respectively (Licker *et al.*, 1998; Puig *et al.*, 2011). *Dekkera bruxellensis* and *D. anomala* are the most potent producers of these off-flavors (Conterno *et al.*, 2013). An initial load of six cells per mL of *D. bruxellensis* can lead to 1 mg/L 4-ethylphenol in a 4.5-month cask maturation (Barata *et al.*, 2013). It is therefore of utmost importance that this yeast is detected as soon as possible in wine fermentations before these off-flavors are produced in too high amounts (Puig *et al.*, 2011). Several

publications have dealt with the detection or isolation of these yeasts, including the use of selective and specific media (Couto *et al.*, 2005; Rodrigues *et al.*, 2001). PCR- or antibody-based detection techniques show high specificity and selectivity (Puig *et al.*, 2011; Renouf *et al.*, 2007) and are preferred over the agar-based culture media, because *Dekkera* spp. may survive under harsh conditions in a VBNC state (Agnolucci *et al.*, 2010; Millet & Lonvaud-Funel, 2000). The problem of spoilage mostly exists in red wines, as these are preferentially aged in wooden barrels (Puig *et al.*, 2011). A test of 86 commercial wines in Spain revealed that in 16 out of the 86 bottled wines *Dekkera* spp. could be detected either through culture or via quantitative PCR (Puig *et al.*, 2011).

Some studies demonstrate the cooperage as main source of winery contamination with *Dekkera* spp. (Licker *et al.*, 1998). New wooden barrels or materials have generally not been reported to be contaminated with *Dekkera* cells (Renouf, 2006; Renouf *et al.*, 2007). For that reason, it has been stated that the presence of *Dekkera* spp. in the cooperage is merely a consequence of an initial *Dekkera* contamination from another source (Renouf *et al.*, 2007). Some studies however report contaminations with *Dekkera* yeasts in new barrels, but this could be explained by the use of must infected with *Dekkera* cells or poor barrel management (Oelofse *et al.*, 2008; Renouf *et al.*, 2007). *Dekkera* spp. are present on grapes, more preferentially at the time of harvesting than on green and immature grapes (Renouf & Lonvaud-Funel, 2007) and therefore grapes are thought to be the main agent of *Dekkera* contaminations in wineries (Renouf *et al.*, 2007). The detection of *Dekkera* contamination in concrete and steel wine fermentors that have not been in contact with the cooperage supports this hypothesis (Rodrigues *et al.*, 2001). The cooperage, although not responsible for the initial contamination with *Dekkera* yeasts, is probably sustaining the infection, due to the difficult sanitation of wooden barrels and tools and the porosity of the wood, in which the yeasts might find a safe haven during cleaning (Suárez *et al.*, 2007). Additionally, *Dekkera* spp. assimilate cellobiose, which is the basic building block of cellulose and hence of wooden barrels (Licker *et al.*, 1998; Suárez *et al.*, 2007). Due to their economical importance, the 13.4 Mb genome of the wine spoilage species *D. bruxellensis* was recently sequenced and annotated (Woolfit *et al.*, 2007) and revealed that it comprises 5600 genes

(Piskur *et al.*, 2012), including recently duplicated genes encoding for alcohol dehydrogenases that are responsible for the production of alcohol from sugars and for the synthesis of higher alcohols and precursors for aromatic esters (Piskur *et al.*, 2012). Additionally, five genes for nitrate assimilation were annotated (Piskur *et al.*, 2012; Woolfit *et al.*, 2007).

2.4.3 Biochemical and physiological properties of *Dekkera*

Dekkera yeasts exhibit the so-called "Custers effect", also called negative "Pasteur effect", which refers to the absence of fermentation or to a minimal fermentation under anaerobic conditions (Licker *et al.*, 1998). In the anaerobic metabolism of *Dekkera*, less glycerol is produced for the oxidation of NAD(P)H compared to *Saccharomyces* yeasts (Blomqvist *et al.*, 2010; de Barros Pita *et al.*, 2011). *Dekkera* yeasts grow slowly and are generally not competitive in industrial fermentations when *Saccharomyces* species are pitched as starter cultures (Abbott *et al.*, 2005; Blomqvist *et al.*, 2010). However, some fermentation conditions enable *Dekkera* species to outcompete the initially pitched *Saccharomyces* production strain (Bassi *et al.*, 2013; Passoth *et al.*, 2007). A consortium of *D. bruxellensis* and *Lactobacillus vini* is able to replace a *Saccharomyces* production strain in a bio-ethanol production process, without production decline (Passoth *et al.*, 2007). *Dekkera bruxellensis* is better adapted to grow under conditions of continuous fermentation with substrate limitation and yeast recirculation (Passoth *et al.*, 2007). *Dekkera* strains use substrates more efficiently and tolerate inhibitors compared to *Saccharomyces* strains in such industrial fermentations (Bassi *et al.*, 2013). Additionally, *Dekkera* strains can use nitrate as an alternative electron acceptor, thus reoxidizing NAD(P)H by the reduction of nitrate to ammonium (de Barros Pita *et al.*, 2011). A full nitrate assimilation gene cluster has been found in the genome of *D. bruxellensis* (de Barros Pita *et al.*, 2011; Woolfit *et al.*, 2007).

Dekkera strains are resistant to a large variety of physiological stresses. They survive cycloheximide concentrations up to 100 ppm (Licker *et al.*, 1998; Morneau *et al.*, 2011) and resist high concentrations of SO_2, which is generally applied in winemaking by the addition of potassium metabisulphite ($K_2S_2O_5$) to inhibit growth of yeasts (Barata *et al.*, 2008). The level of SO_2 at a given $K_2S_2O_5$ concentration is

dependent on several parameters, *e.g.*, pH, ethanol concentration and temperature of the must or wine (Oelofse *et al.*, 2008). The resistance towards SO_2 causes a real threat to the wine industry, since *Dekkera* contaminations cannot be stopped by adding normal amounts of $K_2S_2O_5$ (Barata *et al.*, 2008). Levels of 0.5-0.8 mg/L SO_2 have been reported for the inhibition of *Dekkera* strains (Licker *et al.*, 1998; Oelofse *et al.*, 2008); the use of 1 mg/L SO_2 has been suggested for successful inhibition of *Dekkera* strains (Barata *et al.*, 2008). In addition, it is not feasible to reach sufficient SO_2 levels everywhere in the barrels, especially near the bung hole (Licker *et al.*, 1998). Next to their resistance to exogenous chemical compounds such as cycloheximide and SO_2, these yeasts also resist higher acetic acid and ethanol levels and changes in pH compared to *Saccharomyces* (Blomqvist *et al.*, 2010; Puig *et al.*, 2011; Renouf *et al.*, 2007). It is indeed known that *Dekkera* strains produce and assimilate ethanol and acetic acid. Therefore, these molecules are both products and substrates for these yeasts (Renouf *et al.*, 2007). Further, its growth and activities are related to the carbohydrate and oxygen concentrations in the environment (Tiukova *et al*., 2013).

The production of 4-ethylphenol (which is responsible for the so-called horse sweat smell) and 4-ethylguaiacol (the spicy, clove-like odor) from *p*-coumaric acid and ferulic acid, respectively, is catalyzed by the action of two enzymes (Suárez *et al.*, 2007). The first enzyme, hydroxycinnamate decarboxylase, converts hydroxycinnamic acids to their corresponding hydrostyrenes or vinylphenols, which in turn are converted to ethylphenols by vinylphenol reductase (Suárez *et al.*, 2007). The ability to produce vinylphenols from hydroxycinnamic acids is widely distributed among yeasts and bacteria, but only a few yeasts, including *D. bruxellensis*, *D. anomala*, some strains of *Pichia guilliermondii* and some *Candida* species, can produce ethylphenols from vinylphenols (Dias *et al.*, 2003; Guzzon *et al.*, 2011; Suárez *et al.*, 2007). Ethylphenol is more harmful to the wine flavor compared to vinylphenols because of its lower sensory thresholds (Guzzon *et al.*, 2011; Puig *et al.*, 2011). However, its presence in certain beers, so-called “brett” beers, is desirable (Bokulich & Bamforth, 2013).

Chapter 3. The identification of bacteria and yeasts using MALDI-TOF MS

3.1 Mass spectrometry

3.1.1 History

The principle of mass spectrometry or the separation of ionized particles based on their mass-to-charge (*m/z*) ratio was already described in 1897 by Thompson (Liyanage & Lay, 2006). Based on this principle, the first mass spectrometer was built by Aston in 1919 (Liyanage & Lay, 2006). Until the mid-1980s, mass spectrometry was mainly used in organic chemistry, since the applied ionization methods were highly energetic and only small molecules could be ionized without fragmentation (Liyanage & Lay, 2006). Macromolecular biomolecules instantly fragmented after ionization and could not be analyzed using mass spectrometry. The coupling of mass spectrometry to gas chromatography (GC) for the analysis of fatty acid methyl esters (FAME) was the first application of mass spectrometry for bacterial identification (Krásný *et al.*, 2013). The advantage of mass spectrometry in this set-up is only minor, since the analysis of GC data is more straightforward compared to the analysis of mass spectra (Krásný *et al.*, 2013). In addition, the fatty acid composition of bacterial cells is dependent on the culture conditions and has limited resolution in bacterial analysis (Fox, 2006; Krásný *et al.*, 2013).

3.1.2 Soft ionization techniques

The analysis of intact proteins and other macromolecular biomolecules became feasible through the invention of the soft ionization techniques, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), whose development marked a major advance in the proteomics era (Aebersold & Mann, 2003). ESI is the most used ionization technique for the identification of proteins in complex protein mixtures. It enables in-line protein mass determination when directly coupled to a high-performance liquid chromatography (HPLC) device, which separates the proteins based on their hydrophobicity, charge or other

characteristics. Although widely used in proteomics, ESI is not suited for whole-cell analysis of bacteria, since the capillaries of the system would clog and, additionally, the deconvolution of the mass spectra is difficult (Fenselau & Demirev, 2001; Krásný *et al.*, 2013). This latter issue would interfere with the analysis of peak pattern fingerprints. MALDI however proved to be widely applicable in microbiology.

3.2 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

3.2.1 General overview

MALDI is a soft ionization technique (Hillenkamp & Karas, 2007) and enables the ionization of large biomolecules such as proteins and carbohydrates through the use of an organic matrix substance that absorbs light energy at a specific wavelength; this energy is subsequently used for the desorption and ionization of an analyte (Figure 3.2.1) (Liyanage & Lay, 2006). All MALDI matrices contain at least one aromatic ring, the specific electron distribution of which allows the matrix to absorb light energy (Liyanage & Lay, 2006). The light energy elevates the electron energy to an excited state (Liyanage & Lay, 2006). The processes by which this leads to the ionization of the analyte is still a topic of discussion in the field of physics (Knochenmuss, 2010). Depending on the electrical potential present on the target plate, negative or positive ions are formed by proton loss or gain, respectively. These ions are accelerated into a field-free analyzer, after which they are recorded by the detector. MALDI is mostly combined with a time-of-flight (TOF) tube as mass analyzer, since both operate in a pulsed manner (Liyanage & Lay, 2006). In the TOF tube, the time needed to reach the detector is inversely proportional to the *m/z* ratio of the ions. The *m/z* ratio can be calculated by the use of a calibration curve. The TOF time measurement is initiated by a pulse of the laser light.

Although MALDI-TOF MS was first described in 1985, it was not successfully applied for proteins before 1987. Tanaka *et al.* (1988) reported the ionization of the protein carboxypeptidase-A (34 kDa) by means of soft laser desorption and Karas and Hillenkamp (1988) subsequently reported MALDI-TOF MS of even larger proteins, up to 67 kDa. From then onwards, MALDI-TOF MS gained much attention in the field of proteomics and later also in bacteriology and bacterial taxonomy. The technique

is appreciated for its simplicity, mass accuracy, high resolution and sensitivity (Aebersold & Mann, 2003). The interpretation of the mass spectra produced is straightforward, since the ions are mostly single charged, in contrast to ESI spectra, which are mostly difficult to deconvolute because of the multiple charged ions (Fenselau & Demirev, 2001; Krásný *et al.*, 2013).

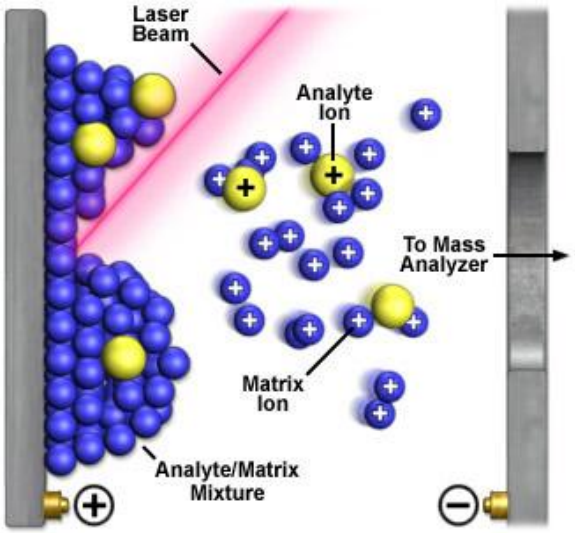


Figure 3.2.1 Schematic representation of the MALDI process. Figure from the National High Magnetic Field Laboratory of The Florida State University (with the permission from Mr. Michael W. Davidson).
(http://www.magnet.fsu.edu/education/tutorials/tools/images/ionization-maldi.jpg)

3.2.2 MALDI-TOF MS for bacterial identification

The usefulness of MALDI-TOF MS for bacterial identification was first verified by Cain *et al.* (1994), who analyzed bacterial cell extracts (cited in Holland et al., 1996). The use of MALDI-TOF MS for the identification of bacteria based on intact cells was simultaneously described by Holland *et al.* (1996) and several other researchers (Claydon *et al.*, 1996; Krishnamurthy *et al.*, 1996) and is now commonly used in the field of medical microbiology (Croxatto *et al.*, 2012). The speed and ease of sample handling and the superior identification capacity compared to traditional biochemical tests has been acknowledged as one of the major factors for the fast adoption of this technique in microbial identification (Croxatto *et al.*, 2012). Additionally, this technique is cost-effective in a clinical laboratory setting (Bille *et al.*, 2012; Cherkaoui *et al.*, 2010; Neville *et al.*, 2011; Tan *et al.*, 2012). Mass spectra based on whole cells and whole-cell extracts typically contain peaks of ribosomal or

nucleic acid-binding proteins (Barbuddhe *et al.*, 2008; Croxatto *et al.*, 2012; Dieckmann *et al.*, 2008; Ryzhov & Fenselau, 2001; Suarez *et al.*, 2013).

The development of MALDI-TOF MS applications for bacterial identification has been driven by the research of its applicability in the identification of biological warfare agents and pathogenic bacteria (Jarman *et al.*, 1999, 2000; Keys *et al.*, 2004; Liu *et al.*, 2007; Seng *et al.*, 2009; Wahl *et al.*, 2002). Obtaining a good reproducibility and the development of universal sample preparation protocols have been major hurdles to implement this methodology for routine identification of microorganisms (Keys *et al.*, 2004; Liu *et al.*, 2007; Vargha *et al.*, 2006). The early reports and applications of MALDI-TOF MS for the identification of bacteria described a multitude of sample preparations (Hettick *et al.*, 2004; Jackson *et al.*, 2005; Liu *et al.*, 2007; Ruelle *et al.*, 2004; Smole *et al.*, 2002; Vargha *et al.*, 2006; Williams *et al.*, 2003). Many of these sample preparation methods were considered ideal for the group of microorganisms tested, which was however mostly rather limited. The use of intact cells and cell extracts was thoroughly compared, including the preparation of cell extracts on-plate (McElvania TeKippe *et al.*, 2013) or in a separate lysis step (Freiwald & Sauer, 2009). In addition, a range of different organic and inorganic solvents have been used, which were combined with a variety of sample deposition methods, matrices and organic solvents (Andres-Barrao *et al.*, 2013; Dieckmann *et al.*, 2005; Jackson *et al.*, 2005; Kuda *et al.*, 2014; Kuehl *et al.*, 2011; Liu *et al.*, 2007; Madonna *et al.*, 2000; Ruelle *et al.*, 2004; Williams *et al.*, 2003).

Cell extracts have been prepared by means of mechanical disruption, enzyme treatment and/or organic solvent-aided cell lysis (Drevinek *et al.*, 2012; Giebel *et al.*, 2008; Liu *et al.*, 2007; Ruelle *et al.*, 2004; Salplachta *et al.*, 2013; Smole *et al.*, 2002; Vargha *et al.*, 2006; Williams *et al.*, 2003). The comparison of these cell extraction techniques is very difficult, since most studies focus only on a limited number of strains of only a few species and genera (Drevinek *et al.*, 2012; Jackson *et al.*, 2005; Liu *et al.*, 2007; Ruelle *et al.*, 2004; Williams *et al.*, 2003), which resulted in a huge and obscure list of so-called “optimal” and “universal” sample preparation protocols. In these studies, Gram-positive and slime-forming bacteria are commonly regarded as the most challenging bacteria to yield a high-quality mass spectrum (Krásný *et al.*, 2013; Liu *et al.*, 2007; Smole *et al.*, 2002; Vargha *et al.*, 2006). In addition to the

different solvents used for sample preparation and measurements, the number of cells used for sample preparation has a major impact on the quality of the mass spectra derived. In general, approximately 10^6 cells are required for MALDI-TOF MS analysis but cell numbers as low as 10^2 (Stackebrandt *et al.*, 2005), $5*10^3$ (Fenselau, 2013) and even up to 10^7 (Furukawa *et al.*, 2013) have been reported (Croxatto *et al.*, 2012; Drancourt, 2010). Too few or too much bacterial cells can both yield poor mass spectra (Petersen *et al.*, 2009; Williams *et al.*, 2003).

Currently, two sample preparation procedures are widely used, mainly because these are the default protocols in most commercial systems used (Freiwald & Sauer, 2009). The first consists of an extraction of the proteins from a small amount of cells using formic acid and acetonitrile, preceded by an ethanol inactivation step (FA/ACN extraction) (Freiwald & Sauer, 2009). The second is even more straightforward and reduces handling time; a bacterial colony is simply smeared on the plate, after which it can additionally be lysed on the plate using formic acid (McElvania TeKippe *et al.*, 2013). Although the smear method is sometimes referenced as an intact cell method, it was reported that the cells are lysed by the matrix solvent (Fenselau & Demirev, 2001; Pennanec *et al.*, 2010). The latter sample preparation technique is mostly used as the default sample preparation for MALDI-TOF MS-based microorganism identification in medical microbiology because of its reduced sample preparation time. However, when this technique does not yield reliable identification scores, the residual colonies on the plate can be subjected to a more elaborate extraction procedure (Bessède *et al.*, 2011; Bizzini *et al.*, 2010; van Veen *et al.*, 2010).

Parameters other than the variation in sample preparation method may affect the reproducibility, including bacterial growth conditions and the MALDI-TOF MS instrument used. The effect of growth conditions has been studied to some degree but comprehensive studies are scarce. Some studies report a clear impact for species level identification purposes (Karger *et al.*, 2013; Wunschel *et al.*, 2005a), while others minimize this effect (De Bruyne *et al.*, 2011; Mellmann *et al.*, 2008; Pennanec *et al.*, 2010; Sedo *et al.*, 2013; Valentine *et al.*, 2005; Wieme *et al.*, 2014). In addition, not only different types of mass spectrometers but also the different parameter settings and operational procedures can produce quality differences between mass spectra (Drevinek *et al.*, 2012; Saenz *et al.*, 1999; Schumaker *et al.*, 2012; Toh-Boyo

et al., 2012). The quality assessment of the mass spectra obtained differs widely. Some studies focus on specific characteristics of the mass spectra, such as signal-to-noise ratio, signal intensity and peak resolution (Goldstein *et al.*, 2013; Schumaker *et al.*, 2012; Toh-Boyo *et al.*, 2012), while others focus on the applicability of the mass spectra for species identification (McElvania TeKippe *et al.*, 2013; Sedo *et al.*, 2013). However, the comparison of distantly related species often compromises the value of the conclusions derived from such studies (Hsieh *et al.*, 2008; Liu *et al.*, 2007).

The inter-laboratory reproducibility can be analyzed more easily by the use of commercial systems that facilitate the use of the same instrument and parameter settings (Barbuddhe *et al.*, 2008; Garner *et al.*, 2013; Karger *et al.*, 2013; Keys *et al.*, 2004; Mellmann *et al.*, 2009), which was previously not always possible (Wunschel *et al.*, 2005b).

Recently, MALDI-TOF MS has been introduced in the field of food microbiology for the detection and identification of food pathogens. These studies have analyzed not only pure cultures but also food products with minimal preprocessing. MALDI-TOF MS has proved suitable for the identification of, *e.g.*, salmonellae (Dieckmann *et al.*, 2008), *E. coli* and *Yersinia enterocolitica* from bovine samples (Parisi *et al.*, 2008) and fish spoilage bacteria (Böhme *et al.*, 2011a; Böhme *et al.*, 2011b). An overview of the potential of MALDI-TOF MS for the identification of food pathogens was recently presented by Böhme *et al.* (2012a). In addition, MALDI-TOF MS can be used for the detection and quantification of microbial spoilage in milk and pork (Nicolaou *et al.*, 2012) and for the identification of beer spoilage bacteria (Kern *et al.*, 2013).

MALDI-TOF MS has also been applied to identify beneficial bacteria in food microbiology, such as AAB used in the production of vinegar (Andres-Barrao *et al.*, 2013). The technique has also been applied for the identification of LAB and probiotic bacteria in food microbiology (Angelakis *et al.*, 2011; De Bruyne *et al.*, 2011; Doan *et al.*, 2012; Dušková *et al.*, 2012; Kuda *et al.*, 2014; Sedo *et al.*, 2013; Snauwaert *et al.*, 2013; Zeller-Péronnet *et al.*, 2013). The Sepsityper™ kit (Bruker Daltonics) was originally developed for direct analysis of bacteria in positive blood cultures and urine (Drancourt, 2010; Loonen *et al.*, 2012; Schubert *et al.*, 2011; Stevenson *et al.*, 2010). This kit has been used for the direct identification of bacteria in artificially contaminated milk and it turned out that a minimum of 10^6 colony

forming units (CFU) per mL is necessary before good quality mass spectra are generated (Barreiro *et al.*, 2012). Mass spectra suitable for species identification after a short incubation step (4 h at 37°C) when the initial bacterial load is approximately 10^4 CFU/mL have also been obtained. Further, an enrichment step has been used to detect bacterial contaminants present in processed soybean products (Furukawa *et al.*, 2013). It is therefore possible to identify the most predominant species present in a food product when cells are directly analyzed after an FA/ACN extraction (Furukawa *et al.*, 2013).

3.2.3 MALDI-TOF MS for yeast and mold identification

The application of MALDI-TOF MS for the identification of yeasts was already described by Amiri-Eliasi and Fenselau (2001). In this early study, the peaks identified from the mass spectra originate from ubiquitin and ribosomal and mitochondrial proteins. Although published in 2001, yeast analysis by means of MALDI-TOF MS was not widely applied due to the difficulties with the generation of high-quality mass spectra (Qian *et al.*, 2008; Sherburn & Jenkins, 2003; Valentine *et al.*, 2002). Marklein *et al.* (2009) have reported the identification of human medical yeast isolates by means of a commercial MALDI-TOF MS identification system (Bruker Biotyper) and presented a modification of the standard FA/ACN extraction through the use of a higher amount of cell mass (they used five colonies rather than one). This approach has been optimized by the use of a single colony with reduced amounts of organic solvents to obtain good quality spectra (Goyer *et al.*, 2012). Since the study of Marklein *et al.* (2009), several additional studies have been published, but mainly in the field of human medical microbiology (Alshawa *et al.*, 2012; De Carolis *et al.*, 2012; Dhiman *et al.*, 2011; Kemptner *et al.*, 2009; Seyfarth *et al.*, 2012; van Veen *et al.*, 2010).

In food microbiology, a commercial MALDI-TOF MS system has been used for the identification of *S. cerevisiae* isolates from chica fermentation (Vallejo *et al.*, 2013). Only one study reports the use of MALDI-TOF MS for the identification of food spoilage yeasts (Usbeck *et al.*, 2013). The analysis of molds can be hampered by the production of melanin, which can inhibit the ionization process (Bader, 2013).

3.2.4 MALDI-TOF MS-based dereplication

Most studies in medical and food microbiology use MALDI-TOF MS for the identification of pathogens, as described above. Few biodiversity studies, however, have used this technique without the initial construction of a reference database. MALDI-TOF MS is nevertheless also suitable as a dereplication tool, in which isolates from complex communities are tested to recognize and eliminate the ones representing the same species. The use of MALDI-TOF MS as a dereplication tool coupled with subsequent identification of representative strains through comparative sequence analysis of 16S rRNA or housekeeping genes allows the creation of niche- or product-specific databases, while such biodiversity studies are performed (Doan *et al.*, 2012; Ghyselinck *et al.*, 2011).

Two approaches for validating MALDI-TOF MS as a dereplication tool have been used. In a first approach, clusters of isolates are delineated at an empirically determined cut-off value based on the similarity of their MALDI-TOF MS profiles, followed by gene sequence analysis of multiple isolates per cluster. Dieckmann *et al.* (2005) have performed a dereplication study of bacterial isolates from marine sponges and grouped isolates together if their spectra shared at least five of the most intense peaks. The validation consists of partial 16S rRNA gene sequence analysis of 65% of the isolates, which demonstrates that both techniques resolve the strains into identical groups (Dieckmann *et al.*, 2005). Similar approaches have been used by Nguyen *et al.* (2013) and Munoz *et al.* (2011). In contrast, a second approach was applied by Ghyselinck *et al.* (2011) and Doan *et al.* (2012), who compared the dereplication potential of MALDI-TOF MS with that of repetitive element sequence primed PCR (rep-PCR), a well-established dereplication technique (De Vuyst *et al.*, 2008; Gevers *et al.*, 2001). Ghyselinck *et al.* (2011) have analyzed part of the isolates in triplicate to determine the reproducibility of the technique and an appropriate cut-off value for cluster delineation was calculated based on this reproducibility assessment. A similar approach has been used by Stets *et al.* (2013) and Stafsnes *et al.* (2013).

3.2.5 MALDI-TOF MS for infraspecific identification and typing applications

Hinse *et al.* (2011) have reported the successful application of MALDI-TOF MS for the identification of members of the *Streptococcus bovis*/*equinus* complex to the subspecies level. MALDI-TOF MS results are comparable with the results obtained by sequence analysis of *sodA* (the manganese-dependent superoxide dismutase), which is the most reliable method for the identification of members of this complex (Hinse *et al.*, 2011). Typing commonly refers to the differentiation of strains of the same species but is also used for the differentiation of groups of strains with specific characteristics, *e.g.*, antibiotic resistance or the capacity to cause infections. Typing applications of MALDI-TOF MS are primarily reported in the field of medical microbiology. The differentiation of *Staphylococcus aureus* strains based on their capacity to produce β-lactamase and α-hemolysin has been reported (Kornienko *et al.*, 2013). Antifungal drug susceptibility tests have been performed using MALDI-TOF MS (Vella *et al.*, 2013). Williamson *et al.* (2008) have applied MALDI-TOF MS for the differentiation of *Streptococcus pneumoniae* strains capable of causing conjunctivitis from non-infectious strains and MALDI-TOF MS has been reported useful for the differentiation of morphotypes or metabolic states of the bacterial cells (Kuehl *et al.*, 2011; Sousa *et al.*, 2013). In food microbiology, Barbuddhe *et al.* (2008) have reported the differentiation of clonal lineages of *Listeria monocytogenes*, Moothoo-Padayachie *et al.* (2013) have used MALDI-TOF MS for the typing of industrial *S. cerevisiae* yeasts and Ruiz-Moyano *et al.* (2012) have applied MALDI-TOF MS for the discrimination of *Bifidobacterium animalis* subspecies.

3.2.6 Commercial MALDI-TOF MS systems for microorganism identification

MALDI-TOF MS-based identification of bacteria and yeasts decreases the identification time and cost (Dhiman *et al.*, 2011; Tan *et al.*, 2012). Currently, three commercial systems are available (Bruker Biotyper, Vitek® MS RUO and Andromas MS), each with a proprietary database of mainly medically relevant microorganisms, including spectra of 4500, 3000 and 700 unique species, respectively (Krásný *et al.*, 2013). The Bruker Biotyper (Bremen, Germany) is the current market leader and has the largest number of species included in the database. Identification scores are

calculated by comparing spectra of novel isolates with main spectra present in the database (Sauer *et al.*, 2008). Each main spectrum combines multiple spectra of a single strain (Figure 3.2.2). The scoring algorithm takes three parameters into account: the ratio of the number of matching peaks to the total number of peaks of the novel mass spectrum, the ratio of the number of matching peaks to the total number of peaks of the main spectrum and an intensity correlation between the matching peaks (Welker, 2011). These factors are multiplied and normalized to a value of 1000, after which the value obtained is log transformed (Welker, 2011). The Bruker Biotyper algorithm therefore does not focus on species-specific peaks, but tries to find the best pattern match in the database (Carbonnelle *et al.*, 2011; Freiwald & Sauer, 2009). The Anagnostec SARAMIS™ (Zossen, Germany) system was acquired by Shimadzu company (Manchester, UK) in 2008, after which bioMérieux (Marcy l'Etoile, France) entered in a partnership with Shimadzu in 2010. The SARAMIS™ system can now be purchased in combination with a Shimadzu Axima mass spectrometer as SARAMIS™@AXIMA (which has recently been referred to as Vitek® MS RUO and is primarily marketed for research use) (Dubois *et al.*, 2012) or as an *in vitro* diagnostic tool as Vitek® MS, for which a novel database has been developed (Figure 3.2.2). The Vitek® MS database includes a smaller number of species and strains per species compared to the Bruker Biotyper database and incorporates more spectra of strains grown under different conditions. Therefore, the spectra in the former database are considered to be more representative for the growth conditions of clinical isolates, as the database includes spectra of strains grown on different media and for different incubation times (Dubois *et al.*, 2012; Reich, 2013). The third system is Andromas MS (Paris, France), founded in 2010 as a spin-off of the Paris Necker hospital – AP-HP (Assistance Publique – Hôpitaux de Paris) and is based on the open-source BGP software (Carbonnelle *et al.*, 2007). The database of the latter is built in a way similar to that of the Vitek® MS system (Figure 3.2.2).

In contrast to the Bruker Biotyper scoring system, the Superspectra of the SARAMIS™ algorithm use a limited number of species-specific peaks for identification purposes and thus have an inherent quality control system to exclude the use of misclassified reference strains from the database (Bader *et al.*, 2011;

Carbonnelle *et al.*, 2011; Emonet *et al.*, 2010; van Belkum *et al.*, 2012). In this system, consensus spectra result from the selection of conserved mass peaks taken from multiple mass spectra of multiple strains (Welker, 2011). Subsequently, a comparison of such consensus spectra with a large number of individual spectra allows to select only those peaks that are species-specific for inclusion in the Superspectrum of a particular species (Welker, 2011). Identification is then based upon a comparison of a novel spectrum with a database of Superspectra, by calculating the sum of peak weights for matching peaks in the novel spectrum (Welker, 2011). The Vitek® MS system uses an even more advanced scoring system and the database contains additional clinical isolates (Dubois *et al.*, 2012). Mass peaks are distributed into bins and per species each bin receives a weight, depending on its frequency of occurrence within that species and its specificity for that species (*i.e.*, its absence in other species) (Welker, 2011). Mass peaks of a novel spectrum are distributed into the same bins and the identification is based on the sum of the bins present (Welker, 2011). The Andromas MS system comprises a database of species-specific peak patterns, taking into account a possible MS peak variation of ± 10 *m/z* (Farfour *et al.*, 2012). A species identification is considered valid if the percentage of common peaks between the reference and unknown mass spectra is ≥ 68% and the difference between the best two matches is at least 10% (Farfour *et al.*, 2012). The formula used can be described as: 100 x (number of peaks common between the peaks of the isolate tested and the peaks of the species-specific spectral fingerprint/total number of peaks specific to the species-specific spectral fingerprint) (Alshawa *et al.*, 2012). In addition, the Andromas MS system provides comments for the reason of identification failure (*e.g.*, poor spectrum quality) (Emonet *et al.*, 2010). Since the Bruker Biotyper system is the most widely used system at the moment, its scoring system has been adopted as a standard in the field. Some studies do not consider the quality of the mass spectra and report only the Biotyper identification scores [*e.g.*, Mellmann *et al.* (2008)]. Yet, a low score can also be caused by a poor quality of the mass spectra generated (Seng *et al.*, 2009). Numerous studies have reported that the species level identification performance of the different commercial systems is similar (Bader *et al.*, 2011; Cherkaoui *et al.*, 2010; Justesen *et al.*, 2011; Lohmann *et al.*, 2013; Marko *et al.*, 2012; Martiny *et al.*,

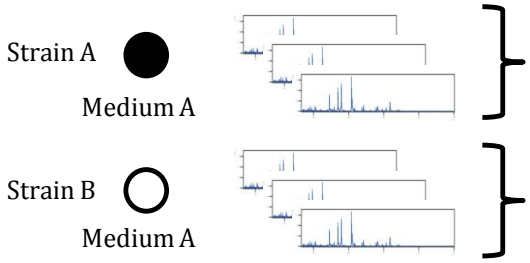
2012). The main differences between the systems are inherent to the database content differences (Fang *et al.*, 2012).

Small scale initiatives for creating public databases of MALDI-TOF MS spectra (*e.g.*, SpectraBank) along with free data analysis tools have been reported [*e.g.*, Böhme *et al.* (2012b)]. Böhme *et al.* (2012b) have focused primarily on food pathogens related to fishery products and have used the non-commercial online data analysis tool Speclust (Alm *et al.*, 2006; Böhme *et al.*, 2011a, 2011b, 2012a; Fernández-No *et al.*, 2010). The latter tool has also been used for the dereplication of bacterial isolates from wheat roots (Stets *et al.*, 2013). Such a public database of peak lists offers the advantage that data can be shared more easily, compared with private databases that are constructed using commercial software systems (Emami *et al.*, 2012; Ferreira *et al.*, 2011; Gaia *et al.*, 2011; Normand *et al.*, 2013; Pennanec *et al.*, 2010; Veloo *et al.*, 2011; Yang *et al.*, 2014). However, before a public database of peak lists can be created, additional research on universal sample preparations, data acquisition and peak-picking algorithms is needed and standardization of all these parameters is mandatory (Toh-Boyo *et al.*, 2012). Another promising initiative is the freeware tool BIOSPEAN, which was recently launched by Raus and Šebela (2013). This tool is now in the beta testing phase but does not include an identification database. BIOSPEAN combines key features of commercial software, such as a peak-picking algorithm, a mySQL database for spectral data storage and a scoring system that resembles the Bruker Biotyper scoring (Raus & Šebela, 2013). Moreover, the software is web-based and can be run at low operational costs and can be used with every MS platform (Raus & Šebela, 2013).

Schematic database build-up for 1 species

Identification of unknown spectra (U)

Bruker Biotyper

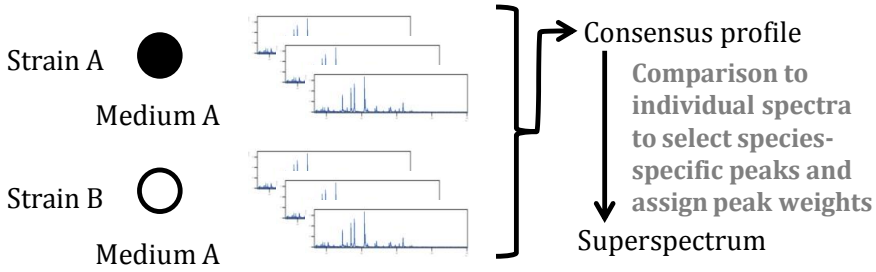


Unknown spectra are matched to the individual MSP's
Score formula:

$$\log\left(1000 * \frac{A}{B} * \frac{A}{C} * I\right)$$

A: Total of matching peaks
B: Total number of peaks in matching MSP
C: Total number of peaks in unknown spectrum
I: Intensity correlation of matching peaks

SARAMIS™@AXIMA / Vitek® MS RUO



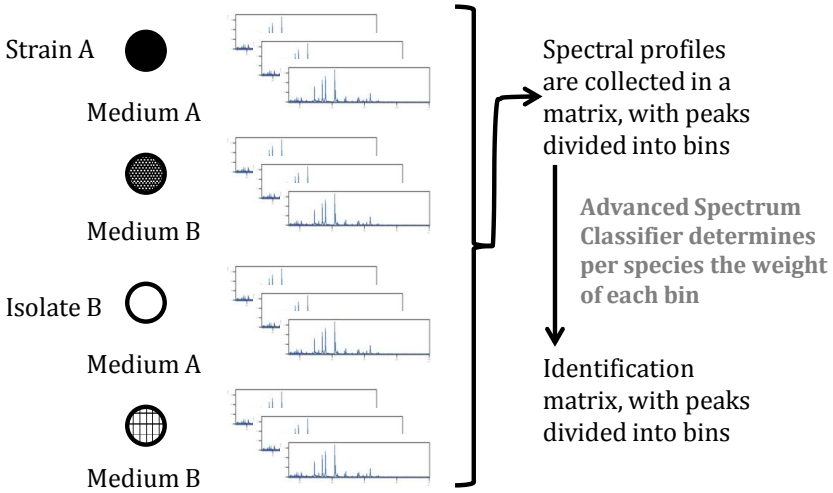
Unknown spectra are matched to the Superspectra present in the database
Score formula:
Sum of peak weights for the matching peaks

Figure 3.2.2 Schematic overview of the different approaches in database construction and identification algorithms of commercial MALDI-TOF MS bacterial identification systems. The figure is based on Alshawa *et al.* (2012), Dubois *et al.* (2012) and Welker (2011).

Schematic database build-up for 1 species

Identification of unknown spectra

Vitek® MS (IVD)



Unknown spectra are binned and a score for each species is calculated

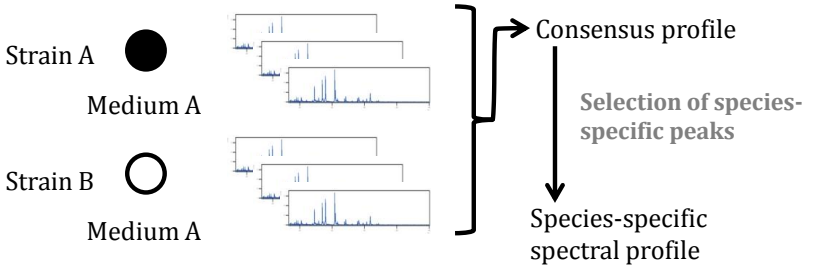
Score formula:

$$\sum_{n=1}^{n} S_n * \frac{\log I_x}{\sum_{x=1}^{x} \log I_x}$$

S_n: Score of n-th bin in the species-specific bin combination

I_x: Intensity of the x-th binned peak of the unknown spectum

Andromas MS



Unknown spectra are matched to the species-specific spectral profile present in the database

Score formula:

$$100 * \frac{A}{B}$$

A: Total of matching peaks

B: Total number of peaks in matching species-specific spectral profile

Figure 3.2.3 (Continued)

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Part III
Experimental work

Chapter 4. Microbial diversity of spontaneously fermented lambic beer

Preamble

The microbiology of lambic beer fermentation was described in several studies performed between 1977 and 1995. However, these studies were limited in the number of samples and the number of isolates examined. Moreover, the samples originated from different casks and different fermentation batches. Also, the taxonomy of the microbial groups described changed considerably since these early reports. Hence, using MALDI-TOF MS as a dereplication tool and sequence analysis of rRNA and housekeeping genes, a more in-depth study of the cultivable microbiota could be performed. Additionally, the bacterial and yeast communities were monitored using culture-independent PCR-DGGE.

In **Chapter 4**, the microbiology of spontaneous lambic beer fermentation processes is described. This chapter consists of two parts. In **Chapter 4.1,** the microbial diversity of the lambic beer fermentation process is described using samples of two batches of the most traditional, still active, lambic beer brewery in Belgium, Cantillon. The lambic beer fermentation process was monitored over a period of two years. In **Chapter 4.2**, the microbial diversity during an industrial lambic beer fermentation process is investigated. Since the industrial fermentation process is more closely monitored, only one batch was sampled over a period of one year. A second batch was monitored during a period of three months. Similarities and differences between the two breweries are presented in Table 4.0.1.

Table 4.0.1 The similarities and differences between the traditional and industrial breweries studied.

	Traditional brewery studied	**Industrial brewery studied**
Similarities	Use of unmalted wheat	
	Overnight cooling of the wort in an open cooling tun	
	Use of wooden casks for fermentation	
	Long fermentation (> 12 months)	
Differences	Turbid mashing	Infusion mashing
	3 h wort boiling	1.5 h wort boiling
	Old whole hop bells	Hop pellets
	No additives	Addition of protein coaggulation product and lactic acid => pH 4
	Wort directly to cooling tun	Wort is centrifuged and pre-chilled
	Brewing between October and April	Brewing all year round
	Old wine or cognac barrels	Custom-made new barrels

4.1 The microbial diversity of traditional spontaneously fermented lambic beer

Redrafted from: Freek Spitaels, Anneleen D. Wieme, Maarten Janssens, Maarten Aerts, Heide-Marie Daniel, Anita Van Landschoot, Luc De Vuyst and Peter Vandamme, The microbial diversity of traditional spontaneously fermented lambic beer, Plos One **9**, e95384.

Author contributions: conceived and designed the experiments: FS, MJ, AVL, LDV and PV; performed the experiments: FS; analyzed the data: FS and HMD; contributed reagents/materials/analysis tools: ADW, MA, and HMD; wrote the manuscript: FS; critically reviewed the manuscript: ADW, MA, HMD, AVL, LDV and PV.

The Genbank/EMBL accession numbers for the sequences generated in this study are KJ186115-KJ186128.

Abstract

Lambic sour beers are the products of a spontaneous fermentation that lasts for one to three years before bottling. The present study determined the microbiota involved in the fermentation of lambic beers by sampling two fermentation batches during two years in the most traditional lambic brewery of Belgium, using culture-dependent and culture-independent methods. From 14 samples per fermentation, over 2000 bacterial and yeast isolates were obtained and identified. Although minor variations in the microbiota between casks and batches and a considerable species diversity were found, a characteristic microbial succession was identified. This succession started with a dominance of *Enterobacteriaceae* in the first month, which were replaced at 2 months by *Pediococcus damnosus* and *Saccharomyces* spp., the latter being replaced by *Dekkera bruxellensis* at 6 months fermentation duration.

Lambic sour beers are among the oldest types of beers still brewed and are the products of a spontaneous fermentation process that lasts for one to three years (De Keersmaecker, 1996). The fermentation process is not initiated through the inoculation of yeasts or bacteria as starter cultures. Rather, microbial growth starts during the overnight cooling of the cooked wort in a shallow open vessel, called the cooling tun or coolship. Lambic beers are traditionally brewed in or near the Senne river valley, an area near Brussels, Belgium. Brewing for the production of lambic traditionally takes place only during the colder months of the year (October to March), since cold nights are needed to lower the wort temperature to about 20°C in one night. The morning following the wort cooking, the cooled wort is assumed to be inoculated with a specific air microbiota of the Senne river valley and is transferred into wooden casks which are stored at cellar or ambient temperatures, *i.e.*, typically between 15 and 25°C. Subsequently, the wort ferments and the lambic beer matures in these same casks. The end product is a noncarbonated sour beer that mainly serves as a base for gueuze or fruit lambic beers. The sour character of the beer originates from the metabolic activities of various yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995).

Previous studies of the lambic beer fermentation process identified four phases: the *Enterobacteriaceae* phase, the main fermentation phase, the acidification phase, and the maturation phase, each characterized by the isolation of specific microorganisms (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). The *Enterobacteriaceae* phase starts after 3 to 7 days of fermentation, proceeds until 30 to 40 days, and is characterized by *Enterobacter* spp., *Klebsiella pneumoniae*, *Escherichia coli* and *Hafnia alvei* as the most frequently isolated bacteria (Martens *et al.*, 1991), along with the cycloheximide-resistant yeasts *Hanseniaspora uvarum* [asexual form *Kloeckera apiculata* (Meyer *et al.*, 1978)] and *Naumovia (Saccharomyces) dairensis* (Kurtzman, 2003) as well as *Saccharomyces uvarum* [synonym *S. globosus* (Nguyen & Gaillardin, 2005)] (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). The main fermentation starts after 3 to 4 weeks of fermentation and is characterized by the isolation of *S. cerevisiae, S. bayanus/pastorianus* and *S. uvarum* (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). After 3 to 4 months of fermentation, the

acidification phase occurs and is characterized by the increasing isolation of *Pediococcus* spp. and occasionally *Lactobacillus* spp., while *Brettanomyces* spp. become prevalent after 4 to 8 months of fermentation (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). The final maturation phase, during which the wort is gradually attenuated, starts after 10 months of fermentation and is characterized by a decrease of LAB (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). AAB are isolated throughout the fermentation period (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995).

Sour beers are currently attracting interest outside Belgium, especially in the USA. In the American craft-brewing sector, American coolship ales mimic the lambic beer production method (Bokulich *et al.*, 2012), and such beers 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 (Bokulich *et al.*, 2012). A similar microbial succession as described above was recently revealed using culture-independent and culture-dependent techniques for the American coolship ales, whereby 16S rRNA gene sequence analysis was used to identify some morphologically distinct isolates (Bokulich *et al.*, 2012). Although the latter approach is widely applied as part of bacterial identification studies, it lacks resolution between many of the species belonging to the AAB, LAB, and *Enterobacteriaceae* family, and accurate species level identifications can only be obtained after subsequent sequence analysis of more variable protein-encoding genes (Cleenwerck *et al.*, 2010; De Bruyne *et al.*, 2008; De Bruyne *et al.*, 2007; Naser *et al.*, 2007). Except for this American brewery study, previous microbial studies on lambic beers used phenotypic identification techniques only, which are nowadays known to have an inadequate taxonomical resolution for the species-level identification of yeasts, LAB, and AAB (Cleenwerck & De Vos, 2008; Cleenwerck *et al.*, 2008; Kurtzman & Robnett, 1998; Latouche *et al.*, 1997; Van Oevelen *et al.*, 1977; Vandamme *et al.*, 1996; Verachtert & Iserentant, 1995). In addition, the discovery of novel species and of many synonymies in these groups of microorganisms confounds the interpretation of literature data. For instance, "*Pediococcus cerevisiae*" was reported as a key organism in lambic beer

fermentation, but this species name has no standing in bacterial nomenclature and has been used for at least two of the currently known *Pediococcus* species, *i.e.*, *P. damnosus* and *P. pentosaceus* (Garvie, 1974; Judicial Commission of the International Committee on Systematic Bacteriology, 1976). Such "*P. cerevisiae*" isolates likely represent *P. damnosus*, as suggested by Van Oevelen *et al.* (1977). Also, Kufferath and Van Laer (1921) first isolated and described the yeast recognized to confer the characteristic taste to lambic beer as *Brettanomyces bruxellensis* and *B. lambicus*. After the observation of the sexually reproducing form, the name *Dekkera bruxellensis* was introduced (Van der Walt, 1964). *B. bruxellensis* and *B. lambicus* were later recognized as synonyms of the same species (Smith *et al.*, 1990).

The present study aimed at the characterization of the microbial communities in two batches of a traditional lambic beer during the first two years of the fermentation process by means of culture-dependent and culture-independent techniques.

Materials and methods

Brewery

Samples were obtained from the Cantillon brewery (http://www.cantillon.be). This brewery is the most traditional, still active, lambic brewery in Brussels and uses the same infrastructure and most of the equipment since 1900, when the brewery was founded.

Sampling

Mash was prepared and boiled according to the brewer's recipe. After 3 h of boiling, the hot wort was pumped, without the removal of the hot trub, into the cooling tun, which was cleaned using hot water and a 500 mL sample was taken aseptically. The pH of the boiled wort was 5.6. Subsequent 500 mL samples were taken after overnight cooling in the cooling tun (the wort temperature was about 20°C) and 15 min; 1, 2 and 3 weeks; and 1, 2, 3, 6, 9, 12, 18 and 24 months after the transfer of the cooled wort into the multiple wooden casks; all these samples were taken from four casks of each of two batches of brews. The brews started on February 25, 2010 (batch 1), and March 23, 2010 (batch 2). Batch 1 was fermented at cellar temperature (ranging from 12°C in winter to 20°C in summer), batch 2 in a different room at ambient temperature (10-30°C). The wooden casks had a volume of approximately 400 L and had two apertures: a bung hole at the top of the cask, which was inaccessible for sampling due to the piling of the casks, and a sampling hole at the front of the cask. The latter was positioned about 10 cm above the cask bottom, plugged by a cork and was used for sampling. After removal of the cork plug, approximately 100 mL of fermenting wort were discarded before collection of the sample. Homogenization of the samples in the casks was not possible and may

have introduced a sampling bias towards microbiota that settled onto the bottom of the cask and those at the wort/air interphase. All casks were used at least one time for the production of lambic beer and were made of oak or acacia wood. The casks were cleaned by filling them with hot water and a chain was inserted through the bung hole, after which the cask was mounted on a gyroscope-like device. The cask was turned in all directions for about 15-20 min. After the water was drained and the chain was removed, a steam hose was inserted in the bung hole and the cask was steamed for about 20 min. Samples were transported on ice to the laboratory and were processed the same day. One cask per batch was chosen for culture-dependent sampling throughout the whole fermentation period and the microbiota of all eight casks was studied using denaturing gradient gel electrophoresis (DGGE) of the V3 region of the bacterial 16S rRNA genes and the D1/D2 region of the yeast 26S rRNA genes.

DGGE analysis

Crude beer samples were centrifuged at 8000 × *g* for 10 min (4°C) on the day of sampling and cell pellets were stored at -20°C until further processing. DNA was prepared from the pellets as described by Camu *et al.* (2007). The DNA concentration, purity, and integrity were determined using 1% (wt/vol) agarose gels stained with ethidium bromide and by optical density (OD) measurements at 234, 260, and 280 nm. The quality of the DNA was assessed as good, when absorbance ratios were $OD_{260}/OD_{280} > 1.8$ and $OD_{234}/OD_{260} > 0.5$. Total DNA solutions were diluted to an OD_{260} of 1. Amplification of about 200 bp of the V3 region of the 16S rRNA genes with the F357 and R518 primers (with a GC clamp attached to the F357 primer), followed by DGGE analysis, and processing of the resulting fingerprints was performed, as described previously (Duytschaever *et al.*, 2011), except that DGGE gels were run for 960 min instead of 990 min. For the amplification of about 200 bp of the D1/D2 region of the 26S rRNA genes, NL1 and LS2 primers (NL1 with GC clamp) were used, as previously reported by Cocolin *et al.* (2000). Similarities in fingerprint patterns were analyzed by means of Dice coefficient analysis, using the BioNumerics 5.1 software package (Applied Maths, Sint-Martens-Latem, Belgium). Gels were also examined using a moving window analysis, in which the percentage change (expressed as 100% - Dice similarity) between two consecutive sample profiles was plotted as a function of time (Marzorati *et al.*, 2008).

All DNA bands were assigned to band classes using the BioNumerics 5.1 software. Dense DNA bands and/or bands that were present in multiple fingerprints were excised from the polyacrylamide gels by inserting a pipette tip into the band and subsequent overnight elution of the DNA from the gel slice in 40 µL 1 x TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 8) at 4°C. The position of each extracted DNA band was confirmed by repeat DGGE experiments using the excised DNA as template. The extracted DNA was subsequently re-amplified and sequenced using the same protocol and primers (but without GC-clamp). EzBioCloud and BLAST (Altschul *et al.*, 1997; Kim *et al.*, 2012) analyses were performed to determine the most similar sequences in the public sequence databases.

Culture media, enumeration and isolation

The samples were serially diluted in 0.9% (wt/vol) saline and 50 µL of each dilution was plated in triplicate on multiple agar isolation media. The set of isolation media used was selected based on preliminary testing of samples of lambic beers of different ages by comparing DGGE profiles of the original samples with those of all cells that were harvested from the agar isolation media tested (data not shown). A total of twenty-three combinations of different growth media and incubation conditions [20°C vs. 28°C and aerobic vs. anaerobic atmosphere] were tested and this resulted in a set of 7 isolation conditions (see below), which together yielded a community profile that reflected best the diversity obtained in the DGGE profiles of the original beer samples and excluded isolation conditions that yielded redundant results.

All bacterial agar isolation media were supplemented with 5 ppm amphotericin B (Sigma-Aldrich, Bornem, Belgium) and 200 ppm cycloheximide (Sigma-Aldrich) to inhibit fungal growth and were incubated aerobically at 28°C, unless stated otherwise. Samples were incubated after plating on de Man-Rogosa-Sharpe (MRS) agar (Oxoid, Erembodegem, Belgium) (De Man *et al.*, 1960) at 28°C aerobically and at 20°C anaerobically for the isolation of LAB. Violet red bile glucose (VRBG) agar (Mossel *et al.*, 1962, 1978) was used for the isolation of *Enterobacteriaceae* and acetic acid medium (AAM) agar (Lisdiyanti *et al.*, 2003) was used for the isolation of AAB.

Yeast isolation media were first supplemented with 30 ppm ampicillin (Sigma-Aldrich), which proved inefficient to inhibit bacterial growth. All samples starting from 3 weeks in batch 1 were subcultured in the presence of 100 ppm chloramphenicol (Sigma-Aldrich). All yeast isolation media were incubated aerobically at 28°C. DYPAI (2% glucose, 0.5% yeast extract, 1% peptone and 1.5% agar; wt/vol) was used as a general yeast agar isolation medium or was supplemented with an additional 50 ppm cycloheximide (DYPAIX) to favor slow-growing *Dekkera/Brettanomyces* spp. (Abbott *et al.*, 2005; Licker *et al.*, 1998; Suárez *et al.*, 2007). Furthermore, universal beer agar (Oxoid) was supplemented with 25% (vol/vol) commercial gueuze (Belle-Vue - AB Inbev, Anderlecht, Belgium) as recommended by the manufacturer and was used as an additional general yeast agar isolation medium (UBAGI).

Colonies on plates comprising 25 to 250 colony forming units (CFU) were counted after 3 to 10 days of incubation and for each of the seven isolation conditions about 20-25 colonies, or all colonies if the counts were lower, were randomly picked up.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) dereplication and identification

Isolates were subcultured twice using the respective isolation conditions, and MALDI-TOF MS was performed using the third generation of pure cultures by means of a 4800 Plus MALDI TOF/TOF™ Analyzer (AB SCIEX, Framingham, MA, USA), as described previously (Wieme *et al.*, 2012). In short, Data Explorer 4.0-software (AB SCIEX) was used to convert the mass spectra into .txt-files to import

them into a BioNumerics 5.1 (Applied Maths) database. Spectral profiles were compared using Pearson product moment correlation coefficient and a dendrogram was built using the unweighted pair group method with arithmetic mean (UPGMA) cluster algorithm. Homogeneous clusters consisting of isolates with visually identical and/or virtually identical mass spectra were delineated. From each cluster, isolates were chosen randomly for further identification through sequence analysis of 16S rRNA genes and other molecular markers. Sequence analysis of *dnaJ* and *rpoB* genes was performed to identify members of the *Enterobacteriaceae* (Mollet *et al.*, 1997; Nhung *et al.*, 2007), of the *pheS* gene to identify LAB (De Bruyne *et al.*, 2007, 2008; Naser *et al.*, 2005, 2007) and of *dnaK*, *groEL* and *rpoB* genes to identify AAB (Cleenwerck *et al.*, 2010). Yeast isolates were identified through sequence analysis of the D1/D2 region of the 26S rRNA gene (Kurtzman & Robnett, 1998) and, whenever needed, also by determination of *ACT1* and/or ITS sequences (Daniel & Meyer, 2003).

All PCR assays were performed as described by Snauwaert *et al.* (2013). Bacterial DNA was obtained via the protocol as described by Niemann *et al.* (1997), whereas yeast DNA was obtained using the protocol of Harju *et al.* (2004).

Analysis of the microbiota of the brewery environment

To analyze the microbiota of the brewery environment, samples were taken from the cooling tun, the roof above the cooling tun, the walls and ceiling of the cellar, and the outside of the casks by swabbing about 100 cm^2 using a moist swab that was transferred into 5 mL of saline and transported to the laboratory. The inside of a cask was sampled by rinsing it with 5 L of saline. In the laboratory, 5-10 mL portions of each sample were subsequently filtered over a 0.45-µm filter that was transferred into 30 mL of MRS, VRBG, AAM, DYPAI and DYPAIX broth, each, and incubated as described above. Enrichment cultures that showed growth after 3-10 days of incubation were plated on their respective agar media and different morphotypes were selected for further analysis. Isolates were identified as described above. Additionally, the swabs and water sample were directly streaked or plated on the agar isolation media. Air samples were taken using a MAS-100 air sampler (Merck, Darmstadt, Germany) with a flow rate of 0.1 m^3/min placed about 1 m above the floor, for one or ten minutes using yeast and bacterial agar isolation media, respectively.

Results

DGGE analysis

Bacterial and yeast DNA was successfully extracted from most samples and PCR amplicons were generated subsequently. As expected, none of the cooling tun samples collected directly after boiling the wort yielded DNA (the wort temperature at the time of sampling was about 90°C). The samples of the overnight-cooled wort yielded DNA, but this was of low quality (data not shown) and no amplicons could be obtained. The first amplicons were obtained from the cask samples immediately

after the transfer of the wort into the casks. For both batches, bacterial and yeast community fingerprints were generated for each of the four casks. Analysis of these community fingerprints revealed highly similar to identical community fingerprints for each sampling moment (Supplementary Figure S 4.1.1). DGGE banding patterns of both bacterial and yeast communities of the casks that were used in the culture-dependent analysis of batch 1 and 2 (see below) are shown in Figure 4.1.1.

Visual inspection of the bacterial community profiles revealed differences primarily during the first 12 months of the fermentation process, both in terms of presence and intensity of DNA bands. With the exception of two amplicons in the high % G+C region of the fingerprints (Figure 4.1.1, band classes marked 3 and 4), the bacterial community profiles generated after 18 months were virtually identical in both batches. This bacterial community profile was reached in batch 1 after 18 months of fermentation, compared to 6 months in batch 2. The latter may be due to the incubation of batch 2 casks at ambient temperature, which was higher during the summer months compared to batch 1 casks that were incubated at more constant but lower temperatures in the cellar. In batch 1, a very dense band disappeared after 1 month of fermentation (Figure 4.1.1, band class 1), while another band appeared in the subsequent sample taken after 2 months of fermentation time (Figure 4.1.1, band class 2).

Visual inspection of the yeast community profiles revealed more simple fingerprints comprising one to six DNA bands throughout the fermentation process. Again, the communities in both batches reached a fairly stable and highly similar composition after 6 months in batch 2 compared to 18 months in batch 1, with two amplicons in the central % G+C region of the fingerprints that were consistently present (Figure 4.1.1, band classes 5 and 6).

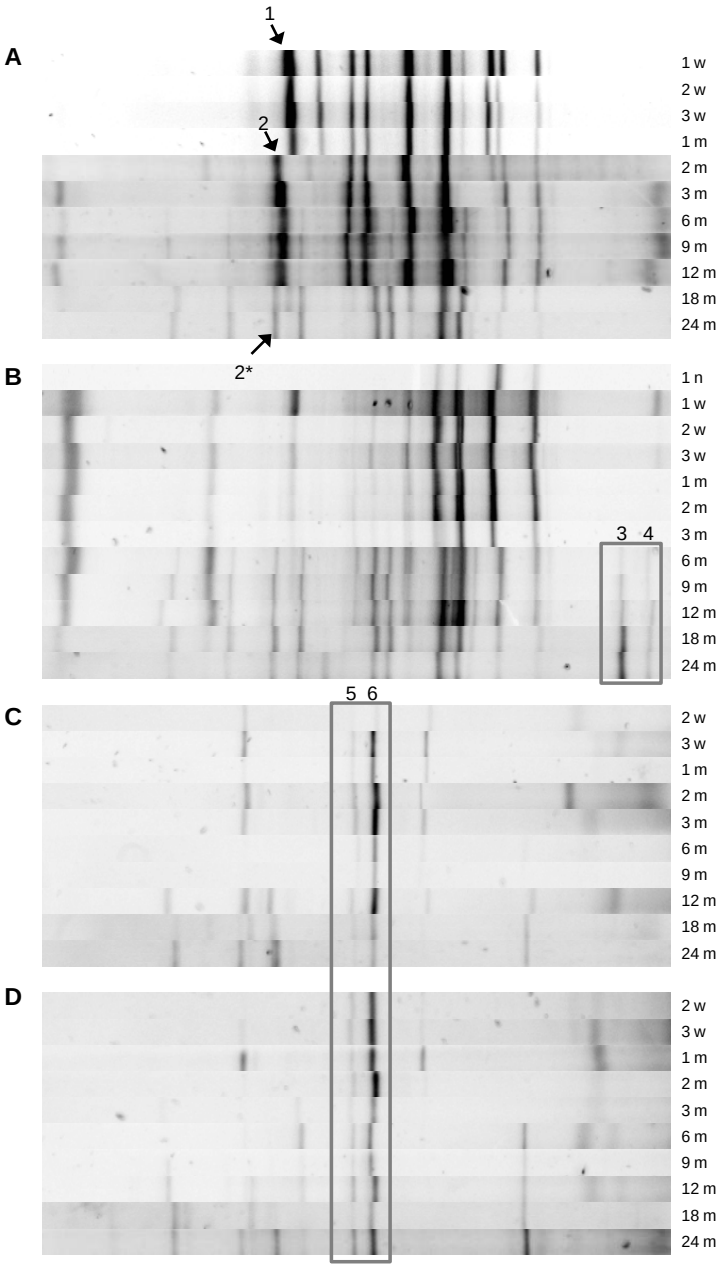

Figure 4.1.1 DGGE banding patterns of bacterial and yeast communities of the plated samples. DGGE banding patterns of the bacterial and yeast communities of batch 1, cask 1 (A and C, respectively) and batch 2, cask 2 (B and D, respectively) n, night; w, week(s); m, month(s). Band classes 1-6 are indicated on the figure. Samples after one night in cask 1 of batch 1 did not yield any amplicons with the V3 primer, the other casks yielded banding patterns highly similar to the pattern of the one-week sample (data not shown). Yeast community profiles were obtained from 2 weeks onwards for all casks. Nevertheless, some samples also yielded amplicons after wort transfer to the casks and after one week; these profiles were comparable to the profiles obtained after 2 weeks for all casks (data not shown).

The moving window analysis of the Dice similarity values between DGGE profiles (Figure 4.1.2A and Figure 4.1.2C) demonstrated that the bacterial community profiles of the four casks of both batches showed a similar evolution in diversity.

Consecutive samples displayed few changes. After 2 months, the appearance and disappearance of two dense bands (Figure 4.1.1, band classes 1 and 2) resulted in a higher percentage change. The major transition in bacterial community profile appeared to occur after 18 months in batch 1, whereas the bacterial community profile changed after 6 months in batch 2 (Figure 4.1.2A and Figure 4.1.2C).

The moving window analysis of the yeast community profiles (Figure 4.1.2B and Figure 4.1.2D) revealed a higher variability. These higher percentages of change are most likely explained by the higher impact of changes in band presence or intensity in these profiles that comprised fewer bands.

A total of 64 bands (28 from yeast community fingerprints and 36 from bacterial community fingerprints) were excised (Supplementary Figure S 4.1.2) and sequenced to tentatively assign these band classes to microbial taxa (Supplementary Table S 4.1.1E). Because of the short length of the sequences (about 200 bp), EzBioCloud and BLAST analyses resulted in genus or family level identifications only. An overview of these identification data is shown in Supplementary Table S 4.1.1 and demonstrates that members of the *Enterobacteriaceae* family could be detected throughout the fermentation process in both batches. Both band class 1 and 2 (Figure 4.1.1) were assigned to members of the *Enterobacteriaceae* family. Band class 2* (Figure 4.1.1) that migrated at nearly the same position as band class 2 was assigned to *Pediococcus*/*Lactobacillus* (which could not be distinguished by using this short rRNA gene fragment). Also, additional band classes in a higher % G+C region of the profile were assigned to LAB, which were rarely found before month 3 in batch 1 samples, but which were nearly consistently present in batch 2 samples (Supplementary Table S 4.1.1A and Table S 4.1.1B). Band classes 3 and 4 (Figure 4.1.1) were assigned to AAB, which were detected from month six onwards in batch 2 samples and primarily during year 2 in batch 1 (Supplementary Table S 4.1.1A and Table S 4.1.1B). Several DNA bands of the bacterial community fingerprints were assigned to yeast taxa (Supplementary Table S 4.1.1A and Table S 4.1.1B), confirming that the V3 primers were not specific for bacteria (Scheirlinck *et al.*, 2008; Van der Meulen *et al.*, 2007).

The yeast band classes 5 and 6 were assigned to the genus *Saccharomyces* (Supplementary Table S 4.1.1C and Table S 4.1.1D) and were present throughout the

fermentation. Bands originating from other yeast taxa (*Candida*, *Dekkera/Brettanomyces, Hanseniaspora, Kregervanrija*, *Naumovia* and *Wickerhamomyces*) were found frequently, albeit on an irregular basis.

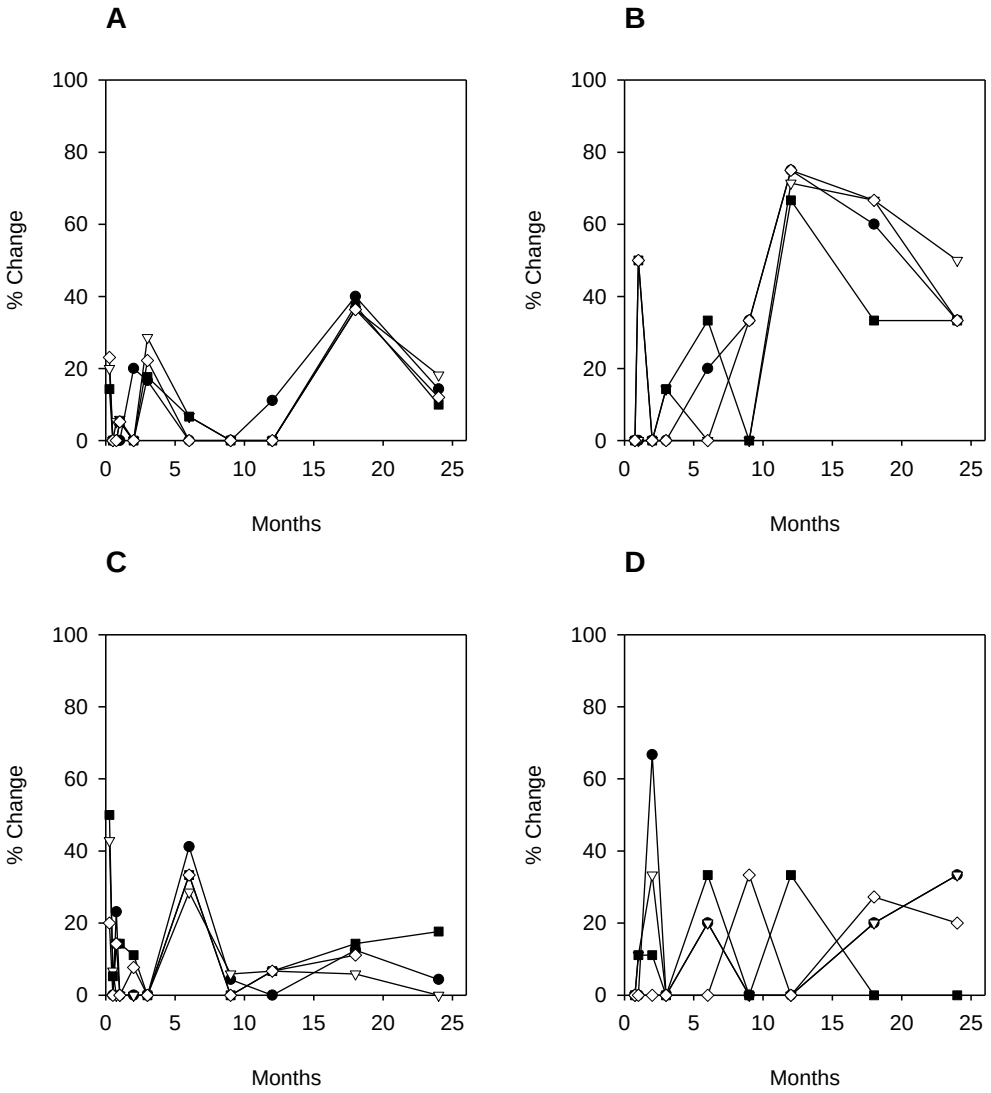

Figure 4.1.2 Moving windows analysis of the DGGE bacterial and yeast community profiles. Moving window analysis of the Dice-based similarity values between DGGE analyses of 4 casks from batches 1 and 2. (A) and (C) represent the bacterial diversity in batches 1 and 2, respectively, (B) and (D) visualize the yeast diversity of both batches 1 and 2. The last data point of the bacterial community profile analysis of batch 2, cask 4 was omitted due to the poor quality of the banding patterns. ● Cask 1; ▽ Cask 2; ■ Cask 3; ◇ Cask 4.

Enumeration and identification of bacteria and yeasts

Table 4.1.1 presents an overview of the enumeration analyses and Supplementary Table S 4.1.2 presents the identifications of the MALDI-TOF MS clusters. A total of

1304 bacterial and 892 yeast isolates were obtained from the 2 batches. The freshly boiled wort did not allow microbial growth. However, both batches were spontaneously inoculated overnight in the cooling tun, as shown by the colony counts on MRS and VRBG agars, but no colonies were found on AAM agar. All cooling tun isolates (48 from batch 1 [Figure 4.1.3] and 77 from batch 2 [data not shown]) were identified as members of the *Enterobacteriaceae* family. These bacteria were also isolated from MRS agar, which was thus not fully specific for the isolation of LAB. Both MRS and VRBG supported the growth of *Enterobacteriaceae*, but the relative species distribution differed (Figure 4.1.3). Batch 1 isolates were identified as *Escherichia*/*Shigella [Escherichia coli* and *Shigella* species are extremely closely related (Brenner, 1984) and cannot be distinguished by sequence analysis of conserved genes (Lan & Reeves, 2002; Pupo *et al.*, 2000)], *Enterobacter hormaechei* or *Enterobacter kobei*, whereas only the latter two were identified in batch 2 samples (31 and 46 of the 77 isolates, respectively).

Enterobacteriaceae counts reached up to 10^7–10^8 CFU/mL after one to two weeks of fermentation. A total of 415 isolates from batch 1 samples taken during the first month were identified. *E. hormaechei* was no longer isolated after the transfer of the wort into the cask (performed 15 min after the sampling of the cooling tun), whereas *Klebsiella oxytoca* was then first isolated (Figure 4.1.3). In the following weeks, the number of isolates identified as *Escherichia*/*Shigella* and *E. kobei* decreased, while the numbers of *Hafnia paralvei* and *Klebsiella oxytoca* isolates increased until the end of the first month, after which *Enterobacteriaceae* were no longer isolated. In batch 2, from which a total of 398 isolates were identified, a similar evolution was found: the major occurrence of *H. paralvei* from week 1 onwards was confirmed and members of the *Enterobacteriaceae* were again no longer isolated after one month of fermentation (data not shown). However, batch 2 *Enterobacteriaceae* were more diverse and included also *Citrobacter gillenii* and *Raoultella terrigena* (data not shown).

Table 4.1.1 Results of plate counts on different agar isolation media. VRBG agar was used for the growth of *Enterobacteriaceae*, MRS agar was used for the growth of LAB, AAM agar was used for the growth of AAB, DYPAI and UBAGI agars were used as global yeast growth media and DYPAIX agar was used to favor the growth of *Dekkera* species. The values represent log CFU/mL. ULD: under limit of detection (< 20 CFU/mL); ULQ: under limit of quantification (the estimated CFU/mL is provided between brackets); ND: no data.

Batch 1	**VRBG 28°C**	**MRS 28°C**	**MRS 20°C AN**	**AAM 28°C**	**DYPAI 28°C**	**UBAGI 28°C**	**DYPAIX 28°C**
Freshly boiled wort	ULD	ULD	ULD	ULD	ULD	ULD	ULD
1 night cooling tun	6.03	ULD	5.9	ULD	ND	ND	ND
1 night cask	6.51	5.25	6.42	ULD	ND	ND	ND
1 week	6.72	6.98	8.05	ULD	ND	ND	ND
2 weeks	7.73	7.68	7.77	ULD	ND	ND	ND
3 weeks	6.92	7.2	7.41	3.72	6.36	6.31	2.9
1 month	4.63	4.92	4.83	ULQ (466)	6.33	6.47	4.02
2 months	ULQ (40)	ULQ (80)	ULQ (180)	3.39	5.73	5.58	3.29
3 months	ULD	3.23	ULQ (33)	3.28	5.78	5.62	ULQ (273)
6 months	ULD	ULQ (300)	ULQ (447)	ULD	4.56	4.6	4.03
9 months	ULD	3.51	4.38	3.01	3.2	3.24	2.87
12 months	ULD	ULD	2.79	ULQ (26)	4.3	4.35	3.16
18 months	ULD	2.83	2.93	ULD	2.8	ULQ (347)	ULQ (293)
24 months	ULD	3.08	4.19	2.96	3.74	3.83	2.94
Batch 2							
Freshly boiled wort	ULD	ULD	ULD	ULD	ULD	ULD	ULD
1 night cooling tun	5.12	6.71	6.92	ULD	ULQ (50)	ULQ (253)	ULQ (40)
1 night cask	6.13	6.79	6.11	ULD	3.29	3.42	3
1 week	7.91	8.41	8.29	ULD	4.36	4.33	ULQ (140)
2 weeks	7.54	7.67	7.5	ULD	6.21	6.18	2.72
3 weeks	6.92	6.78	7	ULQ (40)	5.51	5.49	ULQ (120)
1 month	4.88	4.91	4.86	ULQ (270)	5.18	5.17	ULQ (67)
2 months	ULD	4.46	4.58	3.54	5.37	5.31	ULQ (13)
3 months	ULD	6.42	6.35	4.73	4.5	4.46	ULQ (353)
6 months	ULD	4.58	5.02	ULD	4.26	4.3	4.34
9 months	ULD	5.45	5.48	ULQ (40)	4.09	3.02	3.07
12 months	ULD	5.8	5.77	ULD	3.15	2.72	3.51
18 months	ULD	3.81	4.38	ULD	ULQ (173)	ULQ (300)	ULQ (240)
24 months	ULD	4.07	4.26	ULQ (66)	3.08	3.18	3.18

From months 2 until 24, *Pediococcus damnosus* was consistently the only microorganism isolated from MRS agar (batch 1 [Figure 4.1.3]; batch 2, n = 124 [data not shown]). The bacterial counts on MRS agar remained stable at about 10^4 CFU/mL until the end of the fermentation. Colony counts on AAM agar were generally low (below 10^4 CFU/mL; Table 4.1.1). AAM counts of the samples up to 3 months of fermentation were influenced by the presence of yeasts, which was due to the apparent loss of activity of amphotericin B under acidic conditions (te Dorsthorst *et al.*, 2005). Amphotericin B was also reported to be unstable in other media with a composition similar to AAM (Cheung *et al.*, 1975). A combination of

amphotericin B and cycloheximide was subsequently found to be more effective in inhibiting yeast growth under all isolation conditions used.

AAB were isolated from batch 1 samples at 9 and 24 months (n = 35) and from batch 2 samples at 3, 9 and 24 months (n = 17). All but one of the isolates were identified as a novel *Acetobacter* species, for which the name *Acetobacter lambici* has been proposed (Spitaels *et al.*, 2014b). One batch 2 isolate represented a novel *Gluconobacter* species, for which the name *Gluconobacter cerevisiae* has been proposed (Spitaels *et al.*, 2014a). This erratic isolation of AAB was not in accordance with the consistent presence of AAB-derived DNA bands in the DGGE profiles from 6 months of fermentation onwards in batch 2 (Figure 4.1.1).

An overview of the identified yeast species of batch 1 is graphically represented in Figure 4.1.4 and Supplementary Figure S 4.1.3. Isolation and accurate enumeration of yeasts during the first two weeks of fermentation of batch 1 was not possible, due to an insufficient suppression of bacterial growth. In batch 2 samples (data not shown), yeasts could not be detected in the wort after one night in the cooling tun, but increased in numbers directly after the wort was transferred into the casks (not more than 15 min after the cooling tun was sampled). Maximal counts (10^6 CFU/mL) were reached after 2 weeks to 1 month of fermentation. *Debaryomyces hansenii* (17/18 isolates examined) and *S. cerevisiae* (1/18) were the sole species isolated directly after the transfer of the wort into the cask in batch 2. *S. cerevisiae* (22/44), *S. pastorianus* (21/44) and *Naumovia castellii* (1/44) were isolated after 1 week of fermentation. The relative number of *S. pastorianus* isolates increased further during the first three months of fermentation (a total of 198 isolates examined), until it was the only yeast species isolated on DYPAI and UBAGI agars after 2 months (32 isolates examined). After 3 months, *S. pastorianus* was still the predominant yeast (30/31); one isolate was identified as *N. castellii*.

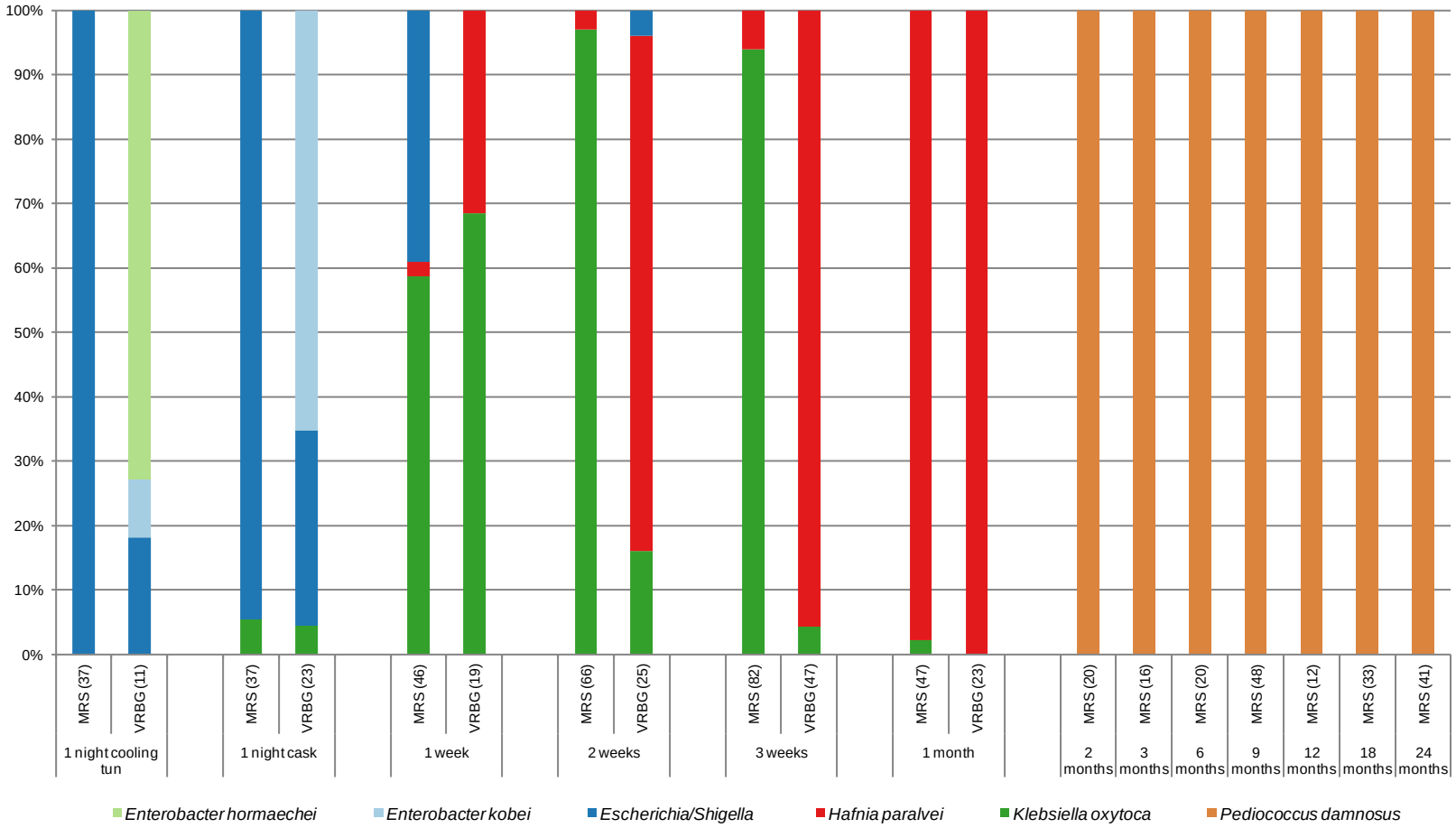

Figure 4.1.3 Identification of random isolates from MRS and VRBG agars of batch 1. The identification of isolates belonging to the *Enterobacteriaceae* are reported to the species level, when reliable identification by housekeeping gene sequences could be obtained. The number of isolates is given between brackets.

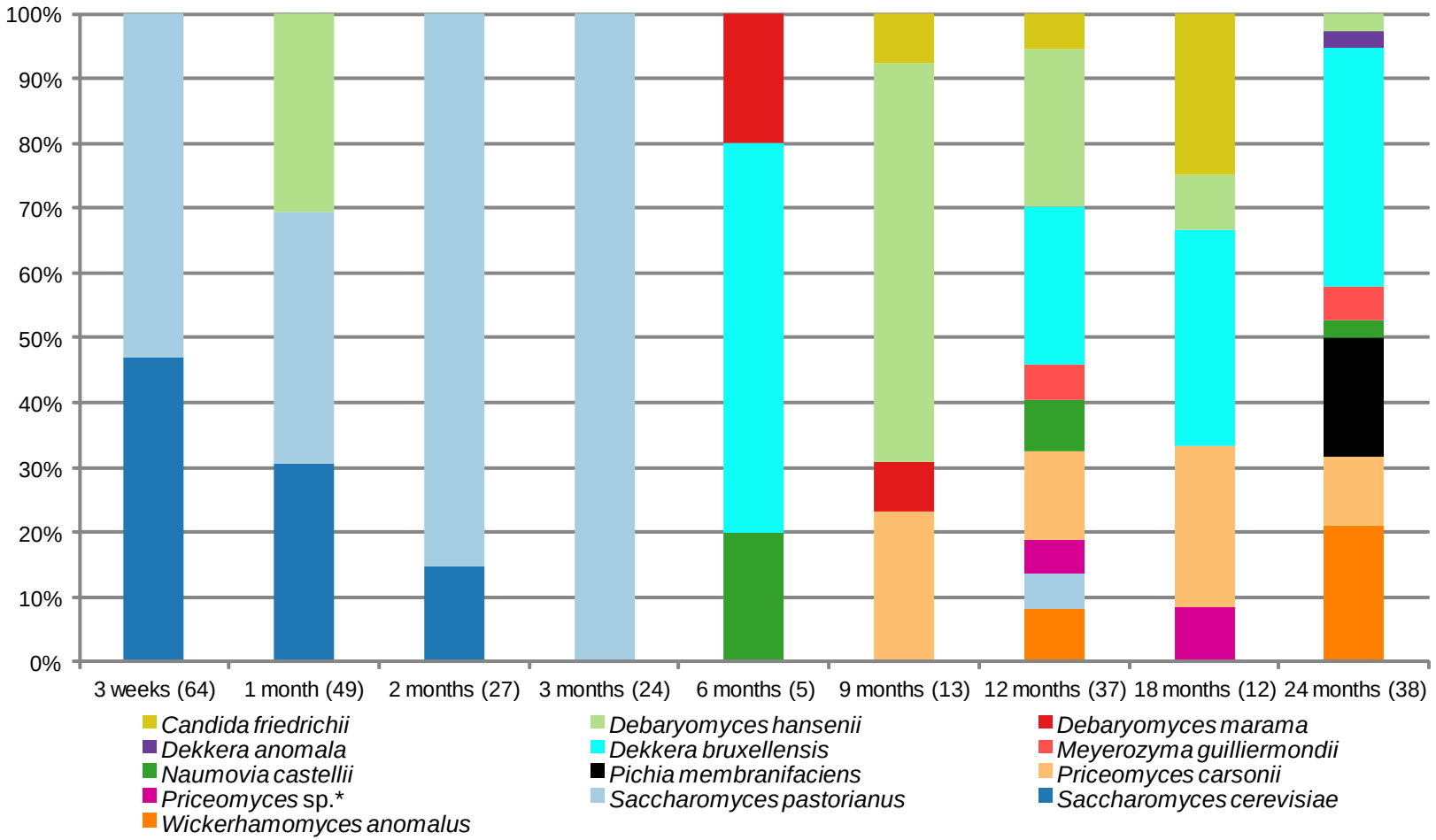

Figure 4.1.4 Identification of random isolates from DYPAI and UBAGI agars of batch 1. The number of isolates is given between brackets. *One yeast cluster from MALDI-TOF MS profiles could not be identified unambiguously (Supplementary Table S 4.1.2).

The same trend occurred during the first three months of fermentation of batch 1 (Figure 4.1.4). *S. cerevisiae* and *S. pastorianus* were the most prevalent species and the latter one was the only yeast species present after three months. Yeast counts on DYPAIX agar were initially lower compared to DYPAI and UBAGI agars, but were comparable from 6 months onwards. The few DYPAIX isolates that were obtained from samples after 2 months (batch 2) or 3 months (batches 1 and 2) failed to grow on the same growth agar medium upon subculture, indicating that there were no cycloheximide-resistant yeast species present in these samples (Supplementary Figure S 4.1.3). DYPAIX isolates obtained from samples of the first 2 months of batch 1 included *N. castellii*, *Kazachstania servazzii* and *Db. hansenii* (Supplementary Figure S 4.1.3), whereby the former was the only species isolated in the first month of batch 2 (n = 58, data not shown).

Saccharomyces spp. were not isolated in large numbers after 6 months of fermentation, while *D. bruxellensis* was isolated at this point for the first time. *D. bruxellensis* was the major yeast species isolated from DYPAI and UBAGI agar media from 6 months until the end of the fermentation of batch 2 (n = 102, data not shown) and the only yeast species isolated from DYPAIX agar in the same period (n = 82). The cultivated yeast diversity in batch 2 was low compared to batch 1 (see below) and the three yeast media yielded the same species diversity from 6 months onwards.

The yeast species distribution in batch 1 samples after 6 months of fermentation (Figure 4.1.4) was more complex than that of samples of the same age in batch 2. The most frequently cultivated species were *D. bruxellensis, Db. hansenii*, *Priceomyces carsonii* and *Wickerhamomyces anomalus* along with other species in lower numbers (Figure 4.1.4 and Supplementary Figure S 4.1.3). In contrast to batch 2 where the three yeast agar isolation media yielded the same species diversity from 6 months onwards, the species diversity recovered from different yeast agar isolation media in batch 1 was not comparable. For example, *D. bruxellensis* was not detected on the non-selective yeast agar media in batch 1 after 9 months, but was detected at this sampling point on DYPAIX agar (Figure 4.1.4 and Supplementary Figure S 4.1.3). The use of DYPAIX agar allowed isolating some unusual species from batch 1, such as *Candida patagonica* and *Yarrowia lipolytica* (Supplementary Figure

S 4.1.3), of which the latter has never been associated with a beer fermentation process. The total yeast and bacterial counts were similar in both batches after 24 months at about 10^3–10^4 CFU/mL (Table 4.1.1).

Air and brewery environment

None of the directly plated samples yielded growth. A total of 139 isolates from the brewery environment were picked up from the bacterial and yeast agar isolation media after enrichment and were identified through MALDI-TOF MS and sequence analysis of 16S rRNA genes or other molecular markers as described above (Table 4.1.2). Several species or taxa that were previously isolated during the fermentation process as described above were also found in environmental samples. *E. hormaechei* and *Escherichia/Shigella* were isolated from the cellar air. *Raoultella terrigena*, *Pichia membranifaciens*, *Debaryomyces marama* and *Db. hansenii* were isolated from the inside of a cask. The latter species was also isolated from the ceiling, the attic and cellar air, along with *S. pastorianus*, *Meyerozyma guilliermondii*, *Candida friedrichii* and *Wickerhamomyces anomalus*. The latter species was also found on the outside of a cask. A considerable number of additional microorganisms that were not detected during the fermentation process were also isolated from environmental samples. These included species previously related to beverage fermentation or spoilage, such as *Brettanomyces custersianus* (Martens *et al.*, 1997), *Pediococcus pentosaceus* (Hutzler *et al.*, 2013), *Lactobacillus malefermentans* (Farrow *et al.*, 1988) and *Acetobacter cerevisiae* (Cleenwerck *et al.*, 2002).

Table 4.1.2 Overview of microorganisms isolated from the brewery environment and their isolation sources. The bacteria and yeasts present in the fermentation were identified based on their MALDI-TOF MS spectra. *One yeast cluster from MALDI-TOF MS profiles could not be identified unambiguously (Supplementary Table S 4.1.2).

Identification	Accession number	Accession number highest hit	Similarity	Present in fermentation	Air attic before cooling	Cooling tun	Roof	Air attic after cooling	Air cellar	Cellar ceiling	Cellar wall	Cask outside	Cask inside
Bacteria[a]													
Acetobacter cerevisiae[b]		KF537492	100%										+
Aerococcus urinaeequi		D87677	100%		+								
Bacillus licheniformis		AE017333	100%				+						
Enterobacter hormaechei				+					+				
Enterococcus faecium[c]	KJ186124	AJ843428	97%		+	+							
Escherichia/Shigella				+					+				
Hafnia alvei		M59155	100%										+
Lactobacillus curvatus		AJ621550	100%						+				
Lactobacillus malefermentans		BACN01000105	100%									+	
Lactobacillus nenjiangensis[c]	KJ186125	HF679044	99%		+								
Leuconostoc mesenteroides		CP000414	100%		+				+		+	+	
Leuconostoc pseudomesenteroides		AEOQ01000906	100%		+								
Pediococcus pentosaceus[c]		AM899822	100%		+								
Pseudomonas azotoformans		D84009	100%		+								
Pseudomonas libanensis		AF057645	100%		+								
Pseudomonas psychrotolerans		AJ575816	100%		+								
Rahnella aquatilis		CP003244	100%										+
Raoultella terrigena				+									+
Staphylococcus hominis		X6601	100%		+			+	+				

Table 4.1.2 (Continued)

Identification	Accession number highest hit	Similarity	Present in fermentation	Air attic before cooling	Cooling tun	Roof	Air attic after cooling	Air cellar	Cellar ceiling	Cellar wall	Cask outside	Cask inside
Yeasts[d]												
Brettanomyces custersianus	DQ406717	100%										+
Candida friedrichii			+	+								
Candida pomicola	AF245400	100%										+
Cryptococcus heveanensis	AF075467	100%										+
Cryptococcus magnus	AF181851	100%		+			+					+
Debaryomyces hansenii			+	+				+	+			+
Debaryomyces marama			+									+
Meyerozyma guilliermondii			+	+								
Pichia membranifaciens			+									+
Priceomyces sp.*			+					+				
Saccharomyces pastorianus			+					+				
Trichosporon gracile	JN939453	100%		+				+				
Trichosporon cutaneum	AF075483	100%									+	
Wickerhamomyces anomalus			+	+				+			+	

[a] Identification is based on 16S rRNA gene sequence.
[b] Identification is based in *rpoB* sequence.
[c] Identification is based in *pheS* sequence.
[d] Identification is based on D1/D2 26S rRNA gene sequence

Discussion

Serious limitations of the few available microbiological studies of the lambic beer fermentation process are the rather low numbers of isolates identified using biochemical methods only (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). Recent polyphasic taxonomic studies revealed that phenotypic identification approaches alone have an inadequate taxonomical resolution for the accurate species level identification of these microorganisms (Cleenwerck & De Vos, 2008; De Bruyne *et al.*, 2008; Kurtzman & Robnett, 1998; Latouche *et al.*, 1997; Nhung *et al.*, 2007; Vandamme *et al.*, 1996). Therefore, the present study revisited the microbiology of the lambic beer fermentation process of the most traditional lambic brewery (Cantillon) in Belgium and identified and monitored the microbiota using MALDI-TOF MS as a high-throughput dereplication technique. This allowed to compare numerous fingerprints and to reduce these isolates to a non-redundant set of different species that were further identified using an array of DNA sequence-based methods (Dieckmann *et al.*, 2005; Vandamme *et al.*, 1996). This approach allowed a more in depth analysis of the culturable microbiota of this ecosystem and resulted in the isolation and description of two novel AAB species, *i.e.*, *Acetobacter lambici* and *Gluconobacter cerevisiae* (Spitaels *et al.*, 2014a, 2014b). The former species was even the most frequently isolated AAB species during the lambic fermentation process of Cantillon. The present study also used DGGE profiles of variable prokaryotic and eukaryotic rRNA gene regions to identify and monitor the microbial communities in two batches of lambic beer during a two-year fermentation period at Cantillon.

In both lambic batches, members of the *Enterobacteriaceae* were isolated during the first month, which corresponded to previous studies on Belgian lambic and American coolship ales (Bokulich *et al.*, 2012; Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). The bacteria identified included *E. hormaechei*, *E. kobei*, *Es. coli*, *H. paralvei*, *K. oxytoca*, *Citrobacter gillenii* and *R. terrigena*, from which some of these were already detected in the cooling tun sample, suggesting their origin from the cooling tun environment. Remarkably, DNA from members of the *Enterobacteriaceae* family was detected in the DGGE experiments throughout the two-year fermentation period. This suggests that DNA from these cells persisted for

a long time or, alternatively, that these bacteria remained present in a VBNC form, even under conditions to which *Enterobacteriaceae* are susceptible, *i.e.*, pH < 4.0 and ethanol concentrations over 2.0% (Priest & Stewart, 2006). This has also been seen during cocoa bean fermentation (Papalexandratou *et al.*, 2011a, 2011b). Yeast isolations during the first three months yielded *Saccharomyces* spp., but no *Hanseniaspora* spp., as expected from previous studies (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). However, *Hanseniaspora* spp. were detected by DGGE profiles over several months in both batches. Species of this genus are frequently found in spontaneously fermenting fruit and their preparations, and a positive contribution to wine flavor development is increasingly recognized (*e.g.*, Medina *et al.,* 2013).

After the initial *Enterobacteriaceae* phase, the effects of ethanol production by the main fermentation were reflected in the dominance of *P. damnosus* at two months, along with some AAB (primarily *Acetobacter lambici*) that were occasionally isolated. AAB may survive in the cask due to the diffusion of oxygen through the wood (Joyeux *et al.*, 1984; Ribéreau-Gayon *et al.*, 2006) or the short vacuum-releasing opening of the bung hole during sampling. Similarly, AAB seem to survive the anaerobic phase of cocoa bean fermentations (Papalexandratou *et al.*, 2011a, 2011b). The irregular isolation of AAB may suggest that they are also present in a VBNC form that could be reversed when oxygen becomes available, as for example in wine production (Millet & Lonvaud-Funel, 2000). In both batches, *P. damnosus* remained present throughout the fermentation process and these bacteria were accompanied by *D. bruxellensis* after the decrease of *Saccharomyces* spp. Remarkably, no other LAB were isolated, while *Lactobacillus* spp. and other LAB species have also been isolated from American coolship ales recently (Bokulich *et al.*, 2012).

The culture-independent detection of microorganisms by DGGE was useful to observe the similar succession of microorganisms in each of the four casks of both lambic batches, and to visualize the relative stability of community profiles over time and their homogenization in the two batches at the advanced stage of the fermentation, but it confirmed some of the established pitfalls of this methodology. For instance, some cultivated yeast genera were not detected by DGGE

(*Debaryomyces, Kazachstania, Meyerozyma, Pichia, Priceomyces, Yarrowia*), while other genera were detected by DGGE but not cultivated (*Hanseniaspora, Kregervanrija*). Also, some organisms were detected by DGGE before appearing in culture or after having disappeared from cultures, such as *Enterobacteriaceae* which were detected throughout the sampling period. Similar observations using T-RFLP and barcoded amplicon sequencing were made in spontaneous fermentations of American coolship ales (Bokulich *et al.*, 2012). Cultivation experiments too can be strongly biased, for instance, by the presence of VBNC cells, the selection of the culture media in the experiment design and by culture media that favor specific organisms. Therefore, a combination of multiple complementary techniques including both culture-based and culture-independent methods and a cautious interpretation of the results remains the best approach for microbial diversity analyses (Lagier *et al.*, 2012).

The microbial community analyses of the present study did not provide evidence for an extended acidification phase (Verachtert & Iserentant, 1995), as after six months *P. damnosus* and *D. bruxellensis* were both present and *Saccharomyces* spp. were no longer isolated. In addition, neither lambic batch showed a clear decrease of LAB. Pending a detailed analysis of the microbial metabolites and other biochemical characteristics, the data of the present study suggest that the acidification took place rapidly at the transition from the main fermentation phase to the long maturation phase, as was also found for American coolship ale fermentations (Bokulich *et al.*, 2012).

The two nearly simultaneously fermented wort batches were inoculated by microorganisms present in the brewery air, equipment or casks. As discussed above, members of the *Enterobacteriaceae* family were present in the wort before its transfer into the casks. These rather adventitious bacteria, *S. pastorianus* and some other yeast species, may have at least partially originated from the brewery air, but the present study failed to isolate the key microorganisms *P. damnosus, S. cerevisiae* and *D. bruxellensis* from environmental samples. These microorganisms were either missed by the sampling protocol or were concealed in niches that were not sampled. Examples of such niches are biofilms in the corners (where the head connects to the staves) and the pores of the wooden casks. Microorganisms may have penetrated

and effectively be immobilized and protected from washing steps in the wood of the cask, as demonstrated previously (Swaffield & Scott, 1995; Swaffield *et al.*, 1997). All casks had been used for lambic production before, preceded by their use in different fermentations, mostly red wine, so they could have retained specific microbiota in spite of cleaning procedures after previous fermentations (Swaffield & Scott, 1995; Swaffield *et al.*, 1997).

This study generally confirmed and extended the microbial diversity and succession known from previous accounts of lambic beers. The more than 2000 microbial isolates from two fermentation batches of the present study showed diverse members of the *Enterobacteriaceae* family during the first month, and *S. cerevisiae* and *S. pastorianus* from the first week until two and three months, respectively. No LAB were recovered during this first phase, which was previously denoted as the 'mixed acid fermentation'. The main fermentation was characterized by *Saccharomyces* spp. and the completion of the shift from *Enterobacteriaceae* to *P. damnosus*, the latter being isolated from 2 months onwards. The increase of LAB in months 2 and 3 and the concomitant decrease of *Saccharomyces* spp. was followed by the highly acid- and ethanol-resistant *D. bruxellensis,* which dominated from 6 months onwards together with *P. damnosus*. *Hanseniaspora* spp. that were previously reported in the first fermentation weeks were not isolated, but their presence was evidenced by DGGE analyses. The role of these and other taxa, such as *N. castellii* and *Kazachstania* spp., both also seen in lambic beer fermentations before, is not known.

Despite apparent differences in the microbial diversity, both batches examined reached similar community profiles at the end of the fermentation. The time needed to reach these final community fingerprints differed between the two batches and it is likely that the lower ambient temperature in the localization of batch 1 explains both the longer period needed to reach the characteristic community fingerprints as well as the larger diversity observed in later phases of the fermentation process.

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Supplementary information

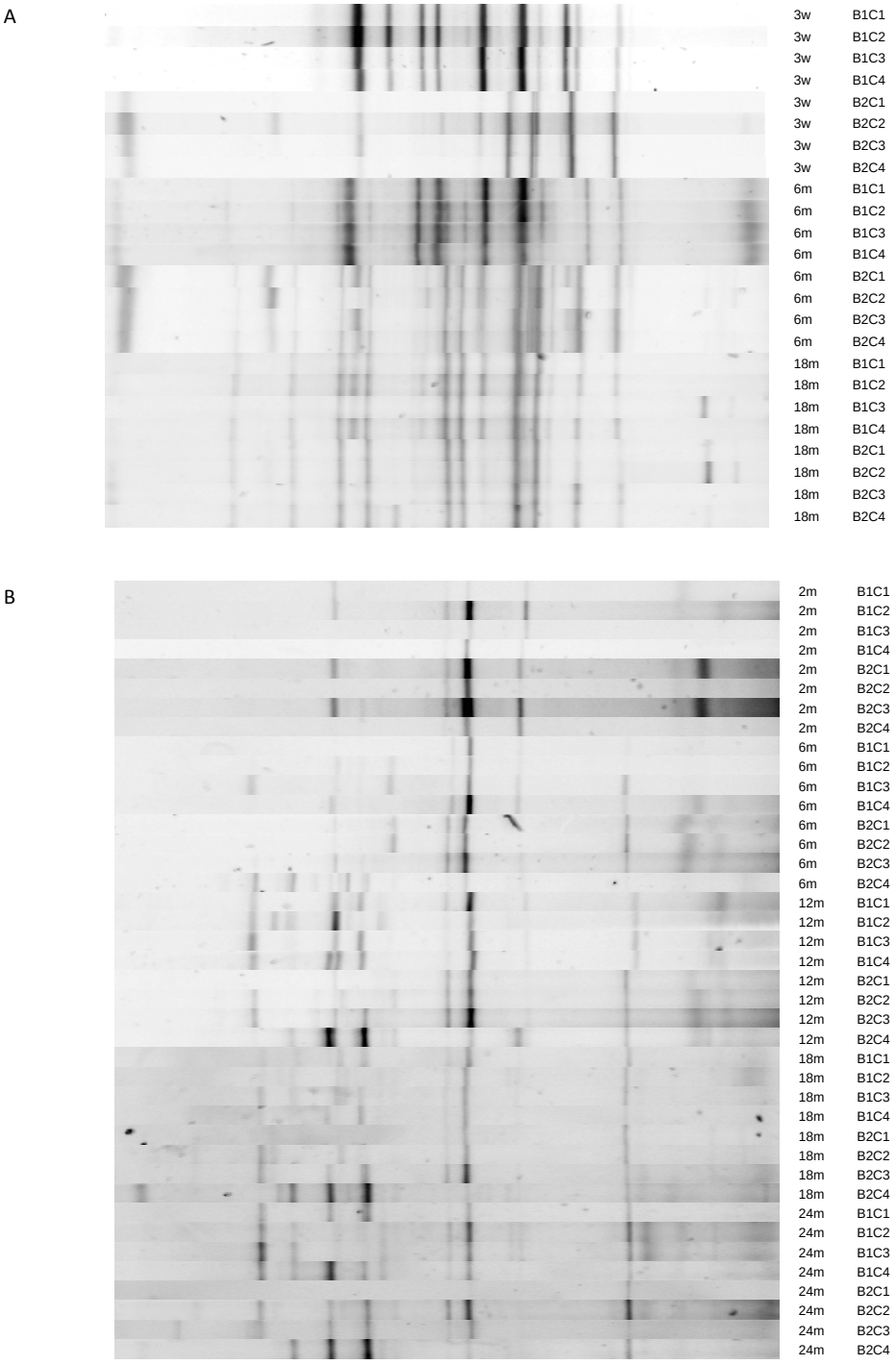

Figure S 4.1.1 Overview of intra-batch DGGE banding pattern differences. Overview of the differences in banding profiles for the DGGE analysis of 4 different casks (C1, C2, C3 and C4) within the same fermentation of batches 1 (B1) and 2 (B2). (A) DGGE banding patterns of the bacterial communities after 3 weeks (3w), 6 months and 18 months of fermentation; (B) DGGE banding patterns of the yeast after 2 months (2m) 6 months (6m), 12 months (12m), 18 months (18m) and 24 months (24m) of fermentation.

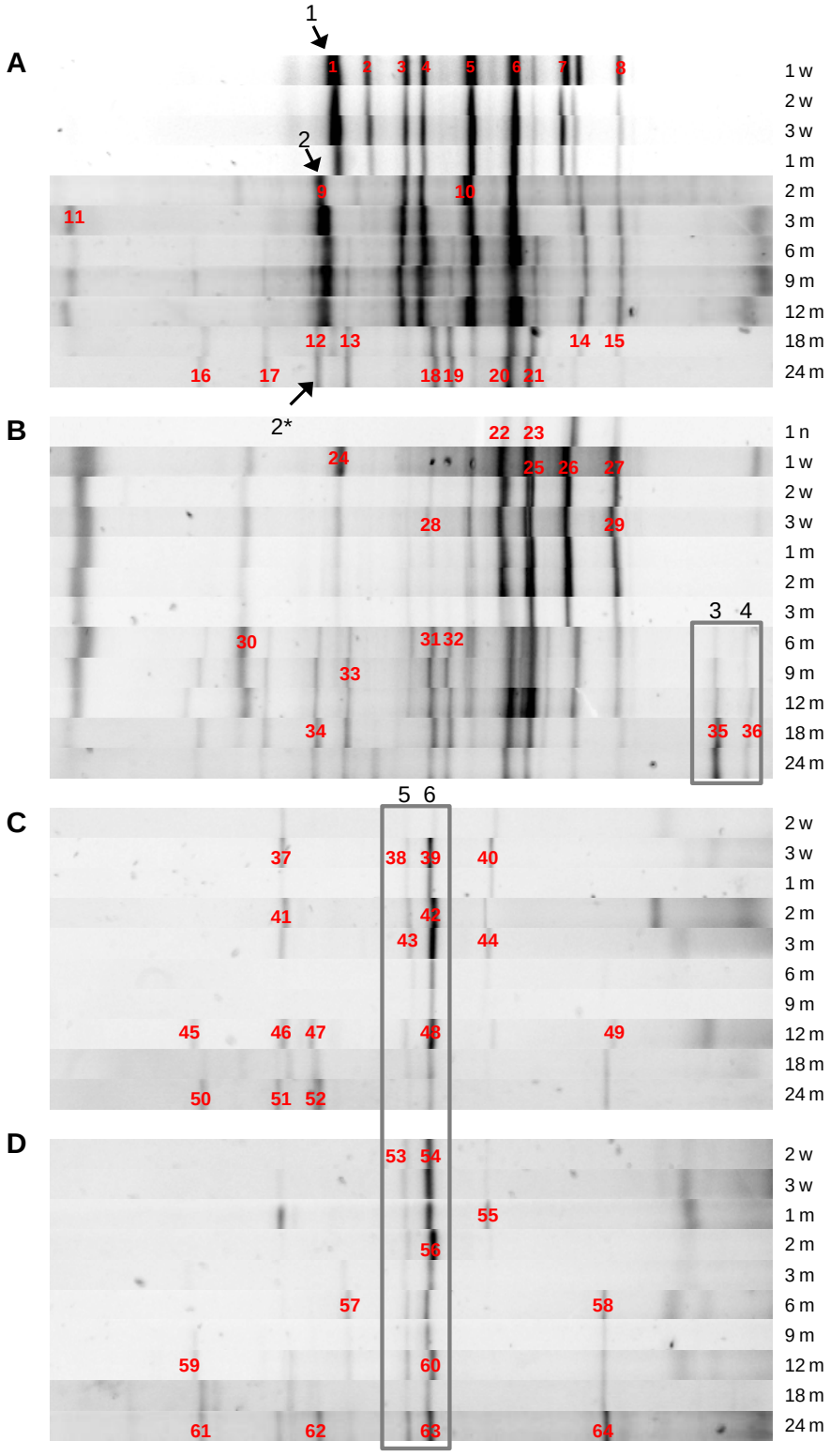

Figure S 4.1.2 Overview of the excised DGGE bands for identification. DGGE banding patterns of the bacterial and yeast communities of batch 1, cask 1 (A and C, respectively) and batch 2, cask 2 (B and D, respectively) n, night; w, week(s); m, month(s). Band classes 1-6 are indicated on the figure. The excised bands are indicated in red and identifications based on the derived DNA sequences of these bands can be found in Supplementary Table S 4.1.1E.

Table S 4.1.1A Occurrence of microbial taxa as identified through sequence analysis of V3 and LSU DGGE bands. Identifications from the V3 DGGE analyses from batch 1. +: taxon is present.

Sampling moment	Sample	*Hanseniaspora*	*Pediococcus /Lactobacillus*	*Enterobacteriaceae*	Acetic acid bacteria
1 night	Batch 1 cask 2			+	
1 night	Batch 1 cask 3			+	
1 night	Batch 1 cask 4			+	
1 week	Batch 1 cask 1			+	
1 week	Batch 1 cask 2		+	+	
1 week	Batch 1 cask 3			+	
1 week	Batch 1 cask 4			+	
2 weeks	Batch 1 cask 1			+	
2 weeks	Batch 1 cask 2			+	
2 weeks	Batch 1 cask 3			+	
2 weeks	Batch 1 cask 4			+	
3 weeks	Batch 1 cask 1			+	
3 weeks	Batch 1 cask 2			+	
3 weeks	Batch 1 cask 3			+	
3 weeks	Batch 1 cask 4			+	
1 month	Batch 1 cask 1			+	
1 month	Batch 1 cask 2			+	
1 month	Batch 1 cask 3			+	
1 month	Batch 1 cask 4			+	
2 months	Batch 1 cask 1		+	+	+
2 months	Batch 1 cask 2			+	
2 months	Batch 1 cask 3	+		+	
2 months	Batch 1 cask 4			+	
3 months	Batch 1 cask 1	+	+	+	
3 months	Batch 1 cask 2	+	+	+	
3 months	Batch 1 cask 4	+	+	+	
6 months	Batch 1 cask 1	+	+	+	
6 months	Batch 1 cask 2	+	+	+	
6 months	Batch 1 cask 3	+	+	+	
6 months	Batch 1 cask 4	+	+	+	
9 months	Batch 1 cask 1	+	+	+	
9 months	Batch 1 cask 2	+	+	+	
9 months	Batch 1 cask 3	+	+	+	
9 months	Batch 1 cask 4	+	+	+	
12 months	Batch 1 cask 1	+	+	+	
12 months	Batch 1 cask 2	+	+	+	
12 months	Batch 1 cask 3		+	+	+
18 months	Batch 1 cask 1		+	+	
18 months	Batch 1 cask 2		+	+	
18 months	Batch 1 cask 3		+	+	+
18 months	Batch 1 cask 4		+	+	
24 months	Batch 1 cask 1		+		
24 months	Batch 1 cask 2		+		
24 months	Batch 1 cask 3		+		+
24 months	Batch 1 cask 4		+	+	+

Table S 4.1.1B Occurrence of microbial taxa as identified through sequence analysis of V3 and LSU DGGE bands. Identifications from the V3 DGGE analyses from batch 2. +: taxon is present.

Sampling moment	Sample	*Hanseniaspora*	*Saccharomyces*	*Pediococcus/Lactobacillus*	*Enterobacteriaceae*	Acetic acid bacteria
1 night	Batch 2 cask 2				+	
1 night	Batch 2 cask 3				+	+
1 night	Batch 2 cask 4				+	+
1 week	Batch 2 cask 1	+		+	+	+
1 week	Batch 2 cask 2	+	+	+	+	+
1 week	Batch 2 cask 3	+	+	+	+	+
1 week	Batch 2 cask 4	+	+	+	+	+
2 weeks	Batch 2 cask 1	+		+	+	+
2 weeks	Batch 2 cask 2	+	+	+	+	+
3 weeks	Batch 2 cask 1	+			+	+
3 weeks	Batch 2 cask 2	+	+	+	+	+
3 weeks	Batch 2 cask 3	+	+		+	+
3 weeks	Batch 2 cask 4	+			+	+
1 months	Batch 2 cask 1	+	+		+	+
1 months	Batch 2 cask 2	+	+	+	+	+
1 months	Batch 2 cask 3	+	+	+	+	+
1 months	Batch 2 cask 4	+	+		+	+
2 months	Batch 2 cask 1	+			+	+
2 months	Batch 2 cask 2	+	+		+	+
2 months	Batch 2 cask 3	+			+	+
2 months	Batch 2 cask 4	+			+	+
3 months	Batch 2 cask 1	+	+	+	+	+
3 months	Batch 2 cask 2	+	+	+	+	+
3 months	Batch 2 cask 3	+		+	+	+
3 months	Batch 2 cask 4	+		+	+	+
6 months	Batch 2 cask 1		+	+	+	+
6 months	Batch 2 cask 2		+	+	+	+
6 months	Batch 2 cask 3		+	+	+	
6 months	Batch 2 cask 4		+	+	+	
9 months	Batch 2 cask 1		+	+	+	+
9 months	Batch 2 cask 2		+	+	+	+
9 months	Batch 2 cask 3		+	+	+	
9 months	Batch 2 cask 4		+	+	+	
12 months	Batch 2 cask 1		+	+	+	
12 months	Batch 2 cask 2		+	+		+
12 months	Batch 2 cask 3		+	+	+	
12 months	Batch 2 cask 4		+	+	+	
18 months	Batch 2 cask 1	+		+	+	+
18 months	Batch 2 cask 2	+		+	+	+
18 months	Batch 2 cask 3	+		+	+	
18 months	Batch 2 cask 4			+	+	+
24 months	Batch 2 cask 1			+	+	+
24 months	Batch 2 cask 2			+	+	+
24 months	Batch 2 cask 3			+	+	
24 months	Batch 2 cask 4		+	+	+	

Table S 4.1.1C Occurrence of microbial taxa as identified through sequence analysis of V3 and LSU DGGE bands. Identifications from the LSU DGGE analyses from batch 1. +: taxon is present.

Sampling moment	Sample	*Candida*	*Dekkera/Brettanomyces*	*Kregervanrija*	*Naumovia*	*Saccharomyces*	*Wickerhamomyces*
1 week	Batch 1 cask 2					+	
1 week	Batch 1 cask 4	+				+	+
2 weeks	Batch 1 cask 1					+	
2 weeks	Batch 1 cask 2	+				+	
2 weeks	Batch 1 cask 3					+	
2 weeks	Batch 1 cask 4					+	
3 weeks	Batch 1 cask 1					+	
3 weeks	Batch 1 cask 2					+	
3 weeks	Batch 1 cask 3					+	
3 weeks	Batch 1 cask 4					+	
1 month	Batch 1 cask 1					+	
1 month	Batch 1 cask 2					+	
1 month	Batch 1 cask 3					+	
1 month	Batch 1 cask 4					+	
2 months	Batch 1 cask 1					+	
2 months	Batch 1 cask 2					+	
2 months	Batch 1 cask 3					+	
2 months	Batch 1 cask 4					+	
3 months	Batch 1 cask 1					+	
3 months	Batch 1 cask 2	+		+		+	
3 months	Batch 1 cask 3	+		+		+	
3 months	Batch 1 cask 4	+				+	
6 months	Batch 1 cask 1					+	
6 months	Batch 1 cask 2	+		+		+	
6 months	Batch 1 cask 3			+		+	+
6 months	Batch 1 cask 4					+	
9 months	Batch 1 cask 1					+	
9 months	Batch 1 cask 3					+	
9 months	Batch 1 cask 4					+	
12 months	Batch 1 cask 1		+		+	+	+
12 months	Batch 1 cask 2	+	+			+	+
12 months	Batch 1 cask 3	+	+		+	+	+
12 months	Batch 1 cask 4	+	+		+	+	+
18 months	Batch 1 cask 1	+			+	+	
18 months	Batch 1 cask 2					+	
18 months	Batch 1 cask 3				+	+	
18 months	Batch 1 cask 4				+	+	+
24 months	Batch 1 cask 1		+		+	+	+
24 months	Batch 1 cask 2					+	
24 months	Batch 1 cask 3					+	
24 months	Batch 1 cask 4				+	+	

Table S 4.1.1D Occurrence of microbial taxa as identified through sequence analysis of V3 and LSU DGGE bands. Identifications from the LSU DGGE analyses from batch 2. +: taxon is present.

Sampling moment	Sample	*Candida*	*Dekkera/Brettanomyces*	*Kregervanrija*	*Naumovia*	*Saccharomyces*	*Wickerhamomyces*
1 night	Batch 2 cask 4					+	
1 week	Batch 2 cask 1					+	
1 week	Batch 2 cask 4					+	
2 weeks	Batch 2 cask 1					+	
2 weeks	Batch 2 cask 2				+	+	
2 weeks	Batch 2 cask 3				+	+	
2 weeks	Batch 2 cask 4					+	
3 weeks	Batch 2 cask 1					+	
3 weeks	Batch 2 cask 2					+	
3 weeks	Batch 2 cask 3					+	
3 weeks	Batch 2 cask 4					+	
1 month	Batch 2 cask 1					+	
1 month	Batch 2 cask 2					+	
1 month	Batch 2 cask 3				+	+	
1 month	Batch 2 cask 4				+	+	
2 months	Batch 2 cask 1					+	
2 months	Batch 2 cask 2					+	
2 months	Batch 2 cask 3					+	
2 months	Batch 2 cask 4				+	+	+
3 months	Batch 2 cask 1					+	
3 months	Batch 2 cask 2			+		+	+
3 months	Batch 2 cask 3					+	
3 months	Batch 2 cask 4		+	+		+	+
6 months	Batch 2 cask 1			+		+	
6 months	Batch 2 cask 2			+		+	
6 months	Batch 2 cask 3					+	
6 months	Batch 2 cask 4	+			+	+	+
9 months	Batch 2 cask 1					+	+
9 months	Batch 2 cask 2					+	+
9 months	Batch 2 cask 3				+	+	+
9 months	Batch 2 cask 4	+		+	+	+	+
12 months	Batch 2 cask 1	+				+	
12 months	Batch 2 cask 2	+		+		+	+
12 months	Batch 2 cask 3	+			+	+	+
12 months	Batch 2 cask 4		+	+	+	+	+
18 months	Batch 2 cask 1					+	
18 months	Batch 2 cask 2					+	+
18 months	Batch 2 cask 3		+		+	+	+
18 months	Batch 2 cask 4	+	+		+	+	
24 months	Batch 2 cask 1					+	
24 months	Batch 2 cask 2				+	+	+
24 months	Batch 2 cask 3		+		+	+	+
24 months	Batch 2 cask 4				+	+	

Table S 4.1.1E Identifications of the excised DNA bands (Supplementary Figure S 4.1.2).

Band number	Accession number highest hit*	Similarity	Identification
1	AB004754	100%	*Enterobacteriaceae*
2	AB681728	100%	*Enterobacteriaceae*
3	AY696662	100%	*Enterobacteriaceae*
4	AY696662	100%	*Enterobacteriaceae*
5	AB681728	100%	*Enterobacteriaceae*
6	AB681728	100%	*Enterobacteriaceae*
7	AJ853891	100%	*Enterobacteriaceae*
8	HE978272	100%	*Enterobacteriaceae*
9	AJ853891	100%	*Enterobacteriaceae*
10	AB681728	100%	*Enterobacteriaceae*
11	AY046257	100%	*Hanseniaspora*
12	AB681216	100%	*Pediococcus/Lactobacillus*
13	AB681216	100%	*Pediococcus/Lactobacillus*
14	AJ853891	100%	*Enterobacteriaceae*
15	HE978272	100%	*Enterobacteriaceae*
16	AJ318414	100%	*Pediococcus/Lactobacillus*
17	AJ271383	100%	*Pediococcus/Lactobacillus*
18	AJ271383	100%	*Pediococcus/Lactobacillus*
19	AB681216	100%	*Pediococcus/Lactobacillus*
20	AB681216	100%	*Pediococcus/Lactobacillus*
21	AB681216	100%	*Pediococcus/Lactobacillus*
22	AJ853891	100%	*Enterobacteriaceae*
23	AJ853891	100%	*Enterobacteriaceae*
24	U78183	100%	*Enterobacteriaceae*
25	AF025367	100%	*Enterobacteriaceae*
26	AJ853891	100%	*Enterobacteriaceae*
27	HE978272	100%	*Enterobacteriaceae*
28	AJ318414	100%	*Pediococcus/Lactobacillus*
29	HE978272	100%	*Enterobacteriaceae*
30	AY497740	100%	*Saccharomyces*
31	AJ318414	100%	*Pediococcus/Lactobacillus*
32	AJ318414	100%	*Pediococcus/Lactobacillus*
33	AB681216	100%	*Pediococcus/Lactobacillus*
34	AJ318414	100%	*Pediococcus/Lactobacillus*
35	AB680026	100%	Acetic acid bacteria
36	AB680026	100%	Acetic acid bacteria
37	AJ279065	100%	*Saccharomyces*
38	AJ279065	100%	*Saccharomyces*
39	AJ279065	100%	*Saccharomyces*
40	BR000309	100%	*Saccharomyces*
41	AJ279065	100%	*Saccharomyces*
42	AJ279065	100%	*Saccharomyces*
43	AJ279065	100%	*Saccharomyces*
44	BR000309	100%	*Saccharomyces*
45	EU057562	100%	*Wickerhamomyces*
46	DQ406717	100%	*Dekkera/Brettanomyces*
47	AY007880	100%	*Naumovia*
48	AJ279065	100%	*Saccharomyces*
49	AY969049	100%	*Dekkera/Brettanomyces*
50	EU057562	100%	*Wickerhamomyces*
51	DQ406717	100%	*Dekkera/Brettanomyces*
52	AY007880	100%	*Naumovia*
53	AJ279065	100%	*Saccharomyces*
54	AJ279065	100%	*Saccharomyces*
55	BR000309	100%	*Saccharomyces*
56	AJ279065	100%	*Saccharomyces*
57	U70247	100%	*Kregervanrija*
58	AY969049	100%	*Dekkera/Brettanomyces*
59	AB365475	100%	*Candida*
60	AJ279065	100%	*Saccharomyces*
61	EU057562	100%	*Wickerhamomyces*
62	AY007880	100%	*Naumovia*
63	AJ279065	100%	*Saccharomyces*
64	AY969049	100%	*Dekkera/Brettanomyces*

*Highest hit with first type strain in BLAST results

Table S 4.1.2 Overview of MALDI-TOF MS clusters and the identifications of the representative isolates. The number of isolates in each MALDI-TOF MS cluster is given in parentheses. The accession number of the cluster representative sequence is given when sequence similarity with a known sequence was below 100%. B: bacterial MALDI-TOF MS cluster, Y: yeast MALDI-TOF MS cluster.

MALDI-TOF MS cluster identifier	Strain number of representative isolate	Accession number[a]*	Sequence type	Identification	Accession number highest hit	Similarity
B-1 (1)[a]	LMG 27882			*Gluconobacter cerevisiae*		
B-2 (43)	R-47374; R-49023	KJ186115	*rpoB*	*Enterobacter hormaechei*	AJ543724	99%
B-3 (159)	R-47368; R-47377; R-49018	KJ186116	*rpoB*	*Enterobacter kobei*	JX494753	99%
B-4 (21)	R-49013		*rpoB*	*Raoultella terrigena*	KF057939	100%
B-5 (32)	R-49012	KJ186117	*rpoB*	*Citrobacter gillenii*	KF057931	99%
B-6 (194)	R-49008	KJ186118	*rpoB*	*Klebsiella oxytoca*	AJ871804	97%
B-7 (102)	R-49019;	KJ186120	*dnaJ*	*Escherichia/Shighella*	AB272648	98%
	R-49020	KJ186119	*rpoB*	*Escherichia/Shighella*	EU010107	99%
B-8 (387)	R-47375; R-47380-R-47386; R-49024-R-49031; R-49033-R-49039; R-49555		16S rRNA gene	*Hafnia paralvei*	FM179943	100%
B-9 (51)[b]	LMG 27440			*Acetobacter lambici*		
B-10 (314)	R-49097; R-49102		*pheS*	*Pediococcus damnosus*	AM899820	100%
Y-1 (16)	R-49565; R-49568; R-49827		D1/D2 26S rRNA gene	*Priceomyces carsonii*	U45743	100%
Y-2 (14)	R-49569; R-49824; R-49826		D1/D2 26S rRNA gene	*Wickerhamomyces anomalus*	U74592	100%
Y-3 (255)	R-49830; R-49831		D1/D2 26S rRNA gene	*Dekkera bruxellensis*	JQ689028	100%
Y-4 (111)	R-49654; R-49655; R-49662; R-49821; R-52120; R-52121		D1/D2 26S rRNA gene	*Saccharomyces cerevisiae*	JQ689017	100%
Y-5 (89)	R-49564; R-49820		D1/D2 26S rRNA gene	*Naumovia castellii*	HE576754	100%
Y-6 (299)	R-49562; R-49653; R-49661	KJ186121	*ACT1*	*Saccharomyces pastorianus*	ALJS01000103	99%
					ABPO01000006	99%
Y-7 (29)	R-49837; R-49838		ITS	*Kazachstania servazzii*	AY046153	100%
Y-8 (7)	R-49647; R-49648		D1/D2 26S rRNA gene	*Candida friedrichii*	HQ283384	100%
Y-9 (62)	R-49652; R-49844;	KJ186123	*ACT1*	*Debaryomyces hansenii*	CR382136	99%
	R-49570; R-49825	KJ186122	*ACT1*	*Debaryomyces hansenii*	CR382136	98%
Y-10 (7)	R-49650		*ACT1*	*Meyerozyma guilliermondii*	AJ389063	100%
Y-11 (3)	R-49567	KJ186127	D1/D2 26S rRNA gene	*Priceomyces* sp.	AB568341	99%
		KJ186126	ITS	*Priceomyces carsonii*	AJ586521	99%
Y-12 (5)	R- 49657		D1/D2 26S rRNA gene	*Dekkera anomala*	EF550258	100%
Y-13 (7)	R- 49649		D1/D2 26S rRNA gene	*Pichia membranifaciens*	EU057561	100%
Y-14 (1)	R-49839	KJ186128	D1/D2 26S rRNA gene	*Candida nemodendra*	EU011629	98%
Y-15 (1)	R-49840		D1/D2 26S rRNA gene	*Candida patagonica*	DQ841165	100%
Y-16 (1)[c]			D1/D2 26S rRNA gene	*Yarrowia lipolytica*	JQ689067	100%
Y-17 (2)	R-49843		D1/D2 26S rRNA gene	*Debaryomyces marama*	JN940502	100%

[a]Isolates of this cluster were characterized in a polyphasic taxonomic study as the new species *Gluconobacter cerevisiae* (Spitaels et al., 2014a).

[b]Isolates of this cluster were characterized in a polyphasic taxonomic study as the new species *Acetobacter lambici* (Spitaels et al., 2014b).

[c]Cluster Y-16 consisted of one isolate from the Cantillon brewery and three isolates from a second brewery. One of the latter isolates was chosen as representative for sequence-based identification.

*Accession numbers are given for the unique isolate sequences within the same MALDI-TOF MS cluster.

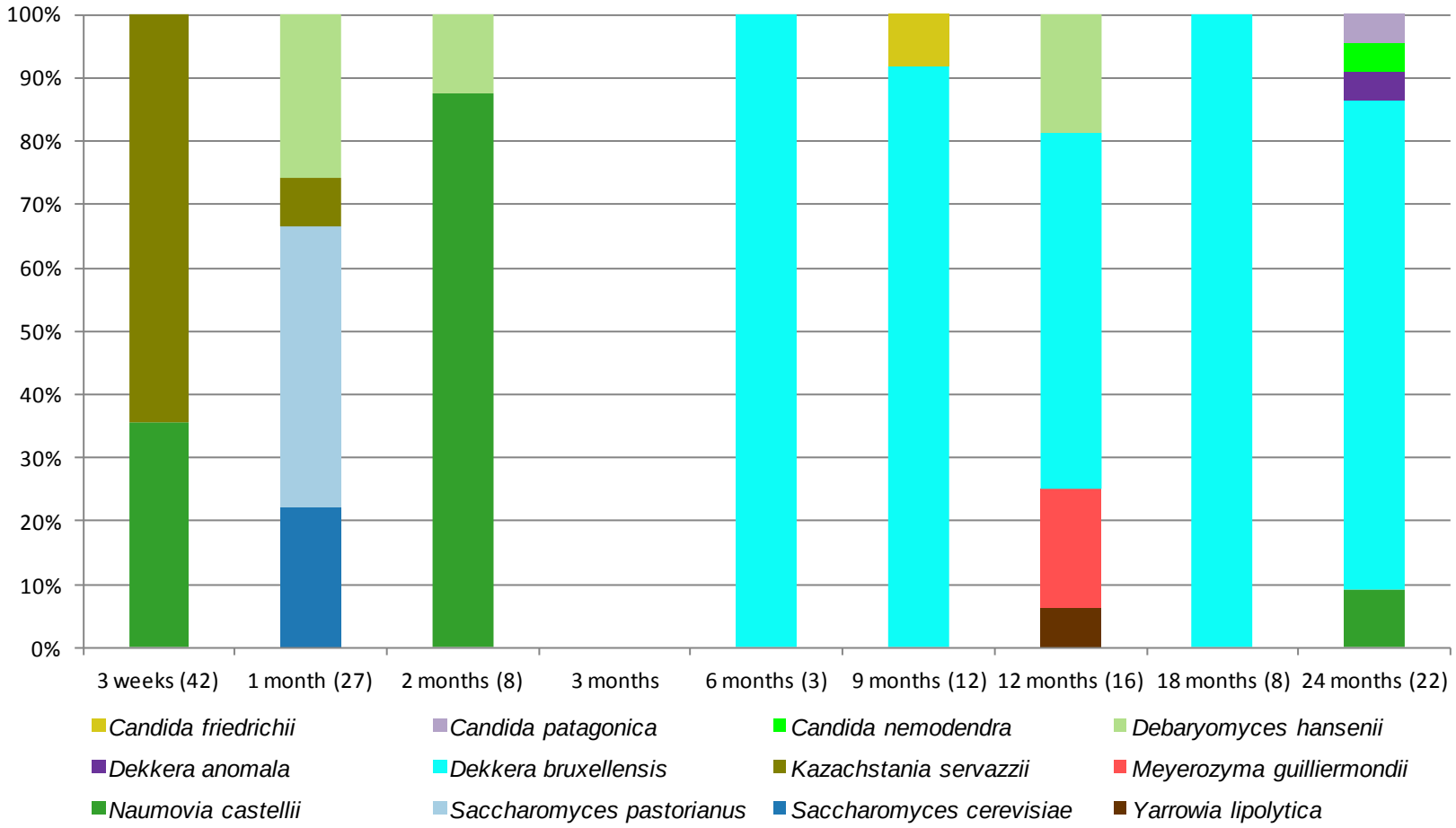

Figure S 4.1.3 Identification of random isolates from DYPAIX agar of batch 1. Empty bars represent isolates that could not be recovered after isolation. The number of isolates is given between brackets.

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4.2 The microbial diversity of an industrially produced lambic beer shares members of a traditionally produced fermented lambic beer and reveals a core microbiota for lambic beer fermentation

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Author contributions: conceived and designed the experiments: FS, MJ, AVL, LDV and PV; performed the experiments: FS; analyzed the data: FS; contributed reagents/materials/analysis tools: ADW and MA; wrote the manuscript: FS; critically reviewed the manuscript: ADW, MA, AVL, LDV and PV.

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Abstract

The microbiota involved in lambic beer fermentations in an industrial brewery in West-Flanders, Belgium, was determined through a detailed study using culture-dependent and culture-independent techniques. More than 1300 bacterial and yeast isolates from 13 samples collected during a one-year fermentation process were dereplicated and identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry followed by sequence analysis of 16S rRNA and various protein-coding genes. The bacterial and yeast communities of the same samples were further analyzed using denaturing gradient gel electrophoresis of PCR-amplified V3 regions of the 16S rRNA genes and of PCR-amplified D1/D2 regions of the 26S rRNA genes, respectively. In contrast to traditional lambic beer fermentations, no *Enterobacteriaceae* phase was found and a larger variety of acetic acid bacteria were found. Similar to traditional lambic beer fermentations, *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*, *Dekkera bruxellensis* and *Pediococcus damnosus* were the responsible microorganisms for the main fermentation and maturation phases and were therefore considered as the core microbiota of lambic beer fermentations. They originated most probably from the wood of the casks.

Lambic sour beers are among the oldest types of beers still brewed. They are the weakly carbonated products of a spontaneous fermentation process that lasts for one to three years before bottling (De Keersmaecker, 1996). The sour character of the beer originates from the metabolic activities of lactic acid bacteria (LAB), acetic acid bacteria (AAB) and various yeasts (Spitaels *et al.*, 2014c; Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). These beers can be drunk as such or are used to produce gueuze or fruit lambic beers. Except for an American coolship ales study based on 16S rRNA gene sequence analysis (Bokulich et al., 2012), previous microbial studies on lambic beers used phenotypic identification techniques only (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). A recent in-depth analysis, based on both culture-dependent and culture-independent analysis of lambic brew samples, of the most traditional lambic brewery of Belgium revealed a characteristic microbial succession of *Enterobacteriaceae* in the first month (representing the first phase of lambic beer fermentation), *Pediococcus damnosus* and *Saccharomyces* spp. after two months, reflecting the main fermentation phase, and *Dekkera bruxellensis* after six months, characteristic for the maturation phase (Spitaels *et al.*, 2014c).

Although lambic beers were originally only brewed in the Senne river valley and southeast of Brussels, they are now also brewed elsewhere in Belgium. In West-Flanders, the most western province of Belgium and thus outside the small region of the Senne river valley, two independent breweries produce lambic beers. In the past, both breweries obtained the necessary lambic wort from breweries located in the Senne river valley: one bought wort to graft the casks to be able to produce an own lambic beer and the other blended lambic beers to produce gueuze beers. Because of the growing interest in beers of spontaneous fermentation, both breweries started to brew a lambic-type of beer, conform to the lambic beer production process, besides their regular ales and lager beer brands and stopped buying lambic wort or beer from traditional lambic breweries.

The production activities of American craft breweries, including American coolship ales and other types of beers, resemble the activities of the industrial lambic beer breweries (Bokulich *et al.*, 2012). Industrial lambic breweries do not only produce lambic beers and products derived thereof, but also the more typical ales and lager beer brands. Industrial lambic breweries mostly filter, pasteurize and carbonate

their spontaneously fermented beers, which are sometimes also sweetened (Van Oevelen *et al.*, 1976). Moreover, they can brew lambic-type beers all year round, because they have the capacity to prechill the wort before its transfer into the cooling tun and hence do not need the cold winter months to properly cool their wort in one night as traditional lambic breweries do. Also, industrial brewers generally do not use old, small wine or cognac casks for fermentation (2-6 hL); instead, their wooden casks are usually larger and custom-made on-site (about 170-200 hL).

The present study aimed to determine the microbial succession in a lambic beer fermentation process during one year in an industrial lambic beer brewery outside the Senne river valley and to unravel the relation with the succession of microorganisms of a lambic beer fermentation in a traditional lambic beer brewery.

Materials and methods

Brewery

The selected brewery was an industrial lambic brewery located in West-Flanders, approximately 70 kilometers to the west of Brussels. This brewery started to produce own lambic beers in 1981. Before 1981, this brewery produced gueuze based on the blending of lambic beers purchased from traditional lambic breweries.

Brewing process and sampling to study the succession of the microbiota

Mash was prepared and boiled in the brewery according to the brewer's recipe. This recipe included acidification of the wort to pH 4 by the addition of lactic acid at the end of the 1.5-h wort boiling before wort chilling. After the acidification, the wort was prechilled to 40°C and centrifuged to remove the hot break. The prechilled wort was then transferred into a cleaned cooling tun and a 500-mL sample was taken aseptically. A second 500-mL sample was taken from the wort in the cooling tun after overnight cooling at the start of the wort transfer into the 170 hL cask. The transfer process required about 8 h. Near the end of the wort transfer into the cask, the cooling tun was sampled a third time. From the wooden cask, samples were taken after the transfer of the cooled wort and after 1, 2 and 3 weeks and 1, 2, 3, 6, 9 and 12 months. Two batches were followed. Batch A started on January 4, 2011 and was sampled at all time points mentioned above. The wort temperature of batch A after overnight cooling was about 22°C. Batch B started on July 27, 2010 and was sampled at the same time points for three months only. The wort temperature of batch B after overnight cooling was about 29°C. Two weeks after transfer of the batch A wort into the cask there was no apparent production of foam, indicating no initiation of the fermentation, hence the brewer decided to mix batch A (which is further referred to as the acceptor batch A) with a 3-months old fermenting lambic

wort from another batch (further referred to as the donor batch A) to initiate the fermentation. Mixing occurred through the bottom apertures of the casks and was performed in a ratio of 5 hL to 165 hL (± 3%, vol/vol). Both the donor and acceptor batches A were sampled at the time of mixing, further referred to as the mixing point. The acceptor batch A was sampled prior to and 15 min after mixing, enabling debris to settle.

All casks were located in a single, separate building of the brewery at ambient temperature and contained three apertures: a manhole at the top, closed with a loose panel, a valve at the bottom to fill and empty the cask, and a sampling tap located at about 1/3 of the total height of the cask. Before every sampling, the sampling tap was cleaned with 70% (vol/vol) ethanol and approximately 100 mL of fermenting wort were discarded. Samples (500 mL) were collected in a sterile bottle and transported on ice to the laboratory to be processed on the same day.

Denaturing gradient gel electrophoresis (DGGE) analysis

Crude brew samples were centrifuged at 8000 × *g* for 10 min (4°C) at the day of sampling and cell pellets were stored at -20°C until further processing. DNA was prepared from the pellets as described by Camu *et al.* (2007). The DNA concentration, purity, and integrity were determined using 1% (wt/vol) agarose gels stained with ethidium bromide and by optical density (OD) measurements at 234, 260, and 280 nm. The quality of the DNA was assessed as good, when absorbance ratios were $OD_{260}/OD_{280} > 1.8$ and $OD_{234}/OD_{260} > 0.5$. Total DNA solutions were diluted to an OD_{260} of 1. Amplification of about 200 bp of the V3 region of the 16S rRNA genes with the F357 (with a GC clamp) and R518 primers, followed by denaturing gradient gel electrophoresis (DGGE) analysis, and processing of the resulting fingerprints was performed, as described previously (Duytschaever *et al.*, 2011), except that DGGE gels were run for 960 min instead of 990 min. For the amplification of about 200 bp of the D1/D2 region of the 26S rRNA genes, NL1 (with GC clamp) and LS2 primers were used, as previously reported by Cocolin *et al.* (2000).

All DNA bands were assigned to band classes using the BioNumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium). Dense DNA bands and/or bands that were present in multiple fingerprints were excised from the polyacrylamide gels by inserting a pipette tip into the bands and subsequent overnight elution of the DNA from the gel slices in 40 µL 1 x TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 8) at 4°C. The position of each extracted DNA band was confirmed by repeat DGGE experiments using the excised DNA as template. The extracted DNA was subsequently re-amplified and sequenced using the same protocol and primers (but without GC clamp). EzBioCloud and BLAST (Altschul *et al.*, 1997; Kim *et al.*, 2012) analyses were performed to determine the most similar sequences in the public sequence databases.

Culture media, enumeration and isolation

The samples were serially diluted in 0.9% (wt/vol) saline and 50 µL of each dilution was plated in triplicate on multiple agar isolation media. The bacterial agar isolation media were incubated under different conditions [selected as described before; (Spitaels *et al.*, 2014c)], namely on de Man-Rogosa-

Sharpe (MRS) agar (Oxoid, Erembodegem, Belgium) (De Man *et al.*, 1960) incubated at 28°C aerobically and at 20°C anaerobically for the isolation of LAB; violet red bile glucose (VRBG) agar (Mossel *et al.*, 1962, 1978) incubated at 28°C aerobically for the isolation of *Enterobacteriaceae*; and acetic acid medium (AAM) agar (Lisdiyanti *et al.*, 2003) incubated at 28°C aerobically for the isolation of AAB. All bacterial agar isolation media were supplemented with 5 ppm amphotericin B (Sigma-Aldrich, Bornem, Belgium) and 200 ppm cycloheximide (Sigma-Aldrich) to inhibit fungal growth.

All yeast agar isolation media were supplemented with 100 ppm chloramphenicol (Sigma-Aldrich) to inhibit bacterial growth and were incubated aerobically at 28°C. DYPAI agar (2.0% glucose, 0.5% yeast extract, 1.0% peptone and 1.5% agar; wt/vol) was used as a general yeast agar isolation medium. To favor the slow-growing *Dekkera*/*Brettanomyces*, DYPAI was supplemented with an additional 50 ppm cycloheximide (DYPAIX) (Abbott *et al.*, 2005; Licker *et al.*, 1998; Suárez *et al.*, 2007). Furthermore, universal beer agar (Oxoid) was supplemented with 25% (vol/vol) commercial gueuze (Belle-Vue; AB Inbev, Anderlecht, Belgium) as recommended by the manufacturer and was used as an additional general yeast agar isolation medium (UBAGI).

Colonies on plates comprising 25 to 250 colony forming units (CFU) were counted after 3 to 10 days of incubation and for each of the seven isolation conditions about 20-25 colonies, or all colonies if the counts were lower, were randomly picked up.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) dereplication and identification

Isolates were subcultured twice using the respective isolation conditions and MALDI-TOF MS was performed using the third generation of pure cultures by means of a 4800 Plus MALDI TOF/TOF™ Analyzer (AB SCIEX, Framingham, MA, USA), as described previously (Wieme *et al.*, 2012). In short, Data Explorer 4.0 software (AB SCIEX) was used to convert the mass spectra into .txt-files to import them into a BioNumerics 5.1 (Applied Maths) database. The spectral profiles were compared using the Pearson product-moment correlation coefficient (PPMCC) and a dendrogram was built using the unweighted pair group method with arithmetic mean (UPGMA) cluster algorithm. Homogeneous clusters consisting of isolates with visually identical or virtually identical mass spectra were delineated. From most clusters, isolates were randomly selected for further identification through sequence analysis of 16S rRNA genes and other molecular markers. Sequence analysis of the *pheS* gene was performed to identify LAB (De Bruyne *et al.*, 2007, 2008; Naser *et al.*, 2005, 2007) and of *dnaK*, *groEL* and *rpoB* genes to identify AAB (Cleenwerck *et al.*, 2010). Yeast isolates were identified through sequence analysis of the D1/D2 region of the 26S rRNA gene (Kurtzman & Robnett, 1998) and, whenever needed, also by determination of *ACT1* gene sequences (Daniel & Meyer, 2003). Some isolates of the present study grouped in clusters of lambic isolates that were examined in a previous study using the same polyphasic approach and were therefore considered identified (Spitaels *et al.*, 2014c).

All PCR assays were performed as described by Snauwaert *et al.* (2013). Bacterial DNA was obtained via the protocol described by Niemann *et al.* (1997), whereas yeast DNA was obtained using the protocol of Harju *et al.* (2004).

Analysis of the microbiota in the brewery environment

To analyze the microbiota of the brewery environment, two samples were taken from the cooling tun, the ceiling above the cooling tun, the walls and ceiling of the cellar and the inside and outside of the casks, each by swabbing a surface of about 100 cm^2, using a moist swab. A first swab was streaked on each of the agar isolation media; a second sample was transferred into 5 mL of saline and filtered over a 0.45-µm filter that was transferred into 30 mL of MRS, VRBG, AAM, DYPAI and DYPAIX broth each, and incubated as described above. Enrichment cultures that showed growth after 3-10 days of incubation were subcultured on their respective agar media and morphologically distinct colonies were selected for further analysis. Isolates were identified as described above. Air samples were taken using a MAS-100 air sampler (Merck, Darmstadt, Germany) with a flow rate of 0.1 m^3/min placed about 1 m above the floor, for 1 or 10 min using yeast and bacterial agar isolation media, respectively.

Results

In neither batches A and B the cooling tun samples nor the first wort sample obtained from the cask yielded DNA for DGGE analysis. Bacterial and yeast DNA was successfully extracted from all subsequent samples and DGGE amplicons were generated from samples taken from one week onwards (Figure 4.2.1). Table 4.2.1 presents an overview of the enumeration analyses. For both batches, the freshly boiled wort sample did not yield growth and there was no apparent inoculation of the cooling tun samples taken after overnight cooling. Moreover, the early samples of both batches nor those of the remainder of the fermentation process yielded growth of *Enterobacteriaceae* nor were these bacteria detected in the DGGE community profiles.

Microbiota succession in batch A

First week fermentation samples. The bacterial community profiles of the batch A sample taken after one week showed only one dense band in the low % G+C region (Figure 4.2.1A, band class 1). Several DNA bands were excised from the DGGE gels and sequenced to tentatively assign these band classes to microbial taxa (Supplementary Figure S 4.2.1 and Supplementary Table S 4.2.1). Sequence analysis demonstrated that this band originated from a yeast, *Hanseniaspora* sp., which

confirmed that the V3 primers also amplify some eukaryotic DNA (Scheirlinck *et al.*, 2008; Spitaels *et al.*, 2014c; Van der Meulen *et al.*, 2007). Two additional faint DNA bands in the bacterial DGGE community profile did not yield amplicons after excision and subsequent amplification (Figure 4.2.1A, bands marked with an asterisk). The corresponding yeast community profiles contained two dense DNA bands that were assigned to *Hanseniaspora* (Figure 4.2.1B, band class 6) and *Candida*/*Pichia* (Figure 4.2.1B, band class 7). A full overview of the microbiota identified, using DGGE band sequencing, can be found in Supplementary Table S 4.2.2; similarly, an overview of the identification results of isolates (including numbers of isolates investigated) per MALDI-TOF MS cluster is presented in Supplementary Table S 4.2.3. Cultivation experiments of the one-week old sample yielded primarily yeasts while bacterial counts were low to zero (Table 4.2.1); yeast counts on DYPAI, UBAGI and DYPAIX agars were comparable (about 10^6 CFU/mL), indicating growth of cycloheximide-resistant yeasts. Yeast isolates were mainly identified as *Hanseniaspora uvarum* (Figure 4.2.2), which confirmed the results obtained by DGGE analysis. These isolates were cycloheximide-resistant, since *H. uvarum* was the only species isolated from DYPAIX agar (Supplementary Figure S 4.2.2). *Pichia fermentans* was isolated as minor part of the yeast communities (Figure 4.2.2).

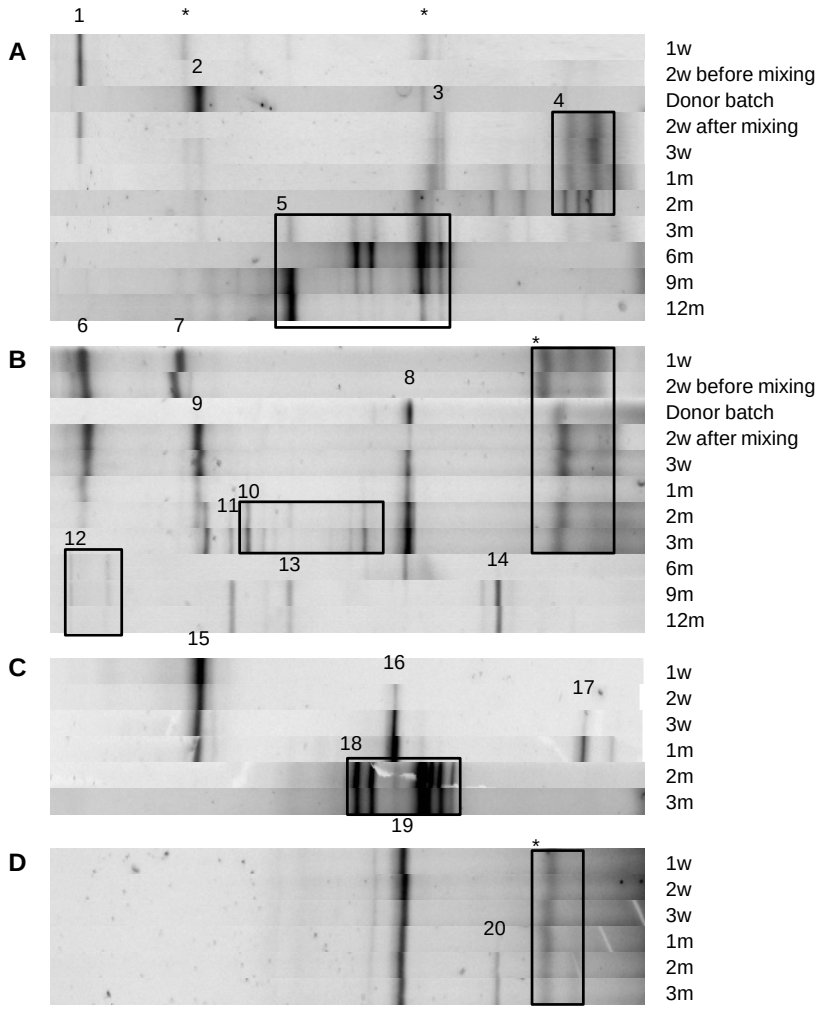

Figure 4.2.1 DGGE banding patterns of the bacterial and yeast communities of batch A (A and B, respectively) and batch B (C and D, respectively): w, week(s); m, month(s). Band classes 1-18 are indicated with numbers and some are grouped in a band class box. Samples only yielded DNA and PCR amplicons after one week of fermentation. *These bands did not yield PCR amplicons after band excision and subsequent DNA amplification. The 35-70 % denaturing gradient is represented from left to right on the gels.

Table 4.2.1 Results of plate counts on different agar isolation media. MRS agar was used for the growth of LAB, VRBG agar was used for the growth of *Enterobacteriaceae*, AAM agar was used for the growth of AAB, DYPAI and UBAGI agars were used as general yeast growth media and DYPAIX agar was used to favor the growth of *Dekkera* species. The values represent log CFU/mL. ULD: under limit of detection (< 20 CFU/mL); ULQ: under limit of quantification (the estimated CFU/mL is provided between brackets).

Batch A	**MRS 28°C**	**MRS 20°C AN**	**VRBG 28°C**	**AAM 28°C**	**DYPAI 28°C**	**UBAGI 28°C**	**DYPAIX 28°C**
Freshly boiled wort	ULD	ULD	ULD	ULD	ULD	ULD	ULD
1 night cooling tun	ULD	ULD	ULD	ULD	ULD	ULD	ULD
1 night cask	ULQ (20)	ULD	ULD	ULD	ULD	ULD	ULD
1 week	ULQ (20)	ULD	ULD	ULD	6.85	6.88	6.83
2 weeks (before mixing point)	3.13	ULD	ULD	3.03	6.51	6.42	6.63
Donor batch	3.61	3.8	ULD	ULQ (440)	5.57	5.62	3.9
2 weeks (after mixing point)	4.95	ULQ (40)	ULD	5.12	6.72	6.87	6.46
3 weeks	5.37	ULQ (50)	ULD	5.35	6.62	6.66	6.48
1 month	5.31	ULQ (200)	ULD	5.92	6.06	5.97	5.5
2 months	4.35	3.38	ULD	5.05	5.98	5.95	3.25
3 months	6.98	6.78	ULD	3.98	6.37	6.44	ULQ (300)
6 months	6.59	6.53	ULD	ULQ (20)	4.76	4.75	4.94
9 months	ULQ (480)	2.83	ULD	ULD	3.84	3.83	3.73
12 months	4.38	4.33	ULD	ULD	3.9	3.3	2.88

Batch B	**MRS 28°C**	**MRS 20°C AN**	**VRBG 28°C**	**AAM 28°C**	**DYPAI 28°C**	**UBAGI 28°C**	**DYPAIX 28°C**
Freshly boiled wort	ULD	ULD	ULD	ULD	ULD	ULD	ULD
1 night cooling tun	ULD	ULD	ULD	ULD	ULD	ULD	ULD
1 night cask	ULQ (70)	ULD	ULD	ULD	3	3	ULQ (100)
1 week	ULD	ULD	ULD	ULQ (20)	5.92	5.92	ULQ (20)
2 weeks	ULQ (390)	ULD	ULD	ULQ (410)	6.24	6.33	ULD
3 weeks	5.37	ULQ (200)	ULD	5.28	6.05	6.13	ULD
1 month	5.56	5.49	ULD	5.46	5.95	5.93	ULQ (100)
2 months	7.56	6.63	ULD	4.81	5.04	4.51	5.02
3 months	7.4	7.44	ULD	ULQ (20)	4.62	4.62	4.64

Mixing point. The bacterial and yeast community profiles of the acceptor batch A prior to the mixing point were comparable to the profiles of the 1-week old sample, except for the disappearance of the faint bands in the bacterial community profiles (Figure 4.2.1A). Also the yeast counts were similar (Table 4.2.1) and *H. uvarum* remained the most isolated species, in addition to *Pi. fermentans* and *Dekkera bruxellensis* (Figure 4.2.2). A single isolate identified as *Candida parapsilosis* was likely a contaminant, since this species was not isolated from subsequent samples. Only AAB were isolated from aerobically incubated MRS and AAM agars before the mixing point. MRS agar isolates were identified as *Gluconobacter cerinus* (Figure 4.2.3). AAM agar isolates represented a novel *Acetobacter* species (Figure 4.2.3), for which the name *Acetobacter lambici* was recently proposed (Spitaels *et al.*, 2014b). The bacterial and yeast community profiles of the donor batch A comprised a single dense band each (Figure 4.2.1A, band class 2 and Figure 4.2.1B, band class 8, respectively), which both originated from *Saccharomyces* strains (Supplementary Table S 4.2.2). Immediately after the mixing point, the acceptor batch A sample

yielded several DNA bands that originated from AAB (Figure 4.2.1A, bands grouped in band class box 4) and one faint, reproducibly fuzzy band that originated from *Pediococcus*/*Lactobacillus* (Figure 4.2.1A, band class 3). In the yeast community profiles, band class 7 (assigned to *Candida*/*Pichia*) disappeared and two new bands (band classes 8 and 9) appeared, which both originated from *Saccharomyces* strains (Figure 4.2.1B). Bacterial counts of the donor batch A were generally equal to or lower than those of the acceptor batch A, except for counts on anaerobically incubated MRS agar (Table 4.2.1). *Acetobacter fabarum* was isolated from the donor cask A sample from aerobically incubated MRS agar, but represented only a minor fraction of the MRS agar isolates (Figure 4.2.3). The majority of the latter isolates were identified as *Pediococcus damnosus* (Figure 4.2.3), which was also the only species isolated from anaerobically incubated MRS agar. AAM agar isolates belonged to *Acetobacter orientalis* and *A. fabarum* (Figure 4.2.3). Yeast counts were comparable in both the acceptor batch A and donor batch A, except for DYPAIX agar counts, as the donor batch A contained more cycloheximide-sensitive yeasts (as revealed by the difference in colony counts on DYPAI and UBAGI *versus* DYPAIX agars; Table 4.2.1). DYPAI and UBAGI agar isolates of the donor batch A sample yielded the cycloheximide-sensitive *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* (Figure 4.2.2), while DYPAIX agar isolates were identified as *D. bruxellensis*, *Hanseniaspora meyeri* and *H. uvarum* (Supplementary Figure S 4.2.2). Immediately after the mixing point, bacterial counts on aerobically incubated MRS and AAM agars increased, which is likely explained by the presence of microbiota settled in either the donor or acceptor batch A that were at least partially resuspended, due to shearing forces during the mixing. *Hanseniaspora uvarum* and *Pi. fermentans* remained the only yeast species isolated from the acceptor batch A immediately after the mixing point (Figure 4.2.2). A novel *Gluconobacter* species (Figure 4.2.3), for which the name *Gluconobacter cerevisiae* was recently proposed (Spitaels *et al.*, 2014a), was the only isolated species from AAM agar. This species was not isolated from previous samples nor from the donor batch A. Aerobically incubated MRS agar yielded *G. cerinus* isolates only; anaerobically incubated MRS agar yielded *P. damnosus* isolates only (Figure 4.2.3).

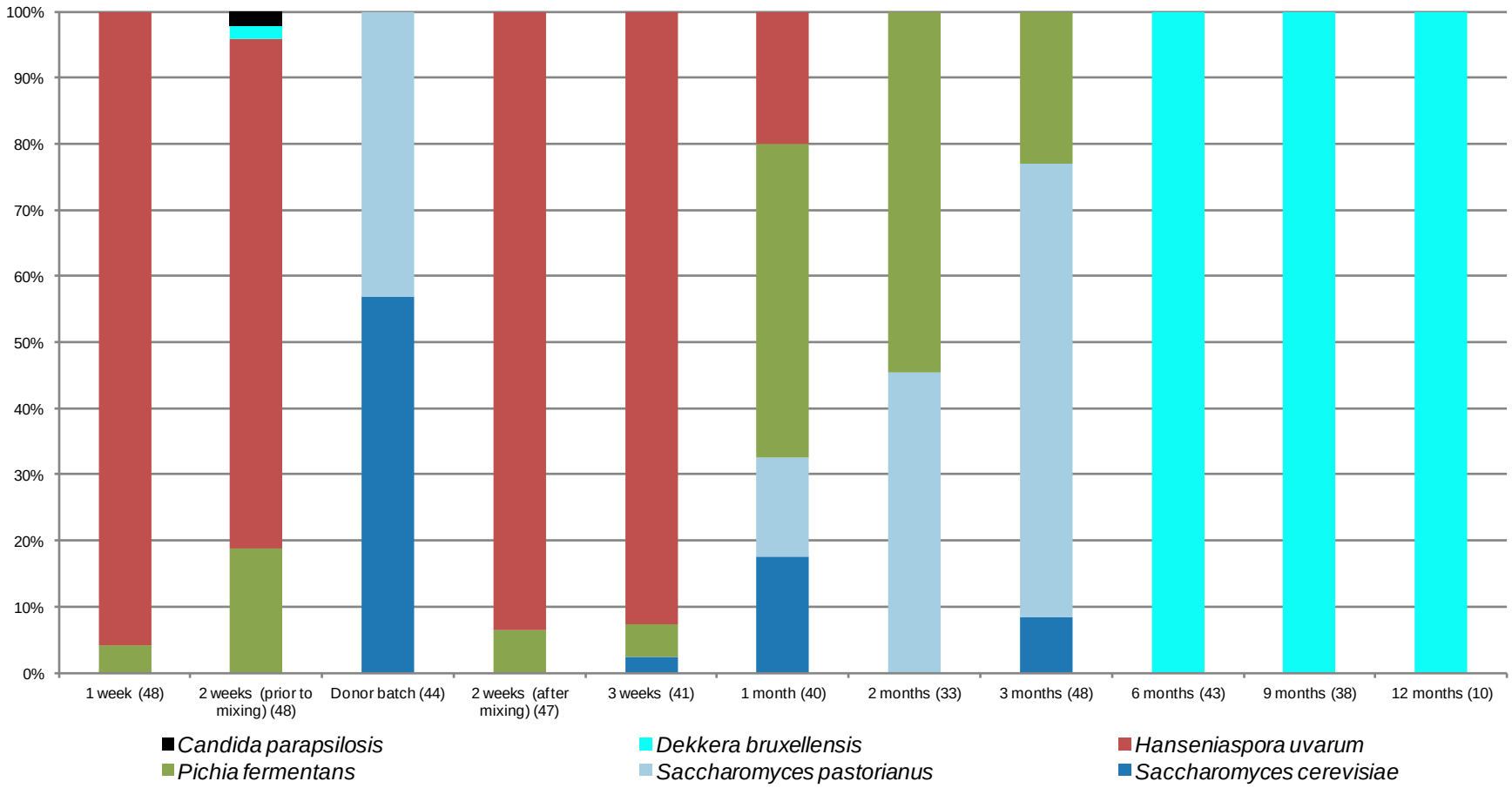

Figure 4.2.2 Identification of random isolates from DYPAI and UBAGI agars of batch A cask samples. The number of isolates is given between brackets.

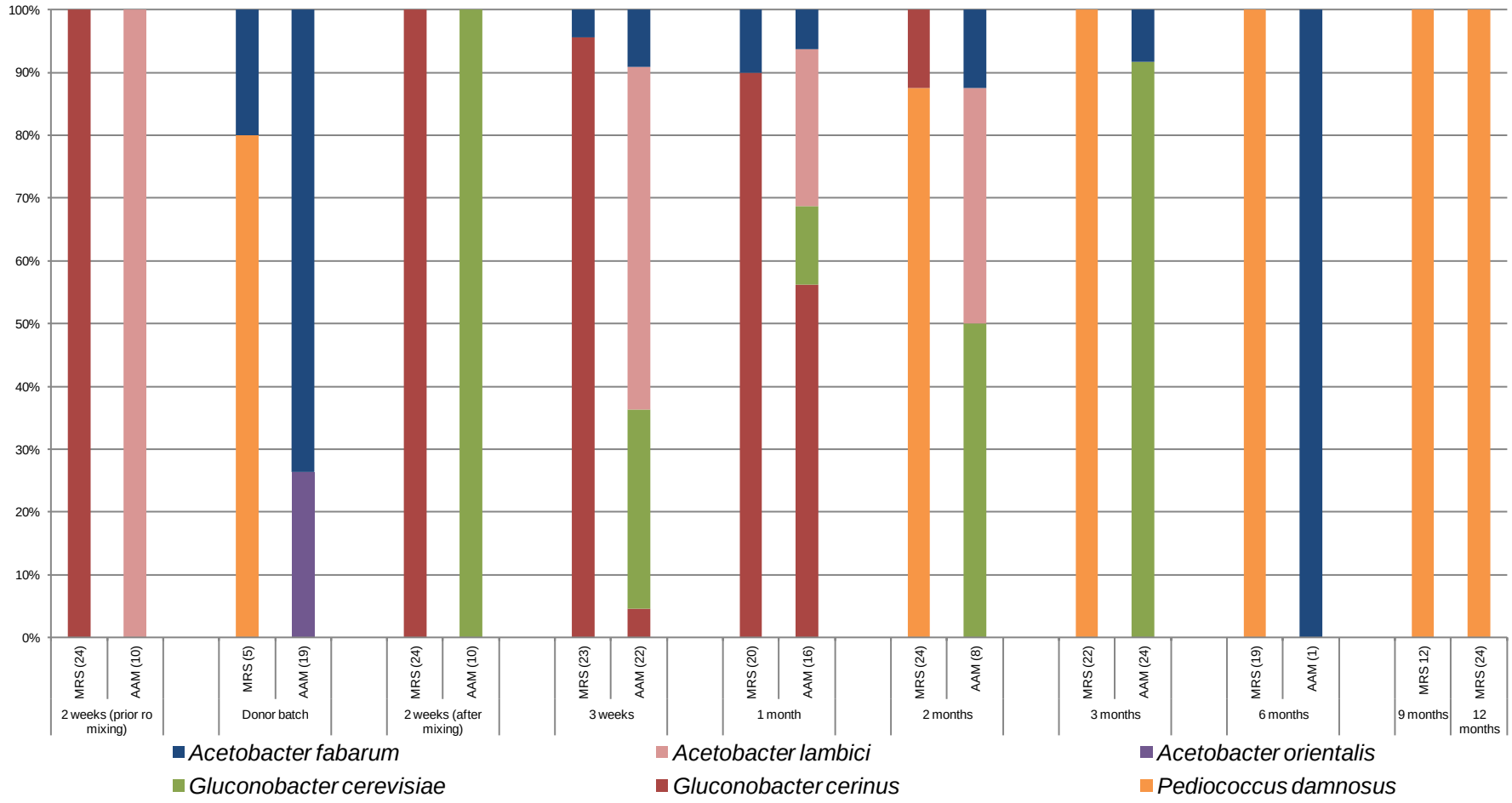

Figure 4.2.3 Identification of random isolates from MRS and AAM agars of batch A cask samples. The identification of anaerobically incubated MRS agar isolates is not shown, as all isolates were identified as *Pediococcus damnosus*. The number of isolates is given between brackets.

Three-weeks fermentation samples. Although foam was produced and the fermentation thus started one week after the mixing point, the enumeration results showed no profound changes (Table 4.2.1). Not unexpectedly because of its presence in the donor cask A, *S. cerevisiae* in addition to *H. uvarum* and *Pi. fermentans* was isolated from the yeast agar isolation media (Figure 4.2.2). AAB seemed more diverse one week after the mixing point, with *A. lambici*, *G. cerevisiae*, *G. cerinus* and *A. fabarum* being isolated from AAM agar, and the latter two species also from MRS agar (Figure 4.2.3). The few colonies from anaerobically incubated MRS (n = 4) were identified as *P. damnosus*.

One month fermentation samples. One month after brewing, the composition of the cultivable microbiota of the samples and the corresponding DGGE fingerprints (Figure 4.2.1 and Figure 4.2.2) changed remarkably, with *Pi. fermentans*, *S. cerevisiae*, *S. pastorianus* and *H. uvarum* being isolated from DYPAI and UBAGI agars. *H. uvarum* remained the only yeast species isolated from DYPAIX agar, but it was no longer the major isolated yeast species (Supplementary Figure S 4.2.2). Aerobically incubated MRS and AAM agars yielded largely the same diversity as found one week after the mixing point (Figure 4.2.3). Again, only *P. damnosus* was isolated from anaerobically incubated MRS agar.

Second- and third-month fermentation samples. During the second and third month of fermentation, the number of colonies on anaerobically incubated MRS agar gradually increased to reach 10^6 CFU/mL (Table 4.2.1). In contrast, colony counts on aerobically incubated MRS agar showed a decrease during the second month, followed by an increase during the third month (Table 4.2.1). At the 2-months sampling point, primarily *P. damnosus* was isolated from both aerobically and anaerobically incubated MRS agar; a small fraction of the isolates from aerobically incubated MRS agar was identified as *G. cerinus* (Figure 4.2.3). Counts on AAM agar decreased after 2 months and were below the limit of quantification at 6 months (Table 4.2.1). *Acetobacter lambici*, *G. cerevisiae* and *A. fabarum* were isolated from the 2-months old sample, whereas only the latter two species were isolated at 3 months of fermentation (Figure 4.2.3). The decrease and subsequent increase in colony counts on aerobically incubated MRS agar are likely to be explained by the decrease of AAB and the subsequent increase of LAB, as shown by the colony counts

on anaerobically incubated MRS agar (Table 4.2.1). Changes in the microbial communities led to the appearance of new bands in the bacterial community profiles of batch A after 3 months of fermentation (Figure 4.2.1A, bands grouped in band class box 5). These new bands all originated from *Pediococcus*/*Lactobacillus* (Supplementary Table S 4.2.2). Yeast counts on DYPAIX agar were stable until month 1, after which the counts decreased (Table 4.2.1). In the yeast community profiles, band class 6 (*Hanseniaspora*, Figure 4.2.1B) and two *Saccharomyces* DNA bands (Figure 4.2.1B, band classes 8 and 9) were present during the main fermentation phase, although band classes 6 and 9 disappeared at 3 months. In the 2-months old sample, multiple new DNA bands appeared, which again all originated from *Saccharomyces* strains (Figure 4.2.1B, bands grouped in band class box 10). At 2 months, *Pi. fermentans* and *S. pastorianus* were isolated from DYPAI and UBAGI agars (Figure 4.2.2), whereas *H. uvarum* remained the only species isolated from DYPAIX agar (Supplementary Figure S 4.2.2). The former two species were the main yeast species isolated at 3 months, in addition to a small number of *S. cerevisiae* isolates (Figure 4.2.2). Both *D. bruxellensis* and *H. uvarum* were isolated from DYPAIX agar (Supplementary Figure S 4.2.2). Band class 11 (Figure 4.2.1B) originated from *Dekkera* strains and was first detected in the yeast DGGE community profiles at 3 months.

Six-months, nine-months, and one-year fermentation samples. After the 3-months sampling point, the relative intensity of the bands grouped in band class box 5 varied (Figure 4.2.1A), but each of these DNA bands was assigned to *Pediococcus*/*Lactobacillus* and thus this taxon was the single dominant bacterium during the remainder of the fermentation. Bacterial counts on MRS agar remained high (10^6 CFU/mL) after 6 months of fermentation, but then started to decrease (Table 4.2.1). *Pediococcus damnosus* was the only isolated species from both aerobically and anaerobically incubated MRS agars in samples taken at months 6, 9 and 12 (Figure 4.2.3). Only a single colony, identified as *A. fabarum*, was found on AAM agar at 6 months of fermentation (Figure 4.2.3). Yeast counts started to decrease from 3 months onwards. In the 6-months old and subsequent samples, *D. bruxellensis* was the only yeast species isolated from DYPAI and UBAGI agars (Figure 4.2.2). This yeast species was also isolated from DYPAIX agar and *Dekkera anomala*

was only found once in the 9-months old sample (Supplementary Figure S 4.2.2). Most bands of the yeast community profiles, except for band class 8 (Figure 4.2.1) disappeared at 6 months and concomitantly two faint bands (Figure 4.2.1B, bands grouped in band class box 12) appeared in the low % G+C region. The latter bands originated from *Hanseniaspora* strains. The yeast DGGE community profiles of the samples at 9 and 12 months were similar: band class 8 disappeared, band class 11 reappeared and two new bands, originating from *Kregervanrija* (band class 13) and *Dekkera* (band class 14) strains were present. After one year, not only *D. bruxellensis* but also *Wickerhamomyces anomalus* and *Yarrowia lipolytica* were isolated from DYPAIX agar (Supplementary Figure S 4.2.2).

Microbiota succession in batch B

The early bacterial and yeast DGGE community profiles of batch B contained only one dense band each (Figure 4.2.1C, band class 15; Figure 4.2.1D, band class 19), which again were both assigned to *Saccharomyces* strains. Only DYPAI and UBAGI agars yielded substantial growth immediately after the transfer of the wort into the cask (10^3 CFU/mL; Table 4.2.1); yeast isolates from these samples were identified as *Pichia kudriavzevii* (n = 8 isolates) and *Debaryomyces hansenii* (n = 1). Isolates from MRS agar were all identified as *A. orientalis* (n = 7).

After one week, yeast counts increased to 10^5 CFU/mL. The yeasts present were primarily cycloheximide-sensitive, as shown by the low counts on DYPAIX agar (Table 4.2.1). Isolates from DYPAI and UBAGI agars were identified as *S. cerevisiae* (n = 38) and *Pi. kudriavzevii* (n = 2), whereas only one isolate, identified as *D. bruxellensis*, was obtained from DYPAIX agar. Bacterial counts were again low and only one bacterial isolate was obtained from MRS agar, which was identified as *A. orientalis*, the AAB species that was readily present after the transfer of the wort into the cask.

Band class 16 (originating from *Pediococcus*/*Lactobacillus*) appeared in the 2-weeks old sample and increased in intensity in later samples until it virtually disappeared after one month (Figure 4.2.1C). In the 3-weeks and 1-month old samples, one band originating from AAB could be detected in the high % G+C region (Figure 4.2.1C, band class 17). The bacterial DGGE community profiles changed during the second

month of the fermentation. The profiles of the 2-months old sample contained multiple bands (Figure 4.2.1C, bands grouped in band class box 18), which all originated from *Pediococcus*/*Lactobacillus*. Yeast DGGE community profiles were all nearly identical and bands originated from *Saccharomyces* (Figure 4.2.1D, band class 19) and *Dekkera* (Figure 4.2.1D, band class 20) strains. The bacterial community profiles after 3 months of fermentation were highly similar to the bacterial community profiles obtained from the sample of the 6-months fermented batch A.

Colony counts on all yeast agar isolation media were comparable after three months and were all about 10^4 CFU/mL. *Saccharomyces cerevisiae* (n = 46) and *Pi. kudriavzevii* (n = 17) were the major yeast species isolated during the first month. *Dekkera bruxellensis* (n = 5) was increasingly recovered from one month of fermentation onwards and was the only isolated yeast species (n = 36) in the samples taken after 2 and 3 months of fermentation. From 2 weeks onwards, *A. fabarum* was the sole AAB species that could be isolated from both AAM agar (n = 23) and aerobically incubated MRS agar (n = 48). This species was isolated up to 3 months of fermentation and counts on AAM agar reached a maximum of 10^5 CFU/mL after 3 weeks, but decreased below the level of quantification at 3 months (Table 4.2.1). From 3 weeks onwards, *P. damnosus* was isolated from MRS agar and it was the most isolated bacterial species during the remainder of the fermentation (n = 57). Similarly to batch A, the increase of LAB resulted in higher colony counts on anaerobically incubated MRS agar (Table 4.2.1).

Microbiota of the brewery environment

No yeasts or bacteria could be recovered from samples of the brewery ceilings, walls and cooling tun surface; in addition, the microorganisms that were isolated from air samples were not found in the lambic beer fermentation process, such as *Klebsiella oxytoca*, *Bacillus* spp. and *Staphylococcus* spp. (Table 4.2.2). In contrast, swab samples taken from both inside and outside of the casks yielded several species found in the fermenting lambic beer. *Pediococcus damnosus*, *D. bruxellensis* and *D. anomala* (Table 4.2.2), microorganisms isolated frequently from 6 months of fermentation onwards, were isolated from the inside of a cleaned cask and were thus readily present when the wort entered the cask. *Saccharomyces cerevisiae* and *S. pastorianus* were not isolated from air samples nor from the casks.

Discussion

Whereas for traditional lambic beer fermentations inoculation starts during the overnight cooling of the wort in the cooling tun and acidification of the wort by the action of acid-producing microorganisms, the industrial lambic beer fermentations of the present study were steered or started spontaneously as soon as the acidified chilled wort received microorganisms from the surroundings when it was transferred into the cask. Due to acidification of the wort with lactic acid to pH 4 after boiling, *Enterobacteriaceae* were not present because of their sensitivity to low pH values (Priest & Stewart, 2006). This contrasted with traditional lambic and American coolship ale fermentations, where this group of bacteria is dominantly present from the cooled wort sample in the cooling tun until the end of the first month of fermentation (Bokulich *et al.*, 2012; Spitaels *et al.*, 2014c; Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). However, sometimes a lambic beer fermentation is too sluggish, which was the case for batch A of the present study, although high counts of *H. uvarum,* a yeast isolated during the *Enterobacteriaceae* phase of traditional lambic beers (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995), were present. Such a sluggish fermentation is steered by adding fermenting wort from another batch of more or less the same age, which was the case for batch A of the present study, to stimulate the onset of the fermentation. In contrast to batch A, the fermentation process of batch B started spontaneously within the first week of the start of the brewing process. The identification of *H. uvarum* from industrial lambic beer samples of batch A confirms the findings of previous studies of lambic beer (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995), wherein its asexual name *Kloeckera apiculata* was used. *Hanseniaspora uvarum* was not found in batch B nor in a recent study of the lambic beer fermentation in a traditional brewery (Spitaels *et al.*, 2014c). Consequently, whether this yeast species is necessary for lambic beer fermentation is not clear up to now. This species has however a low fermentative capacity and is commonly found during the spontaneous fermentation of wines and cider, where its contribution to flavor complexity is increasingly appreciated (Bezerra-Bussoli *et al.*, 2013; de Arruda Moura Pietrowski *et al.*, 2012; Valles *et al.*, 2007).

Table 4.2.2 Overview of the microorganisms isolated from the brewery environment and their isolation sources.

	Accession number	Accession number closest hit	Similarity (%)	Present in fermentation	Air attic after	Air attic before	Air cellar	Cask exterior	Cask interior
Bacteria[a]									
Aerococcus urinaeequi		D87677	100				+		
Bacillus aerophilus		AJ831844	100		+				
Bacillus aryabhattai		EF114313	100				+		
Bacillus licheniformis		AE017333	100			+			
Bacillus simplex		AB363738	100			+			
Bacillus subtilis		AMXN01000021	100			+			
*Klebsiella oxytoca**		AB004754	100			+			
Kocuria kristinae		X80749	100			+			
Lactococcus lactis		AE005176	100			+			
Leuconostoc citreum	KJ541152	AF111948	99			+			
Lysinibacillus macroides		AJ628749	100			+			
Pediococcus damnosus				+					+
Pediococcus pentosaceus[b]		AM749815	100			+			
Propionibacterium cyclohexanicum	KJ541151	D82046	99			+			
Propionibacterium thoenii	KJ541153	AJ704572	98			+			
Pseudomonas azotoformans		D84009	100				+		
Rumeliibacillus pycnus		AB271739	100				+		
Staphylococcus aureus		D83355	100				+		
Staphylococcus caprae		AB009935	100				+		+
Staphylococcus epidermidis		L37605	100			+			
Staphylococcus haemolyticus		L37600	100			+			
Staphylococcus hominis		X6601	100		+		+		
Staphylococcus petrasii		AY953148	100				+		
Staphylococcus saprophyticus		AP008934	100		+				
Staphylococcus succinus		AF004220	100				+		
Staphylococcus warneri		L37603	100		+				
Streptococcus parauberis		NR_043001	100			+			
Yeasts[c]									
Blastobotrys arbuscula		DQ442689	100						+
Cryptococcus carnescens		AB035054	100		+				
*Debaryomyces hansenii**		JQ689041	100					+	
Dekkera anomala				+					+
Dekkera bruxellensis				+					+
Trichosporon domesticum		JN939449	100			+			

[a]Identification was based on the 16S rRNA gene sequence.

*Identification was confirmed by MALDI-TOF MS, clustering together with isolates obtained during a previous study (Spitaels et al., 2014).

[b]Identification was based on the *pheS* gene sequence.

[c]Identification was based on the D1/D2 26S rRNA gene sequence.

Saccharomyces cerevisiae, *S. pastorianus*, *D. bruxellensis*, and *P. damnosus* were dominating the lambic beer fermentation processes studied, although much more AAB species could be isolated from the industrial lambic brew samples of the present study compared with spontaneous lambic brew samples (Spitaels *et al.*, 2014c). In both cases, most AAB belonged to new species (Spitaels *et al.*, 2014a, 2014b). This indicates a possible role for AAB too, as these new species may have been missed during former studies. Further, the counts of the yeasts were higher than those of the bacteria in the main fermentation phase and vice versa in the maturation phase. As the DGGE technique is known to detect community members that represent more than 1% of the total communities (Muyzer *et al.*, 1993), the low bacterial counts could explain the absence of bacterial bands in the bacterial DGGE profiles of the batch A samples prior to the mixing point. Alternatively, the isolation of AAB species from MRS agar indicates the non-selectiveness of this agar medium for LAB, as reported increasingly (Papalexandratou *et al.*, 2011; Spitaels *et al.*, 2014c). However, aerobically incubated MRS agar allowed the growth of both LAB and AAB, whereas anaerobically incubated MRS agar favored the growth of LAB.

Since the *Enterobacteriaceae* phase was absent, the first phase of these industrial lambic fermentation processes studied was the main fermentation phase, characterized by the presence of *Saccharomyces* spp., which were isolated from batch A until the third month. The ratio of *S. pastorianus* to *S. cerevisiae* increased for a reason that is not known but may be related to fermentation temperature adaptation (Cousseau *et al.*, 2013; Vidgren *et al.*, 2010), and this phenomenon was also seen during the traditional lambic beer fermentation studied (Spitaels *et al.*, 2014c). The slow onset and pace of the main fermentation phase of batch A caused a late onset of the growth of *D. bruxellensis* and hence the maturation phase in batch A. In batch B, *S. cerevisiae* was the most isolated yeast species during the main fermentation phase, until *D. bruxellensis* became predominant after one month. According to the endorsement 'traditional specialty guaranteed' of the European Commission, lambic beer is defined as a spontaneously fermented beer in which *D. bruxellensis* plays a crucial role during the maturation phase (European Commission, 1997a, b).

The different start of the two lambic beer batches is difficult to explain. All casks in the industrial brewery were similar in height, were exclusively used for lambic beer fermentation, and were stored at ambient temperature at the same location. Thus, the environmental factors in the industrial lambic brewery were more uniform than those of a traditional lambic brewery where casks are smaller, had a previous use in wine or cognac production, and are often located in different rooms (Spitaels *et al.*, 2014c). However, the temperature of the chilled wort may vary between batches and ambient temperatures may differ considerably as well. Noteworthy to mention that batch A started in January (winter) and batch B started in July (summer). The present study hypothesizes that both temperature factors are likely influencing the successful initiation and the pace of the main fermentation phase. Further, the onset of the maturation phase differed. Since the industrially brewed lambic wort was acidified at the end of the wort boiling, the pH drop caused by the production of lactic acid by LAB was less pronounced compared to a non-acidified wort. In agreement with this, the start of the maturation phase occurred after one month of fermentation in batch B, since only *D. bruxellensis* was present in the samples after 2 and 3 months. Simultaneously, *P. damnosus* was the only bacterium isolated. This effect was not apparent in batch A where the maturation phase occurred from 6 months onwards, but was most probably masked by the delayed start of the fermentation altogether. Similarly, recent microbiological studies of spontaneous beer fermentation processes did not reveal an extended acidification phase (Bokulich *et al.*, 2012; Spitaels *et al.*, 2014c). In contrast, the acidification and maturation phases seemed to proceed simultaneously and hence it is more appropriate to consider this part of the lambic beer fermentation as a long maturation phase. In the traditional and industrial breweries, *P. damnosus* was the only isolated bacterium during this long maturation phase. This stresses the impact of the microbiota on the lambic beer characteristics.

Opportunistic contaminants were occasionally isolated, such as *C. parapsilosis*, *D. anomala*, *W. anomalus*, and *Y. lipolytica*. *Candida parapsilosis* was previously reported as a wild yeast from lager beers, but failed to grow in wort or beer, and was therefore regarded as a contaminant (Van der Aa Kühle & Jespersen, 1998). The yeast species *W. anomalus* and *Y. lipolytica* were also found at the end of a lambic

beer fermentation process in a traditional lambic brewery (Spitaels *et al.*, 2014c). *Yarrowia lipolytica* is primarily found in dairy and meat products, but also in soil and wastewaters (Knutsen *et al.*, 2007). The typical presence of this yeast at a late stage of the lambic beer fermentation process may suggest a specific role and deserves further attention.

The observation of *D. bruxellensis* and not that of *Saccharomyces* spp., as suggested by the DGGE results, as the most isolated yeast species from 6 months onwards, confirmed the known pitfalls of a culture-independent technique such as DGGE (Spitaels *et al.*, 2014c). *Saccharomyces* reached high counts (about 10^6 CFU/mL) compared to the counts of *Dekkera* (about 10^4 CFU/mL) and the latter may be absent in the DGGE community profiles due to the abundant presence of DNA from dead cells or from VBNC *Saccharomyces* cells.

Lambic beers are assumed to be spontaneously inoculated by the air microbiota of the Senne river valley during the overnight cooling in the cooling tun (Martens *et al.*, 1991; Verachtert & Iserentant, 1995). However, whereas air samples harbored microorganisms not relevant for lambic beer fermentation, none of the cooling tun samples of the industrial brewery studied yielded DNA or microbial growth. This indicates that the cooling tun samples were sterile or very low numbers of microorganisms were present and thus the microbiota must have been inoculated once the wort entered the cask. Consequently, they should originate from the cask wood or from residues of the previous fermentation batches. Indeed, casks are cleaned superficially using only a pressure washer to remove yeast and bacterial clumps from the ceiling, sides and bottom of the casks. Unlike in traditional breweries, no efforts are made to kill the residual microbiota, using for instance steam or other sanitizing agents. Again contrasting with traditional lambic breweries, the industrial brewery uses anti-fungal paint on all walls and ceilings in the brewery. *Saccharomyces cerevisiae* and *S. pastorianus* are responsible for the main fermentation phase although not isolated at the end of the fermentation process. Yet, these yeasts and bacteria may penetrate into the wood of the casks and effectively form a biofilm there (Swaffield & Scott, 1995; Swaffield *et al.*, 1997). Therefore, the present study hypothesizes that *Saccharomyces* yeasts may remain present in the cask wood and thus survive the maturation phase to re-emerge when

fresh wort enters the cask. Likewise, AAB may survive in the cask wood. As *P. damnosus*, *D. bruxellensis* and *D. anomala* were isolated from the inside of the casks, these species could enter the wort directly after the transfer from the cooling tun into the casks.

Conclusion

The present study demonstrates that industrial and traditional lambic beer fermentations involve the same main actors, including *S. cerevisiae*, *S. pastorianus*, *P. damnosus* and *D. bruxellensis,* which confirms and extends previous observations (Spitaels *et al.*, 2014c; Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). These species could therefore be regarded as the core microbiota of lambic beer fermentation. Although these main actors were the same in both breweries, the present data showed a different fermentation profile in the industrial brewery compared to the traditional brewery studied. This was mainly due to the absence of the *Enterobacteriaceae* phase, as the industrially produced wort was acidified at the end of the wort boiling. Further, *P. damnosus* was found as the only LAB present, indicating an adaptation of this species to grow under the harsh conditions of the lambic beer fermentation process.

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Supplementary information

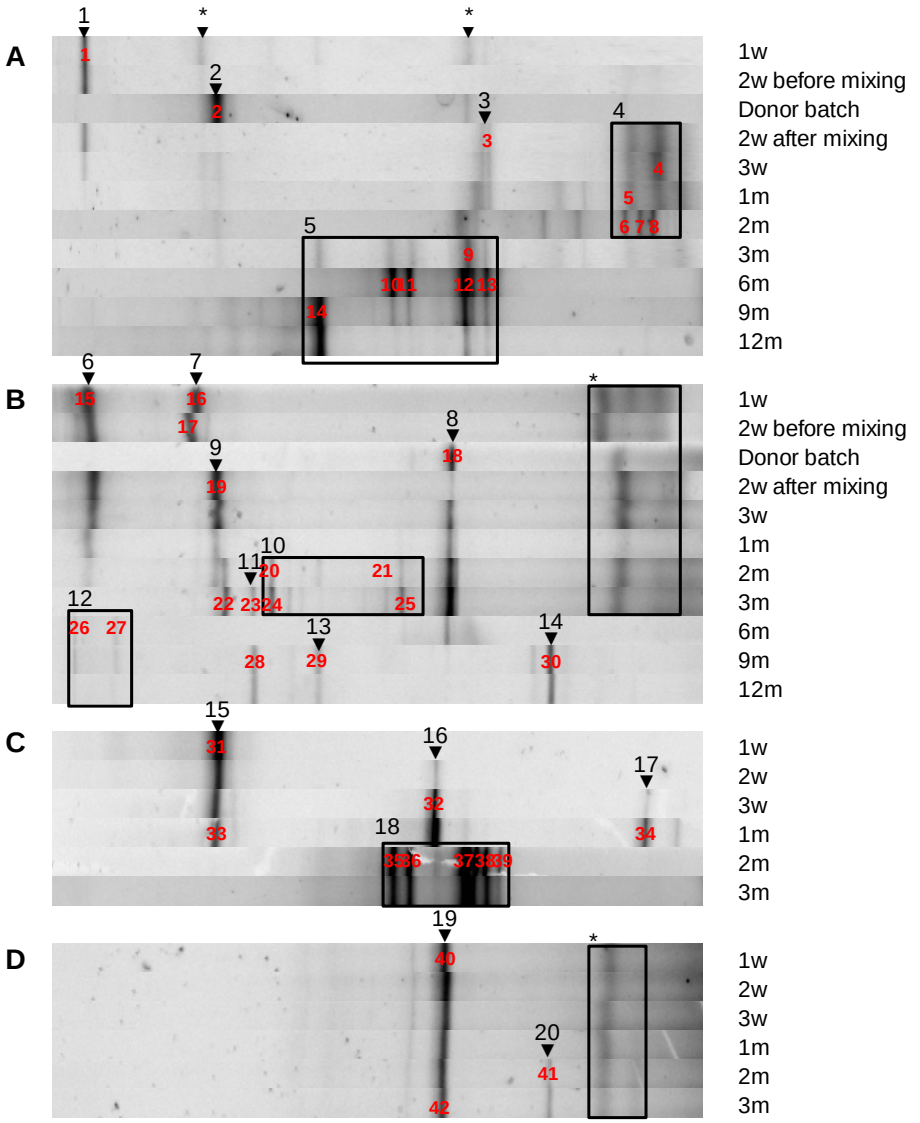

Figure S 4.2.1 DGGE banding patterns of the bacterial and yeast communities of batch A (A and B, respectively) and batch B (C and D, respectively): w, week(s); m, month(s). Band classes 1-18 are indicated with numbers. Samples only yielded DNA and PCR amplicons after one week of fermentation. *These bands did not yield PCR amplicons after band excision and subsequent DNA amplification. The 35-70 % denaturing gradient is represented from left to right on the gels. The excised bands are indicated in red and identifications based on the derived DNA sequences of these bands can be found in Supplementary Table S 4.2.1.

Table S 4.2.1 Identifications of the excised V3 and LSU DGGE DNA bands used for sequence analysis.

Band number	Accession number highest hit*	Similarity	Identification
1	AY046257	100%	*Hanseniaspora*
2	AY497740	100%	*Saccharomyces*
3	AB681216	100%	*Pediococcus/Lactobacillus*
4	NR_041445	100%	Acetic acid bacteria
5	NR_041445	100%	Acetic acid bacteria
6	NR_041445	100%	Acetic acid bacteria
7	NR_041445	100%	Acetic acid bacteria
8	NR_041445	100%	Acetic acid bacteria
9	AB681216	100%	*Pediococcus/Lactobacillus*
10	AB681216	100%	*Pediococcus/Lactobacillus*
11	AB681216	100%	*Pediococcus/Lactobacillus*
12	AB681216	100%	*Pediococcus/Lactobacillus*
13	AB681216	100%	*Pediococcus/Lactobacillus*
14	FR683099	100%	*Pediococcus/Lactobacillus*
15	AY046257	100%	*Hanseniaspora*
16	AB365475	100%	*Candida/Pichia*
17	AB365475	100%	*Candida/Pichia*
18	AJ279065	100%	*Saccharomyces*
19	BR000309	100%	*Saccharomyces*
20	BR000309	100%	*Saccharomyces*
21	BR000309	100%	*Saccharomyces*
22	AJ279065	100%	*Saccharomyces*
23	BR000309	100%	*Saccharomyces*
24	DQ406717	100%	*Dekkera*
25	BR000309	100%	*Saccharomyces*
26	U84230	100%	*Hanseniaspora*
27	U84230	100%	*Hanseniaspora*
28	DQ406717	100%	*Dekkera*
29	U70247	100%	*Kregervanrija*
30	AY969049	100%	*Dekkera*
31	AY497740	100%	*Saccharomyces*
32	AB626053	100%	*Pediococcus/Lactobacillus*
33	NR_041445	100%	Acetic acid bacteria
34	AY497740	100%	*Saccharomyces*
35	AB681216	100%	*Pediococcus/Lactobacillus*
36	AB681216	100%	*Pediococcus/Lactobacillus*
37	AB681216	100%	*Pediococcus/Lactobacillus*
38	AB681216	100%	*Pediococcus/Lactobacillus*
39	AB681216	100%	*Pediococcus/Lactobacillus*
40	AJ279065	100%	*Saccharomyces*
41	AY969049	100%	*Dekkera*
42	AJ279065	100%	*Saccharomyces*

*Highest hit with first type strain in BLAST results

Table S 4.2.2 Occurrence of microbial taxa in batch A and batch B cask samples as identified through sequence analysis of V3 and LSU DGGE bands. +: taxon is present.

	Saccharomyces	*Dekkera / Brettanomyces*	*Candida/Pichia*	*Hanseniaspora*	*Kregervanrija*	*Pediococcus / Lactobacillus*	Acetic acid bacteria
Batch A / 1 week			+	+			
Batch A / 2 weeks (prior to mixing)			+	+			
Batch A / Donor batch	+						
Batch A / 2 weeks (after mixing)	+			+		+	+
Batch A / 3 weeks	+			+		+	+
Batch A / 1 month	+	+				+	+
Batch A / 2 months	+	+			+	+	+
Batch A / 3 months	+	+				+	
Batch A / 6 months	+			+		+	
Batch A / 9 months		+		+	+	+	
Batch A / 12 months		+			+	+	
Batch B / 1 week	+						
Batch B / 2 weeks	+					+	
Batch B / 3 weeks	+					+	+
Batch B / 1 month	+					+	+
Batch B / 2 months	+	+				+	
Batch B / 3 months	+	+				+	

Table S 4.2.3 Overview of MALDI-TOF MS clusters and the identification of the representative isolates. The number of isolates in each MALDI-TOF MS cluster is given in parentheses. The accession number of the cluster representative sequence is given when sequence similarity with a known sequence was below 100%. B: bacterial MALDI-TOF MS cluster, Y: yeast MALDI-TOF MS cluster.

MALDI-TOF MS cluster	Strain number of representative isolate	Accession number*	Identification technique/gene	Identification	Accession number closest hit	Similarity (%)
B-1 (101)	R-49088	KJ541146	*rpoB*	*Gluconobacter cerinus*	FN391790	98
B-2 (45)[a]				*Gluconobacter cerevisiae*		
B-3 (13)	R-49663; R-49740		*groEL*	*Acetobacter orientalis*	KC176391	100
B-4 (96)	R-49664; R-49666; R-49667; R-49741; R-49743	KJ541148 KJ541149	*dnaK*	*Acetobacter fabarum*	HG329536	99
B-5 (29)[b]				*Acetobacter lambici*		
B-6 (267)	R-49092; R-49096; R-49101		*pheS*	*Pediococcus damnosus*	AM899820	100
Y-1 (27)	R-49641; R-49845	KJ541147	D1/D2 26S rRNA gene	*Pichia kudriavzevii*	EF550222	99
Y-2 (1)[c]			MALDI-TOF MS	*Wickerhamomyces anomalus*	U74592	100
Y-3 (218)	R-49640; R-49643; R-49828; R-49829		D1/D2 26S rRNA gene	*Dekkera bruxellensis*	JQ689028	100
Y-4 (121)	R-49642; R-49644; R-49814; R-49815		D1/D2 26S rRNA gene	*Saccharomyces cerevisiae*	JQ689017	100
Y-5 (76) [c]			MALDI-TOF MS	*Saccharomyces pastorianus*		
Y-6 (65)	R-49639; R-49813; R-49817; R-49818		D1/D2 26S rRNA gene	*Pichia fermentans*	GQ458040	100
Y-7 (304)	R-49645; R-49646; R-49811; R-49812; R-49816; R-49819	KJ541150	*ACT1*	*Hanseniaspora uvarum*	AM039456	99
Y-8 (6)	R-49561		*ACT1*	*Hanseniaspora meyeri*	AM039466	100
			growth at 30°C	*Hanseniaspora meyeri*		
Y-9 (1) [c]			MALDI-TOF MS	*Debaryomyces hansenii*		
Y-10 (1) [c]			MALDI-TOF MS	*Dekkera anomala*		
Y-11 (1)	R-49836		D1/D2 26S rRNA gene	*Candida parapsilosis*	U45754	100
Y-12 (3)	R-49846		D1/D2 26S rRNA gene	*Yarrowia lipolytica*	JQ689067	100

[a]Isolates of this cluster were characterized in a polyphasic taxonomic study as the new species *Gluconobacter cerevisiae* (Spitaels et al., 2014a).

[b]Isolates of this cluster were characterized in a polyphasic taxonomic study as the new species *Acetobacter lambici* (Spitaels et al., 2014b).

[c]The isolates of this cluster were identified by means of MALDI-TOF MS. These isolates clustered together with isolates that were obtained and identified previously (Spitaels et al., 2014c).

*Accession numbers are given for the unique isolate sequences within the same MALDI-TOF MS cluster.

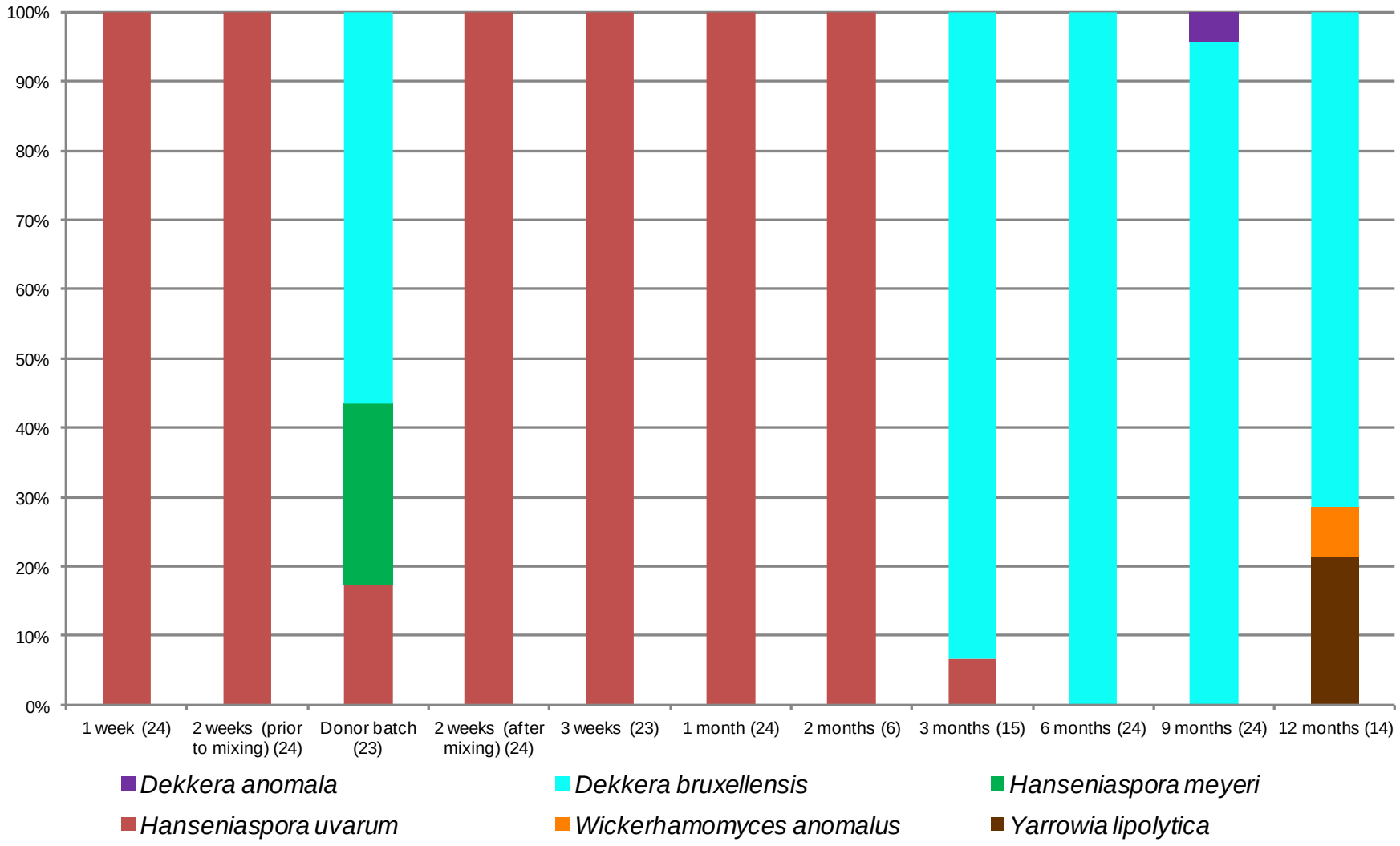

Figure S 4.2.2 Identification of random isolates from DYPAIX agar of batch A cask samples. The number of isolates is given between brackets.

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Chapter 5. Description of two novel acetic acid bacteria

Preamble

Both the traditionally and industrially produced lambic beers appeared to contain new acetic acid bacteria. All but one isolate from the traditional brewery were identified as the new species *Acetobacter lambici* sp. nov., described in **Chapter 5.1**. This species was also isolated from industrial lambic beer samples and all isolates showed the same RAPD profiles, indicating their clonality.

Gluconobacter cerevisiae sp. nov. is described in **Chapter 5.2** and was the second novel acetic acid bacteria isolated during these diversity studies. *Gluconobacter cerevisiae* was primarily isolated from the industrial lambic beer samples, but one additional isolate was obtained from a traditional lambic beer sample. Furthermore, this species was isolated from a spoiled brewer's yeast in a third brewery that did not produce lambic beers.

5.1 *Acetobacter lambici* sp. nov., isolated from fermenting lambic beer

Redrafted from: Freek Spitaels, Leilei Li, Anneleen Wieme, Tom Balzarini, Ilse Cleenwerck, Anita Van Landschoot, Luc De Vuyst and Peter Vandamme, *Acetobacter lambici* sp. nov. isolated from fermenting lambic beer, *International Journal of Systematic and Evolutionary Microbiology* **64**, 1083-1089.

Author contributions: conceived and designed the experiments: FS, LL, ADW, IC and PV; performed the experiments: FS, LL and ADW; determination of the production of keto-D-gluconic acids: TB and FS; analyzed the data: FS, LL and ADW; wrote the manuscript: FS; critically reviewed the manuscript: IC, AVL, LDV and PV.

The Genbank/EMBL accession numbers for the 16S rRNA, *dnaK*, *groEL* and *rpoB* gene sequences generated in this study are HF969863, HG329567-HG329569 for the 16S rRNA gene sequences; HG329531-HG329542 for *dnaK* gene sequences; HG329543-HG329554 for *groEL* gene sequences and HG329555-HG329566 for *rpoB* gene sequences.

Abstract

An acetic acid bacterium, strain LMG 27439^T, was isolated from fermenting lambic beer. The cells were Gram-stain-negative, motile rods, catalase-positive and oxidase-negative. Analysis of the 16S rRNA gene sequence revealed the strain was closely related to *Acetobacter okinawensis* (99.7% 16S rRNA gene sequence similarity towards the type strain of this species), *A. ghanensis* (99.6%), *A. syzygii* (99.6%), *A. fabarum* (99.4%) and *A. lovaniensis* (99.2%). DNA-DNA hybridization with the type strains of these species revealed moderate DNA-DNA hybridization values (31–45%). Strain LMG 27439^T was unable to grow on glycerol or methanol as the sole carbon source, on yeast extract with 10% ethanol or on glucose-yeast extract medium at 37°C. It did not produce acid from L-arabinose, D-galactose and D-mannose, nor did it produce 2-keto-D-gluconic, 5-keto-D-gluconic or 2,5-diketo-D-gluconic acid from D-glucose. It did not grow on ammonium as the sole nitrogen source and ethanol as the sole carbon source. These genotypic and phenotypic data distinguished strain LMG 27439^T from established species of the genus *Acetobacter*, and therefore we propose this strain represents a novel species of the genus *Acetobacter*. The name *Acetobacter lambici* sp. nov. is proposed, with LMG 27439^T (= DSM 27328^T) as the type strain.

The genus *Acetobacter* belongs to the family *Acetobacteraceae* within the class *Alphaproteobacteria* and currently comprises 23 species with validly published names. The ability of species of the genus *Acetobacter* and all other acetic acid bacteria (AAB), except for members of the genus *Asaia,* to oxidize ethanol to acetic acid or to carbon dioxide and water under neutral or slightly acidic conditions enables their growth in fermented foods and beverages (Cleenwerck *et al.*, 2002). This growth capacity can be detrimental, for instance when it leads to spoilage of lager or ale beers, wines or ciders, as well as beneficial, for instance in the production of vinegar, fermented cocoa, kombucha, red sour ales or lambic beers (Bartowsky & Henschke, 2008; Bokulich *et al.*, 2012; Martens *et al.*, 1991, 1997; Papalexandratou *et al.*, 2011; Raspor & Goranovic, 2008; Vaughan *et al.*, 2005).

Strain LMG 27439^T was isolated during a study of the fermentation process of acidic lambic beers. The latter beers are the product of a spontaneous fermentation, which progresses for at least two years in wooden casks. Strain LMG 27439^T was isolated on acetic acid medium (AAM), an AAB enrichment medium which consists of 1.0% (wt/vol) glucose, 0.5% (vol/vol) ethanol, 1.5% (wt/vol) peptone, 0.8% (wt/vol) yeast extract and 0.3% (vol/vol) acetic acid (Lisdiyanti *et al.*, 2003). The medium had a pH of 3.5 and contained 5 ppm amphotericin B and 200 ppm cycloheximide to prevent fungal growth. Isolates grown on AAM were subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as described previously (Wieme *et al.*, 2012). MALDI-TOF MS was shown to be useful for the identification of AAB involved in the production of vinegar (Andres-Barrao *et al.*, 2013) and was used as a dereplication tool in the present study. A total of 187 AAB lambic beer isolates obtained from two different lambic beer breweries (an industrial and a traditional type) had identical mass spectra that differed from those of established AAB species, which suggested a unique taxonomic position. Random amplified polymorphic DNA (RAPD) analysis of a selection of 13 isolates representing the two lambic beer breweries was performed as described by Williams *et al.* (1990) and revealed that all isolates were clonal derivatives of a single strain (data not shown). Subsequently, two isolates of the industrial type of lambic beer brewery (LMG 27439^T and R-50194) and two from the traditional type

of lambic beer brewery (LMG 27440 and R-50193) were chosen as representatives for further analyses.

The 16S rRNA gene sequence of strain LMG 27439^T was determined as described previously (Snauwaert *et al.*, 2013). EzBioCloud analysis (Kim *et al.*, 2012) of the obtained sequence revealed that it was an AAB strain, closely related to *Acetobacter okinawensis* (99.7%), *Acetobacter ghanensis* (99.6%), *Acetobacter syzygii* (99.6%), *Acetobacter fabarum* (99.4%) and *Acetobacter lovaniensis* (99.2%); values in parentheses are pairwise similarity values towards the type strains of these species. All sequences were aligned using the SILVA Incremental Aligner (SINA v1.2.11) (http://www.arb-silva.de/aligner/) (Pruesse *et al.*, 2012), with the corresponding SILVA SSURef 111 database (Pruesse *et al.*, 2007) and a dendrogram was constructed using the MEGA 5.2 software package (Tamura *et al.*, 2011). The tree topologies were statistically analyzed using 1000 bootstrapping replications. The maximum-likelihood and maximum-parsimony method trees (data not shown) showed the same topology as the neighbour-joining tree (Figure 5.1.1).

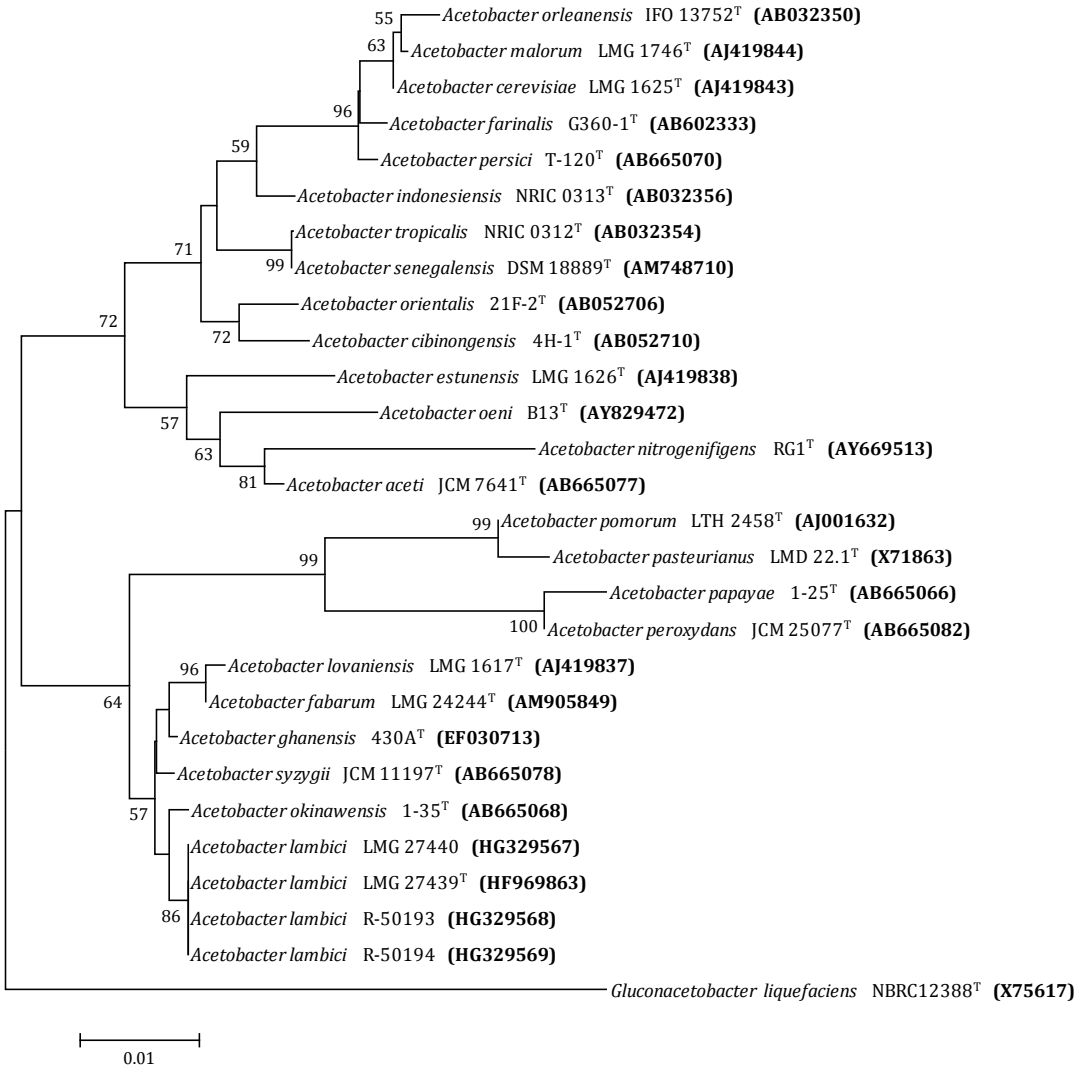

Figure 5.1.1 Neighbour-joining tree based on the nearly full-length 16S rRNA gene sequences (1401 bp) showing the phylogenetic relationship of isolates LMG 27439^T, LMG 27440, R-50193 and R-50194 and of the type strains of all species of the genus *Acetobacter*. *Gluconacetobacter liquefaciens* NBRC 12388^T was used as an outgroup. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. GenBank accession numbers are given in parentheses. Bootstrap percentages (>50%) are shown next to the branch points. Bar, 1% sequence divergence.

Due to the limited taxonomic resolution of the 16S rRNA gene in this group of bacteria, the phylogenetic position of the taxon represented by strains LMG 27439^T and LMG 27440 and of type and other taxonomic reference strains of its nearest phylogenetic neighbours (*A. okinawensis, A. ghanensis, A. syzygii*, *A. fabarum* and *A. lovaniensis*) were analyzed using sequence analysis of the housekeeping genes *dnaK* (encoding chaperone protein DnaK), *groEL* (encoding for 60 kDa chaperonin) and

rpoB (encoding DNA-directed RNA polymerase subunit beta) (Cleenwerck *et al.*, 2010). Sequences of at least 627 nt, 715 nt and 582 nt were generated for *dnaK*, *groEL* and *rpoB*, respectively. All gene sequences were aligned at the amino acid level using the MEGA 5.2 software (Tamura *et al.*, 2011). The trees were built using the maximum-likelihood model. A discrete gamma distribution was used to model evolutionary rate differences among sites and the rate variation model allowed for some sites to be evolutionarily invariable. Tree topologies were analyzed statistically using 1000 bootstrapping replications. Numerical analysis of the individual (Supplementary Figure S 5.1.1, Figure S 5.1.2 and Figure S 5.1.3) and concatenated (Figure 5.1.2) gene sequences revealed that the novel taxon represented by strains LMG 27439^T and LMG 27440 could be clearly differentiated from its nearest neighbours.

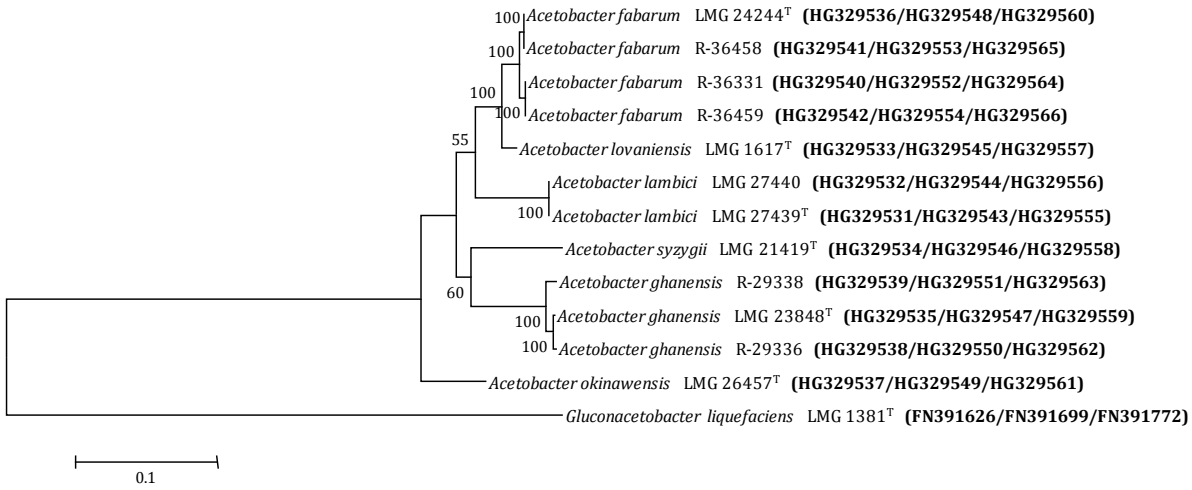

Figure 5.1.2 Maximum-likelihood tree based on concatenated *dnaK*, *groEL* and *rpoB* gene sequences (a total of 1911 bp) showing the phylogenetic relationship of isolates LMG 27439^T and LMG 27440 and their closest phylogenetic neighbours. *Gluconacetobacter liquefaciens* LMG 1381^T was used as an outgroup. The substitution model used was the Tamura-Nei model (Tamura & Nei, 1993). GenBank accession numbers for *dnaK*, *groEL* and *rpoB* gene sequences are given in this order in parentheses. Bootstrap percentages (>50%) are shown next to the branch points. Bar, 10% sequence divergence.

DNA-DNA hybridizations were performed between strain LMG 27439^T and the type strains of its nearest phylogenetic neighbours as described previously (Cleenwerck *et al.*, 2008). DNA-DNA relatedness values are presented as means of reciprocal reactions (A × B and B × A), where each reciprocal reaction was performed at least in three-fold. The level of DNA-DNA relatedness between strain LMG 27439^T and the

type strains of its nearest neighbours was intermediate: 42% towards *A. okinawensis* LMG 26457^T, 35% towards *A. ghanensis* LMG 23848^T, 31% towards *A. syzygii* LMG 21419^T, 41% towards *A. fabarum* LMG 24244^T, and 45% towards *A. lovaniensis* LMG 1617^T. The DNA G+C content of strain LMG 27439^T was determined as described previously (Cleenwerck *et al.*, 2008) and was 56.2 mol%.

The phenotypic characteristics of strain LMG 27439^T and of three additional isolates (LMG 27440, R-50193 and R-50194) were determined as described previously (Cleenwerck *et al.*, 2002). Type strains of closely related AAB were included as positive or negative controls. For microscopy and colony morphology, strains were grown aerobically at 28°C for 48 h on AAM agar. The biochemical characteristics tested included Gram-stain reaction, analysis of catalase and oxidase activity, growth on glucose (30%), glycerol (0.3%), or methanol (0.3%) as the sole carbon sources, growth on ammonium as the sole nitrogen source and ethanol as the sole carbon source, and growth at 37°C on GY agar medium (5% glucose, 1% yeast extract and 1.5% agar). In addition, acid production from 1% L-arabinose, D-galactose, D-mannose and D-glucose was determined as described previously (Asai *et al.*, 1964). We observed that *Acetobacter farinalis* LMG 26772^T did not exhibit catalase activity in contrast with previously reported data (Tanasupawat *et al.*, 2011). Analysis of additional *A. farinalis* strains, *i.e.*, LMG 27045 and LMG 27046, confirmed that strains of this species lack catalase activity. We also observed that *A. farinalis* LMG 26772^T exhibited strong growth at 37°C instead of weak growth as reported previously (Tanasupawat *et al.*, 2011).

For testing the production of 2-keto-D-gluconic acid and 5-keto-D-gluconic acid, cells were grown as described by Gosselé *et al.* (1980). The presence of 2-keto-D-gluconic acid and 5-keto-D-gluconic acid was determined using high-performance anion-exchange chromatography (HPAEC) with conductivity under ion suppression (CIS), using an ICS 3000 chromatograph (Dionex) equipped with an AS-19 column (Dionex). The mobile phase, at a flow rate of 1.0 mL min^{-1}, consisted of ultrapure water (0.015 µS cm^{-1}; eluent A) and 100 mM KOH (eluent B), with the following gradient: 0.0 min: 96% eluent A and 4% eluent B; 20.0 min: 96% eluent A and 4% eluent B; 50.0 min: 60% eluent A and 40% eluent B; and 60.0 min: 0% eluent A and 100% eluent B. To remove proteins from the samples, 500 µL of acetonitrile was

added. After centrifugation (16060 × *g* for 15 min), the supernatants were filtered (0.2 µm filters; Minisart high-flow, Sartorius AG), and injected (10 µL) into the column. All four isolates (LMG 27439^T, LMG 27440, R-50193 and R-50194) produced D-gluconic acid but not 2-keto-D-gluconic acid or 5-keto-D-gluconic acid. *Acetobacter nitrogenifigens* LMG 23498^T produced 5-keto-D-gluconic acid but not 2-keto-D-gluconic acid or 2,5-diketo-D-gluconic acid (production of keto-D-gluconic acids from D-glucose was previously not reported for this strain).

Isolates LMG 27439^T, LMG 27440, R-50193 and R-50194 could be differentiated from *A. okinawensis*, *A. ghanensis*, *A. syzygii*, *A. fabarum* and *A. lovaniensis* by means of multiple biochemical characteristics (Table 5.1.1).

Whole-cell fatty acid methyl esters (FAME) were extracted and analyzed as described by Cleenwerck *et al.* (2007) using the TSBA50 identification library 5.0 (MIDI). Type strains of established species of the genus *Acetobacter* and the isolates LMG 27439^T, LMG 27440, R-50193 and R-50194 were grown on AAM agar for 24-72 h at 28°C under aerobic conditions. The most predominant fatty acid, which accounted for approximately 60% of the total fatty acid content was the straight-chain, unsaturated $C_{18:1}\omega 7c$. Other fatty acids that were present in lower percentages were $C_{16:0}$ (11%), $C_{16:0}$ 2-OH (8%), $C_{18:0}$ (7%) and $C_{14:0}$ (4%). Species of the genus *Acetobacter* could not be differentiated based on these data. A full overview of the obtained FAME data is shown in Table 5.1.2.

Finally, numerical comparison by means of the Pearson product moment correlation coefficient of the MALDI-TOF MS mass spectra (data not shown) of the isolates LMG 27439^T, LMG 27440, R-50193 and R-50194 and those of reference strains of their nearest phylogenetic neighbours allowed a very straightforward separation of all taxa. The differences between the spectra could also be examined visually (Figure 5.1.3). The four novel isolates could be differentiated from all established species of the genus *Acetobacter* by the consistent presence of six biomarker peaks characterized by *m/z* values of 3196.3 ± 4.6, 5226.9 ± 6.2, 6392.1 ± 7.1, 6576.7 ± 7.3, 8414.7 ± 8.7 and 10070.5 ± 10.1 (Figure 5.1.3); some of the peaks were present in the mass spectra of other species of the genus *Acetobacter*, but never all six simultaneously.

In conclusion, the present study provides polyphasic information that demonstrates that the taxon represented by LMG 27439^T, LMG 27440, R-50193 and R-50194 could be differentiated from its nearest phylogenetic neighbours, *A. okinawensis*, *A. ghanensis, A. syzygii, A. fabarum* and *A. lovaniensis,* by multiple genotypic and phenotypic characteristics and methodologies. We therefore propose to name this taxon *Acetobacter lambici* sp. nov., with LMG 27439^T (= DSM 27328^T) as the type strain.

Description of *Acetobacter lambici* sp. nov.

Acetobacter lambici (*lam'bi.ci*, N.L. gen. n. *lambici* of lambic beer, an acidic spontaneously fermented beer)

Cells are Gram-stain-negative, motile rods and are approximately 0.7 µm wide and 1.5-4.0 µm long. Cells occur separately or in pairs. Catalase-positive but oxidase-negative. After incubation for 48 h on AAM agar at 28°C, colonies are round, rough, brownish-beige and slightly raised, with a diameter of approximately 1 mm. Ethanol is oxidized to acetic acid. D-Gluconic acid is produced from D-glucose but not 2-keto-D-gluconic acid or 5-keto-D-gluconic acid. Unable to grow on glycerol or methanol as the sole carbon source, on 30% glucose or on GY medium at 37°C. Unable to produce acid from L-arabinose, D-galactose and D-mannose. No growth with ammonium as the sole nitrogen source and on glycerol or on yeast extract with 10% ethanol.

The type strain is strain LMG 27439^T (= DSM 27328^T), which was isolated from fermenting lambic beer. The G+C content of strain LMG 27439^T is 56.2 mol%.

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Table 5.1.1 Differential characteristics for *Acetobacter lambici* sp. nov. and established species of the genus *Acetobacter*.
Taxa: 1, *Acetobacter lambici* sp. nov. (n=4); 2, *A. okinawensis* (n=7); 3, *A. ghanensis* (n=3); 4, *A. syzygii* LMG 21419^T; 5, *A. fabarum* (n=4); 6, *A. lovaniensis* LMG 1617^T; 7, *A. aceti* (n=4); 8, *A. peroxydans* (n=2); 9, *A. cerevisiae* (n=4); 10, *A. cibinongensis* LMG 21418^T; 11, *A. estunensis* (n=3); 12, *A. orleanensis* (n=4); 13, *A. persici* (n=2); 14, *A. malorum* LMG 1746^T; 15, *A. orientalis* LMG 21417^T; 16, *A. farinalis* (n=3); 17, *A. tropicalis* (n=2); 18, *A. indonesiensis* (n=2); 19, *A. oeni* B13^T; 20, *A. papayae* (n=2); 21, *A. pomorum* LMG 18848^T; 22, *A. pasteurianus* (n=7); 23, *A. senegalensis* (n=3); 24, *A. nitrogenifigens* RG1^T. n is the number of strains; the type strain is included for all taxa. +, Positive; -, negative; w, weakly positive; v, strain-dependent (the result of the type strain is given in parentheses). Data for taxon 1 were obtained in the present study; data for taxa 2, 13 and 20 and the data for acid production of different carbon sources were taken from Iino *et al.* (2012); data for taxon 16 were taken from *Tanasupawat et al.* (2011); and data for taxa 3-12, 14-15, 17-19 and 21-24 were taken from Cleenwerck *et al.* (2008).

Feature	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Formation from D-glucose:																								
5-keto-D-gluconic acid	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+°
2-keto-D-gluconic acid	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	v (-)	+	-°
Growth in ammonium with ethanol	-	-	-	-	v (+)	+	+	+	-	w	+	-	-	-	-	w	-	-	-	-	-	-	+	+
Growth in 10 % ethanol	-	v (+)	v (-)	-	v (-)	-	-	-		-	-	-	+	+	-	-	-	-	+	+	-	+	+	+
Growth on YE+30 % D-glucose	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	v (-)	+	+
Growth at 37°C on GY agar	-	+	+	+	+°	+	w[a]	+[a]		+[b]	+[a]	+[a]	+		+[b]	+°	+[a]	+[a]		+		+[a]		-[b]
Growth on carbon sources:																								
Glycerol	-	+°	w	+	+	+	+	-	+	+	v	+	+°	+	+	+	+	+	+	w°	+	v	+	+
Methanol	-	+°	-	-	+	+	-	-	-	-	-	-		+	w	-	-	-	-		-	-	-	-
Acid production from																								
L-arabinose	-	-	+	v (-°)	-	v (+)	+	-	-			v (-)	+	-		+				+		+		
D-galactose	-	-	-	-	-	v (+)	+	-	+			v (-)	+	+		+				+				
D-mannose	-	-	+	v (+°)	-	v (+)	+	-	+			v (+)	+	-		+				+				
Catalase	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-°	+	+	+	+	+	+	+	+
G+C content of DNA (mol%)	56.2	59.2-59.4	56,9-57,3	54.3-55.4	56.8-58.0	57.1-58.9	56.9-58.3	59.7-60.7	56.0-57.6	53.8-54.5	59.2-60.2	55.7-58.1	58.7-58.9	57.2	52.0-52.8	56.3-56.5	55.6-56.2	54.0-54.2	58.1	60.5-60.7	52.1	53.2-54.3	55.6-56.0	64.1

°Data obtained in present study.
[a] Data obtained from Lisdiyanti *et al.* (2000).
[b] Data obtained from the original species description.

Table 5.1.2 Cellular fatty acid contents (%) of *Acetobacter lambici* sp. nov. and type strains of all established species of the genus *Acetobacter*. Taxa: 1-4, *A. lambici* sp. nov. (LMG 27439^T, LMG 27440, R-50193 and R-50194, respectively); 5, *A. okinawensis* LMG 26457^T; 6, *A. ghanensis* LMG 23848^T; 7, *A. syzygii* LMG 21419^T; 8, *A. fabarum* LMG 24244^T; 9, *A. lovaniensis* LMG 1617^T; 10, *A. aceti* LMG 1504^T; 11, *A. peroxydans* LMG 1635^T; 12, *A. cerevisiae* LMG 1625^T; 13, *A. cibinongensis* LMG 21418^T; 14, *A. estunensis* LMG 1626^T; 15, *A. orleanensis* LMG 1583^T; 16, *A. persici* LMG 26458^T; 17, *A. malorum* LMG 1746^T; 18, *A. orientalis* LMG 21417^T; 19, *A. farinalis* LMG 26772^T; 20, *A. tropicalis* LMG 19825^T; 21, *A. indonesiensis* LMG 19824^T; 22, *A. oeni* LMG 21952^T; 23, *A. papayae* LMG 26456^T; 24, *A. pomorum* LMG 18848^T; 25, *A. pasteurianus* LMG 1262^T; 26, *A. senegalensis* LMG 23690^T; 27, *A. nitrogenifigens* LMG 23498^T. -, Not detectable or trace amount (<1%). All data were generated in the present study.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
$C_{14:0}$	4.05	4.6	4.25	4.05	4.62	4.46	6.06	5.96	5.98	5.55	2.03	0.94	1	3.63	1.18	1.07	-	2.22	-	1.46	1.91	1.2	5.48	6.32	3.89	2.81	-
$C_{14:0}$2-OH	1.18	1.6	1.24	1.08	2.57	3.75	2.53	2.1	1.11	21.3	9.21	3.91	2.26	5.43	5.05	4.39	5.16	6.54	3.19	9.24	6	8.74	9.41	15.38	16.47	14.13	16.22
$C_{16:0}$	10.45	11.49	11	11.25	9.51	9.31	10.67	8.47	9.04	10	9.73	11.06	10.98	11.63	11.22	11.38	10.66	10.3	13	10.04	10.96	9.82	11.4	8.21	8.22	8.01	10.33
$C_{16:0}$2-OH	8.83	8.4	8.01	8.36	10.32	9.64	7.93	9.97	9.11	17.45	10.17	5.38	4.52	3.97	7.36	6.6	6.42	6.32	8.23	8.32	10.22	10.58	13.32	12.49	12.97	13.2	23.13
$C_{16:0}$3-OH	1.92	2.06	1.68	1.57	2.94	2.58	2.39	3.1	2.16	4.44	2.23	2.22	3.82	2.61	2.58	2.3	2.63	4.71	2.37	4.48	4.57	4.37	3.74	7.6	6.85	7.35	5.46
C18:0	8.29	7.09	7.13	7.55	4.39	3.47	3.25	2.6	1.95	-	2.21	5.88	5.09	4.15	3.95	3.89	4.72	3.1	6.07	2.53	4.08	4.4	3.45	-	1.43	1.86	-
$C_{18:1}\omega7$	59.33	57.74	60.49	59.83	59.28	61.43	60.92	61.47	65.43	35.12	60.03	62.98	62.08	61.7	64.57	64.8	61.49	61.93	58.12	52.77	53.83	48.08	46.27	41.44	42.75	33.82	33.38
$C_{18:0}$3-OH	1.2	1.24	1.05	1.26	1.5	1.47	1.24	1.02	-	1.57	-	3.58	4.05	3.29	1.82	1.87	3.13	1.64	3.02	3.69	4	8.04	1.55	4.75	2.97	6.11	2.28
$C_{19:0}cyclo\omega8$	2.14	3.6	1.93	2.68	2.22	1.95	2.23	3.06	1.75	-	2.63	1.03	1.87	1.93	0.59	-	-	1.58	3.09	-	1.02	-	2.63	-	-	-	1.76

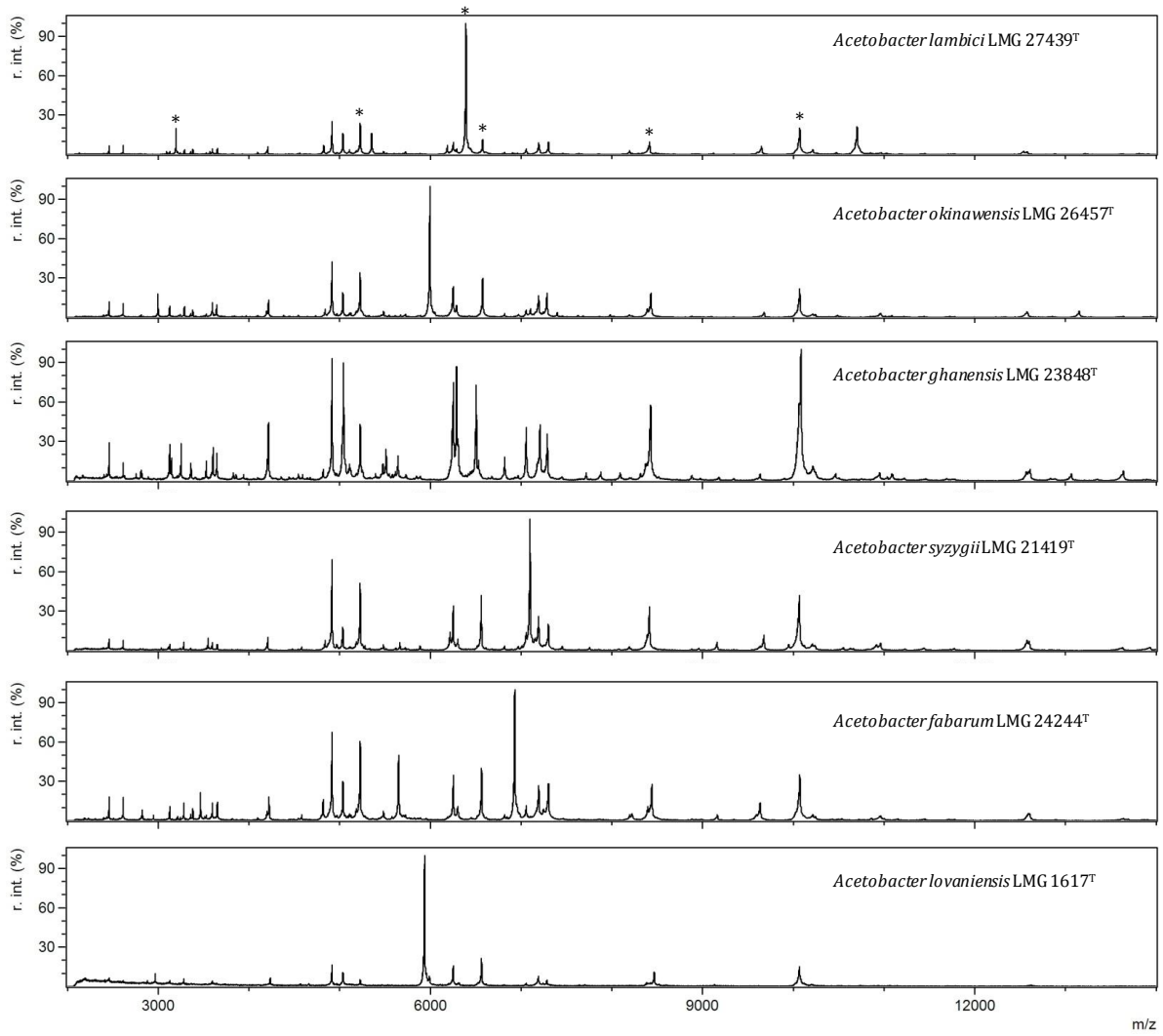

Figure 5.1.3 Visualization of MALDI-TOF MS profiles of *Acetobacter lambici* sp. nov. and its closest phylogenetic neighbours. Asterisks indicate the set of six peaks by which the strains could be differentiated from other species of the genus *Acetobacter*. The profiles are visualized using mMass 5.5.0 (Strohalm et al., 2010).

Supplementary information

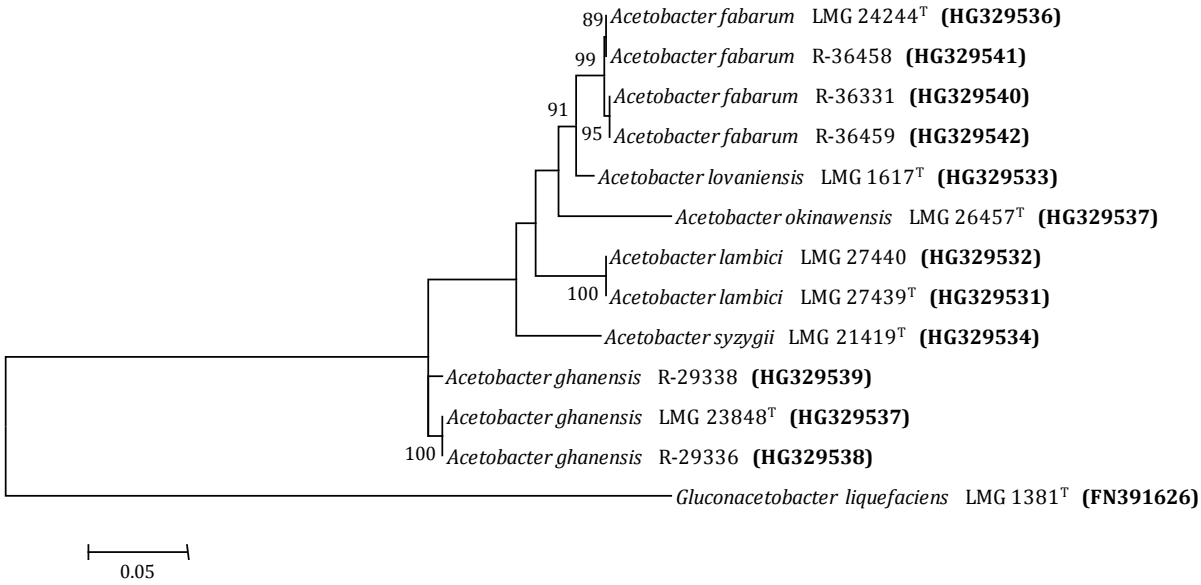

Figure S 5.1.1 Maximum-likelihood tree based on *dnaK* gene sequences (627 bp) showing the phylogenetic relationships of the isolates LMG 27439^T and LMG 27440 and their closest phylogenetic neighbours. *Gluconacetobacter liquefaciens* LMG 1381^T was used as an outgroup. The substitution model used was the Tamura-Nei model (Tamura & Nei, 1993). The sequence accession numbers are given between brackets. Bootstrap percentages (>50%) are shown next to the branch points. Bar, 5% sequence divergence.

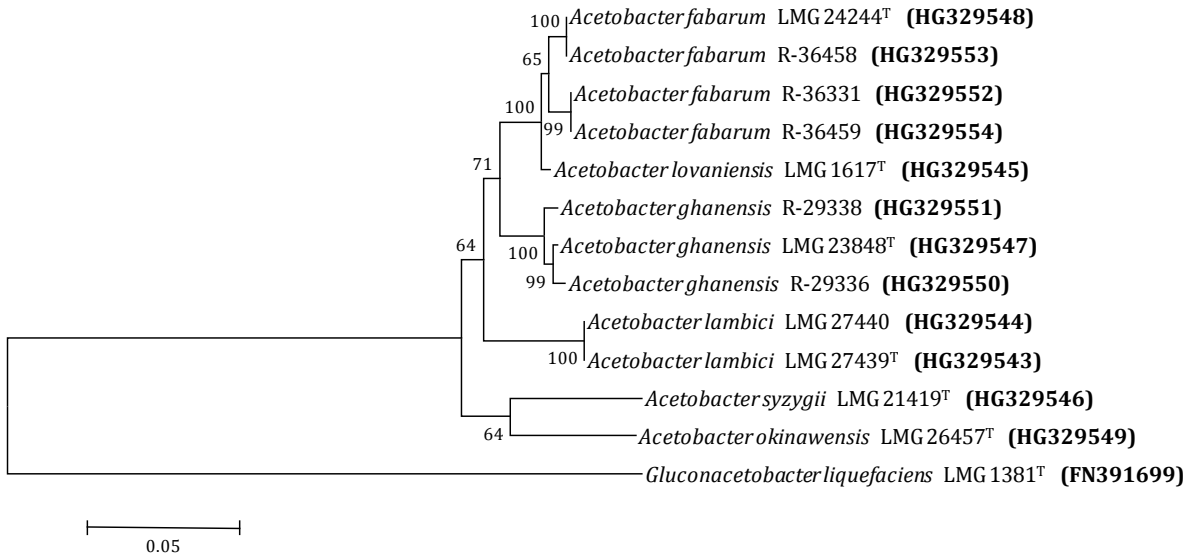

Figure S 5.1.2 Maximum-likelihood tree based on *groEL* gene sequences (715 bp) showing the phylogenetic relationships of the isolates LMG 27439^T and LMG 27440 and their closest phylogenetic neighbours. *Gluconacetobacter liquefaciens* LMG 1381^T was used as an outgroup. The substitution model used was the Tamura-Nei model (Tamura & Nei, 1993). The sequence accession numbers are given between brackets. Bootstrap percentages (>50%) are shown next to the branch points. Bar, 5% sequence divergence.

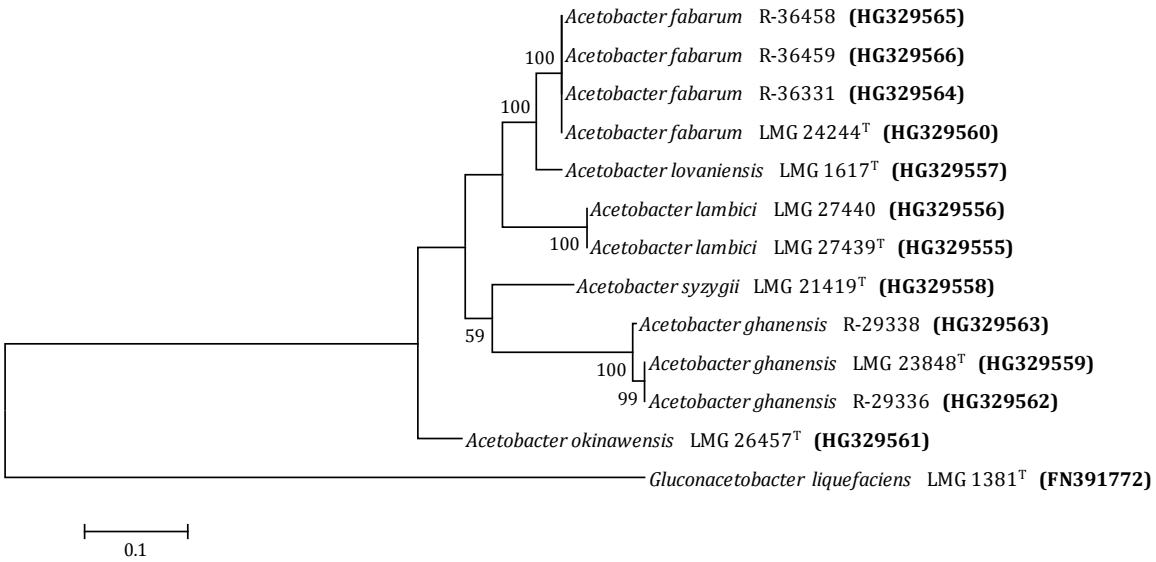

Figure S 5.1.3 Maximum-likelihood tree based on rpoB gene sequences (582 bp) showing the phylogenetic relationships of the isolates LMG 27439^{T} and LMG 27440 and their closest phylogenetic neighbours. *Gluconacetobacter liquefaciens* LMG 1381^{T} was used as an outgroup. The substitution model used was the Tamura-Nei model (Tamura & Nei, 1993). The sequence accession numbers are given between brackets. Bootstrap percentages (≥50%) are shown next to the branch points. Bar, 10% sequence divergence.

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5.2 *Gluconobacter cerevisiae* sp. nov., isolated from the brewery environment

Redrafted from: Freek Spitaels*, Anneleen D. Wieme*, Tom Balzarini, Ilse Cleenwerck, Anita Van Landschoot, Luc De Vuyst and Peter Vandamme, *Gluconobacter cerevisiae* sp. nov. isolated from the brewery environment, *International Journal of Systematic and Evolutionary Microbiology* **64**, 1134-1141.
*These authors contributed equally to this work and are considered joint first authors.

Author contributions: conceived and designed the experiments: FS, ADW and PV; performed the experiments: FS and ADW; determination of the production of keto-D-gluconic acids: TB and FS; analyzed the data: FS and ADW; wrote the manuscript: FS and ADW; critically reviewed the manuscript: LL, ADW, IC, AVL, LDV and PV.

The Genbank/EMBL accession numbers for sequences generated in this study are HG329624, HG329625, HG424633 and KF700364 for the 16S rRNA gene sequences; HG424630-HG424632 for the 16S-23S ITS gene sequence; HG329570-HG329587 for the *dnaK* gene sequences; HG329588-HG329605 for the *groEL* gene sequences and HG329606-HG329623 for the *rpoB* gene sequences.

Abstract

Three strains, LMG 27748^T, LMG 27749 and LMG 27882 with identical MALDI-TOF mass spectra were isolated from samples from the brewery environment. Analysis of the 16S rRNA gene sequence of strain LMG 27748^T revealed that the taxon it represents was closely related to the type strains of *Gluconobacter albidus* (100% sequence similarity), *Gluconobacter kondonii* (99.9%), *Gluconobacter sphaericus* (99.9%) and *Gluconobacter kanchanaburiensis* (99.5%). DNA-DNA hybridization experiments towards the type strains of these species revealed moderate DNA relatedness values (39-65%). The three strains used D-fructose, D-sorbitol, *meso*-erythritol, glycerol, L-sorbose, ethanol (weakly), sucrose and raffinose as the sole carbon source for growth (weak growth on the latter two carbon sources was obtained for strains LMG 27748^T and LMG 27882). The strains were unable to grow on glucose-yeast extract medium at 37°C. They produced acid from *meso*-erythritol and sucrose, but not from raffinose. D-Gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid were produced from D-glucose, but not 2,5-diketo-D-gluconic acid. These genotypic and phenotypic characteristics distinguish strains LMG 27748^T, LMG 27749 and LMG 27882 from species of the genus *Gluconobacter* with validly published names and, therefore, we propose to classify them formally as representatives of a novel species, *Gluconobacter cerevisiae* sp. nov., with LMG 27748^T (=DSM 27644^T) as the type strain.

The genus *Gluconobacter* belongs to the family *Acetobacteraceae* within the class *α-Proteobacteria* and currently comprises 13 validly named species. *Gluconobacter* strains oxidize glucose to gluconic acid (De Ley & Frateur, 1970; Gammon *et al.*, 2007) rather than ethanol to acetic acid, differentiating them from most acetic acid bacteria (AAB) (Andres-Barrao *et al.*, 2013; De Ley & Frateur, 1970). They are unable to oxidize acetate to carbon dioxide and water (Yamada & Yukphan, 2008). Strains of the species of the genus *Gluconobacter* thus prefer carbohydrates as carbon sources, whereas other AABs such as members of the genus *Acetobacter* thrive in alcohol-rich environments (Vaughan *et al.*, 2005). Strains of the species of the genus *Gluconobacter* are able to grow in highly concentrated sugar solutions and at low pH values (Deppenmeier *et al.*, 2002). This capacity for growth can be detrimental, for instance when it leads to spoilage of lager or ale beers, soft drinks, wines and ciders, but beneficial to the production of vinegar, red sour ales and lambic beers (Andres-Barrao *et al.*, 2013; Bokulich & Bamforth, 2013; Gammon *et al.*, 2007; Raspor & Goranovic, 2008; Sakamoto & Konings, 2003; Van Oevelen *et al.*, 1977; Vaughan *et al.*, 2005).

Strain LMG 27748^T was isolated during a study of the fermentation process of acidic lambic beers. The latter beers are the product of a spontaneous fermentation, which progresses for at least two years in wooden casks. Strain LMG 27748^T was isolated on acetic acid medium (AAM), an AAB enrichment medium that consists of 1.0% (wt/vol) D-glucose, 0.5% (vol/vol) ethanol, 1.5% (wt/vol) peptone, 1.5% (wt/vol) agar, 0.8% (wt/vol) yeast extract and 0.3% (vol/vol) acetic acid (Lisdiyanti *et al.*, 2003). The medium was adjusted to a pH of 3.5 and supplemented with 5 ppm amphotericin B and 200 ppm cycloheximide to prevent fungal growth. Isolates grown on AAM were subjected to matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) as described previously (Wieme *et al.*, 2012). MALDI-TOF MS was shown useful for the identification of AAB involved in the production of vinegar (Andres-Barrao *et al.*, 2013) and was used as a dereplication tool in the present study. A total of 14 AAB isolates obtained from two different lambic breweries (an industrial and a traditional type located 74 km apart in Belgium) and a spoiled brewer's yeast starter culture of a third brewery displayed

identical mass spectra that differed from those of established AAB species, which suggested a unique taxonomic position (data not shown).

Random amplified polymorphic DNA (RAPD) analysis of a selection of six AAB isolates representing the three breweries was performed as described by Williams *et al.* (1990), using primers RAPD-270 and RAPD-272 (Mahenthiralingam *et al.*, 1996). The results revealed three RAPD patterns, corresponding with the three breweries (Figure 5.2.1) and thus indicated the presence of three genetically distinct strains. Subsequently, one isolate from each brewery was chosen for further analyses: strain LMG 27748^T representing isolates of the industrial lambic brewery, strain LMG 27749 originating from the spoiled brewer's yeast starter culture and strain LMG 27882 isolated in a traditional lambic brewery.

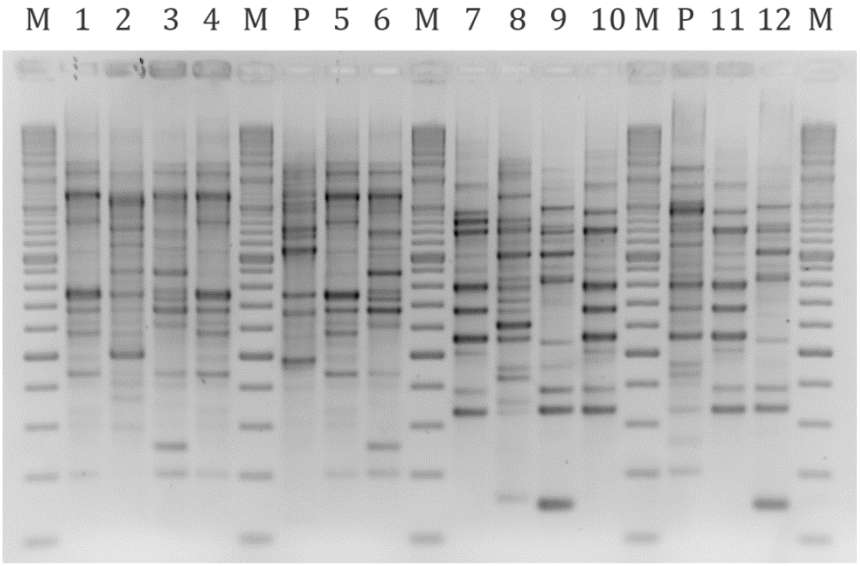

Figure 5.2.1 Patterns of the RAPD analysis of six representative AAB isolates using primers RAPD-270 (1-6) and RAPD-272 (7-12). Lanes: 1/7, LMG 27748^T; 2/8, LMG 27882; 3/9, LMG 27749; 4/5/10/11, two additional isolates from an industrial lambic brewery; 6/12, additional isolate from a spoiled brewer's yeast starter culture. M denotes the size marker and P represents a positive control sample.

The 16S rRNA gene sequence of strain LMG 27748^T was determined as described previously (Snauwaert *et al.*, 2013). EzBioCloud analysis (Kim *et al.*, 2012) of this 16S rRNA gene sequence revealed similarity to those of *Gluconobacter albidus* NBRC 3250^T (100%), *Gluconobacter kondonii* NBRC 3266^T (99.9%), *Gluconobacter sphaericus* NBRC 12467^T (99.9%) and *Gluconobacter kanchanaburiensis* BCC 15889^T

(99.5%) (pairwise similarity values in parentheses). All 16S rRNA gene sequences were aligned using the SILVA Incremental Aligner (SINA v1.2.11) (http://www.arb-silva.de/aligner/) (Pruesse *et al.*, 2012), with the corresponding SILVA SSURef 115 database (Pruesse *et al.*, 2007), and phylogenetic trees were reconstructed using the MEGA 5.2 software package (Tamura *et al.*, 2011). Tree topologies were analyzed statistically using 1000 bootstrapping replications. The maximum-likelihood and maximum-parsimony method trees (data not shown) showed the same topology as the neighbour-joining method tree (Figure 5.2.2).

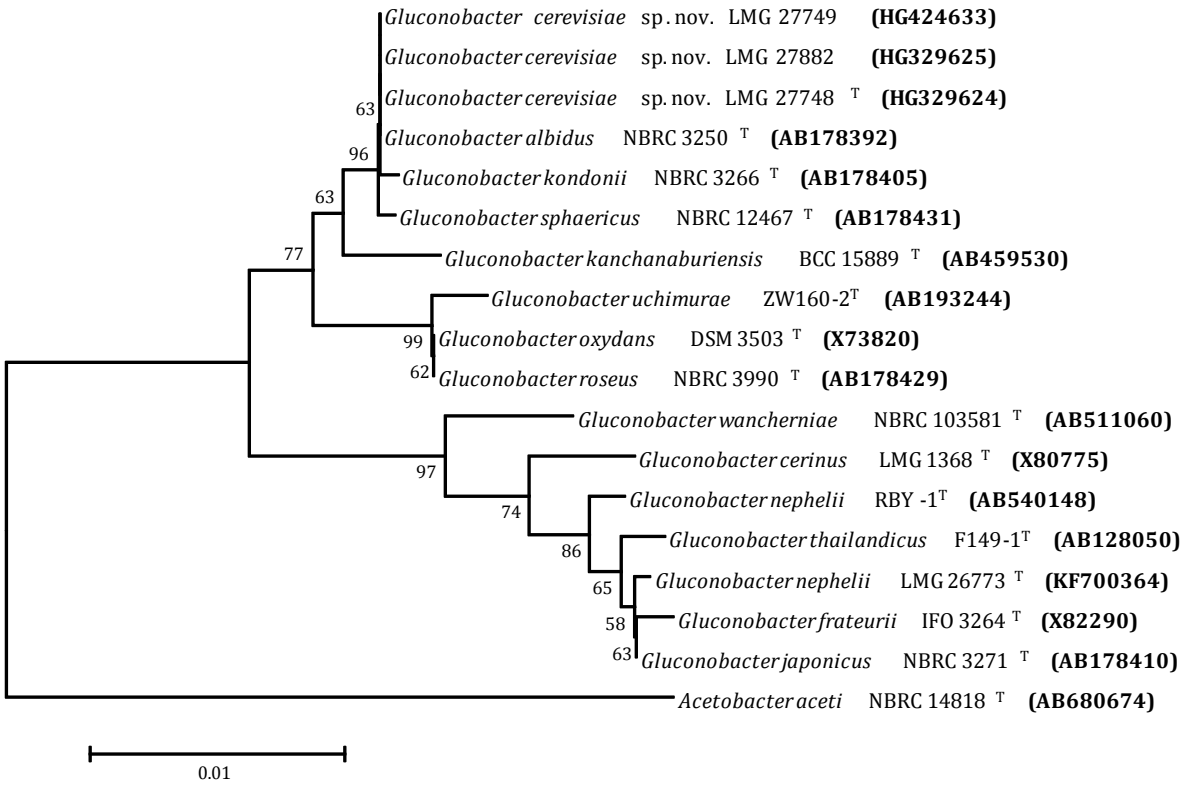

Figure 5.2.2 Neighbour-joining tree based on nearly full-length 16S rRNA gene sequences (1363 bp) showing the phylogenetic relationship of isolates LMG 27748^T, LMG 27749 and LMG 27882 and of the type strains of all species of the genus *Gluconobacter* with validly published names. *Acetobacter aceti* NBRC 14818^T (= LMG 1504^T) was used as an outgroup. Evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004). Sequence accession numbers are given in parentheses. Bootstrap percentages (> 50%) are shown next to the branch points. Bar, 1% sequence divergence.

Because of the limited taxonomic resolution of the 16S rRNA gene in the AAB group of bacteria, the 16S-23S rRNA gene internal transcribed spacer (ITS) sequence of strain LMG 27748^T was determined using the same protocol as used for the 16S

rRNA gene sequence (Snauwaert *et al.*, 2013). The 16S-23S rRNA gene ITS primers 16S-23S-ITS-1F 5'-TGCGGCTGGATCACCTCCT-3' (positions 1522–1540 on the 16S rRNA gene, *Escherichia coli* numbering) and 16S-23S-ITS-2R 5'-GTGCCAAGGCATCCACCG-3' (positions 38–22 on the 23S rRNA gene, *E. coli* numbering) were used. BLAST analysis (Altschul *et al.*, 1997) of the 16S-23S rRNA gene ITS sequence revealed that the LMG 27748^T ITS sequence was similar to that of *Gluconobacter kondonii* NBRC 3266^T (96.0%) and *Gluconobacter albidus* NBRC 3250^T (94.0%) (pairwise similarity values in parentheses). Phylogenetic trees were reconstructed using the MEGA 5.2 software package (Tamura *et al.*, 2011). Tree topologies were analyzed statistically using 1000 bootstrapping replications. The maximum-likelihood and maximum-parsimony trees (data not shown) showed the same topology as the neighbour-joining method tree (Supplementary Figure S 5.2.1).

Additionally, the phylogenetic position of the taxon represented by strains LMG 27748^T, LMG 27749 and LMG 27882 was analyzed using partial sequences of the housekeeping genes *dnaK* (encoding the chaperone protein DnaK), *groEL* (encoding a 60-kDa chaperonin) and *rpoB* (encoding the DNA-directed RNA polymerase beta subunit) (Cleenwerck *et al.*, 2010). Sequences of type and additional taxonomic reference strains of the genus *Gluconobacter* were determined to make a comprehensive multilocus sequence analysis MLSA dataset for the entire genus. Sequences of at least 654, 534 and 510 nt were generated for *dnaK*, *groEL* and *rpoB*, respectively. All gene sequences were aligned at the amino acid level using the MEGA 5.2 software (Tamura *et al.*, 2011). Trees were reconstructed using the maximum-likelihood model. A discrete gamma distribution was used to model evolutionarily rate differences among sites and the rate variation model allowed for some sites to be evolutionarily invariable. Tree topologies were analyzed statistically using 1000 bootstrapping replications. Numerical analysis of the individual (Supplementary Figure S 5.2.2, Figure S 5.2.3 and Figure S 5.2.4) and concatenated (Figure 5.2.3) gene sequences revealed that strains LMG 27748^T, LMG 27749 and LMG 27882 could be clearly differentiated from their nearest neighbours, *G. kondonii* LMG 1367^T t1 and *G. albidus* LMG 1356^T. The concatenated MLSA data revealed that most species of the genus *Gluconobacter* were well separated, with the exception of *G. nephelii* LMG 26773^T that grouped with *G. japonicus* strains. A pairwise comparison

of the 16S rRNA gene sequence of *G. nephelii* LMG 26773^T with that of *G. nephelii* RBY-1^T (AB540148) revealed a sequence similarity value of 99.8%, *i.e.*, a difference of 3 out of 1410 nt. With *G. japonicus* NBRC 3271^T (AB178410) a sequence similarity value of 99.9% was found, *i.e.*, a difference of 1 out of 1406 nt. *Gluconobacter nephelii* strain RBY-1^T was originally deposited as NBRC 106061^T in the NITE Biological Resource Center (NBRC, Japan) and *G. nephelii* strain LMG 26773^T is a direct subculture of the *G. nephelii* strain NBRC 106061^T culture. The 16S rRNA gene sequences of the subcultures LMG 26773^T and NBRC 106061^T (16S rRNA gene sequence retrieved from the NBRC website, http://www.nbrc.nite.go.jp/NBRC2/NBRCCatalogueDetailServlet?ID=NBRC&CAT=00106061) are fully identical, suggesting that LMG 26773^T and NBRC 106061^T represent the same strain. Therefore, it is likely that the sequence of RBY-1^T with accession number AB540148 contains sequencing errors or that the biological material that was deposited in the NBRC culture collection does not correspond to strain RBY-1^T (Figure 5.2.2) (Kommanee *et al.*, 2011).

DNA-DNA hybridizations were performed between strains LMG 27748^T and LMG 27749 and the type strains of their nearest phylogenetic neighbours as described previously (Cleenwerck *et al.*, 2008). DNA-DNA hybridization values are presented as means of reciprocal reactions (A×B and B×A, values are indicated between parentheses), where each reciprocal reaction was performed at least in three-fold. Strains LMG 27748^T and LMG 27749 showed 80% (81% and 80%) DNA-DNA relatedness. The DNA-DNA relatedness between strain LMG 27748^T and the type strains of its nearest phylogenetic neighbours was 65% (66% and 64%) towards *G. kondonii* LMG 1367^T t1, 54% (60% and 49%) towards *G. albidus* LMG 1356^T, 45% (56% and 36%) towards *G. sphaericus* LMG 1414^T and 41% (52% and 30%) towards *G. kanchanaburiensis* LMG 26774^T. The DNA G+C content of strains LMG 27748^T and LMG 27749 were determined as described previously (Cleenwerck *et al.*, 2008) and were 58.0 mol% and 57.7 mol% respectively.

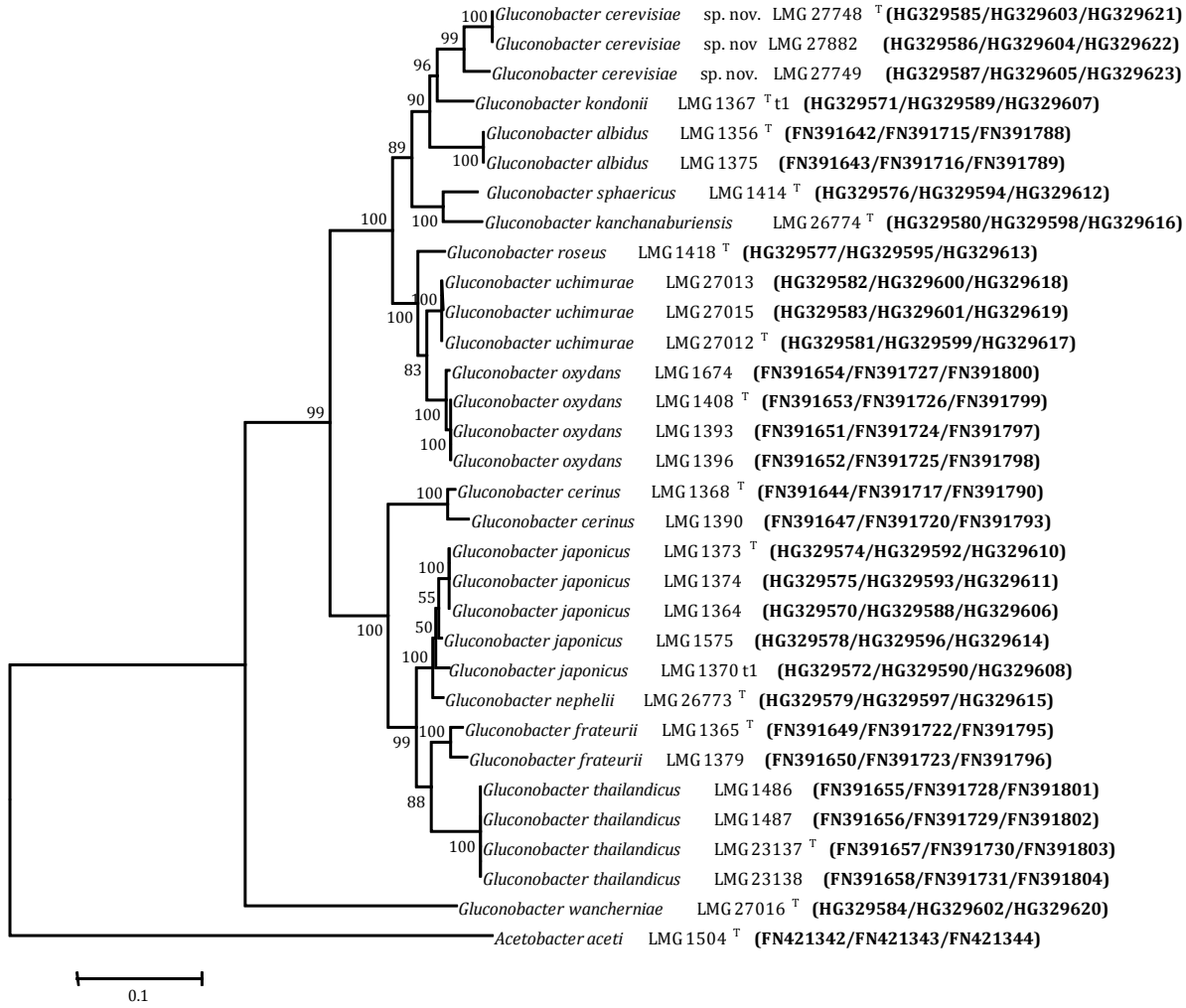

Figure 5.2.3 Maximum-likelihood tree based on concatenated *dnaK*, *groEL* and *rpoB* gene sequences (a total of 1698 bp) showing the phylogenetic relationship of isolates LMG 27748^T, LMG 27749 and LMG 27882 and all species of the genus *Gluconobacter* with validly published names. *Acetobacter aceti* LMG 1504^T (= NBRC 14818^T) was used as an outgroup. The substitution model used was the General Time Reversible model (Nei & Kumar, 2000). Sequence accession numbers for *dnaK*, *groEL* and *rpoB* gene sequences are given in parentheses in that order. Bootstrap percentages (> 50%) are shown next to the branch points. Bar, 10% sequence divergence.

The phenotypic characteristics of strains LMG 27748^T, LMG 27749 and LMG 27882 were determined as described previously (Cleenwerck *et al.*, 2002). Type strains of closely related AAB (*G. albidus* LMG 1356^T, *G. kondonii* LMG 1367^T t1, *G. sphaericus* LMG 1414^T and *G. kanchanaburiensis* LMG 26774^T) were included as positive or negative controls. For microscopy and morphological examination of colonies, strains were grown aerobically on AAM agar at 28°C for 48 h. The biochemical characteristics tested included a Gram-stain reaction, analysis of catalase and

oxidase activities, growth on 0.3% D-fructose, D-sorbitol, *meso*-erythritol, glycerol, sucrose, raffinose, L-sorbose or ethanol as the sole carbon sources, growth at 37°C on GY agar (5% D-glucose, 1% yeast extract and 1.5% agar). In addition, acid production from 1% *meso*-erythritol, sucrose and raffinose was determined as described previously (Asai *et al.*, 1964), results are shown in the species description.

For testing the production of 2-keto-D-gluconic acid and 5-keto-D-gluconic acid, cells were grown as described by Gosselé *et al.* (1980) and the presence of both keto-D-gluconic acids was determined as described by Spitaels *et al.* (2014). All three strains produced D-gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid, but not 2,5-diketo-D-gluconic acid (data not shown).

Strains LMG 27748^T, LMG 27749 and LMG 27882 could be differentiated from *G. kondonii*, *G. albidus*, *G. sphaericus* and *G. kanchanaburiensis* by means of multiple biochemical characteristics, such as acid production from sucrose and raffinose and growth on ethanol as sole carbon source (Table 5.2.1). The biochemical test results did not always correspond to published data. The utilization of L-sorbose and raffinose by *G. kondonii* LMG 1367^T t1 was as reported by Yukphan *et al.* (2010) (positive for L-sorbose and negative for raffinose) and differed from results reported by Malimas *et al.* (2007, 2009b). Similarly, acid production from maltose (absent) and growth on D-arabitol (present) by *G. cerinus* NBRC 3267^T as reported by Malimas *et al.* (2009b), Tanasupawat *et al.* (2004) and Yukphan *et al.* (2010) contradicted results reported by Tanasupawat *et al.* (2011). In addition, Kommanee *et al.* (2011) reported both characteristics as present in *G. cerinus* strains. These discrepant test results were obtained using the same test procedures (Asai *et al.*, 1964; Gosselé *et al.*, 1983; Katsura *et al.*, 2002; Mason & Claus, 1989; Yamada *et al.*, 1969, 1976, 1999). Therefore, these biochemical tests appear to reproduce poorly as observed previously by Yukphan *et al.* (2004) and the inclusion of sufficient and appropriate control strains is warranted when performing them.

Numerical comparison of the MALDI-TOF mass spectra of strains LMG 27748^T, LMG 27749 and LMG 27882, and those of reference strains of their nearest phylogenetic neighbours by means of the Pearson product-moment correlation coefficient allowed a very straightforward separation of these taxa. As described above, the three strains displayed indistinguishable spectra that could be differentiated from

those of species of the genus *Gluconobacter* with validly published names by the consistent presence of five biomarker peaks characterized by *m/z* values of 3253.4 ± 4.6, 4912.4 ± 5.9, 6371.2 ± 7.1, 6506.3 ± 7.2 and 9171.0 ± 9.3 (Figure 5.2.4); some of these peaks were present in the mass spectra of strains of other species of the genus *Gluconobacter*, but never all five simultaneously.

In conclusion, the present polyphasic study provides taxonomic data demonstrating that the taxon represented by strains LMG 27748^T, LMG 27749 and LMG 27882 could be differentiated, by means of multiple genotypic [*i.e.*, 16S-23S rRNA gene ITS sequence analysis (Supplementary Figure S 5.2.1), MLSA (Figure 5.2.3) and DDH] and phenotypic characteristics [*i.e.*, MALDI-TOF MS analysis (Figure 5.2.4), acid production and growth on several carbon sources (Table 5.2.1)] using various methodologies, from its nearest phylogenetic neighbours. We, therefore, propose to assign these strains to a novel species, *Gluconobacter cerevisiae* sp. nov., with LMG 27748^T (=DSM 27644^T) as the type strain.

Table 5.2.1 Differential characteristics for *Gluconobacter cerevisiae* and the type strains of the validly named *Gluconobacter* species.
Taxa: 1, LMG 27748^T; 2, LMG 27749; 3, LMG 27882; 4, *G. albidus* (LMG 1356^T); 5, *G. kondonii* (LMG 1367^T); 6, *G. sphaericus* (LMG 1414^T); 7, *G. kanchanaburiensis* (LMG 26774^T); 8, *G. uchimurae* (ZW 160-2^T); 9, *G. oxydans* (NBRC 14819^T); 10, *G. roseus* (NBRC 3990^T); 11, *G. wancherniae* (BCC 15775^T); 12, *G. cerinus* (NBRC 3267^T); 13, *G. japonicus* (NBRC 3271^T); 14, *G. frateurii* (NBRC 3264^T); 15, *G. thailandicus* (BCC 14116^T); 16, *G. nephelii* (NBRC 106061^T). Data for taxa 1-3 were generated in this study. +, positive; -, negative; w, weakly positive; vw, very weakly positive; ND, not determined. *Data taken from: a, Tanasupawat *et al.* (2011); b, this study; c, Malimas *et al.* (2009a); d, the original species description; e, Malimas *et al.* (2007); f, Kommanee *et al.* (2011).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water-soluble brown pigment[a*]	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-	-
2,5-Diketo-D-gluconic acid production[a]	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-	-
Growth at 37°C[a]	-	-	-	-	-	-	-	+	+	-	-	-	-	-	w	-
Acid production from:																
Sucrose[a]	+	+	+	+	-[b]	+	-[b]	-	-	+	w	+	w	-	-	w
Raffinose[c]	-	-	-	+	+	+	w	-[a]	w	+	vw[d]	w	+	+	w	+[f]
meso-erythritol[e]	+	+	+	+[b]	+	-[b]	w	ND	+	-	-	+	+	w	+	+
Growth on:																
D-Fructose[f]	+	+	+	+[b]	+	+[b]	w[d]	ND	+	+[d]	+[d]	+	+[c]	+	+	+
D-Sorbitol[f]	+	+	+	+[b]	+[b]	+[b]	+[d]	ND	+	vw[d]	+[d]	+	+[c]	+	-	+
Glycerol[f]	+	+	+	+[b]	+	-[b]	+[d]	ND	+	vw[d]	+[d]	+	+[c]	+	+	+
Sucrose[f]	w	+	w	+[b]	-[b]	vw[d]	+[d]	ND	-	+[d]	-[d]	w	+[c]	+	+	+
meso-erythritol[e]	+	+	+	+[b]	+	-[b]	+[d]	+[a]	w	-[d]	w	+	+	-	w	w
Raffinose[c]	w	+	w	+	-[b]	-	w[d]	ND	-	+[d]	-[d]	-	w	+	w	+[f]
L-Sorbose[c]	+	+	+	-	+[b]	-	+	ND	-	-	+[d]	-	+	-	-	+[f]
Ethanol	w	w	w	-[d]	-[e]	-[d]	w[d]	-[a]	ND	-[d]	vw[d]	+[d]	-[c]	ND	ND	-[f]
G+C (%)[a]	58	57.7	ND	60	59.8	59.5	59.5	60.5	60.3	60.5	56.6	55.9	56.4	55.1	55.8	57.2

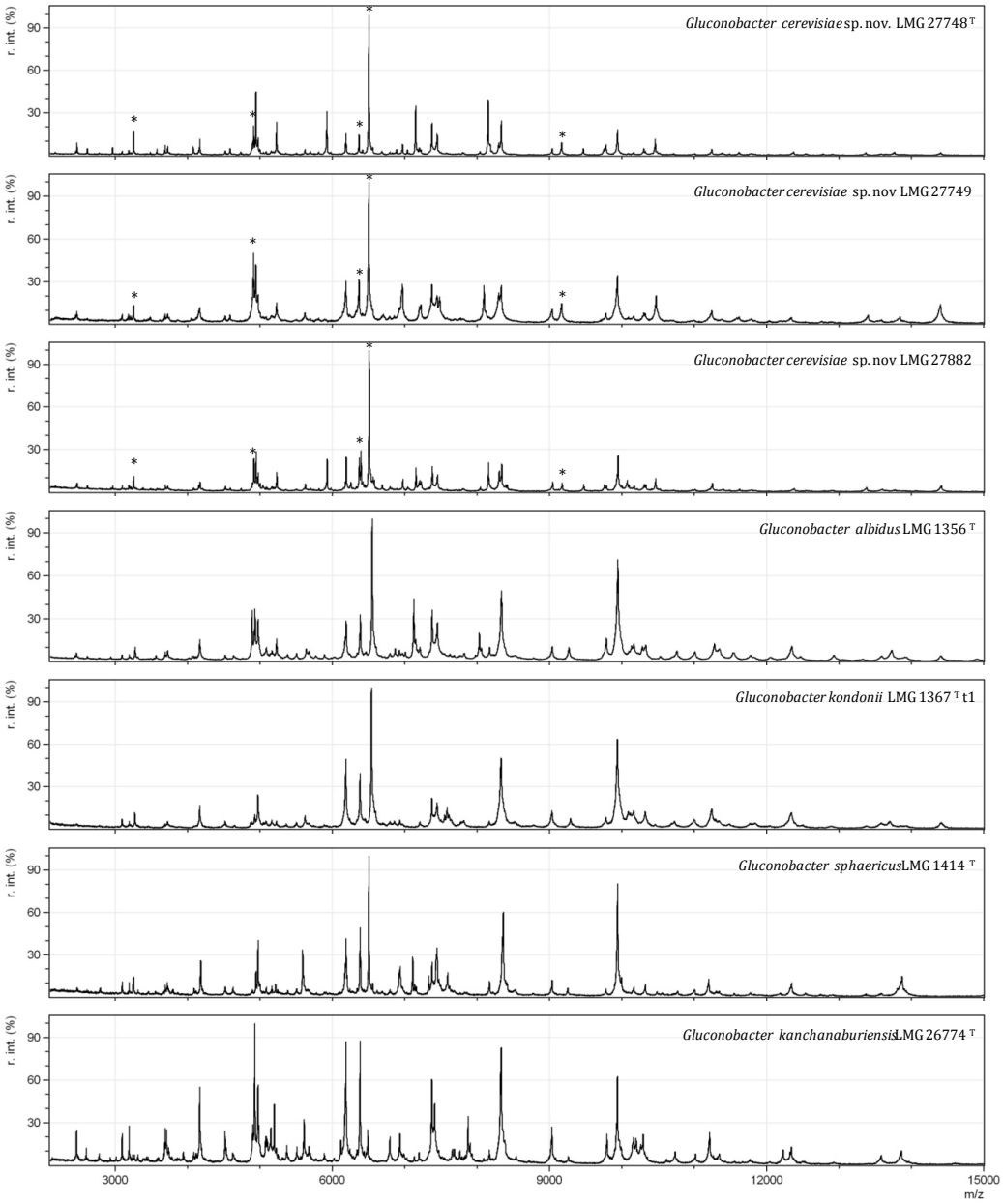

Figure 5.2.4 MALDI-TOF MS profiles of *Gluconobacter cerevisiae* sp. nov. and its close phylogenetic neighbours. Asterisks indicate the set of five peaks (*m/z* 3253.4 ± 4.6, 4912.4 ± 5.9, 6371.2 ± 7.1, 6506.3 ± 7.2 and 9171.0 ± 9.3) by which the strains could be differentiated from the species of the genus *Gluconobacter* with validly published names. The profiles are visualized using mMass 5.5.0 (Strohalm *et al.*, 2010).

Description of *Gluconobacter cerevisiae* sp. nov.

Gluconobacter cerevisiae (ce.re.vi'si.a.e. L. fem. gen. n. *cerevisiae* of beer, referring to the source from which the three cultures have been isolated)

Cells are Gram-stain-negative, non-motile rods and are approximately 1 µm × 2-3 µm long. Cells occur separately or in pairs. Catalase activity is exhibited, but no oxidase activity. After 48 h of incubation on AAM agar at 28°C colonies are round, rough, brownish beige and slightly raised, with a diameter of approximately 1-2 mm. D-Gluconic acid is produced from D-glucose as well as 2-keto-D-gluconic acid and 5-keto-D-gluconic acid. Able to grow on D-fructose, D-sorbitol, *meso*-erythritol, glycerol, L-sorbose and ethanol (weakly) as the sole carbon source. Growth on sucrose and raffinose as the sole carbon source is variable, ranging from weak (LMG 27748^T and LMG 27882) to strong (LMG 27749). Unable to grow on glucose-yeast extract medium at 37°C. Acid is produced from *meso*-erythritol and sucrose, but not from raffinose.

The type strain is strain LMG 27748^T (=DSM 27644^T), which was isolated from fermenting lambic beer. The DNA G+C content of strain LMG 27748^T is 58.0 mol%.

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Supplementary information

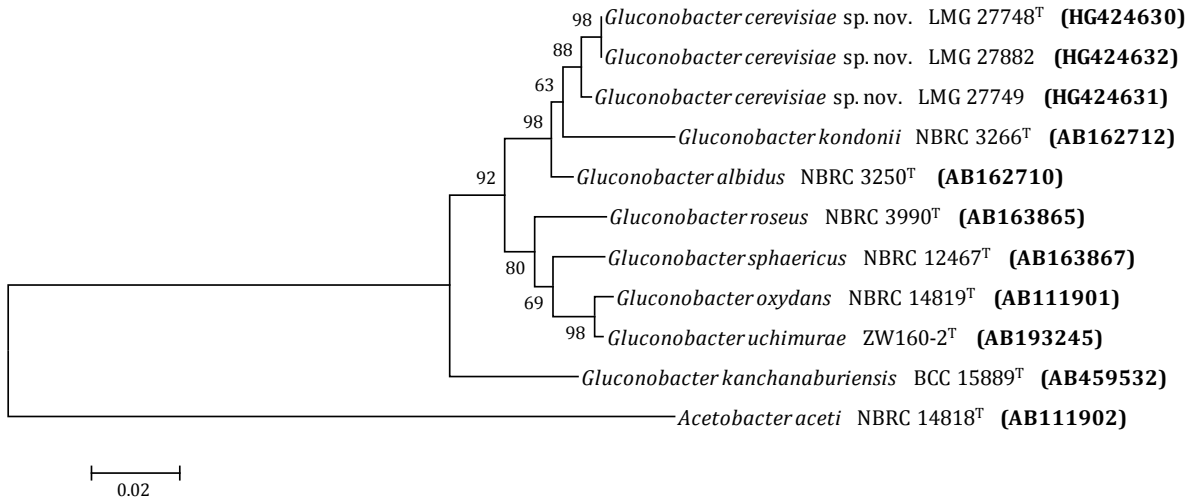

Figure S 5.2.1 Neighbour-joining tree based on 16S–23S rRNA gene ITS sequences (627 bp) showing the phylogenetic relationship of strains LMG 27748^T, LMG 27749 and LMG 27882 and of their closest phylogenetic neighbours. *Acetobacter aceti* NBRC 14818^T (= LMG 1504^T) was used as an outgroup. Evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004) and are expressed as the number of base substitutions per site. Sequence accession numbers are given between brackets. Bootstrap percentages (> 50%) are shown next to the branch points. Bar, 2% sequence divergence.

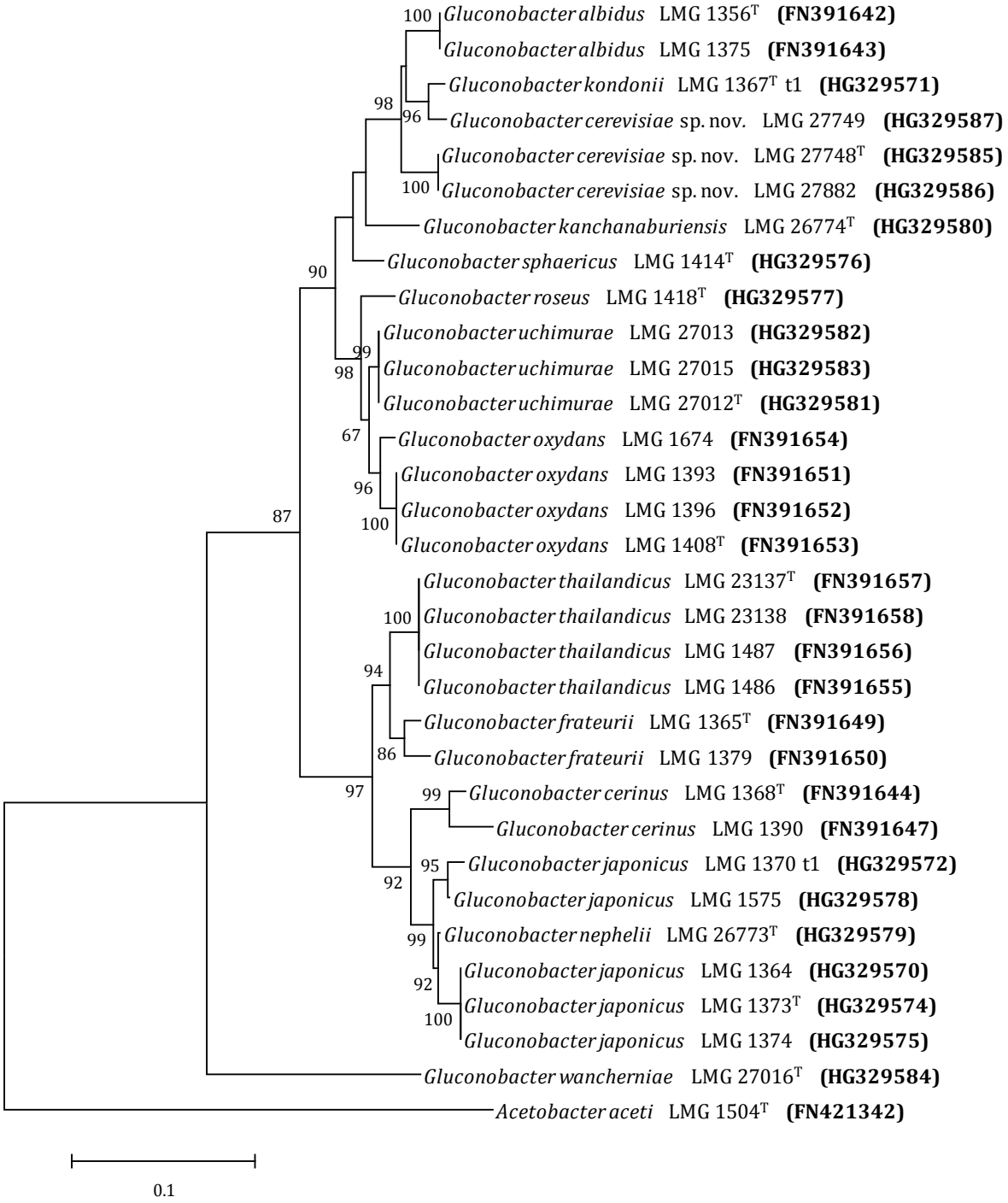

Figure S 5.2.2 Maximum-likelihood tree based on *dnaK* gene sequences (654 bp) showing the phylogenetic relationships of strains LMG 27748T, LMG 27749 and LMG 27882 and all species of the genus *Gluconobacter* with validly published names. *Acetobacter aceti* LMG 1504T (= NBRC 14818T) was used as an outgroup. The substitution model used was the General Time Reversible model (Nei & Kumar, 2000). Sequence accession numbers for the *dnaK* gene sequences are given between brackets. Bootstrap percentages (> 50%) are shown next to the branch points. Bar, 10% sequence divergence.

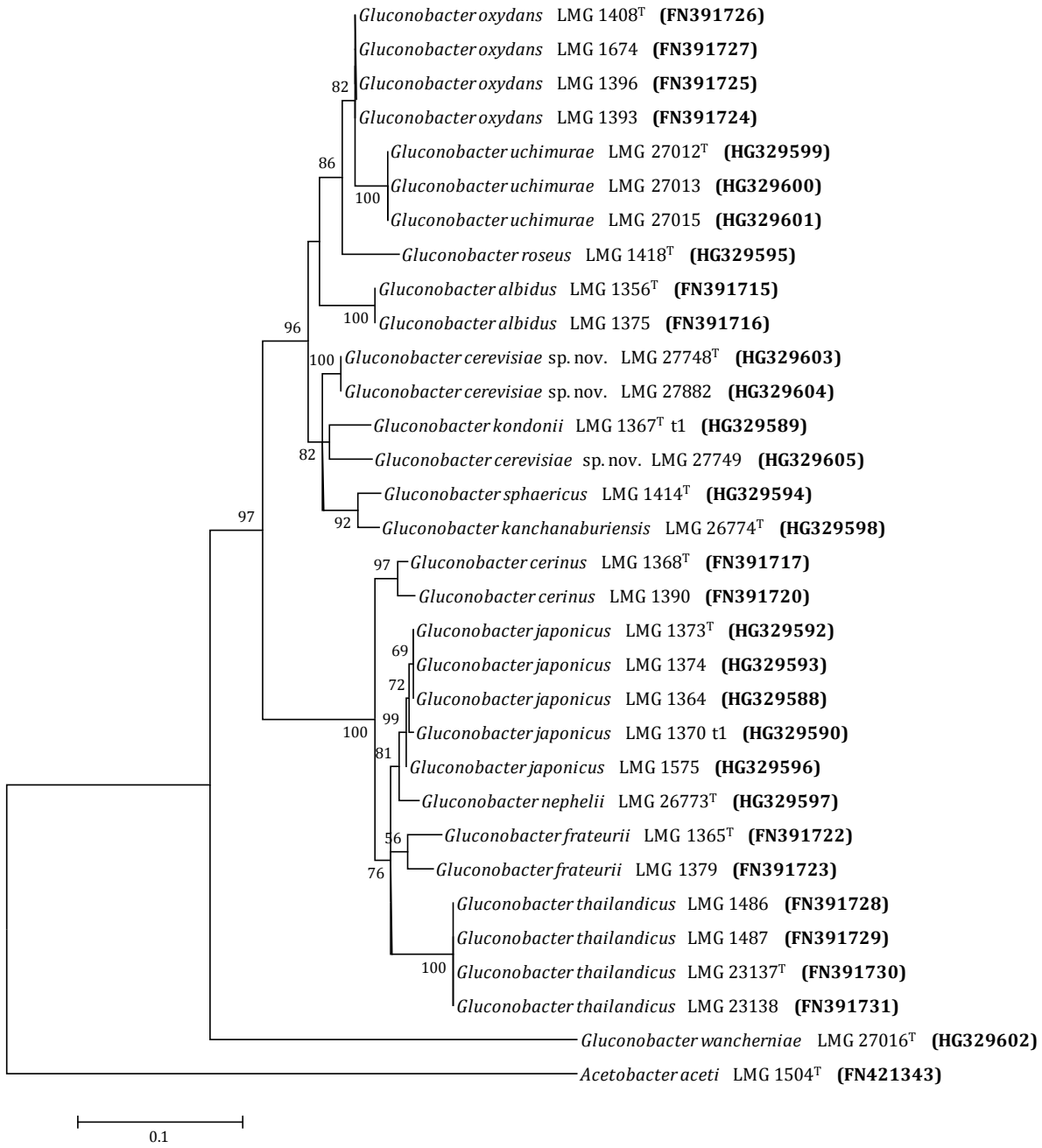

Figure S 5.2.3 Maximum-likelihood tree based on *groEL* gene sequences (534 bp) showing the phylogenetic relationships of strains LMG 27748^T, LMG 27749 and LMG 27882 and all species of the genus *Gluconobacter* with validly published names. *Acetobacter aceti* LMG 1504^T (= NBRC 14818^T) was used as an outgroup. The substitution model used was the General Time Reversible model (Nei & Kumar, 2000). Sequence accession numbers for the *groEL* gene sequences are given between brackets. Bootstrap percentages (> 50%) are shown next to the branch points. Bar, 10% sequence divergence.

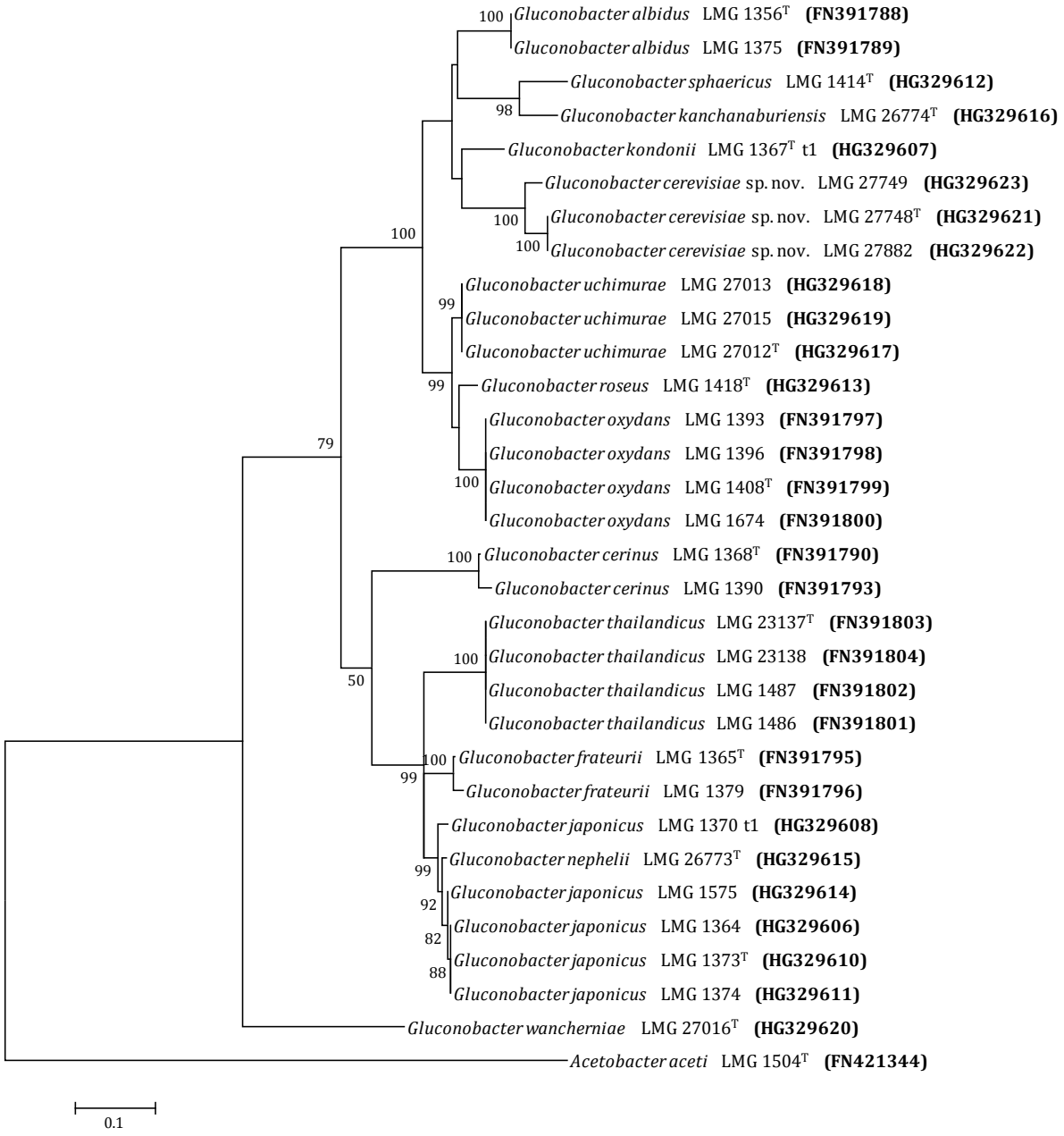

Figure S 5.2.4 Maximum-likelihood tree based on *rpoB* gene sequences (510 bp) showing the phylogenetic relationships of strains LMG 27748^T, LMG 27749 and LMG 27882 and all species of the genus *Gluconobacter* with validly published names. *Acetobacter aceti* LMG 1504^T (= NBRC 14818^T) was used as an outgroup. The substitution model used was the General Time Reversible model (Nei & Kumar, 2000). Sequence accession numbers for the *rpoB* gene sequences are given between brackets. Bootstrap percentages (> 50%) are shown next to the branch points. Bar, 10% sequence divergence.

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Chapter 6. The microbiota and metabolites of aged bottled gueuze beers

Preamble

Lambic beers are commonly blended to produce gueuze beers. These beers are carbonated by the spontaneous bottle refermentation after bottling. These beers are commonly aged for several years, but little is known about the microbial and metabolic processes during the bottle refermentation and therefore their aging process. Hence, several bottled gueuze beers of different ages were obtained from the same traditional lambic brewery. Next to the microbial diversity present in the bottled beers of different ages, targeted metabolites were analyzed to obtain more information about the processes that drive gueuze beer aging.

6.1 Microbiota and metabolites of aged bottled gueuze beers converge to the same composition

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Author contributions: conceived and designed the experiments: FS, SVK, AVL, LDV and PV; performed the experiments: FS and SVK; analyzed the data: FS and SVK; contributed reagents/materials/analysis tools: ADW, IS and MA; wrote the manuscript: FS and SVK; critically reviewed the manuscript: ADW, IS, MA, AVL, LDV and PV.

Abstract

Abstract: Gueuze beers are prepared by mixing young and old lambic beers and are bottle-refermented spontaneously for aging The present study analyzed the microbiota and metabolites present in gueuze beers that were aged between a few months and up to 17 years. Yeasts were cultivated from all beers sampled, but bacteria could not be grown from beers older than 5 years. Lactic acid and ethyl lactate concentrations increased steadily during aging, whereas ethanol concentrations remained constant. The concentrations of isoamyl acetate and ethyl decanoate decreased during the aging process. Hence, ethyl lactate and ethyl decanoate can be considered as positive and negative gueuze beer-aging metabolite biomarkers, respectively. Nevertheless, considerable bottle-to-bottle variation in the metabolite profiles was found, which hindered the generalization of the effects seen during the aging of the gueuze beers examined, but which illustrated the unique character of the lambic beers. The present results further indicate that gueuze beers are preferably aged for less than 10 years

Introduction

Bottle refermentation or conditioning is a common practice in the production of Belgian specialty beers (Van Landschoot *et al.*, 2005). Beer bottles are incubated to enable a secondary fermentation after the addition of yeast cells and an energy source during beer bottling process (Vanderhaegen *et al.*, 2003b). The yeast cells protect the beer from oxidation by scavenging oxygen and can add new flavors to the beer upon maturation (Vanderhaegen *et al.*, 2003b). For some beers, such as Berliner Weisse beer, a starter culture of yeasts and lactic acid bacteria (LAB) is used for refermentation (Verachtert & Derdelinckx, 2005). Other beers, such as gueuze beers, are the refermented products of mixtures of spontaneously fermented lambic beers (Verachtert & Iserentant, 1995). For the production of gueuze beer, a young (typically one-year old) lambic beer with residual dextrin carbohydrates is mixed with old (typically three-years old) lambic beer, which contains the microbiota that can convert the dextrin carbohydrates to more simple fermentable carbohydrates (Verachtert & Iserentant, 1995). Once mixed, the beer referments spontaneously, without the addition of energy sources, yeast or bacterial cells (Verachtert & Iserentant, 1995). *Dekkera* spp. and LAB species are the dominant microorganisms in the refermenting beer, although after 14 months of refermentation only LAB are isolated (Verachtert & Iserentant, 1995). In contrast to the storage of some wines, beer storage is usually considered negative for the flavor quality of the beer (Vanderhaegen *et al.*, 2006). Nevertheless, aging of gueuze beers is a common practice in traditional lambic beer breweries and is well appreciated by the consumers. Aging may last for more than ten years and hence means an investment by the brewery in end-products and space. It is however not clear to what extent this long-lasting aging process contributes to the (flavor) quality of gueuze beers.

Lambic beers are the result of a spontaneous fermentation process that proceeds for up to three years. We previously reported the microbial succession of a traditional lambic beer fermentation process in the Belgian lambic beer brewery, Cantillon (Spitaels *et al.*, 2014). This fermentation process consists of a succession of three phases that starts with an *Enterobacteriaceae* phase, which proceeds for up to 1 month and in which multiple *Enterobacteriaceae* species are dominant. After one month, the main fermentation phase starts, which is characterized by the dominant

presence of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* until month 3. The last phase is the maturation phase, in which the beer obtains its characteristic acidity and dryness, and is characterized by the presence of *Pediococcus damnosus* as the only LAB species and *Dekkera bruxellensis* as the dominant yeast species. Both microorganisms are still cultivable in the lambic beer at the end of a two-years monitoring period (Spitaels *et al.*, 2014). In addition, acetic acid bacteria (AAB) are isolated inconsistently throughout this period.

The flavor of gueuze beers is somewhat different from that of most beers, because of the high concentrations of organic acids (mainly lactic acid) that create a profound acidity (Van Oevelen *et al.*, 1976). As *Dekkera* spp. are commonly the most metabolically active microorganisms in gueuze beers, carbohydrates and oligosaccharides are completely degraded (Shanta Kumara *et al.*, 1993). During aging, both enzymatic and non-enzymatic changes in beer flavor prevail (Vanderhaegen *et al.*, 2006). Esters soften the sour taste and add fruity notes to the beers (Verstrepen *et al.*, 2003). Ethyl decanoate (also referred to as ethyl caprate) is a typical ester present in lambic and gueuze beers (Van Oevelen *et al.*, 1976). In contrast, the concentration of isoamyl acetate, which yields a banana-like flavor, is lower as compared to other beers (Van Oevelen *et al.*, 1976). Additionally, the use of aged hops deprives gueuze beers of the typical hop bitterness, while the maturation in oak barrels imparts additional flavors (Scholtes *et al.*, 2012).

The aim of the present study was to assess the impact of aging on the microbial species diversity and the metabolite profile of gueuze beers. The microbiota was studied using culture-dependent and culture-independent techniques. The metabolite profiles were determined through a metabolomics analysis.

Materials and methods

Brewery

Samples were obtained from the Cantillon brewery (http://www.cantillon.be). This brewery is the most traditional, still active, lambic brewery in Brussels, Belgium, and uses the same infrastructure and still most of the original equipment since 1900, the year when the brewery was founded.

Sampling

In June 2013, gueuze beers of different ages were obtained from the brewery, where they had been stored at cellar temperatures (ranging from 12°C in winter to 20°C in summer) since their bottling in 1996, 2004, 2008, 2010, 2011, and early 2013. Different batches of lambic beer were used for the production of these beers over the years and therefore these bottled beers cannot be regarded as aged replicates. Per bottling year, three bottles prepared from the same mixture of lambic beers were available and analyzed. Bottles were opened and samples were taken aseptically. Per bottling year, one bottle (further referred to as bottle 1) was used for microbiota cultivation, while all three bottles of each bottling year were subjected to polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and metabolite analysis (see below). All cultivation experiments were performed at the day of sampling and cell pellets and supernatants (see below) were stored at -20°C until PCR-DGGE and metabolite analysis, respectively.

Besides the gueuze beer bottle samples, a maturation phase sample of a three-year old lambic beer that was still fermenting in a cask was obtained. The analysis of this lambic beer was the subject of a former study, during which the microbiological characterization was restricted to the first two years of fermentation (Spitaels *et al.*, 2014). The wooden cask had a volume of approximately 400 L and possessed two apertures, namely a bung hole at the top of the cask, which was inaccessible due to the piling of the casks, and a second aperture at the front of the cask. The latter was positioned about 10 cm above the cask bottom and was used for sampling. The opening was plugged with a cork and a 500 mL sample was taken after approximately 100 mL of lambic beer was discarded. The lambic beer sample was transported on ice to the laboratory and was processed the same day. For this sample, only the microbial communities were examined. After the final sampling the lambic beer was used by the brewer for the production of a fruit lambic beer.

PCR-DGGE

The bottled gueuze beers were homogenized by swirling and three crude beer samples (100 mL each) per bottle, *i.e.*, three replicate samples per gueuze beer bottle and thus nine replicate samples per bottling year, were centrifuged at 8000 × *g* for 10 min (4°C). Cell pellets were stored at -20°C until further processing. DNA was prepared from the cell pellets as described by Camu *et al.* (2007). The DNA concentration, purity, and integrity were determined using 1 % (wt/vol) agarose gels, stained with ethidium bromide and by optical density (OD) measurements at 234, 260, and 280 nm. The quality of the DNA was assessed as good, when absorbance ratios were $OD_{260}/OD_{280} > 1.8$ and $OD_{234}/OD_{260} > 0.5$. Total DNA solutions were diluted to an OD_{260} of 1.0. Amplification of about 200 bp of the V3 region of the 16S rRNA genes with the F357 (with a GC clamp attached) and R518 primers, followed by DGGE analysis, and processing of the resulting fingerprints was performed as described previously (Duytschaever *et al.*, 2011), except that DGGE gels were run for 960 min instead of 990 min. For the amplification of about 200 bp of the D1/D2 region of the 26S rRNA gene, the NL1 (with GC clamp) and LS2 primers were used as previously reported by Cocolin *et al.* (2000). However, PCR

amplicons of both 16S rRNA and 26S rRNA genes were not consistently obtained from the gueuze beer samples and a nested PCR approach was therefore applied by means of a second PCR assay using the same primers and the products of the first PCR assay as template.

All DNA bands were assigned to band classes using the BioNumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium). Dense DNA bands and/or bands that were present in multiple fingerprints were excised from the polyacrylamide gels by inserting a pipette tip into the bands and subsequent overnight elution of the DNA from the gel slices in 40 µL 1 x TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 8) at 4°C. The position of each DNA band extracted was confirmed by repeat DGGE experiments using the excised DNA as template. The DNA extracted was subsequently re-amplified and sequenced using the same protocol and primers (without GC clamp). EzBioCloud and BLAST (Altschul *et al.*, 1997; Kim *et al.*, 2012) analyses were performed to determine the most similar sequences in the public sequence databases (NCBI).

Culture media, enumeration and isolation

Samples were serially diluted in 0.9 % (wt/vol) saline and 50 µL of each dilution was plated in triplicate on multiple agar isolation media.

All bacterial agar isolation media were supplemented with 5 ppm amphotericin B (Sigma-Aldrich, Bornem, Belgium) and 200 ppm cycloheximide (Sigma-Aldrich) to inhibit fungal growth and were incubated aerobically at 28°C, unless stated otherwise. Samples were incubated on de Man-Rogosa-Sharpe (MRS) agar (Oxoid, Erembodegem, Belgium) (De Man *et al.*, 1960) at 28°C aerobically and at 20°C anaerobically for the isolation of LAB. Violet red bile glucose (VRBG) agar (Mossel *et al.*, 1962, 1978) was used for the isolation of *Enterobacteriaceae* and acetic acid medium (AAM) agar (Lisdiyanti *et al.*, 2003) was used for the isolation of AAB.

All yeast isolation media were supplemented with 100 ppm chloramphenicol (Sigma-Aldrich) to inhibit bacterial growth and were incubated aerobically at 28°C. DYPAI agar [2.0 % (wt/vol) glucose, 0.5 % (wt/vol) yeast extract, 1.0 % (wt/vol) peptone and 1.5 % (wt/vol) agar] was used as a general yeast isolation medium. To favor the slow-growing *Dekkera*/*Brettanomyces*, DYPAI agar was supplemented with an additional 50 ppm cycloheximide (DYPAIX) (Abbott *et al.*, 2005; Licker *et al.*, 1998; Suárez *et al.*, 2007). Furthermore, universal beer agar (Oxoid) was supplemented with 25 % (vol/vol) commercial gueuze beer (Belle-Vue; AB Inbev, Anderlecht, Belgium), as recommended by the manufacturer, and was used as an additional general yeast agar isolation medium (UBAGI).

Simultaneously, enrichment cultures were prepared. Therefore, 30 mL of gueuze beer was filtered over a 0.45-µm cellulose nitrate filter (Whatman, Maidstone, Kent, UK) and the filter was subsequently incubated in MRS broth at 20°C and 28°C and in AAM broth, VRBG broth, DYPAI broth, and DYPAIX broth at 28°C containing the appropriate antimicrobial agents. Enrichment cultures showing growth were serially diluted in 0.9 % (wt/vol) saline and 50 µL of each dilution was plated on their corresponding agar isolation medium.

Colonies on plates comprising 25 to 250 colony forming units (CFU) were counted after 3 to 10 days of incubation and for each of the seven isolation conditions used, about 20-25 colonies or, if counts were lower, all colonies were randomly picked up.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) dereplication and identification

Isolates were subcultured twice using the respective isolation conditions and MALDI-TOF MS was performed on the third generation of pure cultures using the 4800 Plus MALDI TOF/TOF™ Analyzer (AB/SCIEX, Framingham, MA, USA), as described previously (Wieme *et al.*, 2012). In short, Data Explorer 4.0 software (AB/SCIEX) was used to convert the mass spectra into .txt-files to import them into a BioNumerics 5.1 database (Applied Maths). Spectral profiles were compared using the Pearson product moment correlation coefficient and a dendrogram was built using the unweighted pair group method with arithmetic mean (UPGMA) cluster algorithm. Homogeneous clusters consisting of isolates with visually identical and/or virtually identical mass spectra were delineated. From each cluster, at least one isolate per sample was chosen for further identification through sequence analysis of the 16S rRNA gene and other molecular markers. Sequence analysis of the *pheS* gene was used to identify LAB (De Bruyne *et al.*, 2007, 2008; Naser *et al.*, 2005, 2007). Yeast isolates were identified through sequence analysis of the D1/D2 region of the 26S rRNA gene (Kurtzman & Robnett, 1998). All PCR assays were performed as described by Snauwaert *et al.* (2013). Bacterial DNA was obtained via the protocol described by Niemann *et al.* (1997), whereas yeast DNA was obtained using the protocol of Harju *et al.* (2004).

Metabolite analysis

Determination of the concentrations of carbohydrates

High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used for the determination of the concentrations of glucose, fructose and sucrose, as described previously, but with the following modifications (Van der Meulen *et al.*, 2007). An ICS 3000 chromatograph (Dionex, Sunnyvale, CA, USA) with a CarboPac™ PA10 column (Dionex) was used. The mobile phase, at a flow rate of 1.0 mL/min, consisted of ultrapure water (0.015 µS/cm; eluent A), 167 mM NaOH (eluent B), and 500 mM NaOH (eluent C), and was used with the following gradient: 0.0 min, 87 % A and 13 % B; 20.0 min, 87 % A and 13 % B; 25.0 min, 100 % C; 30.0 min, 100 % C; 31.0 min, 87 % A and 13 % B; and 35.0 min, 87 % A and 13 % B. To remove proteins, 150 µL of Carrez A reagent [3.6 % (wt/vol) $K_4Fe(CN)_6 \cdot 3H_2O$] and 150 µL of Carrez B reagent [7.2 % (wt/vol) $ZnSO_4 \cdot 7H_2O$] were added to 300 µL of the tenfold diluted beer samples. After microcentrifugation (14000 rpm for 10 min), the supernatant was filtered (UNIFLO 13/0.2 µm RC; Whatman) and transferred to an appropriate vial prior to injection. Calibration was performed using external standards.

High-performance liquid chromatography with evaporative light-scattering detection (HPLC-ELSD) was used for the determination of the concentrations of maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, and higher malto-oligosaccharides. A Waters chromatograph (Waters Corporation, Milford, MA, USA) was used, equipped with a 2424 evaporative light-scattering detector, a 600 controller, a 717Plus autosampler, and a Grace Prevail Carbohydrate ES column (250 x 4.6 mm, 5 µm, 35°C; Grace Davison Discovery Sciences, Columbia, MD, USA). The mobile phase, at a flow rate of 1.0 mL/ min, consisted of ultrapure water (0.015 µS/cm; eluent A) and acetonitrile (eluent B), and applied with the following gradient: 0.0 min, 25 % A and 75 % B; 50.0 min, 40 % A and 60 % B; 51.0 min, 25 % A and 75 % B, and 60.0 min, 25 % A and 75 % B. Proteins were removed by adding 500 µL acetonitrile to a 500 µL beer sample. All samples were microcentrifuged (14000 rpm for 10 min), filtered (UNIFLO 13/0.2 µm RC; Whatman) and transferred to an appropriate vial prior to injection. Calibration was performed using external standards.

Determination of the concentration of lactic acid

Concentrations of lactic acid were determined by HPAEC with conductivity under ion suppression (CIS), using the same apparatus as described above, equipped with an AS-19 column (Dionex), according to the procedure described previously, but with the following modifications (Lefeber *et al.*, 2011). Briefly, the mobile phase, at a flow rate of 1.0 mL/min, consisted of ultrapure water (0.015 µS/cm; eluent A) and 0.1 M KOH (eluent B). The following gradient was applied: 0.0 min, 96 % A and 4 % B; 15.0 min, 100 % B; 20.0 min, 100 % B; 21.0 min, 96 % A and 4% B; and 25.0 min, 96 % A and 4 % B. A standard addition protocol was used as described previously (Vrancken *et al.*, 2008), with the following standard solutions (g/L): ultrapure water (0.015 µS/cm; solution A); 7.5 g/L lactic acid (solution B); 15.0 g/L lactic acid (solution C); and 22.5 g/L lactic acid (solution D). All samples were microcentrifuged (14000 rpm for 10 min), filtered (UNIFLO 13/0.2 µm RC; Whatman) and transferred to an appropriate vial prior to injection.

Determination of the concentrations of ethanol, ethyl lactate, and short-chain fatty acids

The concentrations of ethanol, ethyl lactate, and short-chain fatty acids (SCFAs; in particular, acetic acid, propionic acid and isobutyric acid) in the beer samples were measured by GC, using a Focus gas chromatograph (Interscience, Breda, The Netherlands) equipped with a Stabilwax-DA column (Restek, Bellefonte, PA, USA), a flame ionization detector (FID), and an AS 3000 autosampler. Hydrogen gas was used as carrier gas with a constant flow rate of 1 mL/min; nitrogen gas was used as make-up gas. The injector and detector temperatures were set to 240°C and 250°C, respectively, and the following temperature program was used: 0.0 min at 40°C, 10.0 min at 140°C, 12.0 min at 230°C and 22.0 min at 230°C. A volume of 900 µL of a mixture (638:250:12) of acetonitrile:1-butanol (1.0 mL/L):formic acid was added to 300 µL of a non-diluted (in the case of ethyl lactate and SCFAs) or ten times diluted (in the case of ethanol) sample. Prior to injection, the samples were

microcentrifuged (14000 rpm for 10 min), filtered (UNIFLO 13/0.2 µm RC, Whatman) and transferred to an appropriate vial. A volume of 1.0 µL was injected using a split ratio of 40:1. Quantification was performed using external standards. All samples were analyzed in triplicate and the results are presented as means of the three independent measurements with respective standard deviations.

Determination of volatile organic compounds

The targeted analysis of volatile organic compounds (VOCs), in particular esters and higher alcohols, was performed using static headspace gas chromatography-MS (SH-GC-MS; semi-quantitative analysis), solid-phase microextraction GC-MS (SPME-GC-MS; qualitative fingerprinting analysis), and selected ion flow tube-mass spectrometry (SIFT-MS; semi-quantitative fingerprinting analysis). Odor thresholds were taken from Simpson and Miller (1984) and Engan (1972).

SH-GC-MS

A semi-quantitative targeted analysis of VOCs was performed through SH-GC-MS by means of an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 5973N mass spectrometer (Agilent Technologies), equipped with an MPS2 Gerstel autosampler (Gerstel GmbH & Co. KG, Mülheim-an-der-Ruhr, Germany), with the following modifications (Ravyts *et al.*, 2009). The samples were prepared in headspace vials containing 5 mL of beer and 100 µL of an internal standard solution (1-butanol, 1 mL/L). Prior to injection, the samples were equilibrated in appropriate vials in the shaker-incubator of the MPS2 Gerstel autosampler by agitation at 60 °C for 30 min. Depending on the compound to be analyzed, a split of 20:1 (in the case of isobutanol, ethyl acetate, and isoamyl alcohol) or no split (in the case of isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and ethyl hexadecanoate) were applied. The needle temperature was 90 °C. The capillary column was a DB-WAXetr (Agilent Technologies) with a length of 30 m, an internal diameter of 0.25 mm, and a film thickness of 0.5 µm. Helium was used as carrier gas with a flow rate of 1 mL/min. The oven temperature program consisted of an initial step at 40°C for 5 min, followed by a linear increase to 120°C at 20°C/min and a linear increase from 120 to 225°C at 10°C/min. Finally, the temperature was kept constant at 225°C for the rest of the run, which lasted 24.5 min. The temperature of the transfer tube was held at 280°C. Peak identification was done by comparison with pure standard compounds and library information (NIST 08, National Institute of Standards and Technology, Gaithersburg, MD, USA). Quantification was done by comparison with external standards, corrected for the response of the internal standard. Samples were analyzed in triplicate and values are presented as the means of the three independent measurements with respective standard deviations.

SPME-GC-MS

The SPME device (Supelco, Bellefonte, PA, USA) was equipped with a 75-µm divinylbenzene/carboxen/polydimethylsiloxane (DVS/CAR/PDMS) fibre and was mounted on a MPS2 Gerstel autosampler, which was connected to an Agilent 6890 gas chromatograph coupled to

an Agilent 5973N mass spectrometer (Agilent Technologies). The device was operated as described above and reported previously (Ravyts & De Vuyst, 2011). No split was applied. Samples were analyzed at least in triplicate. The results of components positively identified in at least 66 % of the samples analyzed were retained.

SIFT-MS

A selected ion flow tube-mass spectrometer, the Syft Voice 200 (Syft Technologies, Christchurch, New Zealand), was used to record a spectrum (*m/z* of 10-250) of the VOCs present in the headspace of a diluted beer sample. Briefly, 50 µL of gueuze beer was added to 5 mL of ultrapure water (0.015 µS/cm) in a 100 mL Schott bottle capped with a PTFE/silicone septum (VWR, Radnor, PA, USA). The samples were kept at room temperature for 15 min prior to analysis. Upon analysis, the septum was pierced by an inactivated needle connected to the HEX-inlet of the Syft Voice 200 and a full scan was started instantly. To minimize the pressure drop in the closed bottle, the scan length was limited to 100 s. All gueuze beer samples were analyzed in hexaplicate. The concentrations of important volatile gueuze beer metabolites were calculated using the SIFT-MS database provided with the LabSyft software package (version 1.3.1, Syft Technologies) that included 2-phenylethanol, acetic acid, ethanol, ethyl acetate, ethyl hexanoate, ethyl octanoate, isoamyl acetate, and lactic acid. The methodology was validated using three commercial beers differing in VOC fingerprint, namely a Flemish sour ale beer, a gueuze beer, and a lager beer. The results obtained during this study were verified using SH-GC-MS and an additional technique appropriate for every compound.

Statistical analysis

Statistical analysis was performed using the SPSS 20 software package (IBM, Armonk, NY, USA). ANOVA ($p < 0.05$) was performed on quantitative data of the following metabolites: acetic acid, ethanol, ethyl acetate, ethyl lactate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl hexadecanoate, isoamyl acetate, isoamyl alcohol, and isobutanol, using the individual bottles as populations. For lactic acid, however, populations were based on years. Homoscedasticity was tested using Levene's test ($p < 0.05$). In the case of homoscedasticity, Tukey's HSD test was used as a *post hoc* test, while Dunett T3 was used in the case of heteroscedasticity. The correlation coefficients were calculated using the Pearson's correlation coefficient. Significance is reported at the $p \leq 0.01$ and $p < 0.05$ levels using a two-sided test.

Sensory analysis

The gueuze beer samples were subjected to a preliminary descriptive sensory analysis by twelve assessors. The assessors were asked to give a general evaluation of the beers and to score acidity, bitterness, fruitiness, and sweetness on a scale from 0 to 10, both for smell and taste. Crackers were used to clean the palate during all tastings.

Results

Microbiological analysis

PCR-DGGE community fingerprints of the gueuze beers

The community profiles obtained through PCR-DGGE analyses of the bacterial and yeast communities of the gueuze beer samples that aged between a few months and up to 17 years and of the three-years old lambic beer were compared with the bacterial and yeast community profiles of the two-years old lambic beer obtained previously (Spitaels *et al.*, 2014; Figure 6.1.1). An overview of the excised PCR-DGGE bands and the species to which they were assigned through comparative gene sequence analysis is presented in Supplementary Figure S 6.1.1 and Supplementary Table S 6.1.2, respectively. The three sample replicates of each of the gueuze beer bottles examined yielded identical bacterial and yeast PCR-DGGE community profiles (data not shown). With the exception of the yeast community fingerprints of the 2008 production year, the bacterial and yeast community profiles of all gueuze beer bottles of the same bottling year were also highly similar (data not shown). The 2008-2 and 2008-3 PCR-DGGE yeast community profiles differed however strongly from those of all other gueuze beers (Figure 6.1.1B). For the 2008-3 sample, this could be explained by the cork that was disintegrated by mold growth, although the cork of the 2008-2 sample was still intact. In the following, the beers are grouped according to the time of ripening.

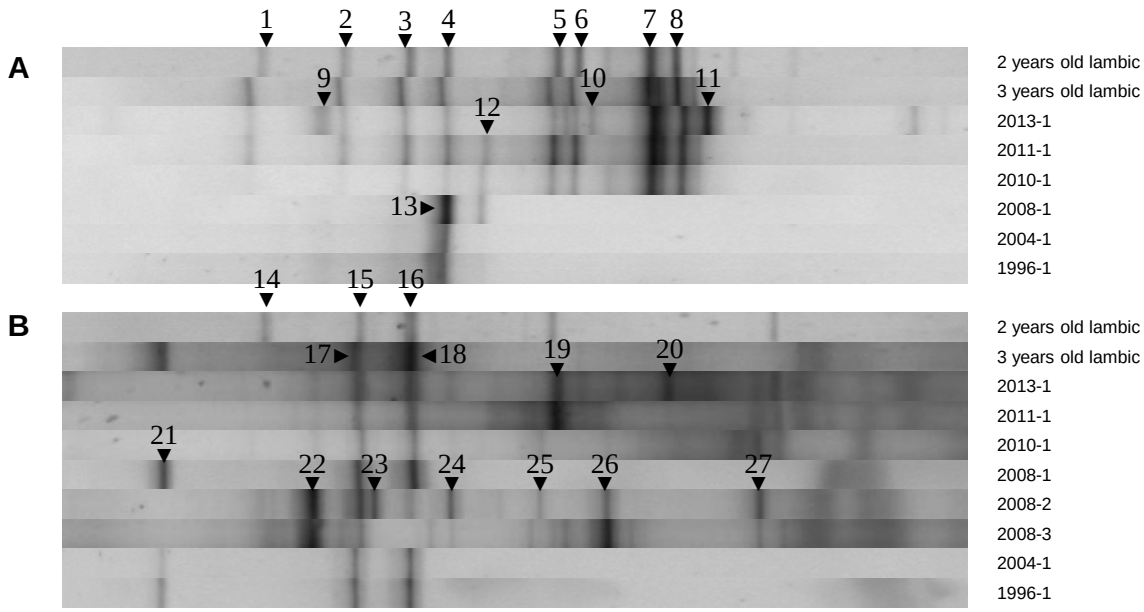

Figure 6.1.1 Representative DGGE profiles of bacterial (A) and yeast (B) communities in lambic and aged gueuze beers. Bottling year, bottle numbers and band classes 1-27 are shown. An overview of the excised DNA bands and their identifications are presented in Supplementary Figure S 6.1.1 and Table S 6.1.2. DGGE fingerprints of the two-years old lambic beer samples were taken from Spitaels *et al.* (2014). The band classes 14, 15 and 16 in this profile were previously assigned to *Wickerhamomyces* strains, *Dekkera* strains and *Naumovia* strains, respectively (Spitaels *et al.*, 2014).

Three-years old lambic beer

The bacterial PCR-DGGE community profiles of the three-years old lambic beer were very similar to the community profiles of the two-years old lambic beer (Figure 6.1.1). Each of the DNA bands (Figure 6.1.1A, band classes 1-8) was assigned to *Pediococcus*/*Lactobacillus*. An overview of the excised DNA bands and their identifications is represented in Supplementary Figure S 6.1.1 and Supplementary Table S 6.1.2. The yeast PCR-DGGE community profiles of the three-years old lambic beer differed to some extent from the yeast profiles of the two-years old lambic beer. Band class 14 (originating from *Wickerhamomyces*) was not present in the profiles of the three-years old lambic beer, which only comprised bands assigned to *Dekkera* (Figure 6.1.1B, band classes 17, 18 and 21). Bacterial counts on AAM and MRS agars of a sample of the three-years old lambic beer were about 10^3-10^4 CFU/mL, whereas yeast counts were about 10^4 CFU/mL (Supplementary Table S 6.1.1). All isolates from AAM agar (n = 22) were identified as *Acetobacter lambici*. All but one of the MRS agar isolates (8 out of 9 isolates) were identified as *P. damnosus*. The remaining

isolate was identified as *A. lambici*. Yeast isolates from DYPAI and UBAGI agars (n = 42) were identified as *Pichia membranifaciens* (23 isolates), *D. bruxellensis* (10 isolates), *Dekkera anomala* (4 isolates), *Candida patagonica* (4 isolates) and *Wickerhamomyces anomalus* (1 isolate).

Gueuze beer samples from 2013

The bacterial PCR-DGGE community profiles of gueuze beers bottled in 2013 contained bands originating from *Pediococcus/Lactobacillus* strains (Figure 6.1.1A, band classes 1-8) and *Enterobacteriaceae* strains (Figure 6.1.1A, band classes 9-11), while the yeast PCR-DGGE community profiles of these samples comprised bands originating from *Dekkera* strains (Figure 6.1.1B, band classes 17 and 18) and *Saccharomyces* strains (Figure 6.1.1B, band classes 19 and 20). Cultivation experiments on isolation agars of this 2013 gueuze beer sample yielded high bacterial counts (about 10^5 CFU/mL) but very low yeast counts (about 10^2 CFU/mL) (Supplementary Table S 6.1.1). On MRS agar (n = 20), *P. damnosus* was the only bacterial species isolated. *Dekkera anomala* was predominantly isolated from DYPAI and UBAGI agars (8 out of 10 isolates), besides *D. bruxellensis* (2 out of 10 isolates). The former was the only species isolated from DYPAIX agar (n = 4). The enrichment cultures also yielded bacterial and yeast growth. Also, *P. damnosus* was the only bacterial species isolated after enrichment, as it was the case after direct plating on MRS agar. The DYPAI enrichment culture gave *S. cerevisiae* (16 out of 20 isolates), *P. membranifaciens* (3 out of 20 isolates), and one isolate of *D. anomala*. DYPAIX agar isolates were primarily *D. anomala* (19 out of 20 isolates); one isolate was *D. bruxellensis*.

Gueuze beer samples from 2011 and 2010

Bacterial PCR-DGGE community profiles of the gueuze beers bottled in 2010 and 2011 did no longer show bands that were assigned to members of the *Enterobacteriaceae*, but comprised an additional band that was also assigned to *Pediococcus/Lactobacillus* (Figure 6.1.1A, band class 12). In the yeast PCR-DGGE community profiles, one of the two *Saccharomyces* bands (Figure 6.1.1B, band class 20) disappeared from the 2011 gueuze beer profiles, while the 2010 gueuze beer profiles only showed two prominent bands originating from *Dekkera* strains, which

were also found in the 2013 and 2011 gueuze beer profiles (Figure 6.1.1B, band classes 17 and 18). *Pediococcus damnosus* was the sole LAB species isolated from the 2011 and 2010 gueuze beer samples (all 48 isolates were recovered from MRS agar), while yeast strains could not be isolated from the 2011 and 2010 gueuze beers. The enrichment cultures showed growth of both bacteria and yeasts. Again, all bacterial isolates were identified as *P. damnosus*. *Brettanomyces custersianus* (26 out of 36 isolates) and *D. bruxellensis* (n = 10) were isolated from the gueuze beer bottled in 2011. All isolates from the 2010 gueuze beer were identified as *D. bruxellensis*.

Gueuze beer samples from 2008

The bacterial PCR-DGGE community profiles of the gueuze beers bottled in 2008 comprised a DNA band assigned to *Lactobacillus* (Figure 6.1.1A, band class 13) and a very faint band assigned to *Pediococcus/Lactobacillus* (Figure 6.1.1A, band class 12). The 2008-1 gueuze beer yeast community profiles were highly similar to those of the previous years, except for a prominent DNA band that was again assigned to *Dekkera* and which was also seen in the three-years old lambic beer sample (Figure 6.1.1B, band class 21). Sequence analysis of DNA bands (Supplementary Figure S 6.1.1 and Supplementary Table S 6.1.2) of the 2008-2 and 2008-3 gueuze beer samples revealed that three bands originated from *Dekkera* strains (Figure 6.1.1B, band classes 17, 26 and 27); other bands originated from *Candida* (Figure 6.1.1B, band classes 22, 23 and 24) and *Saccharomyces* (Figure 6.1.1B, band class 25) strains. Cultivation experiments did no longer yield bacterial isolates, while only *D. bruxellensis* was isolated from an enrichment culture of the gueuze beer bottled in 2008.

Gueuze beer samples from 2004 and 1996

The bacterial PCR-DGGE community profiles of the gueuze beers that were bottled in 2004 and 1996 comprised a single band that was assigned to *Lactobacillus* (Figure 6.1.1A, band class 13). Three yeast bands (Figure 6.1.1B, band classes17, 18 and 21) were detected in the 2004 and 1996 gueuze beer samples, all originating from *Dekkera* yeasts (Supplementary Figure S 6.1.1 and Supplementary Table S 6.1.2). Cultivation experiments again yielded *D. bruxellensis* isolates, after enrichment only, but no bacteria.

Metabolite analysis

None of the gueuze beers examined contained fermentable monosaccharides (glucose and fructose) or disaccharides (sucrose and maltose) at concentrations higher than 0.1 g/L. Maltotriose and other maltodextrins (up to maltoheptaose) were present in the beers, but their concentrations were low to zero in the gueuze beers bottled in 2004 and 1996 (Figure 6.1.2). The ongoing degradation of these carbohydrate sources was reflected in the gradual increase of the lactic acid concentrations for all gueuze beers, except for those bottled in 1996 (Table 6.1.1). Additionally, the bottle-to-bottle variation was remarkable, *i.e.*, the lactic acid concentration in gueuze beer 2004-2 was much higher than the concentrations found in gueuze beers 2004-1 and 2004-3.

The ethanol content of these gueuze beers did not vary significantly (Table 6.1.1). In contrast, the acetic acid concentrations did vary considerably between the beers bottled in different years (Table 6.1.1). An elevated concentration of acetic acid was found in gueuze beer 2013-3, while the concentrations in gueuze beer samples 2008-1, 2008-2 and 2008-3 were lower compared to the concentrations in gueuze beer samples 2004-1, 2004-2 and 2004-3. The significantly lower acetic acid concentrations ($p < 0.05$) in gueuze beer samples 1996-1, 1996-2, and 1996-3 indicated a further conversion of acetic acid. Both propionic acid and isobutyric acid were under the limits of quantification (0.01 g/L) for all gueuze beer samples examined.

The concentrations of ethyl lactate clearly increased during aging (Table 6.1.1). The evolution of the ethyl lactate concentrations strongly correlated with the evolution of the lactic acid concentrations in the gueuze beers of different ages (Supplementary Table S 6.1.4). In contrast, the evolution of the isoamyl acetate concentrations was negatively correlated with the evolution of both the lactic acid and ethyl lactate concentrations ($p < 0.01$) (Supplementary Table S 6.1.4). However, the individual variations between the bottles were again noteworthy. For example, an elevated ethyl lactate concentration was present in gueuze beer sample 2013-3 compared to gueuze beer samples 2013-1 and 2013-2.

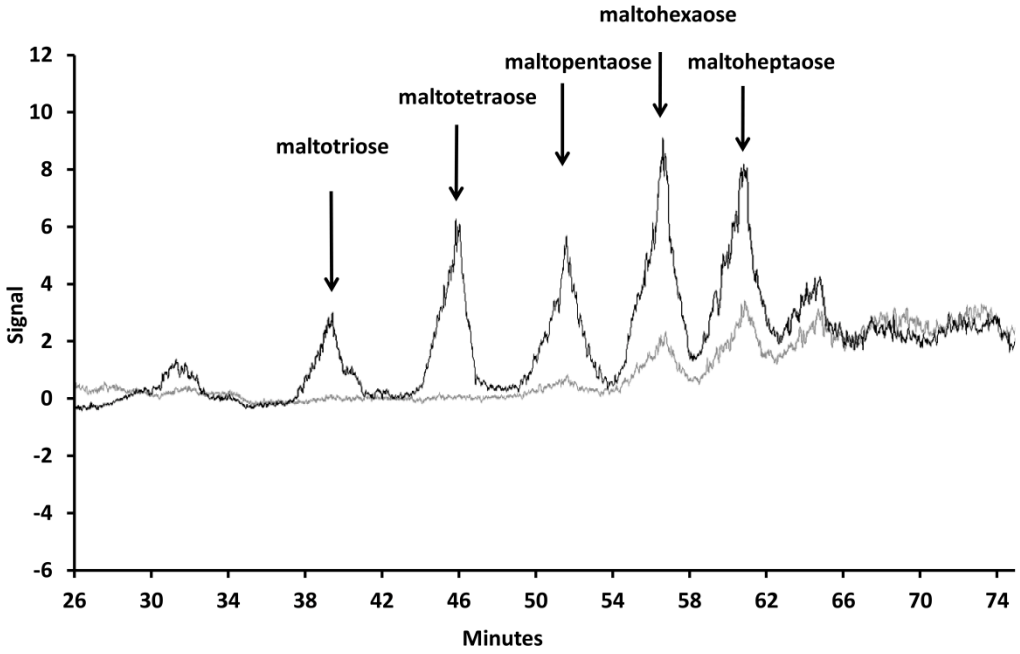

Figure 6.1.2 Comparison of a representative profile of malto-oligosacharides from beers bottled in 2008 (black line) and 2004 (grey line), as monitored by HPLC-ELSD.

Table 6.1.1 Comparison of the concentrations of the major metabolites present in in aged gueuze beers, as determined by HPAEC-CIS (lactic acid), GC-FID (ethanol, acetic acid, and ethyl lactate), and SH-GC-MS (ethyl acetate, ethyl decanoate, and isoamyl acetate). Standard deviations are based on three replicates taken from each bottle.

Component Bottle	Ethanol % (vol/vol)	Lactic acid (g/L)	Acetic acid (g/L)	Ethyl lactate (mg/L)	Ethyl acetate (mg/L)	Ethyl decanoate (mg/L)	Isoamyl acetate (mg/L)
2013-1	4.7±0.5	4.5±0.5	1.36±0.05	68±3	118±28	4.6±0.5	0.25±0.01
2013-2	3.7±0.1	3.8±0.5	1.05±0.02	44±1	163±26	11.8±0.1	0.29±0.02
2013-3	4.9±0.1	6.2±0.9	2.15±0.13	195±15	130±7	11.3±0.5	0.27±0.02
2011-1	4.6±0.3	6.2±0.8	1.89±0.13	166±17	170±110	3.3±1.3	0.26±0.02
2011-2	4.4±0.7	5.7±0.7	1.88±0.09	175±12	252±42	8.7±0.7	0.32±0.01
2011-3	4.4±1.1	5.3±0.6	1.82±0.23	162±28	292±59	7.5±0.4	0.33±0.02
2010-1	4.3±0.4	7.5±1.4	1.79±0.18	249±28	203±6	2.9±0.1	0.19±0.01
2010-2	5.2±1.1	8.5±0.9	2.02±0.20	295±37	283±39	8.2±0.2	0.26±0.01
2010-3	4.9±1.4	10.6±0.6	2.24±0.47	332±75	285±47	6.5±0.1	0.26±0.02
2008-1	4.7±1.1	9.6±0.4	1.22±0.19	371±70	125±13	2.2±0.1	0.06±0.01
2008-2	4.8±0.9	13.8±1.6	1.27±0.22	413±86	319±134	5.7±0.4	0.10±0.01
2008-3	4.8±1.2	11.9±1.2	1.29±0.18	415±63	246±51	5.6±0.4	0.12±0.01
2004-1	5.0±0.8	13.3±0.8	1.85±0.25	630±94	140±10	2.0±0.3	0.11±0.02
2004-2	4.9±1.5	17.6±1.2	1.80±0.50	592±182	197±27	3.4±0.3	0.16±0.01
2004-3	4.6±0.9	11.3±0.8	1.43±0.08	464±38	178±8	2.7±0.3	0.17±0.02
1996-1	4.6±0.4	10.9±2.4	0.72±0.03	526±24	89±20	1.8±0.4	0.04±0.02
1996-2	5.3±0.9	14.1±1.2	0.93±0.06	778±76	150±94	3.9±0.2	0.07±0.03
1996-3	4.2±0.6	10.2±0.1	0.71±0.04	556±33	169±61	2.5±0.3	0.05±0.02

Fatty acid ethyl esters, ranging from ethyl C2 (ethyl acetate) to ethyl C16 (ethyl hexadecanoate) were detected in all gueuze beer samples, using SH-GC-MS and SPME-GC-MS (Supplementary Table S 6.1.3). Ethyl acetate was the most prevalent fatty acid ethyl ester (Table 6.1.1). However, no relationship between the age of the beer and the ethyl acetate concentrations was found. The concentrations of ethyl hexanoate and ethyl octanoate were the lowest in the gueuze beers bottled in 2004. However, gueuze beers bottled in 2011 also showed decreased ethyl hexanoate concentrations, when compared to the gueuze beers bottled in 2013 and 2010. A strong correlation ($p < 0.05$) was found between the evolution of the ethyl octanoate concentrations and the evolution of the ethyl hexanoate concentrations (Supplementary Table S 6.1.4). In addition, significantly lower concentrations ($p < 0.01$) of ethyl decanoate were present in the gueuze beers bottled in 2004 and 1996 compared to the others. The evolution of the ethyl decanoate concentrations were also positively correlated with the evolution of the ethyl octanoate concentrations, whereas they were negatively correlated with the evolution of both the ethyl lactate and lactic acid concentrations ($p < 0.05$) (Table 6.1.1 and Supplementary Table S 6.1.4). In general, low concentrations of isobutanol and isoamyl alcohol were present in all gueuze beer samples (Supplementary Table S 6.1.3). The concentration of isoamyl acetate decreased with the aging of the gueuze beers (Table 6.1.1). Similar fingerprints of the VOC contents of all gueuze beers were obtained by SPME-GC-MS (Supplementary Table S 6.1.3). The compounds 2-phenylethanol, 4-ethylphenol, 4-ethylguaiacol, 4-vinylguaiacol, and diethyl succinic acid were found in all gueuze beers (Supplementary Table S 6.1.3).

Several important volatile gueuze beer metabolites were found by SIFT-MS that were present in the SIFT-MS Labsyft database, the concentrations of which were compared with the concentrations obtained by GC-FID, HPAEC-CIS, or SH-GC-MS (Figure 6.1.3). Ethanol was found to be the most abundant metabolite, followed by ethyl acetate, lactic acid, and acetic acid. However, the overwhelming presence of ethanol limited the detection of other volatile compounds by SIFT-MS. Although the concentration of ethanol measured by SIFT-MS and GC-FID showed similar results (Figure 6.1.3), the concentrations of other volatile compounds found were not comparable when using different techniques, except for the lactic acid

concentrations (Supplementary Table S 6.1.5). The evolution of the lactic acid concentrations showed a significant correlation ($p < 0.01$) when measured with both HPAEC-CIS and SIFT-MS.

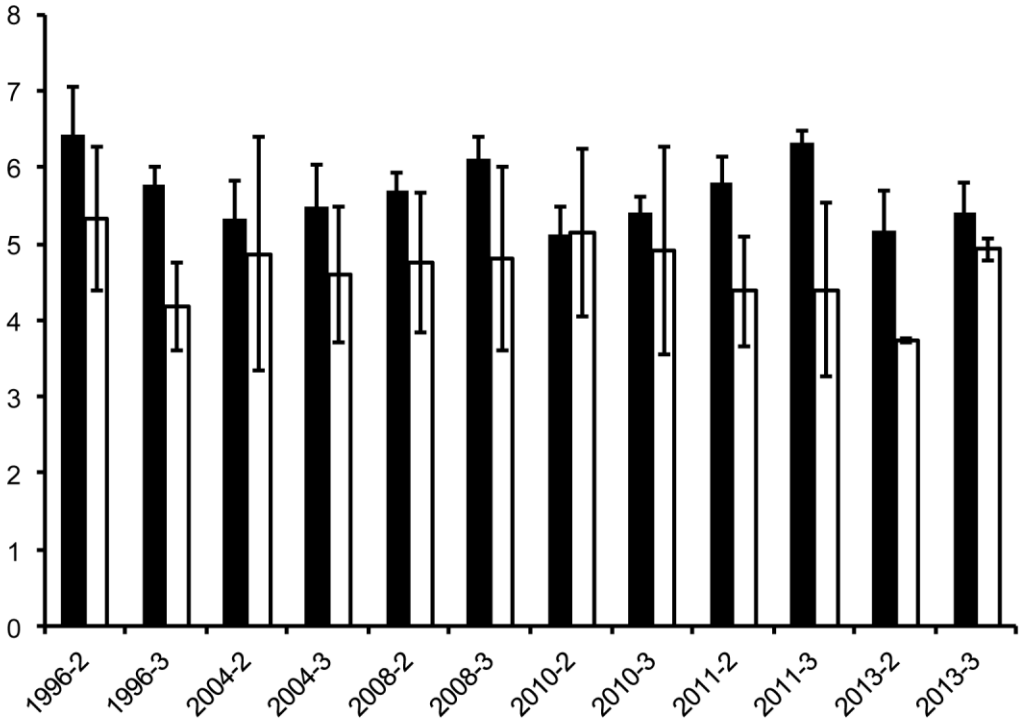

Figure 6.1.3 Comparison of the ethanol concentrations in aged gueuze beers, as measured by SIFT-MS in the headspace of a diluted sample (black bars, ppmv) and by GC-FID with liquid injection (white bars, vol/vol %).

Sensory analysis

The sensory analysis revealed that all beers were assessed as highly acidic. The perceivable fruitiness was the lowest in the beers bottled in 1996. A preference towards the beers bottled in 2008 was noticed.

Discussion

A previous study of the traditional lambic beer fermentation process revealed that *Dekkera bruxellensis* and *Pediococcus damnosus* are the dominant microorganisms in lambic beers (Spitaels *et al.*, 2014). The present study revealed that the microbial communities present in the three-years old lambic beer were highly similar to those in the two-years old lambic beer originating from the same batch (Spitaels *et al.*, 2014). As this lambic beer fermentation is performed in closed wooden casks, inoculation from the cask surroundings does not occur during the fermentation

process, explaining maintenance of the prevailing microorganisms upon maturation. Lambic beer from casks is used to produce gueuze beers through mixing of lambic beers of different ages (Verachtert & Iserentant, 1995). As such, the final gueuze beers in the bottles are the products of a spontaneous bottle refermentation of these mixtures. The present study showed that a bottle maturation process of gueuze beers has a profound influence on the microbiota present in these beers. This was reflected in the bacterial PCR-DGGE community profiles of the gueuze beers, which gradually changed from the presence of multiple DNA bands assigned to *Enterobacteriaceae* and *Pediococcus* in the youngest gueuze beers to the presence of a few bands originating from *Lactobacillus* and *Dekkera* in the 17-years old gueuze beer sample. The observation of DNA bands originating from *Enterobacteriaceae*, long after these bacteria were active and could be isolated from the lambic beer fermentation, is remarkable (Spitaels *et al.*, 2014). Indeed, the DNA extraction method of the present study did not discriminate between live and dead cells, thus enabling the DNA extraction of dead *Enterobacteriaceae* cells (Nocker & Camper, 2006). The bacterial PCR-DGGE community profiles of the oldest gueuze beers converged to a profile that only contained a band originating from *Lactobacillus*. The detection of *Lactobacillus* DNA in the bacterial PCR-DGGE community profiles of the oldest gueuze beers examined was remarkable when considering its absence in gueuze beers which that were bottled in later years. Most likely, this suggests that *Lactobacillus* rather than *Pediococcus* was dominant in the Cantillon lambic beer fermentation process in this period.

Yeast PCR-DGGE community profiles of the old gueuze beers only yielded *Dekkera* bands, whereas bands originating from *Saccharomyces* strains were found in younger gueuze beers. The aberrant yeast profiles of the 2008 gueuze beers may indicate deviations in the gueuze beer production process at the time of bottling. This may explain the bottle-to-bottle variations that can be introduced during the bottling process of these beers. Multiple yeast species could be isolated from gueuze beers that had been aged for a few months and for two years. *Brettanomyces custersianus* was isolated from the gueuze beer bottled in 2011 and was in a previous study also isolated from air samples from this lambic beer brewery in a previous study (Spitaels *et al.*, 2014). The presence of this yeast species in the

gueuze beer bottled in 2011 indicates that it was present during the lambic beer fermentation process in this period or that it was introduced during the bottling process. Only *D. bruxellensis* could be isolated from beers that were matured for more than three years. This suggests that *Dekkera* yeasts are better adapted for survival in bottled beers compared to LAB and other yeasts, although the absence of LAB could also indicate batch-to-batch variations in the production process of the gueuze beers. An enrichment culture was needed to isolate yeast from the gueuze beers bottled prior to 2013. Bottle refermentation conditions and carbon dioxide pressure build-up might have created a selective stress on these yeasts, initiating a VBNC state, which was reversed in the enrichment medium, as shown for aging wine (Divol & Lonvaud-Funel, 2005; Millet & Lonvaud-Funel, 2000). This effect is likely less pronounced in the fermenting lambic beer where the carbon dioxide can escape through the cask wood. Likewise, an enrichment culture enabled the isolation of additional yeast species from the gueuze beer bottled in 2013.

The maturation/refermentation process also influenced the metabolites present in the gueuze beers. No monosaccharides or disaccharides were found. These carbohydrates are used prior to sequential degradation of maltodextrins during lambic beer fermentation (Shanta Kumara *et al.*, 1993). The degradation of maltodextrins might indicate a limit for the ongoing secondary fermentation and aging, due to gradual depletion of these carbohydrate sources to low concentrations, as was the case in the gueuze beers bottled in 2004 and 1996 (Shanta Kumara *et al.*, 1993; Verachtert & Iserentant, 1995). Indeed, the increase of the lactic acid concentrations throughout aging pointed to an ongoing refermentation. The slower increase or even decrease of the concentrations of lactic acid in the gueuze beers bottled prior to 2008 was a further indication for this age limit. The acetic acid concentrations were the lowest in gueuze beers bottled in 1996. Despite some batch-to-batch variation, the acetic acid concentrations found in all gueuze beers were clearly above the estimated odor threshold of 0.175 g/L (Simpson & Miller, 1984), thus introducing a certain desirable tartness in the gueuze beers. The concentrations of propionic acid and isobutyric acid were very low, as reported previously (Van Oevelen *et al.*, 1976). Elevated isobutanol and isoamyl alcohol concentrations can be considered as a defect. The concentration of isobutanol was

below the odor threshold in all gueuze beers and this compound most likely did not play a role in the gueuze beer flavor formation. The concentrations of isoamyl alcohol were around or above the odor threshold (Engan, 1972; Simpson & Miller, 1984).

The concentrations of the ester ethyl lactate increased with the age of the bottled gueuze beers, coinciding with the increase of the lactic acid concentrations. Indeed, the concentrations of the former compound proportionally increased more rapidly compared to the lactic acid concentrations. While the lactic acid concentrations tripled during aging, the ethyl lactate concentrations increased six-fold. Esterification is catalyzed by the esterase of *D. bruxellensis* (Spaepen & Verachtert, 1982) and is enabled by a combination of low pH and high concentrations of lactic acid and ethanol (Van Oevelen *et al.*, 1976). Ethyl lactate is absent in most ale and lager beers, but can be produced during aging of these beers (Vanderhaegen *et al.*, 2003a, 2006, 2007). Ethyl lactate is also associated with the malolactic fermentation in wines (Maicas *et al.*, 1999; Ribéreau-Gayon *et al.*, 2006). It has been defined as an essential flavor compound of gueuze beers (Van Oevelen *et al.*, 1976; Vanderhaegen *et al.*, 2007). Therefore, it could be suggested that ethyl lactate is an important marker compound, indicative for gueuze beer aging, at least up to a certain age. Moreover, considering its estimated odor threshold of 0.25 g/L (Simpson & Miller, 1984), the presence of this compound possibly contributes to a softening of the taste of gueuze beers. The higher concentrations of ethyl lactate in the 2013-3 gueuze beer samples compared to other gueuze beers of the same age clearly demonstrated the bottle-to-bottle variation from the bottling process onwards. A decreasing concentration of isoamyl acetate during the gueuze beer aging process was found; the concentration of this compound is remarkably lower in gueuze beers compared to other beer types, *i.e.*, ales (Langos *et al.*, 2013). A breakdown of isoamyl acetate can also be catalyzed by an esterase that is produced by *D. bruxellensis*, although this compound can also hydrolyze spontaneously during the aging of beers (Spaepen & Verachtert, 1982; Van Oevelen *et al.*, 1976; Vanderhaegen *et al.*, 2003a; Wilhelmson *et al.*, 2012).

Fatty acid ethyl esters possibly contribute to the perceived fruitiness of the gueuze beers (Verstrepen *et al.*, 2003). Ethyl acetate was present in all bottles above the

expected concentration (Van Oevelen *et al.*, 1976). The changes in the concentrations of this compound might however be negligible, compared to the high concentrations that were produced during the lambic beer fermentation process, as no correlations in the evolution of the ethyl acetate concentrations and the evolution of both the acetic acid and ethanol concentrations could be found (Van Oevelen *et al.*, 1976). Nevertheless, ethyl acetate could have a synergistic effect on other esters that were present below their threshold concentrations (Engan, 1972). Other fatty acid ethyl esters, such as ethyl hexanoate, ethyl octanoate, and, in particular, ethyl decanoate are important contributors to the typical flavor of gueuze beers (Spaepen & Verachtert, 1982). The concentrations of ethyl decanoate decreased during aging, while the concentrations of ethyl hexanoate and ethyl octanoate were quite similar for all samples. A decreasing concentration of ethyl decanoate has been reported previously during aging of top-fermented beer (Vanderhaegen *et al.*, 2003a). By the degradation of ethyl decanoate, other flavor volatiles might have prevailed and thus changed the flavor profile of the two oldest gueuze beers sampled (Vanderhaegen *et al.*, 2003a). The strong negative correlations of the evolution of the ethyl decanoate concentrations with the evolution of both the ethyl lactate and lactic acid concentrations, combined with the strong positive correlation with the evolution of the isoamyl acetate concentrations support the identification of ethyl decanoate as a marker for the aging of gueuze beers. Nevertheless, taking into account individual odor thresholds, ethyl hexanoate, ethyl octanoate and ethyl decanoate all contributed to the flavor of all gueuze beers, with the exception for the gueuze beers 2004-2 and 2004-3, in which ethyl octanoate concentrations were below the flavor threshold (Simpson & Miller, 1984). These results are comparable with the evolution during aging of other beer types, such as Belgian ale beers, albeit that these compounds are of lesser importance in the latter beers (Vanderhaegen *et al.*, 2003a, 2006, 2007).

The volatile profile was assessed using a qualitative SPME-GC-MS analysis, as this methodology allows the screening of volatile compounds present in low concentrations, due to the enrichment on the SPME fiber (Rodriguez-Bencomo *et al.*, 2012). Several components commonly associated with *Dekkera* spp. metabolism were found, *i.e.*, 4-ethylphenol, 4-vinylguaiacol, 4-ethylguaiacol, and 2-

phenylethanol (Romano *et al.*, 2008). Additionally, the presence of diethyl succinic acid in all bottles was indicative for aging, since succinic acid and ethanol, both produced during lambic beer fermentation, can lead to the formation of esters during beer storage (Câmara *et al.*, 2006; Martens *et al.*, 1992). Similarly, ethyl isovalerate can also be considered as indicative for beer aging. However, the origin of isovaleric acid is uncertain, as both *Dekkera* spp. and aged hops could be responsible for its presence (Romano *et al.*, 2008; Vanderhaegen *et al.*, 2006).

SIFT-MS analysis was applied to provide more quantitative information concerning the importance of minor volatile compounds. Indeed, SIFT-MS was capable of measuring volatile compounds, as illustrated by the measurement of ethanol. The SIFT-MS technique does not involve prior separation of the volatile sample compounds and as such delivers fast analysis times. However, the lack of prior separation hinders the analysis of complicated matrices (Langford *et al.*, 2014). Possibly, the overwhelming presence of ethanol limited the detection of other volatile compounds, as indicated by the poor agreement between the results obtained by SIFT-MS and those obtained with other techniques.

The low perceived fruitiness in the oldest gueuze beers during the sensory analysis might indicate a loss in flavor complexity upon aging by the abundance of organic acids such as lactic acid and the decrease of ethyl acetate. Together with the data mentioned above, this indicates that gueuze beers are preferably aged for less than 10 years.

Conclusion

The bacterial and yeast communities of bottled gueuze beers converged from *Pediococcus damnosus*, *Dekkera anomala*, *Dekkera bruxellensis*, *Pichia membranifaciens* and *Saccharomyces cerevisiae*, also present in the lambic cask beers they are derived from, to *D. bruxellensis* as the sole isolated species upon aging. These microbiota are present in the casks and can be considered as microbiological biomarkers. Yet, during bottling other microorganisms may enter the beers. The concomitant degradation of residual malto-oligosaccharides might impose a limit on gueuze ageing, as malto-oligosaccharides were absent in bottles aged for more than five years. Similarly, lactic acid and ethyl lactate concentrations increased upon

aging. Ethyl lactate could be considered as a positive gueuze beer-aging metabolite biomarker. Oppositely, the concentrations of isoamyl acetate and ethyl decanoate decreased upon aging. The latter compound could be proposed as a negative gueuze beer-aging metabolite biomarker.

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Supplementary information

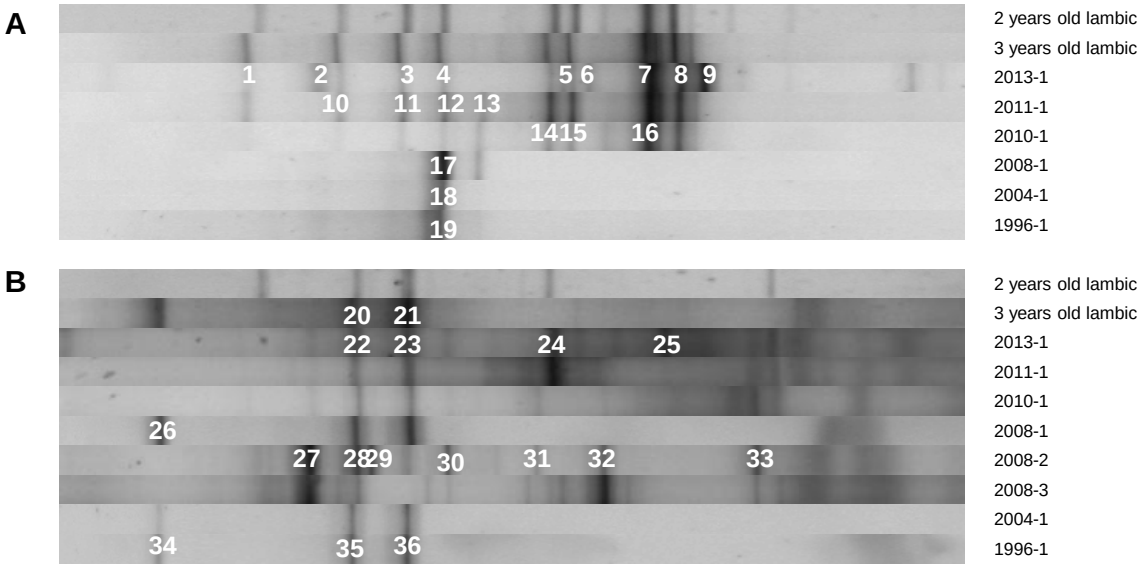

Figure S 6.1.1 Representative DGGE profiles of bacterial (A) and yeast (B) communities in aged gueuze beers. Excised bands that were sequenced are numbered in white font. The assignment of the bands is gathered in Supplementary Table S 6.1.2.

Table S 6.1.1 Results of plate counts on different agar isolation media. MRS agar was used for the growth of LAB, AAM agar was used for the growth of AAB, DYPAI and UBAGI agars were used as global yeast growth media and DYPAIX agar was used for the growth of *Dekkera* species. The values are expressed in log CFU/mL. ULD: under limit of detection (< 20 CFU/mL); ULQ: under limit of quantification (the estimated CFU/mL is provided between brackets); AN: anaerobic growth conditions.

	MRS 20°C AN	MRS 28°C	AAM 28°C	DYPAI 28°C	UBAGI 28°C	DYPAIX 28°C
Three years old lambic	3.90	2.88	3.62	3.91	3.90	ULQ (453)
Gueuze bottled in 2013	5.25	5.31	ULD	ULQ (80)	ULQ (66)	ULQ (80)
Gueuze bottled in 2011	3.87	3.88	ULD	ULD	ULD	ULD
Gueuze bottled in 2010	3.49	3.96	ULD	ULD	ULD	ULD

Table S 6.1.2 Identification of the DGGE bands excised from the polyacrylamide gels (Supplementary Figure S 6.1.1).

Band number	Accession number highest hit*	Similarity	Identification
1	AJ318414	100%	*Pediococcus/Lactobacillus*
2	AJ853891	100%	*Enterobacteriaceae*
3	AB681216	100%	*Pediococcus/Lactobacillus*
4	AB681216	100%	*Pediococcus/Lactobacillus*
5	AB681216	100%	*Pediococcus/Lactobacillus*
6	M59155	100%	*Enterobacteriaceae*
7	AB681216	100%	*Pediococcus/Lactobacillus*
8	AB681216	100%	*Pediococcus/Lactobacillus*
9	AF047186	100%	*Enterobacteriaceae*
10	AJ271383	100%	*Pediococcus/Lactobacillus*
11	AB681216	100%	*Pediococcus/Lactobacillus*
12	AB681216	100%	*Pediococcus/Lactobacillus*
13	AB681216	100%	*Pediococcus/Lactobacillus*
14	AB681216	100%	*Pediococcus/Lactobacillus*
15	AB681216	100%	*Pediococcus/Lactobacillus*
16	AB681216	100%	*Pediococcus/Lactobacillus*
17	FR683099	100%	*Lactobacillus*
18	FR683099	100%	*Lactobacillus*
19	FR683099	100%	*Lactobacillus*
20	AY969049	100%	*Dekkera*
21	AY969049	100%	*Dekkera*
22	AY969049	100%	*Dekkera*
23	AY969049	100%	*Dekkera*
24	BR000309	100%	*Saccharomyces*
25	BR000309	100%	*Saccharomyces*
26	AY969049	100%	*Dekkera*
27	EU011598	100%	*Candida*
28	AY969049	100%	*Dekkera*
29	EF550286	100%	*Candida*
30	EF550286	100%	*Candida*
31	BR000309	100%	*Saccharomyces*
32	BR000309	100%	*Saccharomyces*
33	AY969049	100%	*Dekkera*
34	AY969049	100%	*Dekkera*
35	AY969049	100%	*Dekkera*
36	AY969049	100%	*Dekkera*

*Highest hit with first type strain in BLAST results

Table S 6.1.3 Relevant volatile compounds detected in aged gueuze beers by SPME-GC-MS. The concentration ranges in the aged gueuze beers were determined using the appropriate techniques (HPAEC-CIS, GC-FID, or SH-GC-MS). nq.: not quantified.

Component	Presence in beer bottle	Concentration range (mg/L)
Acids		
2,5-dimethyl-4-hexenoic acid	All bottles	nq.
acetic acid	All bottles	700-2200
octanoic acid	All bottles	nq.
nonanoic acid	Not detected in the bottles of 2011 and 2013	nq.
decanoic acid	All bottles	nq.
Alcohols (aliphatic)		
ethanol	All bottles	3000-4200
1-octanol	All bottles	nq.
2-nonanol	All bottles	nq.
isobutanol	All bottles	10-30
isoamyl alcohol	All bottles	60-150
Aldehydes		
furfural	All bottles	nq.
nonanal	Not detected in the bottles of 1996-2, 1996-3, 2004-2, and 2004-3	nq.
Esters (linear)		
butyl octanoate	All bottles	nq.
ethyl acetate	All bottles	90-320
ethyl butyrate	Bottles of 2004-3	nq.
ethyl valerate	Not detected in the bottles of 1996-2 and 1996-3	nq.
ethyl hexanoate	All bottles	0.38-0.87
ethyl 3-hexenoate	Only detected in the bottles of 2013-2 and 2013-3	nq.
ethyl heptanoate	All bottles	nq.
ethyl octanoate	All bottles	0.5-1.0
ethyl nonanoate	All bottles	nq.
ethyl decanoate	All bottles	nq.
ethyl decanoate	All bottles	2.0-12
ethyl dodecanoate	All bottles	nq.
ethyl tetradecanoate	All bottles	nq.
ethyl hexadecanoate	All bottles	0.13-0.28
ethyl hexadecenoate	Not detected in the bottles of 1996-2 and 1996-3	nq.
Esters (others)		
isoamyl acetate	All bottles	0.04-0.32
isoamyl octanoate	All bottles	nq.
isoamyl decanoate	All bottles	nq.
ethyl isovalerate	All bottles	nq.
ethyl phenylacetate	All bottles	nq.
ethyl lactate	All bottles	40-180
diethyl succinate	All bottles	nq.
Phenolic compounds		
2-phenylethanol	All bottles	nq.
2-phenylethyl acetate	All bottles	nq.
4-ethylguiacol	All bottles	nq.
4-ethylphenol	All bottles	nq.
4-ethyl-1,2-dimethoxybenzene	Not detected in the bottles of 2013-2 and 2013-3	nq.
ethyl benzoate	Not detected in the bottles of 2013-2 and 2013-3	nq.
methyl salicylate	All bottles	nq.
phenol	All bottles	nq.
styrene	All bottles	nq.
4-vinylguaiacol	All bottles	nq.

Table S 6.1.4 Correlations between relevant volatile compounds originating from aged gueuze beers as determined by HPAEC-CIS, GC-FID, or SH-GC-MS. Two-tailed correlations significant at the 0.05 level (*) and 0.01 level are indicated (**) are indicated. N = 12.

		Ethyl lactate	Ethanol	Lactic acid	Acetic acid	Ethyl acetate	Isobutanol	Isoamyl alcohol	Isoamyl acetate	Ethyl hexanoate	Ethyl octanoate	Ethyl decanoate
Ethyl lactate	Pearson Corr.	1	0.536	0.846**	-0.438	-0.238	-0.023	-0.407	-0.860**	-0.429	-0.34	-0.860**
	Sig. (2-tailed)		0.072	0.001	0.155	0.457	0.943	0.19	0	0.164	0.28	0
Ethanol	Pearson Corr.	0.536	1	0.473	0.348	0.117	-0.045	-0.161	-0.267	0.159	0.171	-0.263
	Sig. (2-tailed)	0.072		0.12	0.267	0.716	0.889	0.618	0.402	0.621	0.595	0.409
Lactic acid	Pearson Corr.	0.846**	0.473	1	-0.276	0.017	-0.266	-0.408	-0.742**	-0.279	-0.497	-0.767**
	Sig. (2-tailed)	0.001	0.12		0.385	0.958	0.404	0.188	0.006	0.381	0.1	0.004
Acetic acid	Pearson Corr.	-0.438	0.348	-0.276	1	0.36	0.003	-0.022	0.705*	0.31	0.131	0.408
	Sig. (2-tailed)	0.155	0.267	0.385		0.25	0.991	0.946	0.011	0.327	0.685	0.188
Ethyl acetate	Pearson Corr.	-0.238	0.117	0.017	0.36	1	0.132	0.299	0.239	0.395	0.057	0.013
	Sig. (2-tailed)	0.457	0.716	0.958	0.25		0.683	0.345	0.454	0.203	0.861	0.967
Isobutanol	Pearson Corr.	-0.023	-0.045	-0.266	0.003	0.132	1	0.465	0.243	-0.142	0.391	0.083
	Sig. (2-tailed)	0.943	0.889	0.404	0.991	0.683		0.128	0.447	0.66	0.208	0.798
Isoamyl alcohol	Pearson Corr.	-0.407	-0.161	-0.408	-0.022	0.299	0.465	1	0.23	0.397	0.645*	0.567
	Sig. (2-tailed)	0.19	0.618	0.188	0.946	0.345	0.128		0.472	0.202	0.023	0.054
Isoamyl acetate	Pearson Corr.	-0.860**	-0.267	-0.742**	0.705*	0.239	0.243	0.23	1	0.303	0.333	0.763**
	Sig. (2-tailed)	0	0.402	0.006	0.011	0.454	0.447	0.472		0.338	0.291	0.004
Ethyl hexanoate	Pearson Corr.	-0.429	0.159	-0.279	0.31	0.395	-0.142	0.397	0.303	1	0.712**	0.569
	Sig. (2-tailed)	0.164	0.621	0.381	0.327	0.203	0.66	0.202	0.338		0.009	0.054
Ethyl octanoate	Pearson Corr.	-0.34	0.171	-0.497	0.131	0.057	0.391	0.645*	0.333	0.712**	1	0.653*
	Sig. (2-tailed)	0.28	0.595	0.1	0.685	0.861	0.208	0.023	0.291	0.009		0.021
Ethyl decanoate	Pearson Corr.	-0.860**	-0.263	-0.767**	0.408	0.013	0.083	0.567	0.763**	0.569	0.653*	1
	Sig. (2-tailed)	0	0.409	0.004	0.188	0.967	0.798	0.054	0.004	0.054	0.021	

Table S 6.1.5 Pearson correlation coefficient for relevant volatile compounds from aged gueuze as determined by SIFT-MS and HPAEC-CIS, GC-FID, or SH-GC-MS. Two-tailed correlations significant at the 0.05 level (*) and 0.01 level (**) are indicated. N = 12.

Component	SIFT-MS
Ethanol (GC-FID)	0.152
Lactic acid (HPAEC-CIS)	0.735**
Acetic acid (GC-FID)	0.078
Ethyl acetate (SH-GC-MS)	0.652*
Isoamyl acetate (SH-GC-MS)	0.021
Ethyl hexanoate (SH-GC-MS)	-0.405
Ethyl octanoate (SH-GC-MS)	-0.157

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Part IV
Discussion

Chapter 7. General discussion and future perspectives

Acidic lambic beers are the result of a spontaneous fermentation process that lasts for one to three years (De Keersmaecker, 1996; Verachtert & Iserentant, 1995). Some other acidic beers are produced using backslopping, *i.e.*, the repitching of a mixed culture of yeasts and bacteria from a previous fermentation (Martens *et al.*, 1997; Verachtert & Derdelinckx, 2005). Lambic beers in particular and spontaneously fermented beers in general are currently highly appreciated all over the world since they are trademarks of traditional craftsmanship. Traditionally, Belgian lambic beers were produced in the Senne river valley (southwest of Brussels) and in the southeast of Brussels.

Notwithstanding the increasing popularity of lambic beers, the fermentation process was studied only in some detail in a few microbiological studies between 1976 and 1995 by the research group of Prof. em. Hubert Verachtert (Martens *et al.*, 1991, 1992; Shanta Kumara & Verachtert, 1991; Spaepen *et al.*, 1978, 1979; Van Oevelen *et al.*, 1976, 1977; Verachtert & Iserentant, 1995). These studies were performed using biochemical methods solely and were limited in the number of isolates dealt with and the taxonomical information that was obtained (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). Since the publication of these early studies, the taxonomy of bacteria and yeasts involved in the lambic beer fermentation process underwent several changes and biochemical identification methods were shown inadequate to reliably identify these microorganisms (Cleenwerck & De Vos, 2008; De Bruyne *et al.*, 2008; Kämpfer & Glaeser, 2012; Kurtzman & Robnett, 1998; Nhung *et al.*, 2007).

The present study was initiated for a number of reasons. First, to update our knowledge of the microbial succession in the lambic beer fermentation process and to assess the applicability of MALDI-TOF MS as a dereplication tool to identify hundreds of microbial isolates obtained. Furthermore, to investigate differences between the lambic beer fermentation processes in a traditional and an industrial brewery and in addition to unravel the story of the Senne valley. Finally, as lambic

beers are mostly blended to produce gueuze beers, which are refermented in bottles, these beers can be matured for several years, and therefore the microbiota and metabolites present in several aged gueuze beers were also examined.

7.1 The microbiology of traditional lambic beer fermentation

The present study aimed to exhaustively describe the microbial communities involved in the spontaneous fermentation process of lambic beers. Previous studies examined the microbiota and metabolite profiles of traditional lambic beers over a period of two years. In these studies, samples obtained from multiple brews and multiple casks from a single brewery were analyzed and covered a two-year monitoring period (Van Oevelen *et al.*, 1976, 1977). Moreover, the microbiota present was monitored using culture-dependent techniques only and a limited number of isolates were identified using biochemical identification techniques. In the present study, the traditional lambic beer fermentation process was monitored in two independent batches of lambic beer during a period of two years by sampling the same casks. The microbial communities were studied through state-of-the-art identification of more than 2000 bacterial and yeast isolates, collected at nine time points during the fermentation process and using culture-independent PCR-DGGE of both the bacterial and yeast communities.

Previous research of the microbiota and their metabolites divided the lambic beer fermentation process into four phases: the *Enterobacteriaceae* phase, the main fermentation phase, the acidification phase, and the maturation phase (Van Oevelen *et al.*, 1976; Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). Each phase was characterized by the presence of specific microorganisms and metabolites (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). The culture media used were selected based on previous studies and the observation of increased concentrations of acetic acid and lactic acid, indicating the presence of AAB and LAB (Van Oevelen *et al.*, 1976). *Enterobacteriaceae* were isolated from the cooled wort in the cooling tun and the cask (Martens *et al.*, 1991; Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). The *Enterobacteriaceae* phase was reported to start after 3 to 7 days of fermentation, when cell numbers reached up to 10^8 CFU/mL, to proceed for 30 to 40 days, and to be characterized by *Enterobacter cloacae* and *Klebsiella pneumoniae* as the predominantly isolated *Enterobacteriaceae* species (Martens *et al.*, 1991).

Enterobacter aerogenes, *Citrobacter freundii*, *Escherichia coli* and *Hafnia alvei* were additionally isolated (Martens *et al.*, 1991), along with the cycloheximide-resistant yeasts *Hanseniaspora uvarum* (its asexual form is named *Kloeckera apiculata* [Meyer et al., 1978]) and *Naumovia dairensis (*previously known as *Saccharomyces dairensis)* (Kurtzman, 2003) as well as *S. uvarum* (previously known as *S. globosus* [Nguyen and Gaillardin, 2005]) (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). During this phase, the pH dropped one value, and considerable levels of butanediol and dimethyl sulfide (DMS) were formed, along with formic acid, acetic acid and lactic acid, and ethanol (Verachtert & Iserentant, 1995).

A similar start of the lambic beer fermentation was found in the traditional brewing process (two batches) examined during the present study (**Chapter 4.1**; Figure 7.1.1). Cooled wort already contained high cell numbers of *Enterobacteriaceae* in the cooling tun (10^6-10^7 CFU/mL). The number of *Enterobacteriaceae* was the highest (10^8 CFU/mL) after 1 to 2 weeks. *Escherichia coli* was also isolated in the present study. In contrast to previous studies, however, *Hafnia paralvei* was isolated an opportunistic human and animal pathogen (Huys *et al.*, 2010). It is likely that isolates from previous studies were in fact also *H. paralvei*, as this species was separated from *H. alvei* only very recently (Huys *et al.*, 2010). Other isolates from the *Enterobacteriaceae* phase were identified as *Enterobacter hormaechei*, *Enterobacter kobei*, *Klebsiella oxytoca*, *Citrobacter gillenii* and *Raoultella terrigena*. All these species are coliform bacteria and thus indicator microorganisms for fecal contamination of surface waters and foods. Although these species are considered to be opportunistic pathogens, they are commonly found in various spontaneously fermented foods and beverages, and some were previously isolated from lambic beer as well (Bokulich *et al.*, 2012; Chao *et al.*, 2013; Martens *et al.*, 1991). Remarkably, most of these microorganisms were previously reported as spoilage microorganisms in sweet unfermented wort and pitching yeast (Bokulich & Bamforth, 2013; Van Vuuren & Priest, 2003; Vriesekoop *et al.*, 2012).

Debaryomyces hansenii and *Saccharomyces cerevisiae* were two yeast species isolated immediately after the transfer of the wort into the cask. *Saccharomyces pastorianus* and *Naumovia castelii* were subsequently isolated from the one-week old wort sample. *Debaryomyces hansenii* is a known beer spoilage yeast (Bokulich &

Bamforth, 2013), whereas *N. castellii* was previously known as *Saccharomyces castellii* and part of the *Saccharomyces sensu stricto* group (Kurtzman, 2003). A DNA band originating from *H. uvarum* was detected in the bacterial PCR-DGGE community profiles of the traditionally produced lambic wort samples, but this species was not isolated. *Hanseniaspora uvarum* has a low fermentative capacity and is commonly isolated during the first phases of wine and cider fermentations (Beltran *et al.*, 2002; Morrissey *et al.*, 2004). Sometimes, this species is found at later stages of wine fermentations, since it can survive elevated ethanol concentrations (Ocón *et al.*, 2010; Wang & Liu, 2013). Similar to *Dekkera bruxellensis*, *H. uvarum* is capable of producing ethyl esters, and it was long considered as a wine spoilage yeast (Romano *et al.*, 2003). Currently, this species is increasingly regarded as beneficial for the complex aroma it can add to wines (Moreira *et al.*, 2011). *Hanseniaspora uvarum* originates from the fruit surface in wine and cider fermentation processes (Beltran *et al.*, 2002), but it is known as an environmental contaminant in ales and lagers (Bokulich & Bamforth, 2013; Manzano *et al.*, 2011).

The *Enterobacteriaceae* phase was followed by the main fermentation phase or alcoholic fermentation phase, which started after 3 to 4 weeks. *Pediococcus damnosus* [*P. cerevisiae*] was commonly isolated during and after this main fermentation phase, in addition to low numbers of AAB (*Acetobacter* spp. and *Gluconobacter* [*Acetomonas*] spp.), which were isolated irregularly (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). Unfortunately, the latter isolates were not identified to the species level. The main fermentation phase was however primarily characterized by the predominant isolation of *S. cerevisiae, S. bayanus/S. pastorianus* and *S. uvarum* (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). During this phase, the majority of the ethanol present in the lambic beers was produced, and the level of DMS (produced during the *Enterobacteriaceae* phase) decreased by the exit of CO_2 bubbles produced by the yeasts still present from the fermenting lambic beer (Verachtert & Iserentant, 1995). Simultaneously, higher alcohols, fatty acids and esters, including hexanoate, octanoate, decanoate and their ethyl esters respectively, were formed as well (Spaepen *et al.*, 1978; Van Oevelen *et al.*, 1976; Verachtert & Iserentant, 1995).

In the present study, many samples were collected during this main fermentation phase. *Acetobacter lambici* (**Chapter 5.1**) was first isolated at the end of the main fermentation phase of batch 2 in the traditional brewery (**Chapter 4.1**). This species and one isolate of *Gluconobacter cerevisiae* were occasionally isolated during the lambic beer fermentation process. From 2 months onwards, *P. damnosus* was consistently present (Figure 7.1.1). High numbers of *S. cerevisiae* and *S. pastorianus* were present at the start of the main fermentation phase, but after three months of fermentation most isolates were identified as *S. pastorianus*. Previous studies only reported the presence of *S. cerevisiae, S. bayanus/S. pastorianus* and *S. uvarum* in the main fermentation phase and did not provide detailed information for different sampling moments. It is however not clear why *S. pastorianus* outlives *S. cerevisiae* in the lambic beer fermentation process of the traditional brewery in the present study. The genomic background of the hybrid species *S. pastorianus* was recently elucidated, as discussed in **Chapter 2.2** (Libkind *et al.*, 2011). In *Saccharomyces*, especially the hybridization events between cryotolerant and non-cryotolerant *Saccharomyces* species offer a benefit for the resulting hybrids, because of the capacity of these hybrids to ferment at lower temperature (Peris *et al.*, 2012). Consequently, all commonly used lager-type yeasts are domesticated strains of the initial pastorianus and bayanus hybrids (Libkind *et al.*, 2011). The ambient temperature of the rooms where lambic beers are fermenting is rarely 20°C or higher during the first fermentation months, which may explain the predominance of *S. pastorianus* in the traditional lambic brewery process. Vidgren *et al.* (2010) reported that ale (generally *S. cerevisiae*) and lager (generally *S. pastorianus* or *S. bayanus*) strains exhibit a similar maltose transport activity at 20°C, but at 0°C, the activity of lager strains was higher by the expression of cryotolerant maltose and maltotriose transporters. The different temperature sensitivity of the maltose and maltotriose transporters could have an influence on the survival of different *Saccharomyces* hybrids, since the transport of these molecules is assumed to be the rate-limiting step in the utilization of these sugars (Cousseau *et al.*, 2013).

After 2 to 3 months of main fermentation, an acidification phase has been reported that was characterized by the increasing isolation of *Pediococcus* and occasionally *Lactobacillus* strains (only in breweries with large casks), while *Dekkera* strains

became prevalent after 4 to 8 months of fermentation. Simultaneously, the number of *Saccharomyces* yeasts decreased (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). *Dekkera* and LAB species have a synergistic effect on beer attenuation (Andrews & Gilliland, 1952; Shanta Kumara & Verachtert, 1991). The acidification was characterized by a strong increase in lactic acid and ethyl lactate concentrations (Verachtert & Iserentant, 1995). During the warm summer months, LAB can cause slime in the fermenting lambic beer (Van Oevelen & Verachtert, 1979; Van Oevelen *et al.*, 1977). The final maturation phase, during which the wort was gradually attenuated and *Dekkera* strains were dominant, started after 10 months of fermentation. During this phase, cell-bound esterases of *Dekkera* yeasts can form and degrade several esters in the fermenting lambic beer (Spaepen & Verachtert, 1982). Furthermore, *Dekkera* yeasts can degrade the high-molecular-mass dextrins, which disappeared from the lambic beer (Shanta Kumara *et al.*, 1993; Verachtert & Iserentant, 1995). At the end of this phase, after about 2 years, the number of LAB and *Dekkera* yeasts was reported to decrease (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). AAB were also isolated during these phases.

In the present study, the analysis of the microbiota at three and six months of fermentation did not allow to discriminate between an acidification phase and a maturation phase (Figure 7.1.1). A decrease of *Saccharomyces* yeasts and a consecutive increase of *Dekkera* yeasts was not observed, which is characteristic for the acidification phase. Indeed, yeast isolates from the three-months old sample were identified as *Saccharomyces* spp., those of the six-months old sample were identified as *Dekkera* spp. The ambient temperature of the cask storage room does have an influence on the pace of the fermentation and the ability of *D. bruxellensis* to dominate the fermentation, as observed during the monitoring of the two lambic fermentation batches (**Chapter 4.1**). Isolates of *D. bruxellensis* were predominantly isolated during the maturation phase of batch 2 (**Chapter 4.1**). The yeast species distribution of batch 1, which was fermenting at cellar temperature, was more complex (**Chapter 4.1**) and included *Candida patagonica*, *Dekkera anomala, Pichia membranifaciens*, *Priceomyces carsonii*, and *Wickerhamomyces anomalus*. The number of LAB was elevated in the six-months old sample compared to the three-months old sample, and reached cell numbers that were comparable to those of

Dekkera yeasts. Consequently, the acidification probably occurred very rapidly between the sampling at three and six months and it was therefore considered as a part of the long maturation phase. The same was found in a study of American coolship ales (ACA) (Bokulich *et al.*, 2012). LAB were isolated from all samples after the *Enterobacteriaceae* phase. In contrast to previous studies, *P. damnosus* was the only LAB species found. It is unclear why *P. damnosus* was the only LAB species isolated, while other studies reported the general presence of *Lactobacillus* spp., including *Lactobacillus brevis,* a well-known beer spoilage bacterium (De Cort *et al.*, 1994; Shanta Kumara & Verachtert, 1991; Vriesekoop *et al.*, 2012). Finally, AAB were isolated irregularly during the traditional lambic beer fermentation process, *i.e.*, from 3 months onwards. All isolates belonged to the newly described AAB species, *A. lambici* and *G. cerevisiae* (**Chapter 5**).

As part of a study of the microbiota of aging gueuze beers, an additional maturation phase sample of the same batch of the fermenting lambic beer that was three years old, was obtained (**Chapter 4.1** and **Chapter 6**). The microbial communities present in this three-years old lambic beer were highly similar to those present in the two-years old lambic beer (**Chapter 4.1**) and consisted of *P. damnosus*, *A. lambici*, *P. membranifaciens*, *D. bruxellensis*, *D. anomala, C. patagonica* and *W. anomalus*. This contrasted with the results of Verachtert and Iserentant (1995), who reported a decrease in the number of LAB and yeasts towards the end of the fermentation process and suggested that this microbiota is highly adapted to growth and survival in lambic beer.

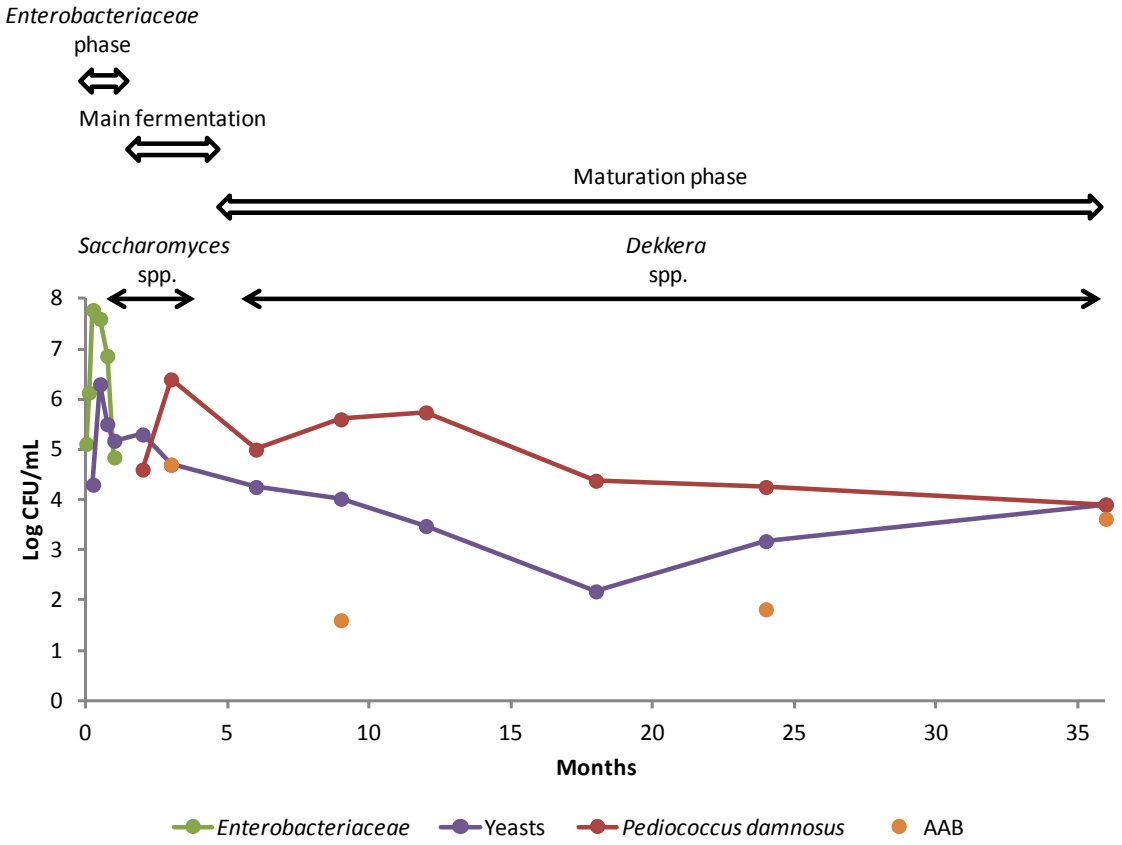

Figure 7.1.1 Schematic overview of the traditional lambic beer fermentation process, as unravelled during the present study.

7.2 The microbiology of industrial lambic beer fermentation

In the present study, the microbiota of a traditional and an industrial lambic beer fermentation process were compared, but also technical characteristics of both production processes differed, which might influence the microbiota and thus the fermentation process. In the industrial brewery, lambic wort was made using an infusion mashing rather than a turbid mashing scheme, the wort was acidified at the end of the wort boiling to pH 4 using lactic acid and, finally, the wort was prechilled after boiling, before it was transferred to the cooling tun. Together, this enables the industrial brewery to produce lambic beers throughout the year.

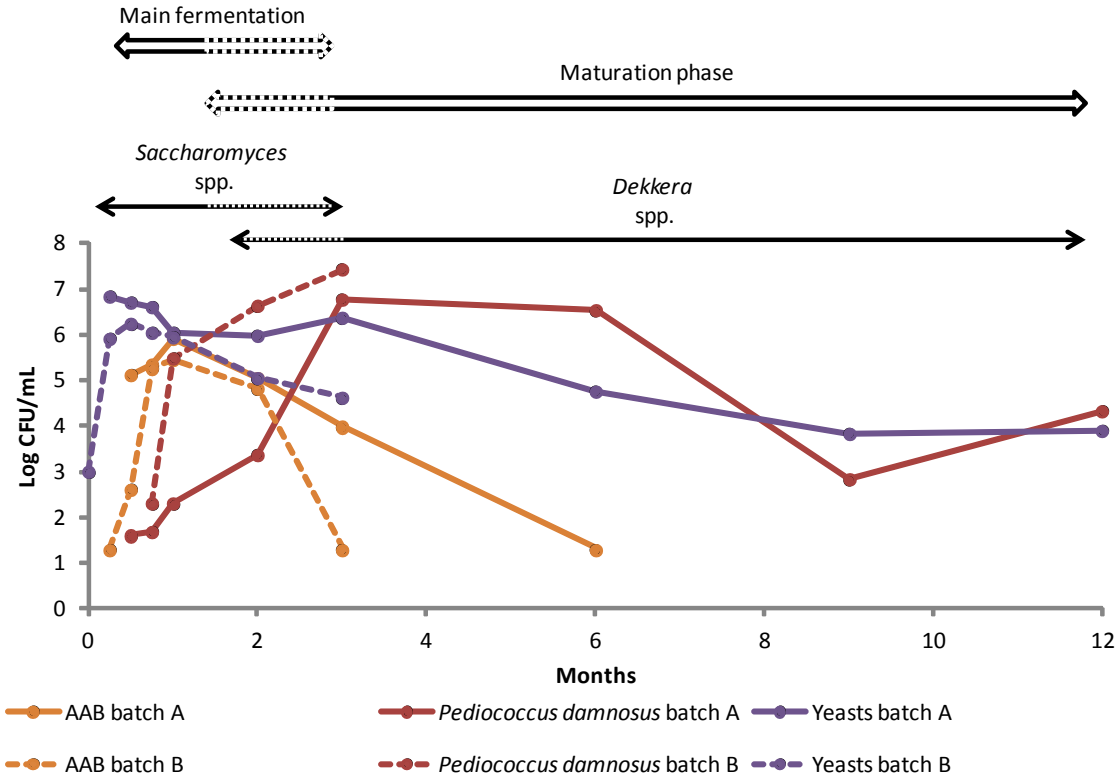

Figure 7.2.1 Schematic overview of the industrial lambic beer fermentation process, as unravelled during the present study.

In contrast to the traditional lambic beer fermentation process, none of the cooling tun samples of the industrial brewery yielded DNA or isolates (**Chapter 4.2**; Figure 7.2.1). Furthermore, members of the *Enterobacteriaceae* could not be isolated during the industrial lambic beer fermentation process, nor could their DNA be detected through PCR-DGGE experiments. Most likely, the acidification of the boiled wort before chilling prevented the growth of *Enterobacteriaceae*, which is known to be inhibited below pH 4 (Priest & Stewart, 2006). Bacteria and yeasts were isolated as soon as the cooled wort was transferred into the cask. These early isolates were identified as *Pichia kudriavzevii*, *D. hansenii* and *Acetobacter orientalis* (**Chapter 4.2**, batch B) and AAB were isolated from the start of the industrial fermentation process up to 6 months, which again contrasted with the traditional fermentation process. *Saccharomyces cerevisiae* was already dominant after one week of fermentation, but also *D. bruxellensis* was isolated from this sample. *Pediococcus damnosus* was present from 3 weeks onwards. If the dominance of *D. bruxellensis* and *P. damnosus* is again used to demarcate the maturation phase, like in the traditional lambic

fermentation process (**Chapter 4.1**) (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995), then the main fermentation phase lasted for one month only (**Chapter 4.2**, batch B). *Hanseniaspora uvarum* was not characteristic, as it was only found in the initial samples of a sluggishly starting fermentation batch (**Chapter 4.2**, batch A), which confirmed that this species has a low fermentative capacity, as discussed above.

Overall, the microbiota present during the maturation phase was the same as the one in the traditional lambic beer fermentation process, although the species diversity was more simple. Like in the traditional lambic beer fermentation, the main fermentation phase of the industrial lambic beer fermentation process was dominated by *Saccharomyces* spp. (**Chapter 4.2**). The dominant isolation of *S. cerevisiae* from batch B samples in the warm summer months (**Chapter 4.2**) supports the hypothesis that the dominance of *S. pastorianus* at the end of the lambic beer fermentation process in the traditional brewery is due to the tolerance of the maltose and maltotriose transporter of this species towards low temperatures.

7.3 The inoculation source of the spontaneous lambic beer fermentation process

The cooling tun sample of the traditional lambic brewery yielded various *Enterobacteriaceae* species (both through cultivation and PCR-DGGE), but no LAB or other microorganisms (**Chapter 4.1**). Additionally, *Dekkera* spp. were isolated from air samples of the traditional brewery, which confirmed the results of previous studies (**Chapter 4.1**) (Verachtert & Iserentant, 1995). LAB and yeasts might have been present in very low numbers in the cooling tun sample, compared to the *Enterobacteriaceae,* thus inhibiting their detection by cultivation or via PCR-DGGE. Nevertheless, sources other than brewery air may still be responsible for the inoculation of the wort.

Besides the brewery air, another potential source of inoculation is the wood used in the brewery. Construction wood that is not covered or treated with paint is present in the truss of the cooling tun room and can be used as ceiling of the cask storage room of traditional breweries. The Belgian Federal Agency for the Safety of the Food Chain published a guide for auto-control of hygienic conditions in the breweries in

2007 (Belgian Federal Agency for the Safety of the Food Chain, 2007). Breweries should have adopted easy to maintain surfaces in the production area and the use of untreated wood was prohibited. However, several exceptions were included for the production of spontaneously fermented beers. Ceilings of the brewery should be free of moisture, except for the cooling tun room when lambic beer is brewed. Additionally, the presence of untreated wood was allowed in the production areas of lambic and red brown ale breweries (Belgian Federal Agency for the Safety of the Food Chain, 2007). These guidelines therefore acknowledge the importance of untreated wooden surfaces in the brewery as a major source of microbiota that could be introduced into the wort. Most likely, the reuse of non-sterile wooden barrels enhances the success of the fermentation, as is the case in natural cider fermentation processes (del Campo *et al.*, 2003). Wooden tools and casks are known as safe harbors for bacteria and yeasts that are present during the spontaneous fermentation of wines and ciders (Swaffield & Scott, 1995; Swaffield *et al.*, 1997). These microorganisms can penetrate the wood in a short period of time, where they are protected from cleaning procedures (Barata *et al.*, 2013; Guzzon *et al.*, 2011). Additionally, these microorganisms can survive for a prolonged time in the pores via micro-oxygenation (De Rosso *et al.*, 2008; Hidalgo *et al.*, 2010; Torija *et al.*, 2009). Clearly, it is conceivable that the lambic beer microbiota too will persist in cask wood after the cleaning procedure, which consists of washing the inside of the cask with cold water and a treatment with low-pressure steam.

Yet, spontaneous fermentation processes were reported to be successful even when new, unused casks and stainless steel fermentors were applied for the production of lambic beers (Verachtert & Derdelinckx, 2005). The microbiota and metabolites of the lambic beers in the latter study were monitored over a period of 18 months and the typical characteristics of lambic beers were found (Verachtert & Derdelinckx, 2005). These characteristics include the presence of *Enterobacteriaceae*, *Saccharomyces* spp., *Dekkera* spp. and LAB, together with the presence of ethanol, acetic acid and lactic acid, and ethyl acetate and ethyl lactate (Verachtert & Derdelinckx, 2005). Furthermore, the wort was highly attenuated and there was a clear drop in pH (Verachtert & Derdelinckx, 2005). The authors also stated that all lambic worts will reach the expected characteristics of a lambic beer after a one-year

fermentation, irrespective of the fermentation profile or initial microbial load of the individual worts (Verachtert & Derdelinckx, 2005). Also, lambic beer fermentation was reported successful and yielded similar products when wort, brewed and cooled in a first lambic brewery, was fermented in other breweries (Verachtert & Derdelinckx, 2005). It should further be noted that a huge amount of carbon dioxide is produced during the main fermentation phase in traditional lambic breweries, which causes an overflow of beer through a temporarily loose plug in the bung hole. The brewer tops the casks off with fermenting wort of other casks to decrease the cask headspace and replaces this temporary plug with a permanent wooden plug or rubber stopper after the main fermentation phase. These practices will influence the microbiota composition and the fermentation process too. Together, these observations demonstrate that the sources of inoculation in the traditional lambic brewery may be diverse and brewery-dependent and/or that the impact of individual microorganisms on the resulting lambic beers may be overestimated. Together, these data also demonstrated that lambic beer fermentation is robust in the Senne river valley (Verachtert & Derdelinckx, 2005).

Traditionally, the production of lambic beers was assumed to be only possible in the Senne river valley and the use of the cooling tun enabled the inoculation of the wort with the microbiota that were uniquely present in the air of the Senne river valley (Verachtert & Iserentant, 1995). However, two breweries in West-Flanders and therefore located outside the Senne river valley, and several American craft breweries have successfully adopted spontaneous fermentation processes for the production of lambic beer and American coolship ale, respectively (Bokulich *et al.*, 2012). Overnight cooled wort samples from the cooling tun of the industrial lambic brewery were not inoculated (**Chapter 4.2**). In contrast, as soon as wort was transferred into the cask, there was a detectable microbiota through cultivation. This unambiguously indicated that in the industrial brewery, the microbiota responsible for the fermentation did not originate from the air and, hence, the Senne river valley is not a *conditio sine qua non*. In addition, the industrial lambic beer fermentation process lacked an *Enterobacteriaceae* phase, while such a phase was present in the traditional lambic beer fermentation process studied (**Chapter 4**) (Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995) and in the ACA

fermentation process (Bokulich *et al.*, 2012). In contrast to the traditional brewery, in the industrial brewery all surfaces are covered or treated with antifungal paint, which renders the surfaces smooth and easy to clean. Hence, there are no untreated wooden surfaces in the industrial brewery, except for the cask wood. Consequently, the influence of the cask wood on the successful inoculation and subsequent lambic beer fermentation is likely to be larger in the industrial brewery than in the traditional brewery. The wort in an industrial lambic brewery is chilled and the air flow in and near the cooling tun is upwards instead of sideways or downwards. The chilling temperature may influence the microbial growth in the cooling tun. Indeed, the wort of the sluggishly fermenting batch A in the industrial brewery (**Chapter 4.2**) was chilled to a lower temperature than normally applied. Batch B of the industrial brewery was correctly chilled and isolates were obtained from the freshly transferred cask wort sample and the fermentation was initiated within one week (**Chapter 4.2**). Hence, the wort did not contain bacteria when it was transferred to the cask and only the low fermentative *H. uvarum* yeasts grew, rather than *Saccharomyces* spp. (**Chapter 4.2**). 'Rebooting' such sluggishly fermenting lambic batches by mixing it with another batch is a commonly applied procedure in this brewery and new barrels are filled with fermenting lambic wort prior to their first use. Most likely, the production of lambic beers in the industrial brewery is facilitated through a lambic core microbiota that is enriched in the cask wood. The close monitoring and mixing of aberrantly fermenting batches enables the brewery to control the fermentation process outcome. The lambic beer fermentation process in the industrial brewery is therefore successful without inoculation in the cooling tun and the transfer of chilled wort into a cask used previously for lambic beer production is sufficient to obtain a normal lambic beer fermentation process. However, when the wort would be cooled and transferred directly into a stainless steel tank, the fermentation probably will not take place, as described in the production of cider (del Campo *et al.*, 2003).

7.4 The microbiota and metabolites of aging gueuze beers

In Chapter 6, the microbiota and selected metabolites of aged gueuze beers that were produced in the traditional brewery studied earlier were analyzed (**Chapter 4.1**). Young and old lambic beers are blended by the brewer to make gueuze beers,

which spontaneously referment after bottling, a process that is referred to as 'aging'. The gueuze beers examined in the present study were bottled between 5 months and 17 years prior to the sampling. Although time constraints prevented to monitor a single production batch for a prolonged period of time, the present study revealed changes in microbiota and metabolites that can be used as a proxy of the processes that occur during gueuze maturation. All gueuze beers showed the characteristic presence of *D. bruxellensis* and comprised acetic acid, lactic acid, ethyl acetate and ethyl lactate as most important metabolites. While pediococci were readily isolated from one-, two- and three-years old lambic beer, and from the gueuze beer bottled a few months before sampling, LAB or any other bacteria were no longer isolated from gueuze beers bottled prior to 2010. Different yeast species including *D. bruxellensis*, *D. anomala*, *P. membranifaciens* and *S. cerevisiae* were isolated from recently bottled gueuze, but this diversity decreased with age until only *D. bruxellensis* was isolated via enrichment culturing, even from the 17-years old gueuze beer. The low nutritional demand of this yeast species probably enabled its long-term survival in this environment (Aguilar Uscanga *et al.*, 2000; Renouf *et al.*, 2007). The latter is also supported by the versatile metabolism of *Dekkera* yeasts, which can both produce and assimilate carbon sources such as acetic acid and ethanol (Renouf *et al.*, 2007). The yeast cells in these old gueuze beers most likely occurred in a VBNC state that could be reversed by the enrichment culture. The VBNC state allows yeast (and bacterial) cells to withstand several stress conditions (Millet & Lonvaud-Funel, 2000).

Further, the metabolite analyses revealed that the aging of gueuze beer is probably limited in time by the depletion of the available malto-oligosaccharides. Malto-oligosaccharide concentrations were very low in the gueuze beer samples of 2004 and 1996 and no further increase in the concentrations of lactic acid was found between the gueuze beer samples of 2004 and 1996. Furthermore, the acetic acid concentrations were the lowest in the gueuze beers bottled in 1996, indicating that acetic acid was further metabolized and that no new acetic acid was produced. Lactic acid concentrations increased steadily for beers aged up to 10 years, but no further increase was noticed in the beers that were aged for 17 years.

Additionally, the typical fruitiness was no longer perceived in the sensory analysis in the oldest gueuze beers examined (**Chapter 6**). This was probably caused by the degradation of the fatty acid ethyl esters, which are known to add fruitiness in beer. The increasing concentrations of ethyl lactate and decreasing concentrations of ethyl decanoate could be considered as positive and negative gueuze beer-aging metabolite biomarkers, respectively.

7.5 The use of culture-dependent and culture-independent techniques in microbial biodiversity studies

Microbial biodiversity studies have long been performed using culture-independent community fingerprint techniques in combination with traditional cultivation methods (Dolci *et al.*, 2010; Scheirlinck *et al.*, 2008; Van der Meulen *et al.*, 2007; Wouters *et al.*, 2013). Some studies focus on specific microbial groups of the communities, *e.g.*, only the LAB or yeast diversity. In recent years, more advanced and high-throughput culture-independent methods, such as bar-coded amplicon sequencing and metagenomics, are gradually replacing community fingerprint methods, such as the DGGE approach applied in the present study. The data obtained are considered superior compared to culture-dependent data. Indeed, methods that involve cultivation of microorganisms are often considered less informative, as some microorganisms can be present in a VBNC state, and isolation media favor the cultivation of specific microorganisms only (Gorski, 2012; Millet & Lonvaud-Funel, 2000). Moreover, these metagenomic techniques are also superior to classical fingerprint-based culture-independent techniques such as PCR-DGGE, because of their ability to detect low abundant species in the communities (Bokulich & Bamforth, 2013).

However, culture-independent techniques also introduce biases, for instance through efficacy of DNA extraction or through PCR-based amplification of target sequences (Hong *et al.*, 2009; Yuan *et al.*, 2012), and potentially detect not only metabolically inactive but also dead cells. So, although these modern culture-independent analyses provide a more in-depth analysis of the microbial community composition, they are not without limitations and also do not reveal which species are metabolically most active. Other tools, not only transcriptomics or meta-

metabolomics but also the availability of pure cultures of the community members will be required to reveal a more complete image of the microbial diversity present.

Ironically, the potential to examine microbial ecosystems by means of modern metagenomics approaches triggered a renewed interest in the development of new approaches to cultivate a large number of microorganisms that are known through the detection of their DNA only (Nature Reviews: Microbiology Editorial, 2013; Rappé, 2013; Teske, 2010). Based on metagenomics information about the genes and metabolic potential present, new culture media are being developed that target the isolation of specific microbial groups (Bomar *et al.*, 2011). An approach referred to as culturomics (Lagier *et al.*, 2012) is currently gaining momentum in the field of gut microbiome research. Culturomics refers to the high-throughput and miniaturized application of numerous classical media for the isolation of microorganisms and is limited only by the rate of identification of the isolates obtained. For this purpose, sequence-based identification methods are too slow and expensive but MALDI-TOF MS has been advocated as an ideal identification technique in culturomics approaches to study microbial diversity (Lagier *et al.*, 2012). Interestingly, culturomics was found to outperform bar-coded sequence analysis for the discovery of the rare microbiota in the gut microbiome of an immunocomprised patient (Dubourg *et al.*, 2013) and another culturomics study of the human microbiome found not less than 31 new species (Lagier *et al.*, 2012).

7.6 MALDI-TOF MS

Since the start of the present study in October 2009, MALDI-TOF MS research and studies applying this technique for the identification of microorganisms are increasingly reported and its range of applications is expanding.

Its widespread application in food microbiology will require purpose-built databases, for instance of microorganisms relevant in food fermentation processes. Its high-throughput capacity can however be exploited already for the dereplication of large numbers of isolates. In the present study, MALDI-TOF MS was only used for the latter purpose. The instrument used, a 4800 Plus MALDI TOF/TOF™ Analyzer (AB Sciex, Framingham, MA, USA), was not designed for the identification of microorganisms and there was no support of the manufacturer for this application.

Spectra were exported into the BioNumerics version 5.1 software package (Applied Maths), which lacked an efficient peak-picking algorithm. Therefore, curve-based analysis methods were used for dereplication and combined with sequence analysis of representative isolates to identify the different MALDI-TOF MS clusters obtained. The development of a proper identification database for the lambic beer microbiota became feasible by the recent availability of the BioNumerics version 7.1 software package (June 2013).

During the present study, in-house sample preparation protocols for bacteria were optimized (Wieme *et al.*, 2014), but sample preparation of yeast cells was more problematic. Finally, good spectra were obtained from yeast cells by increasing the amount of cells used for the cellular extract preparation from one to three 1-µL loops. Increasing the amount of cells used for the cellular extract preparation also proved effective to generate good quality spectra of some problematic bacteria (data not shown), which confirmed the results reported by Marklein *et al.* (2009).

7.7 Protection of lambic beers

Industrial and traditional lambic breweries compete for the same consumers. The production scale and the use of beers other than 100% spontaneously fermented lambic beers for the production of gueuze beers, along with a lagging legislation that fails to clarify the differences between industrially and traditionally produced lambic beers, increasingly threaten traditional lambic beer breweries. In 1997, lambic and gueuze beers were entitled to use the endorsement 'traditional specialty guaranteed product' by the European Commission (1997a, b). Lambic beer was defined as a spontaneously fermented beer, in which *D. bruxellensis* plays a crucial role in the maturation. In addition, specifications for lambic beer were provided and included that the beer should have an initial density of at least 12.7 °P, a maximal pH of 3.8, a maximal color of 25 EBC units (European Brewery Convention) and a maximal bitterness of 20 Bitter Units (European Commission, 1997a, b). However, as in Belgian law (Belgisch Ministerie van Economische Zaken, 1993), threshold percentages for the amount of spontaneously fermented beer in lambic beer were not specified (European Commission, 1997a). Furthermore, a beer is allowed to be named gueuze beer if it is produced by mixing of lambic beers, of which the oldest has aged at least for three years in wooden casks (European Commission, 1997a);

also, the adjective 'Oude' or 'Vieille' (both meaning 'old' in Dutch and French, respectively) can only be used as prefix by traditional brewers if the lambic beer is composed of 100% spontaneously fermented beer or when the gueuze has a mean age of at least one year, is bottle-refermented, and aged for at least six months in the bottle (European Commission, 1997a). As both traditional and industrial lambic beer brewers have their own brewing protocol (*e.g.*, malt types, mashing schemes and process aid products) and procedures for finishing and bottling the beer (*e.g.*, filtration, sweetening, carbonation, bottle refermentation), the end-products can differ significantly in terms of metabolites present and taste. The legislation of lambic beers currently only focuses on a single microbial characteristic (the presence of *D. bruxellensis*) and a limited number of technological parameters, but does not address artificial acidification, chilling or mixing of the wort.

7.8 Perspectives

In the present study, the knowledge of the lambic beer microbiota was revisited and lambic beer fermentation processes were monitored in both a traditional and industrial lambic beer brewery. Both fermentation processes yielded largely the same microbial diversity, but some clear differences were apparent. The industrially produced lambic beer was not inoculated in the cooling tun and the *Enterobacteriaceae* phase that is characteristically present in the traditional fermentation process studied was absent (**Chapter 4.1** and Martens *et al.*, 1991; Van Oevelen *et al.*, 1977; Verachtert & Iserentant, 1995). The presence of these *Enterobacteriaceae* was reported to slow down the pace of the main fermentation phase (Martens *et al.*, 1991; Verachtert *et al.*, 1989), but it is unclear if these bacteria have a substantial role in the lambic beer flavor development.

Similarly to LAB and *Dekkera* strains, *Enterobacteriaceae* can produce biogenic amines (Bover-Cid & Holzapfel, 1999; Oelofse *et al.*, 2008). These amines can cause deleterious effects, as they can be psychoactive (affecting the neural transmitters in the central nervous system) or vasoactive (acting directly or indirectly on the vascular system as vasoconstrictors or vasodilators) (Kalac & Krizek, 2003). Biogenic amines are naturally occurring, but unwanted in fermented foods and beverages (Spano *et al.*, 2010). The levels of biogenic amines commonly found in spontaneously fermented beers are, however, below the levels found in

spontaneously fermented sausages or cheeses (Latorre-Moratalla *et al.*, 2014; Linares *et al.*, 2011; Loret *et al.*, 2005). To reduce the biogenic amine content of lambic beers, lambic beer brewers have adopted an acidification step at the end of the wort boiling process, as it was applied in the industrial brewery during the present study. However, it is unclear to which extent this acidification is applied in all traditional lambic beer breweries, or if an *Enterobacteriaceae* phase is consistently absent in the fermentation processes of other industrial lambic beer breweries. The presence of an *Enterobacteriaceae* phase in American coolship ale breweries (Bokulich *et al.*, 2012) suggests that the latter statement may not always be the case. Such an acidification step effectively inhibits the growth of *Enterobacteriaceae* and thus prevents the production of biogenic amines by these bacteria. However, the effect of this acidification on the total biogenic amine content in the produced lambic beers is unknown, as also other microorganisms are able to produce biogenic amines (Latorre-Moratalla *et al.*, 2014; Linares *et al.*, 2011; Oelofse *et al.*, 2008; Spano *et al.*, 2010). It should, however, be noted that it is unclear if *Enterobacteriaceae* strains affect the flavor of lambic beers, and therefore, the effect of acidification of the wort on the flavor of the lambic beer is not known. All this information is required before it can be considered to use the presence of an *Enterobacteriaceae* phase in the lambic beer fermentation process as a defining factor for the distinction between traditional and industrial lambic beer fermentation processes. The presence of the core microbiota in the lambic beer fermentations of both breweries examined indicated that the lambic beer fermentation process is very robust, since these microorganisms were present under the different environmental, brewing and fermentation conditions. The presence of the same core microbiota could be used to further describe and protect the lambic beer fermentation process.

Further research of the lambic beer fermentation process in other breweries (traditional, industrial or those with intermediate characteristics) will also reveal to which extent the overall microbiota described in the present study is generic, or if additional (novel) species will be discovered as was the case in the present study. AAB were common in the lambic beer fermentation processes studied, although they were isolated only sporadically in the traditional lambic beer brewery. The

occasional isolation of these bacteria could be due to the sampling procedure applied. The cask content could not be homogenized prior to sampling and only one opening at the lower part of the casks could be used. AAB are obligate aerobes and probably survive in the fermenting lambic beer, because of a micro-oxygenation through the wood of the casks. Therefore, they are likely concentrated at the wort/air interphase at the top of the fermenting lambic beer, or attached to the cask inner surfaces. Due to the pressure relief when opening the bung hole, oxygen could be introduced into the wort, enabling the activation and isolation of these bacteria from the traditionally produced lambic brew samples. AAB are commonly regarded as spoilage bacteria in the beer and wine industry, as they can convert ethanol into acetic acid, which has a more stringent acidity compared to lactic acid (Bartowsky & Henschke, 2008; Vaughan *et al.*, 2005). If feasible, sampling of the wort at several sampling points in casks may clarify their presence and role.

The role of yeast species that are not commonly isolated from fermented beverages also deserves further attention. The isolation of *Yarrowia lipolytica* at late phases of the lambic beer fermentation from both the traditional and industrial lambic beer brewery suggests an advantage of these species to survive and prevail in lambic beers. Cheese is the main food product from which this yeast is isolated, next to fermented and raw meat products (Groenewald *et al.*, 2014), but it has also been isolated occasssionally from soft drinks, wines and ciders (Groenewald *et al.*, 2014).

Finally, although the source of the microbiota in the industrial lambic beer brewery seems to be known, it is unclear where the lambic beer microbiota of the traditional lambic beer brewery precisely originates. Several findings indicate a role for both brewery air and the untreated construction wood and cask wood, but this may differ between breweries (Verachtert & Iserentant, 1995; Verachtert & Derdelinckx, 2005). All these breweries produce lambic beers for a long time and the entire environment is probably enriched with the lambic beer microbiota. The precise mode of inoculation might therefore remain part of the mystery and tradition of lambic beer.

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Part V
Summary
Samenvatting

English summary

Acidic lambic beers are the products of a spontaneous fermentation process that lasts for one to three years. The fermentation process is not initiated through the inoculation of yeasts or bacteria as starter cultures. Rather, microbial growth starts during the overnight cooling of the cooked wort in a shallow open vessel, called the cooling tun or coolship. Lambic beers are traditionally brewed in or near the Senne river valley, an area near Brussels, Belgium. However, also breweries outside the Senne river valley, *i.e.*, in West-Flanders, have introduced the lambic beer brewing process. Brewing for the production of lambic beer traditionally takes place only during the colder months of the year (October to March), since cold nights are needed to lower the wort temperature to about 20°C in one night. The morning following the wort cooking, the cooled wort is assumed to be inoculated with a specific air microbiota of the Senne river valley and is transferred into wooden casks, which are stored at cellar or ambient temperatures, *i.e.*, typically between 15 and 25°C. Subsequently, the wort ferments and the lambic beer matures in thesame casks. The end-product is a noncarbonated sour beer. The sour character of the beer originates from the metabolic activities of various yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB).

The present study mainly aimed to update the knowledge on the microbial diversity during the lambic beer fermentation process. Two traditional lambic beer fermentation batches were monitored over a period of two years, using cultivation and culture-independent polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) techniques. More than 2000 bacterial and yeast isolates from 14 samples per batch were obtained and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was used as a fast dereplication tool to decrease this high number of isolates to a non-redundant set of different strains. Three fermentation phases were identified during the traditional lambic beer fermentation process: an *Enterobacteriaceae* phase, which started from the cooling of the wort in the cooling tun onwards and lasted for one month. Several *Enterobacteriaceae* species were isolated, among which *Hafnia*

paralvei was the most isolated species at the end of this phase in both batches. After one month, the main fermentation or alcoholic fermentation phase started and was characterized by an increase in number of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* yeasts. The latter species was the most isolated yeast species at the end of the main fermentation phase after 3 months. In contrast to previous studies, the present investigations were unable to distinguish an acidification and maturation phase. Rather, the acidification phase appeared to be part of a long maturation phase. During the maturation phase, which was initiated after six months, *Pediococcus damnosus* was the only isolated LAB species. *Dekkera bruxellensis* was the most predominant yeast species, although the dominance of this yeast species was highly dependent of the place where the casks were located, and thus dependent of the ambient temperature of the cask room. New AAB species, named *Acetobacter lambici* and *Gluconobacter cerevisiae,* were isolated from lambic beer samples in the course of the present study.

A second objective of the present study was to compare the lambic beer fermentation process in an industrial brewery located outside the Senne river valley to the traditional lambic beer fermentation process described above. Some technical aspects of the lambic beer brewing process differed between the traditional and industrial breweries, which may impact on the lambic beer fermentation process. The main difference was that the industrial lambic beer brewery acidified the lambic beer wort to a pH of 4, using lactic acid, at the end of the boiling process. The lambic beer wort was not inoculated after overnight cooling in the cooling tun, but bacteria and yeasts could be isolated from the wort as soon as it was transferred into the casks. No *Enterobacteriaceae* were isolated from the industrial lambic beer nor was *Enterobacteriaceae* DNA detected using PCR-DGGE analysis. This may have been caused by the addition of lactic acid. Hence, only two fermentation phases were found during the industrial lambic beer fermentation process, *i.e.*, the main fermentation phase and the long maturation phase. The same species as in the traditional lambic beer fermentation process were found, although more AAB were isolated during the industrial lambic beer fermentation process. Lambic beer fermentation has long been considered only to be possible in the Senne river valley, since the air in this region is assumed to contain the microorganisms specific for

gueuze fermentation. However, microorganisms inoculating lambic beer wort could have other sources than the brewery air. Since the wort in the cooling tun yielded no growth, and because the cask samples were readily inoculated, the source of inoculation in the industrial lambic beer brewery seems to be restricted to the cask wood.

Lambic beers are mostly used for the production of other beers, such as gueuze beers and fruit lambic beers. Gueuze beers are the spontaneous bottle-refermented products of the mixtures of lambic beers of different ages. These beers are commonly aged for several years in the breweries or by the consumers. However, the microbial and metabolite changes were not known and nor was it clear how long bottles should be allowed to age before consumption. The third goal of this research aimed to determine the microbial and metabolite changes in aged gueuze beers. The microbial profiles of the beers simplified over the years. While several yeast species, including *D. bruxellensis*, *Dekkera anomala*, *Pichia membranifaciens* and *S. cerevisiae* could be isolated from the youngest gueuze beer sampled, only *D. bruxellensis* could be isolated from old gueuze beers after an enrichment culture. *Pediococcus damnosus* could only be isolated from beers that were aged for a maximum of three years. Aging of gueuze beers appeared to be limited in time. No additional lactic acid was formed after 10 years and malto-oligosaccharide concentrations were low in a nine-years old gueuze beer. Ethyl lactate and ethyl decanoate were identified as potential positive and negative metabolite markers for the aging process of gueuze beers, respectively.

In conclusion, the present study revealed that the lambic beer fermentation process consists of three rather than four phases, when based on bacterial and yeast diversity. Furthermore, the study showed that the microbiota present in both the traditional and industrial lambic beer fermentation processes were comparable, with the exception of an *Enterobacteriaceae* phase that was present in the former and absent in the latter. The precise mode of microorganism inoculation in the traditional lambic beer fermentation process was not elucidated and might therefore remain part of the mystery and tradition of lambic beer.

Nederlandstalige samenvatting

Zure lambiekbieren zijn spontaan gefermenteerde bieren, waarvan de fermentatie één tot drie jaar duurt. Het fermentatieproces start niet door de toevoeging van bacteriën of gisten als starterculturen, maar de microbiële groei start reeds tijdens het overnacht koelen van het wort in een ondiepe open kuip, het zogenaamde koelschip. Traditioneel worden deze bieren geproduceerd in de omgeving van de Zennevallei, een regio rond Brussel, België. Enkele brouwerijen in West-Vlaanderen, en dus buiten de Zennevallei gelegen, passen hetzelfde productieproces toe voor de productie van lambiekbieren. Lambiekbieren worden traditioneel enkel gebrouwen tijdens de koudere wintermaanden (van oktober tot maart), aangezien de koude nachten nodig zijn om het wort overnacht te koelen tot ongeveer 20°C. Na het koelen wordt verondersteld dat het wort geïnoculeerd is door de micro-organismen die specifiek in de lucht van de Zennevallei aanwezig zijn. Hierna wordt het wort overgebracht in houten vaten, die worden gestockeerd bij kelder- of kamertemperatuur, tussen 15 en 25°C. Vervolgens fermenteert en rijpt het wort in deze vaten. Het eindproduct van dit proces is een niet-koolzuurhoudend zuur bier. De zure smaak van het bier komt voort uit de productie en omzetting van metabolieten door verscheidene gisten, melkzuurbacteriën (MZB) en azijnzuurbacteriën (AZB).

Deze studie had als voornaamste doelstelling om de kennis over de microbiologische samenstelling van lambiekbieren tijdens het fermentatieproces op te frissen. Hierbij werden twee lambiekbierbrouwsels gedurende twee jaar opgevolgd in een traditionele lambiekbrouwerij. Hiervoor werden cultivatie en cultuuronafhankelijke polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) technieken gebruikt. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) massaspectrometrie werd toegepast als snelle dereplicatietechniek om de meer dan 2000 bacteriële en gistisolaten te reduceren naar een lager aantal niet-redundante isolaten. Drie fermentatiefasen werden onderscheiden tijdens het traditionele lambiekbierfermentatieproces: een *Enterobacteriaceae* fase duurde ongeveer een maand en startte vanaf het overnacht koelen van het wort in het

koelschip. Verscheidene *Enterobacteriaceae* species werden geïsoleerd, waaronder *Hafnia paralvei* het meest geïsoleerde species was aan het einde van deze fase en dit voor beide brouwsels. Daarna volgde de hoofdfermentatiefase (alcoholische fermentatiefase) die startte na één maand en gekenmerkt was door een stijgend aantal *Saccharomyces cerevisiae* en *Saccharomyces pastorianus* gisten. Isolaten van het laatstgenoemde species werden het meest geïsoleerd aan het einde van de hoofdfermentatiefase (na drie maand). In tegenstelling tot vorige studies was het niet mogelijk om een aparte verzurings- en rijpingsfase waar te nemen. De verzuring leek eerder onderdeel te zijn van een lange rijpingsfase. Tijdens de rijpingsfase (na 6 maand) was *Pediococcus damnosus* het enige aanwezige MZB-species. *Dekkera bruxellensis* was het voornaamste gistspecies, alhoewel de dominantie van dit gistspecies zeer afhankelijk was van de plaats waar de vaten gestockeerd werden, en dus ook van de omgevingstemperatuur van deze vatenzalen. Nieuwe AZB-species, namelijk *Acetobacter lambici* en *Gluconobacter cerevisiae,* werden geïsoleerd uit lambiekbier tijdens deze diversiteitsstudies.

Een tweede doelstelling van dit doctoraatsonderzoek was om het lambiekbierfermentatieproces in een industriële lambiekbrouwerij, gelegen buiten de Zennevallei, te vergelijken met de hoger beschreven resultaten van een traditionele lambiekbrouwerij. De productieprocessen in beide lambiekbrouwerijen vertoonden verschillen in enkele technische aspecten, die een invloed hadden op het lambiekbierfermentatieproces. Het grootste verschil in de industriële lambiekbierbrouwerij was het aanzuren van het wort met melkzuur tot pH 4 aan het einde van het kookproces. Hoewel het lambiekwort in het koelschip niet geïnoculeerd werd na overnacht koelen, werden wel bacteriën en gisten geïsoleerd uit het wortstaal uit het vat, direct nadat het wort hierin was overgebracht. Er werden geen *Enterobacteriaceae* geïsoleerd uit het industrieel geproduceerde lambiekbier noch werd DNA afkomstig van *Enterobacteriaceae* gedetecteerd tijdens de PCR-DGGE-experimenten. De afwezigheid van deze bacteriën kon veroorzaakt zijn door het aanzuren van het wort. Bijgevolg werden slechts twee fermentatiefasen waargenomen in het industriële lambiekbierfermentatieproces, namelijk de hoofdfermentatiefase en een lange rijpingsfase. Dezelfde species als in het traditionele lambiekbierfermentatieproces werden gevonden, hoewel meer

verschillende AZB-species werden geïsoleerd tijdens het industriële lambiekbierfermentatieproces. Lambiekbierfermentatie is lange tijd enkel mogelijk geacht in de omgeving van de Zennevallei, aangezien hier de nodige micro-organismen in de lucht aanwezig zijn. Micro-organismen aanwezig tijdens het lambiekbierfermentatieproces kunnen echter nog afkomstig zijn van andere bronnen, naast de omgevingslucht. Vermits het wort van de industriële brouwerij niet geïnoculeerd werd in het koelschip, maar wel isolaten bekomen werden direct nadat het wort in het vat was overgebracht, lijkt het hout van het vat bijgevolg de enige mogelijke bron van micro-organismen voor de inoculatie van het wort in het industriële lambiekbierfermentatieproces te zijn.

Lambiekbieren worden meestal gebruikt voor de productie van andere bieren, zoals geuzebieren en fruit-lambiekbieren. Geuzebieren worden verkregen door de spontane hergisting op fles van een mengsel van jonge en oude lambiek. Deze bieren worden veelal bewaard om te rijpen, zowel door de brouwerijen als door de consumenten. Zowel de veranderingen in microbiologische samenstelling als in de aanwezige metabolieten werden nog niet in detail bestudeerd. Meer nog, er was geen exacte informatie beschikbaar over hoelang deze bieren het best rijpen. Om deze reden werden in een derde doelstelling de veranderingen in gerijpte geuzebieren onderzocht, zowel op het vlak van microbiologische eigenschappen als op het vlak van aanwezige metabolieten. De microbiologische samenstelling van deze bieren vereenvoudigde naarmate de gueuzebieren langer werden gestockeerd. Verscheidene species, namelijk *D. bruxellensis*, *Dekkera anomala*, *Pichia membranifaciens* en *S. cerevisiae,* werden direct geïsoleerd uit het jongste geteste geuzebier. Uit oudere geuzebieren werd enkel *D. bruxellensis* geïsoleerd na een aanrijkingsstap. Isolaten van *P. damnosus* werden enkel bekomen uit geuzebieren van maximum drie jaar oud. De rijping van de bieren leek ook beperkt in de tijd. Er werd geen additioneel melkzuur aangemaakt na tien jaar rijping en de malto-oligosaccharideconcentraties waren reeds laag in een negenjarig geuzebier. Er werd aangetoond dat ethyllactaat en ethyldecanoaat kunnen worden gebruikt als een potentiële respectievelijk positieve en negatieve metabolietmerker voor de rijping van geuzebieren.

De huidige studie heeft aangetoond dat, gebaseerd op de bacteriële en gistdiversiteit, een traditioneel lambiekbierfermentatieproces uit drie fasen bestaat, in plaats van de eerder gerapporteerde vier fasen. Verder heeft deze studie aangetoond dat de microbiële samenstelling van zowel traditionele als industriële lambiekbierfermentatieprocessen gelijkaardig zijn, met uitzondering van de afwezigheid van een *Enterobacteriaceae* fase in het industriële lambiekbierfermentatieproces. De precieze herkomst van de micro-organismen die verantwoordelijk zijn voor de inoculatie van het lambiekwort werden niet ontrafeld, waardoor dit bijgevolg een deel blijft van het mysterie en de traditie van de productie van lambiekbieren.

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	Ghent University
	Dissertation: MALDI-TOF massaspectrometrie, verkenning van een nieuwe methode voor de identificatie van melkzuurbacteriën
	Promotor: Prof. dr. Peter Vandamme
1998 – 2004	**Wetenschappen – Wiskunde**
	Heilige-Maagd College, Dendermonde

Scientific output

a1-publications

Wieme, A. D., **Spitaels, F.**, Aerts, M., De Bruyne, K., Van Landschoot, A. & Vandamme, P. (2014). Effects of growth medium on matrix-assisted laser desorption–ionization

time of flight mass spectra: A case study of acetic acid bacteria. *Applied and Environmental Microbiology* 80, 1528-1538.

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. *International Journal of Systematic and Evolutionary Microbiology* 64, 1083-1089.

Spitaels*, F., Wieme*, A., Balzarini, T., Cleenwerck, I., Van Landschoot, A., De Vuyst, L. & Vandamme, P. (2014). *Gluconobacter cerevisiae* sp. nov., isolated from the brewery environment. *International Journal of Systematic and Evolutionary Microbiology* 64, 1134-1141.

Spitaels, F., Wieme, A. D., Janssens, M., Aerts, M., Daniel, H.-M., Van Landschoot, A., De Vuyst, L. & Vandamme, P. (2014). The microbial diversity of traditional spontaneously fermented lambic beer. *PLoS ONE* 9, e95384.

Li, L., Wieme, A. D., **Spitaels, F.**, Balzarini, T., Nunes, O. C., Manaia, C. M., Van Landschoot, A., De Vuyst, L., Cleenwerck, I. & other authors (2014). *Acetobacter sicerae* sp. nov., isolated from cider and kefir and identification of *Acetobacter* species by *dnaK* , *groEL* and *rpoB* sequence analysis. *International Journal of Systematic and Evolutionary Microbiology* doi:10.1099/ijs.0.058354-0.

Wieme, A. D.; **Spitaels, F.**; Aerts, M.; De Bruyne, K.; Van Landschoot, A. & Vandamme, P. (2014). Identification of beer spoilage bacteria using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *International Journal of Food Microbiology*. Accepted.

Wieme, A. D.; **Spitaels, F.**; Vandamme, P.; Van Landschoot, A. (2014) Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as monitoring tool for in-house brewer's yeast contamination: a proof of concept. Submitted.

Spitaels, F., Wieme, A. D., Janssens, M., Aerts, M., Van Landschoot, A., De Vuyst, L. & Vandamme, P. (2014). The microbial diversity of an industrially produced lambic beer shares members of a traditionally produced fermented lambic beer and reveals a core microbiota for lambic beer fermentation. Submitted.

Spitaels*, F., Van Kerrebroeck*, S., Wieme, A. D., Snauwaert, I., Aerts, M., Van Landschoot, A., De Vuyst, L. & Vandamme, P. (2014). Microbiota and metabolites of aged bottled gueuze beers converge to the same composition. Submitted.

Wieme, A. D., **Spitaels, F.**, Van Landschoot, A. & Vandamme, P. (2014). Direct detection and identification of bacteria in enrichment cultures of spoiled beer and brewery samples using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: a proof of concept. In preparation.

b2-publication

Wieme, A. D., **Spitaels, F.**, Vandamme, P. & Van Landschoot, A. (2014). The microbial stability of beer and the use of MALDI-TOF MS as fast identification tool for beer spoilage bacteria - a current update, p. 329-346. In Campbell, I., Antkiewicz, P., Tuszyński, T. & Błażewicz, J. (ed.), Beer stability – A Challenge for the 21st Century

Brewers. Ogólnopolskie Stowarzyszenie Wspierania Inicjatyw NAUKA-PRZEMYSŁ, Kraków, Poland ISBN: 978-83-930745-3-2.

Poster presentations

Koninklijke Vlaamse Chemische Vereniging, MS in Food and Feed, Merelbeke, Belgium. June 9, 2011

Health Protection Agenncy, Microbial Diagnostic Applications of Mass Spectrometry, London, United Kingdom. April 4-5, 2012

European Brewery Convention, 34th European Brewery Convention Congress, Luxembourg, Luxembourg. May 26-30, 2013

Workshops and courses

Brouwerijtechnologie, University College Ghent, Ghent, Belgium, February - June 2010

International Basic Training Workshop on BioNumerics and GelCompar II, Applied Maths, Sint-Martens-Latem, Belgium, June 28-29, 2010

Project Management, T.O.M. BVBA, Institute for Continuing Education in Science, Ghent University, Ghent, Belgium

Educational support

Supervision of Master thesis projects

2010-2011	Hendrik Grootaert (Master 2 Biochemistry and Biotechnology)
	Title: De microbiologie van bieren van spontane fermentatie
2011-2012	Alexander Quintelier (Master 2 Biochemistry and Biotechnology)
	Title: Bieren van spontane fermentatie: MALDI-TOF MS als dereplicatie- en identificatietool

Assistance in practical courses of microbiology (2010-2012)

Annex

Strain list

Strainnumber	Genus	Species	Source	Isolation year	Geographical origin
R-47367	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-47368	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-47369	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-47370	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-47371	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-47372	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-47373	*Klebsiella*	*oxytoca*	Fermenting lambic beer	2010	Belgium
R-47374	*Enterobacter*	*hormaechei*	Fermenting lambic beer	2010	Belgium
R-47375	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-47376	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-47377	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-47378	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-47379	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-47380	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-47381	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-47382	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-47383	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-47384	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-47385	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-47386	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49006	*Klebsiella*	*oxytoca*	Fermenting lambic beer	2010	Belgium
R-49007	*Klebsiella*	*oxytoca*	Fermenting lambic beer	2010	Belgium
R-49008	*Klebsiella*	*oxytoca*	Fermenting lambic beer	2010	Belgium
R-49009	*Klebsiella*	*oxytoca*	Fermenting lambic beer	2010	Belgium
R-49010	*Klebsiella*	*oxytoca*	Fermenting lambic beer	2010	Belgium
R-49011	*Klebsiella*	*oxytoca*	Fermenting lambic beer	2010	Belgium
R-49012	*Citrobacter*	*gillenii*	Fermenting lambic beer	2010	Belgium
R-49013	*Raoultella*	*terrigena*	Fermenting lambic beer	2010	Belgium
R-49014	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-49015	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-49016	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-49017	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-49018	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-49019	*Escherichia/Shighella*		Fermenting lambic beer	2010	Belgium
R-49020	*Escherichia/Shighella*		Fermenting lambic beer	2010	Belgium
R-49021	*Escherichia/Shighella*		Fermenting lambic beer	2010	Belgium
R-49022	*Escherichia/Shighella*		Fermenting lambic beer	2010	Belgium
R-49023	*Enterobacter*	*hormaechei*	Fermenting lambic beer	2010	Belgium
R-49024	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49025	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49026	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49027	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49028	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium

Strainnumber	Genus	Species	Source	Isolation year	Geographical origin
R-49029	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49030	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49031	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49032	*Klebsiella*	*oxytoca*	Fermenting lambic beer	2010	Belgium
R-49033	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49034	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49035	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49036	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49037	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49038	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49039	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49040	*Raoultella*	*terrigena*	Fermenting lambic beer	2010	Belgium
R-49041	*Escherichia/Shighella*		Fermenting lambic beer	2010	Belgium
R-49042	*Enterobacter*	*kobei*	Fermenting lambic beer	2010	Belgium
R-49088	*Gluconobacter*	*cerinus*	Fermenting lambic beer	2011	Belgium
R-49089	*Gluconobacter*	*cerinus*	Fermenting lambic beer	2011	Belgium
R-49090	*Acetobacter*	*cerevisiae*	Fermenting lambic beer	2011	Belgium
R-49092	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49093	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49094	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49095	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49096	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49097	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49098	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49099	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49100	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49101	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49102	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49103	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49104	*Pediococcus*	*damnosus*	Fermenting lambic beer	2011	Belgium
R-49551	*Enterobacter*	sp.	Fermenting lambic beer	2010	Belgium
R-49552	*Enterobacter*	sp.	Fermenting lambic beer	2010	Belgium
R-49553	*Pseudomonas*	*azotoformans*	Fermenting lambic beer	2010	Belgium
R-49554	*Pseudomonas*	*azotoformans*	Fermenting lambic beer	2010	Belgium
R-49555	*Hafnia*	*paralvei*	Fermenting lambic beer	2010	Belgium
R-49561	*Hanseniaspora*	*meyeri*	Fermenting lambic beer	2011	Belgium
R-49562	*Saccharomyces*	*bayanus*	Fermenting lambic beer	2010	Belgium
R-49563	*Saccharomyces*	*bayanus*	Fermenting lambic beer	2010	Belgium
R-49564	*Naumovia*	*castelii*	Fermenting lambic beer	2010	Belgium
R-49565	*Priceomyces*	*carsonii*	Fermenting lambic beer	2011	Belgium
R-49566	*Debaryomyces*	*hansenii*	Fermenting lambic beer	2011	Belgium
R-49567	*Priceomyces*	sp.	Fermenting lambic beer	2011	Belgium
R-49568	*Priceomyces*	*carsonii*	Fermenting lambic beer	2012	Belgium
R-49569	*Wickerhamomyces*	*anomalus*	Fermenting lambic beer	2012	Belgium

Strainnumber	Genus	Species	Source	Isolation year	Geographical origin
R-49570	*Debaryomyces*	*hansenii*	Fermenting lambic beer	2012	Belgium
R-49639	*Pichia*	*fermentans*	Fermenting lambic beer	2011	Belgium
R-49640	*Dekkera*	*bruxellensis*	Fermenting lambic beer	2011	Belgium
R-49641	*Pichia*	*kudriavzevii*	Fermenting lambic beer	2011	Belgium
R-49642	*Saccharomyces*	*cerevisiae*	Fermenting lambic beer	2011	Belgium
R-49643	*Dekkera*	*bruxellensis*	Fermenting lambic beer	2011	Belgium
R-49644	*Saccharomyces*	*cerevisiae*	Fermenting lambic beer	2011	Belgium
R-49645	*Hanseniaspora*	*uvarum*	Fermenting lambic beer	2011	Belgium
R-49646	*Hanseniaspora*	*uvarum*	Fermenting lambic beer	2011	Belgium
R-49647	*Candida*	*friedrichii*	Fermenting lambic beer	2011	Belgium
R-49648	*Candida*	*friedrichii*	Fermenting lambic beer	2011	Belgium
R-49649	*Pichia*	*membranifaciens*	Fermenting lambic beer	2012	Belgium
R-49650	*Meyerozyma*	*guilliermondii*	Fermenting lambic beer	2012	Belgium
R-49651	*Saccharomyces*	*bayanus*	Fermenting lambic beer	2010	Belgium
R-49652	*Debaryomyces*	*hansenii*	Fermenting lambic beer	2010	Belgium
R-49653	*Saccharomyces*	*bayanus*	Fermenting lambic beer	2010	Belgium
R-49654	*Saccharomyces*	*cerevisiae*	Fermenting lambic beer	2010	Belgium
R-49655	*Saccharomyces*	*cerevisiae*	Fermenting lambic beer	2010	Belgium
R-49656	*Saccharomyces*	*bayanus*	Fermenting lambic beer	2010	Belgium
R-49657	*Dekkera*	*anomala*	Fermenting lambic beer	2012	Belgium
R-49661	*Saccharomyces*	*bayanus*	Fermenting lambic beer	2010	Belgium
R-49662	*Saccharomyces*	*cerevisiae*	Fermenting lambic beer	2010	Belgium
R-49663	*Acetobacter*	*orientalis*	Fermenting lambic beer	2011	Belgium
R-49664	*Acetobacter*	*fabarum*	Fermenting lambic beer	2011	Belgium
R-49665	*Gluconobacter*	sp.	Fermenting lambic beer	2011	Belgium
R-49666	*Acetobacter*	*fabarum*	Fermenting lambic beer	2011	Belgium
R-49667	*Acetobacter*	*fabarum*	Fermenting lambic beer	2011	Belgium
R-49668	*Acetobacter*	*lambici*	Fermenting lambic beer	2011	Belgium
R-49740	*Acetobacter*	*orientalis*	Fermenting lambic beer	2011	Belgium
R-49741	*Acetobacter*	*fabarum*	Fermenting lambic beer	2011	Belgium
R-49742	*Acetobacter*	*lambici*	Fermenting lambic beer	2011	Belgium
R-49743	*Acetobacter*	*fabarum*	Fermenting lambic beer	2011	Belgium
R-49744	*Gluconobacter*	sp.	Fermenting lambic beer	2010	Belgium
R-49811	*Hanseniaspora*	*uvarum*	Fermenting lambic beer	2011	Belgium
R-49812	*Hanseniaspora*	*uvarum*	Fermenting lambic beer	2011	Belgium
R-49813	*Pichia*	*fermentans*	Fermenting lambic beer	2011	Belgium
R-49814	*Saccharomyces*	*cerevisiae*	Fermenting lambic beer	2011	Belgium
R-49815	*Saccharomyces*	*cerevisiae*	Fermenting lambic beer	2011	Belgium
R-49816	*Hanseniaspora*	*uvarum*	Fermenting lambic beer	2011	Belgium
R-49817	*Pichia*	*fermentans*	Fermenting lambic beer	2011	Belgium
R-49818	*Pichia*	*fermentans*	Fermenting lambic beer	2011	Belgium
R-49819	*Hanseniaspora*	*uvarum*	Fermenting lambic beer	2011	Belgium
R-49820	*Naumovia*	*castelii*	Fermenting lambic beer	2010	Belgium
R-49821	*Saccharomyces*	*cerevisiae*	Fermenting lambic beer	2010	Belgium

Strainnumber	Genus	Species	Source	Isolation year	Geographical origin
R-49822	*Saccharomyces*	*bayanus*	Fermenting lambic beer	2010	Belgium
R-49823	*Saccharomyces*	*bayanus*	Fermenting lambic beer	2010	Belgium
R-49824	*Wickerhamomyces*	*anomalus*	Fermenting lambic beer	2011	Belgium
R-49825	*Debaryomyces*	*hansenii*	Fermenting lambic beer	2011	Belgium
R-49826	*Wickerhamomyces*	*anomalus*	Fermenting lambic beer	2011	Belgium
R-49827	*Priceomyces*	*carsonii*	Fermenting lambic beer	2011	Belgium
R-49828	*Dekkera*	*bruxellensis*	Fermenting lambic beer	2011	Belgium
R-49829	*Dekkera*	*bruxellensis*	Fermenting lambic beer	2011	Belgium
R-49830	*Dekkera*	*bruxellensis*	Fermenting lambic beer	2011	Belgium
R-49831	*Dekkera*	*bruxellensis*	Fermenting lambic beer	2011	Belgium
R-49836	*Candida*	*parapsilosis*	Fermenting lambic beer	2011	Belgium
R-49837	*Kazachstania*	*servazzii*	Fermenting lambic beer	2010	Belgium
R-49838	*Kazachstania*	*servazzii*	Fermenting lambic beer	2010	Belgium
R-49839	*Candida*	sp.	Fermenting lambic beer	2012	Belgium
R-49840	*Candida*	*patagonica*	Fermenting lambic beer	2012	Belgium
R-49843	*Debaryomyces*	*marama*	Fermenting lambic beer	2012	Belgium
R-49844	*Debaryomyces*	*hansenii*	Fermenting lambic beer	2012	Belgium
R-49845	*Pichia*	*kudriavzevii*	Fermenting lambic beer	2012	Belgium
R-49846	*Yarrowia*	*lipolytica*	Fermenting lambic beer	2012	Belgium
R-50193	*Acetobacter*	*lambici*	Fermenting lambic beer	2011	Belgium
R-50194	*Acetobacter*	*lambici*	Fermenting lambic beer	2011	Belgium
R-50447	*Leuconostoc*	*mesenteroides*	Lambic brewery air	2013	Belgium
R-50448	*Leuconostoc*	*mesenteroides*	Lambic brewery air	2013	Belgium
R-50449	*Staphylococcus*	sp.	Lambic brewery air	2013	Belgium
R-50450	*Lactobacillus*	*curvatus*	Lambic brewery air	2013	Belgium
R-50451	*Leuconostoc*	*mesenteroides*	Lambic brewery air	2013	Belgium
R-50452	*Leuconostoc*	*mesenteroides*	Lambic brewery air	2013	Belgium
R-50453	*Leuconostoc*	*mesenteroides*	Lambic brewery air	2013	Belgium
R-50454	*Pseudomonas*	*azotoformans*	Lambic brewery air	2013	Belgium
R-50455	*Pseudomonas*	*azotoformans*	Lambic brewery air	2013	Belgium
R-50543	*Acetobacter*	*lambici*	Fermenting lambic beer	2013	Belgium
R-50544	*Pediococcus*	*damnosus*	Fermenting lambic beer	2013	Belgium
R-50545	*Acetobacter*	*lambici*	Fermenting lambic beer	2013	Belgium
R-50546	*Rahnella*	*aquatilis*	Fermenting lambic beer	2013	Belgium
R-50547	*Enterobacter/Citrobacter*		Fermenting lambic beer	2013	Belgium
R-50548	*Leuconostoc*	*mesenteroides*	Fermenting lambic beer	2013	Belgium
R-50549	*Gluconobacter*	sp.	Fermenting lambic beer	2013	Belgium
R-50550	*Leuconostoc*	*mesenteroides*	Fermenting lambic beer	2013	Belgium
R-50551	*Enterobacter/Citrobacter*	sp.	Fermenting lambic beer	2013	Belgium
R-50552	*Leuconostoc*	*mesenteroides*	Lambic brewery air	2013	Belgium
R-50553	*Pediococcus*	*pentosaceus*	Lambic brewery air	2013	Belgium
R-50554	*Leuconostoc*	*mesenteroides*	Lambic brewery air	2013	Belgium
R-50555	*Lactobacillus*	*nenjiangensis*	Lambic brewery air	2013	Belgium
R-50556	*Pediococcus*	*pentosaceus*	Lambic brewery air	2013	Belgium

Strainnumber	Genus	Species	Source	Isolation year	Geographical origin
R-50557	*Enterococcus*	*faecium*	Lambic brewery air	2013	Belgium
R-50558	*Pseudomonas*	*azotoformans*	Lambic brewery air	2013	Belgium
R-50559	*Hafnia*	*alvei*	Lambic barrel	2013	Belgium
R-50560	*Acetobacter*	sp.	Fermenting lambic beer	2013	Belgium
R-50561	*Acetobacter*	sp.	Fermenting lambic beer	2013	Belgium
R-50562	*Lactobacillus*	*malefermentans*	Fermenting lambic beer	2013	Belgium
R-50563	*Lactobacillus*	*buchneri*	Fermenting lambic beer	2013	Belgium
R-50564	*Lactobacillus*	*buchneri*	Fermenting lambic beer	2013	Belgium
R-50565	*Ophiostoma*	*stenoceras*	Fermenting lambic beer	2013	Belgium
R-50566	*Trichosporon*	*gracile*	Fermenting lambic beer	2013	Belgium
R-50567	*Blastobotrys*	*arbuscula*	Fermenting lambic beer	2013	Belgium
R-50568	*Cryptococcus*	*heveanensis*	Fermenting lambic beer	2013	Belgium
R-50569	*Candida*	*glabrata*	Fermenting lambic beer	2013	Belgium
R-50570	*Pichia*	*membranifaciens*	Fermenting lambic beer	2013	Belgium
R-50571	*Trichosporon*	sp.	Fermenting lambic beer	2013	Belgium
R-50572	*Candida*	*friedrichii*	Fermenting lambic beer	2013	Belgium
R-50573	*Candida*	*patagonica*	Fermenting lambic beer	2013	Belgium
R-50574	*Pichia*	*membranifaciens*	Fermenting lambic beer	2013	Belgium
R-50575	*Trichomonascus*	*apis*	Fermenting lambic beer	2013	Belgium
R-50576	*Saccharomyces*	*cerevisiae*	Fermenting lambic beer	2013	Belgium
R-50578	*Priceomyces*	sp.	Lambic brewery air	2013	Belgium
R-50580	*Meyerozyma*	*guilliermondii*	Lambic brewery air	2013	Belgium
R-50582	*Meyerozyma*	*guilliermondii*	Lambic brewery air	2013	Belgium
R-50584	*Candida*	*boidinii*	Lambic barrel	2013	Belgium
R-50585	*Saccharomyces*	*bayanus*	Fermenting lambic beer	2013	Belgium
R-50586	*Brettanomyces*	*custersianus*	Fermenting lambic beer	2013	Belgium
R-50741	*Pantoea*	sp.	Lambic brewery air	2013	Belgium
R-50742	*Pseudomonas*	sp.	Lambic brewery air	2013	Belgium
R-50743	*Acinetobacter*	*guillouiae*	Lambic brewery air	2013	Belgium
R-50744	*Pseudomonas*	sp.	Lambic brewery air	2013	Belgium
R-50745	*Serratia*	sp.	Lambic brewery air	2013	Belgium
R-50746	*Pseudomonas*	sp.	Lambic brewery air	2013	Belgium
R-50747	*Pseudomonas*	sp.	Lambic brewery air	2013	Belgium
R-50748	*Pseudomonas*	*azotoformans*	Lambic brewery air	2013	Belgium
R-50749	*Azomonas*	sp.	Lambic brewery air	2013	Belgium
R-50750	*Bacillus*	sp.	Lambic brewery air	2013	Belgium
R-50751	*Bacillus*	sp.	Lambic brewery air	2013	Belgium
R-50752	*Serratia*	*marcecens*	Lambic brewery air	2013	Belgium
R-50753	*Bacillus*	sp.	Lambic brewery air	2013	Belgium
R-50754	*Bacillus*	sp.	Lambic brewery air	2013	Belgium
R-50755	*Staphyllococcus*	*sciuri*	Lambic brewery air	2013	Belgium
R-50756	*Pediococcus*	*pentosaceus*	Lambic brewery air	2013	Belgium
R-50757	*Bacillus*	sp.	Lambic brewery air	2013	Belgium
R-50758	*Pseudomonas*	sp.	Lambic brewery air	2013	Belgium

Strainnumber	Genus	Species	Source	Isolation year	Geographical origin
R-50759	*Pseudomonas*	*psychrotolerans*	Lambic brewery air	2013	Belgium
R-50760	*Pseudomonas*	*psychrotolerans*	Lambic brewery air	2013	Belgium
R-50766	*Pichia*	*fermentans*	Lambic brewery air	2013	Belgium
R-50767	*Candida*	*glabrata*	Lambic brewery air	2013	Belgium
R-50768	*Candida*	*glabrata*	Lambic brewery air	2013	Belgium
R-50769	*Candida*	*glabrata*	Lambic brewery air	2013	Belgium
R-50770	*Pichia*	*fermentans*	Lambic brewery air	2013	Belgium
R-50771	*Pichia*	*fermentans*	Lambic brewery air	2013	Belgium
R-50772	*Candida*	*glabrata*	Lambic brewery air	2013	Belgium
R-50773	*Candida*	*glabrata*	Lambic brewery air	2013	Belgium
R-50774	*Pichia*	*fermentans*	Lambic brewery air	2013	Belgium
R-50775	*Candida*	*glabrata*	Lambic brewery air	2013	Belgium
R-50776	*Cryptococcus*	*carnescens*	Lambic brewery air	2013	Belgium
R-50777	*Trichosporum*	*domesticum*	Lambic brewery air	2013	Belgium
R-50778	*Trichosporum*	*domesticum*	Lambic brewery air	2013	Belgium
R-50779	*Staphylococcus*	*caprae*	Lambic brewery air	2013	Belgium
R-50780	*Bacillus*	*mycoides*	Lambic brewery air	2013	Belgium
R-50781	*Bacillus*	*aryabhattai*	Lambic brewery air	2013	Belgium
R-50782	*Rumeliibacillus*	*pycnus*	Lambic brewery air	2013	Belgium
R-50783	*Staphylococcus*	*aureus*	Lambic brewery air	2013	Belgium
R-50784	*Staphylococcus*	*caprae*	Lambic brewery air	2013	Belgium
R-50785	*Staphylococcus*	*caprae*	Lambic brewery air	2013	Belgium
R-50786	*Aerococcus*	*urinaeequi*	Lambic brewery air	2013	Belgium
R-50787	*Staphylococcus*	*hominis*	Lambic brewery air	2013	Belgium
R-50788	*Staphylococcus*	*petrasii*	Lambic brewery air	2013	Belgium
R-50789	*Aerococcus*	*urinaeequi*	Lambic brewery air	2013	Belgium
R-50790	*Staphylococcus*	*succinus*	Lambic brewery air	2013	Belgium
R-50791	*Staphylococcus*	*hominis*	Lambic brewery air	2013	Belgium
R-50792	*Staphylococcus*	*succinus*	Lambic brewery air	2013	Belgium
R-50793	*Staphylococcus*	sp.	Lambic brewery air	2013	Belgium
R-50794	*Staphylococcus*	*caprae*	Lambic brewery air	2013	Belgium
R-50795	*Staphylococcus*	*petrasii*	Lambic brewery air	2013	Belgium
R-50796	*Staphylococcus*	*epidermis*	Lambic brewery air	2013	Belgium
R-50797	*Kocuria*	*rhizophila*	Lambic brewery air	2013	Belgium
R-50798	*Pseudomonas*	*psychrotolerans*	Lambic brewery air	2013	Belgium
R-50799	*Moraxella*	*osloensis*	Lambic brewery air	2013	Belgium
R-50800	*Staphylococcus*	*hominis*	Lambic brewery air	2013	Belgium
R-50801	*Bacillus*	*aerophilus*	Lambic brewery air	2013	Belgium
R-50802	*Staphylococcus*	sp.	Lambic brewery air	2013	Belgium
R-50803	*Staphylococcus*	sp.	Lambic brewery air	2013	Belgium
R-50804	*Staphylococcus*	*hominis*	Lambic brewery air	2013	Belgium
R-50805	*Staphylococcus*	*caprae*	Lambic brewery air	2013	Belgium
R-50806	*Bacillus*	*licheniformis*	Lambic brewery air	2013	Belgium
R-50807	*Staphylococcus*	*epidermis*	Lambic brewery air	2013	Belgium

Strainnumber	Genus	Species	Source	Isolation year	Geographical origin
R-50808	*Bacillus*	*simplex*	Lambic brewery air	2013	Belgium
R-50809	*Lysinibacillus*	*macroides*	Lambic brewery air	2013	Belgium
R-50810	*Staphylococcus*	*haemolyticus*	Lambic brewery air	2013	Belgium
R-50811	*Kocuria*	*kristinae*	Lambic brewery air	2013	Belgium
R-50813	*Streptococcus*	*parauberis*	Lambic brewery air	2013	Belgium
R-50814	*Bacillus*	*subtilis*	Lambic brewery air	2013	Belgium
R-50815	*Bacillus*	*subtilis*	Lambic brewery air	2013	Belgium
R-50816	*Bacillus*	*licheniformis*	Lambic brewery air	2013	Belgium
R-50817	*Klebsiella*	*oxytoca*	Lambic brewery air	2013	Belgium
R-50818	*Serratia*	*grimesii*	Lambic brewery air	2013	Belgium
R-50819	*Serratia*	sp.	Lambic brewery air	2013	Belgium
R-50820	*Cryptococcus*	sp.	Lambic brewery air	2013	Belgium
R-50821	*Trichosporon*	*gracile*	Lambic brewery air	2013	Belgium
R-50990	*Acetobacter*	*fabarum*	Fermenting lambic beer	2011	Belgium
R-51007	*Gluconobacter*	*oxydans*	Fermenting lambic beer	2013	Belgium
R-51008	*Acetobacter*	*orientalis*	Fermenting lambic beer	2013	Belgium
R-51009	*Acetobacter*	*orientalis*	Fermenting lambic beer	2013	Belgium
R-51010	*Gluconobacter*	*japonicus*	Fermenting lambic beer	2013	Belgium
R-51011	*Gluconobacter*	*albidus*	Fermenting lambic beer	2013	Belgium
R-51012	*Acetobacter*	*orientalis*	Fermenting lambic beer	2013	Belgium
R-51013	*Acetobacter*	*orientalis*	Fermenting lambic beer	2013	Belgium
R-51014	*Gluconobacter*	*cerinus*	Fermenting lambic beer	2013	Belgium
R-51015	*Gluconobacter*	*oxydans*	Fermenting lambic beer	2013	Belgium
R-51016	*Lactococcus*	*lactis*	Lambic brewery air	2013	Belgium
R-51017	*Propionibacterium*	*cyclohexanicum*	Lambic brewery air	2013	Belgium
R-51018	*Leuconostoc*	*citreum*	Lambic brewery air	2013	Belgium
R-51019	*Propionibacterium*	*thoenii*	Lambic brewery air	2013	Belgium
R-51020	*Pediococcus*	*damnosus*	Lambic barrel	2013	Belgium
R-51021	*Pediococcus*	*damnosus*	Lambic barrel	2013	Belgium
R-51022	*Lactobacillus*	*malefermentans*	Fermenting lambic beer	2013	Belgium
R-51023	*Lactobacillus*	*malefermentans*	Fermenting lambic beer	2013	Belgium
R-51024	*Leuconostoc*	*pseudomesenteroides*	Fermenting lambic beer	2013	Belgium
R-52105	*Dekkera*	*bruxellensis*	Gueuze beer	2013	Belgium
R-52106	*Dekkera*	*bruxellensis*	Gueuze beer	2013	Belgium
R-52107	*Dekkera*	*bruxellensis*	Gueuze beer	2013	Belgium
R-52108	*Dekkera*	*bruxellensis*	Gueuze beer	2013	Belgium
R-52109	*Dekkera*	*bruxellensis*	Gueuze beer	2013	Belgium
R-52110	*Dekkera*	*bruxellensis*	Gueuze beer	2013	Belgium
R-52111	*Brettanomyces*	*custersianus*	Gueuze beer	2013	Belgium
R-52112	*Dekkera*	*bruxellensis*	Gueuze beer	2013	Belgium
R-52113	*Pichia*	*membranifaciens*	Gueuze beer	2013	Belgium
R-52114	*Saccharomyces*	*cerevisiae*	Gueuze beer	2013	Belgium
R-52115	*Saccharomyces*	*cerevisiae*	Gueuze beer	2013	Belgium
R-52116	*Dekkera*	*anomala*	Gueuze beer	2013	Belgium

Strainnumber	Genus	Species	Source	Isolation year	Geographical origin
R-52117	*Dekkera*	*anomala*	Gueuze beer	2013	Belgium
R-52118	*Dekkera*	*anomala*	Gueuze beer	2013	Belgium
R-52119	*Dekkera*	*anomala*	Gueuze beer	2013	Belgium
R-52120	*Saccharomyces*	*cerevisiae*	Fermenting lambic beer	2013	Belgium
R-52121	*Saccharomyces*	*cerevisiae*	Fermenting lambic beer	2013	Belgium
R-52122	*Pediococcus*	*damnosus*	Gueuze beer	2013	Belgium
R-52123	*Pediococcus*	*damnosus*	Gueuze beer	2013	Belgium
R-52124	*Pediococcus*	*damnosus*	Gueuze beer	2013	Belgium
R-52125	*Pediococcus*	*damnosus*	Gueuze beer	2013	Belgium
R-52126	*Pediococcus*	*damnosus*	Gueuze beer	2013	Belgium
R-52127	*Pediococcus*	*damnosus*	Gueuze beer	2013	Belgium
R-52128	*Pediococcus*	*damnosus*	Gueuze beer	2013	Belgium