Description of the temporal dynamics in microbial community
composition and beer chemistry in sour beer production via barrel
ageing of finished beers

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Abstract

Currently, there is a strong interest in barrel ageing of finished, conventionally fermented beers, as a novel way to produce sour beers with a rich and complex flavour profile. The production process, however, remains largely a process of trial and error, often resulting in profit losses and inconsistency in quality. To improve product quality and consistency, a better understanding of the interactions between microorganisms, wood and maturing beer is needed. The aim of this study was to describe the temporal dynamics in microbial community composition, beer chemistry and sensory characteristics during barrel ageing of three conventionally fermented beers that differed in parameters like alcohol content and bitterness. Beers were matured for 38 weeks in new (two types of wood) and used (one type of wood) oak barrels. Beer samples were taken at the start of the maturation and after 2, 12 and 38 weeks. Microbial community composition, determined using amplicon sequencing of the V4 region of the bacterial 16S rRNA gene and the fungal ITS1 region, beer chemistry and sensory characteristics substantially changed throughout the maturation process. Likewise, total bacterial and fungal population densities generally increased during maturation. PerMANOVA revealed significant differences in the bacterial and fungal community composition of the three beers and across time points, but not between the different wood types. By contrast, significant differences in beer chemistry were found across the different beers, wood types and sampling points. Results also indicated that the outcome of the maturation process likely depends on the initial beer properties. Specifically, results suggested that beer bitterness may restrain the bacterial community composition, thereby having an impact on beer souring. While the bacterial community composition of moderately-hopped beers shifted to a dominance of lactic acid bacteria, the bacterial community of the high-bitterness beer remained fairly constant, with low population

densities. Bacterial community composition of the moderate-bitterness beers also resembled those of traditional sours like lambic beers, hosting typical lambic brewing species like *Pediococcus damnosus*, *Lactobacillus brevis* and *Acetobacter* sp. Furthermore, results suggested that alcohol level may have affected the fungal community composition and extraction of wood compounds. More specifically, the concentration of wood compounds like cis-3-methyl-4-octanolide, trans-3-methyl-4-octanolide, eugenol and total polyphenols was higher in beers with a high alcohol content. Altogether, our results provide novel insights into the barrel ageing process of beer, and may pave the way for a new generation of sour beers.

Key words: Acetic acid bacteria; *Brettanomyces*; Lactic acid bacteria; Maturation; Wood; Yeast

1. Introduction

Sour beers, unique in their acidity, have become very popular in recent years (Bossaert *et al.*, 2019; Shayevitz *et al.*, 2020; Tonsmeire, 2014). Currently, they are produced using diverse methods, which range from modern kettle souring and short-term mixed fermentation to traditional long-term mixed fermentation and maturation in wooden barrels (Bossaert *et al.*, 2019; Tonsmeire, 2014). Whereas kettle souring and short-term mixed fermentation often generate beers with a one-dimensional sourness, long-term ageing in wooden barrels yields extra complexity and distinctive layers of flavour within sour beers (Bossaert *et al.*, 2019; Cantwell and Bouckaert, 2016; Tonsmeire, 2014). The sour character of such beers generally originates from a wide range of organic acids like lactic acid and acetic acid, produced by lactic acid bacteria (LAB), acetic acid bacteria (AAB) and/or various yeasts, including *Brettanomyces* spp. (Spitaels *et al.*, 2015). Likewise, microbial metabolites like 4-vinyl guaiacol and 4-ethyl phenol contribute to the ‘funky’ character of these beers by imparting spicy and barnyard flavour notes (Sterckx *et al.*, 2012), while esters and higher alcohols are mainly responsible for fruity flavours (Dzialo *et al.*, 2017). Additional flavour characteristics may be acquired from other beverages that were previously matured inside the barrel (e.g. wine, sherry and whisky) or from the wood itself (Cantwell and Bouckaert, 2016). During the ageing process, the wood transfers a series of aromatic substances to the maturing beer, whose quantity and extraction rate vary considerably depending on a number of variables, including the wood’s geographical region of origin, the wood species, various parameters involved in the cooperage process (especially the level of toasting), the history of the barrel (e.g. used vs new), the duration of the maturation, the temperature and humidity at which the beers are matured, and intrinsic parameters of the beverage such as alcohol content and pH (De Rosso *et al.*, 2009; Fan *et al.*, 2006; Sterckx *et al.*, 2012).

The best-known sour beers produced by long term ageing in wooden barrels are Belgian lambic beers, traditional beers characterized by a spontaneous fermentation process that lasts for 1 to 3 years before bottling, or blending to produce gueuze or fruit lambic beers (De Keersmaecker,

1996). In lambic brewing, the extraction of wood compounds is of no interest, and the wooden barrels are mainly used to allow microbes and oxygen to perform the fermentation (De Roos *et al.*, 2019b). Another well-known example includes Belgian red ales, which are produced by a mixed-culture fermentation and long periods of ageing in wooden barrels, and subsequent blending to produce a beer with a balanced mix of acidity and sweet fruit and vanilla flavours extracted from the wood (Snauwaert *et al.*, 2016). Currently, there is a strong interest in barrel ageing of finished, conventionally fermented craft beers, as a tool to add supplementary flavours to the beer, including sourness and/or wood-derived flavours (Bossaert *et al.*, 2019; Shayevitz *et al.*, 2020; Tonsmeire, 2014). This trend has inspired several brewers to start experimenting or implementing this approach for the production of a new generation of sour beers. This enables them to expand their portfolio with distinctive products of a noteworthy flavour complexity and nuance without changing their brewing scheme, while minimizing the risk of contaminating the brewing environment. Although the use of wood fragments (e.g. wood shavings, chips, cubes, etc.) or wood extracts can yield beers with various woody flavours, the production of complex sour beers with the characteristics of a traditional sour requires long-term ageing in wooden barrels.

Until now, barrel ageing of beer remains largely a black box process, often resulting in unpredictable outcomes, inconsistency in quality and profit losses. To improve product quality and consistency, an effective method is needed to control the production process by which such high-quality sour beers can be produced. Therefore, a profound knowledge of the interactions between microorganisms, wood and maturing beer is fundamental. The overall aim of this study was to describe the temporal dynamics in microbial community composition, beer chemistry and sensory characteristics during barrel ageing of three conventionally fermented beers that differed in diverse parameters like alcohol content and bitterness. Therefore, a data-driven ecological study was set up in which the microbiological and chemical dynamics of barrel ageing were monitored under industrial conditions. Beers were matured for 38 weeks in two types of new oak barrels and one type of used oak barrels, aiming at the production of sours with a complex flavour profile. Compared to new

barrels, souring is expected to occur faster in used barrels, due to a higher density of souring microorganisms on the interior surfaces of the barrels, even after cleaning (De Roos *et al.*, 2019b). However, a higher extraction rate of wood compounds is expected for the new barrels (Navarro *et al.*, 2016). Beer samples were taken at the start and after 2, 12 and 38 weeks of the maturation process. Changes in microbial community composition and density were assessed using deep sequencing of partial bacterial 16S rRNA gene and fungal internal transcribed spacer 1 (ITS1) amplicons, and quantitative real-time PCR (qPCR), respectively. Compared to traditional culture-dependent methods such molecular methods have several advantages (Justé *et al.*, 2008). It is well known that not all microorganisms can be cultivated in standard laboratory conditions. Furthermore, sometimes important microbial groups are missed due to culture-dependent biases such as the appearance of microorganisms in a viable but non-culturable state and/or the possibility that isolation media favour the cultivation of specific microorganisms, by which others are outcompeted. Additionally, microorganisms may remain undetected when they occur at cell densities below the limit of detection of the plate assays. These drawbacks can be circumvented by culture-independent molecular approaches, enabling an in-depth characterization of microbial communities without resorting to culturing (Oulas *et al.*, 2015). In addition, concentrations of the most important wood-derived flavour compounds and microbial metabolites were measured, and sensory characteristics of the beers were monitored. Integration of the acquired results provides novel insights into the barrel ageing process of distinct beer types. Additionally, obtained results can be used a solid basis to further unravel the interactions between microorganisms, wood and maturing beer using controlled lab experiments.

2. Materials and methods

2.1 Beers, barrels, wood maturation and sampling

The study was conducted in collaboration with three Belgian breweries, further referred to as brewery 1, 2 and 3 providing and maturing beer 1, 2 and 3, respectively. Beer 1 was a stout beer (i.e. a dark, top-fermented beer) with 9.64 % Alcohol By Volume (ABV) and 34.20 ppm iso- α -acids, giving the beer

a strong bitter taste. It was produced using 23.8 % munich malt, 7.9 % special belge malt, 2.4 % debittered chocolate malt, 2 % weyerman black malt, 36% pale ale malt and 20% dark candy sugar. The wort was brewed to an initial density of 21.83 °P and was hopped using German Hallertau Magnum hop (62.5 % Magnum pellets and 37.5 % Magnum hop extract), and no dry-hopping was performed. Primary fermentation was executed by *Saccharomyces cerevisiae* (Lallemand Abbaye yeast, Lallemand Inc., Quebec, Canada) for four days at 26 °C, was not cold-crashed and no filtration nor pasteurization was performed before filling the wooden barrels with this beer. Beer 2 was a top-fermented blond beer with a lower alcohol content of 3.12 % ABV, and an intermediate amount of iso- α -acids equal to 18.44 ppm. The wort was brewed to an initial density of 10 °P using 80 % pilsner malt and 20 % munich malt, supplemented with 0.3 % siromix 80. The added hop was composed of 60 % Magnum pellets, 12.5 % Spalt spalter pellets and 27.5% East Kent Golden pellets, and no dry-hopping was performed. A Belgian ale strain of *S. cerevisiae* was used to perform primary fermentation for about two weeks at 28 °C. The fermentation was not cold-crashed and the resulting beer was filtered before filling the wooden barrels. Beer 3 was a top-fermented blond beer and had an alcohol content of 8.88 % ABV and an intermediate bitterness corresponding to a concentration of 19.85 ppm iso- α -acids. Wort was produced at an initial density of 18.8 °P using pilsner malt supplemented with high maltose syrup. The added hop was Belgian Target hop and no dry-hopping was performed. Primary fermentation was performed by *S. cerevisiae* for five days at 20 °C, was not cold-crashed and the beer was not filtered nor pasteurized before filling the barrels. At each brewery, the respective beers were transferred into six 225-liter medium-toasted oak barrels. Three types of barrels were included with two biological replicates: (i) two new oak barrels from category ‘Sweet’, (ii) two new oak barrels from category ‘Structure’, and (iii) two oak barrels that were previously used for a 4-year Sangiovese (red) wine maturation. According to the cooperage, Garbellotto Spa (Italy), the ‘Sweet’ barrels were characterized by high contents of vanillin and furfural, giving the maturing beverage hints of sweet aroma, while the ‘Structure’ barrels were characterized by high contents of tannins and ellagitannins, providing structure and body. Before filling the barrels with beer, barrels were saturated

with a mixture of water and 0.1 % citric acid for five days to avoid leakage and to extract the most pungent tannins. Next, the barrels were disinfected by burning sulphur, and finally flushed with CO₂ to remove oxygen from the barrel. Burning sulphur is the most common disinfection method used for cleaning wooden barrels (Cantwell and Bouckaert, 2016; Stadler and Fischer, 2020) and was applied to mimic the level of disinfection employed in industrial conditions. However, it should be noted that complete sterilization of the wood is generally not achieved as certain microorganisms may penetrate the porous wood up to depths greater than one cm, and are therefore difficult to remove (Stadler and Fischer, 2020; Swaffield *et al.*, 1995), leaving microbes that can inoculate the beers (De Roos *et al.*, 2019b). Subsequently, beers were matured in industrial conditions in the three respective breweries for 38 weeks at ambient, i.e. uncontrolled, temperature and humidity. Barrels were completely filled with beer and closed off with a fermentation lock to minimize headspace exposure to the air and avoid microbial contamination. At regular time intervals, barrels were replenished with beer from the same batch as the one used for the maturation, but which has been stored in stainless steel kegs, to correct for any beer losses and ensure a complete saturation of the barrel and avoid beer oxidation.

The beer was sampled immediately before transfer to the wooden barrels (reference beer), and after 2, 12 and 38 weeks of maturation. Samples were taken according to De Roos *et al.* (2018). Briefly, sampling was performed at different heights (top, middle, and bottom) in the barrels by applying flame sterilization and using gamma-irradiated jumbo pipettes (Bürkle, Munich, Germany). At each height, samples of 50 ml were taken, which were subsequently mixed to obtain a bulk sample of 150 ml. Following cooled transport to the laboratory, samples were centrifuged (3,500 x g for 15 minutes at 4 °C) and the resulting cell pellets and cell-free supernatants were stored at -20 °C for microbial and chemical analysis, respectively.

2.2 Microbial analyses

DNA extraction was performed on 100 µl of the cell pellet according to the procedure described by Lievens *et al.* (2003). Additionally, a negative control in which the cell pellet was replaced by sterile

water was included to confirm absence of reagent contamination. DNA samples were diluted ten times and subjected to PCR amplification of the hypervariable V4 region of the bacterial 16S rRNA gene (primers 515F and 806R) (Caporaso *et al.*, 2011; Kozich *et al.*, 2013; Wuyts *et al.*, 2018) and the fungal ITS1 region (primers BITS and B58S3) (Bokulich and Mills, 2013) using Illumina barcoded primers, designed according to Kozich *et al.* (2013) (Tables S1 and S2, Supporting Information). In each run, a negative PCR control (in which DNA template was replaced by DNA-free water) and two samples from a mock community (one for bacteria and one for fungi) were included. Both mock communities were composed of a handful of species that were expected to occur in the maturing beers (Table S3, Supporting Information). Amplification was performed in a reaction volume of 40 μ l, comprised of 2 μ l DNA, 0.5 μ M of each primer, 150 μ M of each dNTP, 1x Titanium Taq PCR buffer and 1x Titanium Taq DNA polymerase (Takara Bio, Saint-Germain-en-Laye, France). The reactions were initiated by denaturation at 94 °C for 120 s, followed by 34 cycles of 45 s at 95 °C, 45 s at 59 °C and 45 s at 72 °C, and a final elongation step of 10 min at 72 °C. Next, amplicons were purified and equimolar concentrations were pooled into two libraries, one representing the pool of bacterial V4 amplicons and one representing the pool of ITS1 amplicons. Next, following ethanol precipitation, the amplicon pools were loaded onto a 1.5 % agarose gel, and the bands corresponding to the expected fragment length were excised from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). While the length of the V4 region is widely conserved throughout the bacterial domain, the ITS1 region may vary substantially across fungal species (De Filippis *et al.*, 2017; Zhao *et al.*, 2015). Therefore, as Illumina MiSeq sequencing yields the best results when the fragments to be sequenced have a similar length (Bronner *et al.*, 2013), for fungi, agarose gel pieces corresponding to DNA fragments ranging from 200 to 400 bp and from 400 to 550 bp were separately collected and purified (further referred to as ITS1₂₀₀₋₄₀₀ and ITS1₄₀₀₋₅₅₀). As a result, three distinct libraries were obtained, one for bacteria and two for fungi. After performing gel extraction and dilution of the libraries to 2 nM, all libraries were sequenced using an Illumina MiSeq sequencer with a v2 500-cycle reagent kit (Illumina, San Diego, USA). Further, in order to assess total bacterial and fungal density, bacterial 16S rRNA gene

and fungal ITS1 copy numbers were determined using qPCR with the same primers (but without barcodes) as those used for the sequencing approach (for details, see Borremans *et al.* (2019)). qPCR analyses were performed in duplicate.

Sequences were received as a demultiplexed FASTQ file, with barcodes and primer sequences removed. Paired-end reads for the V4 sequences were merged using USEARCH (v11.0.667) to form consensus sequences (Edgar, 2013) with no more than 10 mismatches allowed in the overlap region, while only forward reads of the ITS1 region were retained. Subsequently, sequences were truncated at the 250th base for the V4 sequences, and the 95th and 250th base for the ITS1₂₀₀₋₄₀₀ and ITS1₄₀₀₋₅₅₀ data sets, respectively. Reads shorter than 95 bp (ITS1₂₀₀₋₄₀₀) or shorter than 250 bp (V4 and ITS1₄₀₀₋₅₅₀), or reads with a total expected error threshold above 0.1 and 1 for the V4 and ITS1 regions, respectively, were discarded using USEARCH (v11.0.667). Next, Mothur's (v1.39.3) commands 'classify.seqs' and 'remove.lineage' or 'get.lineage' in combination with the Silva database (v1.23, for bacteria) and UNITE database (v6, for fungi), respectively, were used to identify and remove potential mitochondrial, chloroplast or other non-target sequences. Next, bacterial sequences were classified into zero-radius operational taxonomic units (zOTUs (Edgar, 2016); also known as amplicon sequence variants (ASVs) (Callahan *et al.*, 2017)) by the UNOISE3 algorithm as implemented in USEARCH (Edgar, 2015). Only zOTUs with a minimum global relative abundance of eight reads were kept and chimeric sequences were removed. Fungal sequences were clustered into operational taxonomic units (OTUs) based on a 3 % sequence dissimilarity cut-off. The advantage of zOTUs is that they enable resolution of closely related taxa that would be incorporated into the same OTU when applying a 3 % dissimilarity cut-off. However, given that many fungal species have intraspecific and intragenomic variations in ITS (De Filippis *et al.*, 2017; Wang and Yao, 2005; Zhao *et al.*, 2015), fungal diversity is still commonly assessed by the use of 97 % OTUs as fungal species proxies (Sielaff *et al.*, 2019). OTU clustering was performed using the UPARSE greedy algorithm in USEARCH, during which chimeric sequences were also removed (Edgar, 2013), as were global singletons (i.e. OTUs with only 1 sequence represented in the entire data set). Next, all data sets were analysed in R (v3.5.2) using decontam (v1.2.0) to control

for the presence of contaminants based on (z)OTU prevalence in beer samples versus the control samples (Davis *et al.*, 2018; R Core Team, 2019). Finally, the number of sequences was rarefied to the sample with the lowest number of reads (812 for bacteria, 1074 and 1000 for fungi for ITS1₂₀₀₋₄₀₀ and ITS1₄₀₀₋₅₅₀, respectively). The taxonomic origin of each bacterial zOTU and fungal OTU was determined with the SINTAX algorithm as implemented in USEARCH based on the SILVA Living Tree Project v123 (LTP v123, for bacteria) and the RDP Warcup trainingset (v2, for fungi). Further, the identity of the most important zOTUs and OTUs was verified with a BLAST search against type materials in GenBank. Analysis of the mock communities demonstrated that all expected taxa were found, and no single contaminant had passed the quality filtering and decontamination steps, indicating that the experimental conditions were met to achieve robust data (Tables S4-6, Supporting Information). Sequence data have been deposited in the Sequence Read Archive under BioProject accession PRJNA681555 (accession numbers SAMN16956109-SAMN16956169, and SAMN16982878-SAMN16982883).

2.3 Chemical analyses and sensory evaluation

Based on the protocols of Sterckx (2011), a total of 20 aroma compounds related to wood maturation (Table S7, Supporting information) were quantified from the cell-free samples using Head Space – Solid Phase Micro Extraction – Gas Chromatography – Mass Spectrometry (HS-SPME-GC-MS). A detailed overview of the sample preparation and HS-SPME-GC-MS settings used in this analysis can be found in Table S8 (Supporting Information), and for all chemical analyses two technical replicates were included per sample. Likewise, a total of 13 fermentation products related to fungal or bacterial activity (such as esters, higher alcohols and aldehydes) were quantified by HS-SPME-GC-MS (Tables S7 and S8, Supporting information). Furthermore, five organic acids were measured using High Pressure Liquid Chromatography (HPLC) (Tables S7 and S8, Supporting information), and four sugars using a Gallery Plus Beermaster (Thermo Fisher Scientific, Austin, USA) (Table S9, Supporting information). Additionally, the pH value was determined using a Consort C832 (Table S7, Supporting information).

Further, ethanol content was determined with an AlcoLyzer beer ME (Anton Paar GmbH, Graz, Austria; Table S9, Supporting information) and polyphenols were measured according to EBC-methods: total polyphenols: 9.11 and flavonoid content: 9.12. Finally, the proanthocyanidin content was quantified according to Bate-Smith (1973) (Table S7, Supporting information).

Sensory evaluation was performed by an in-house trained tasting panel consisting of ten panellists (female: 4, male: 6, age: 20-50 years, average age: 31). All panellists were experienced with sensory evaluations of beers, spirits and raw materials used in brewing and distilling. For this study, the panellists were additionally trained for recognition, description and scoring of different sensory attributes of barrel aged beers. Quantitative descriptive analysis was performed by scoring the samples on a scale from 0 (not present) to 8 (high intensity) for the following attributes: smoky, phenolic, bitter, woody, whisky, astringency, and esters. Training of the tasting panel was executed using reference wood-related compounds (FlavorActiV, Thame, UK and The Siebel Institute of Technology, Chicago, USA).

2.4 Statistical analyses

The amplicon sequencing approach used in this study typically yields a representation of the relative abundance of the (z)OTUs found per sample for each library, and provides no information about absolute abundance. Therefore, the three different sequence datasets obtained are analysed and interpreted separately. To test whether our sampling was adequate to capture the microbial diversity, for each sample a rarefaction curve was generated using the Phyloseq package in R showing the number of observed (z)OTUs as a function of the number of sequences (McMurdie and Holmes, 2013; R Core Team, 2019). To test for significant differences in microbial community composition between the different beer types, wood types and maturation time points, a permutational multivariate analysis of variance (perMANOVA) was carried out. PerMANOVA is a non-parametric permutational multivariate statistical technique that tests whether a set of response variables (in this case (z)OTUs) differs between groups. The analysis was performed with Bray-Curtis distances of the Hellinger-

transformed relative abundance data and was executed with the *adonis* function (vegan package) in R (v3.6.1) (Oksanen *et al.*, 2019) using 1,000 permutations. Further, observed richness was determined by counting the number of observed (z)OTUs, as well as the Simpson's index of diversity (1-D). The latter is a measure that characterizes diversity in a sample, and takes into account the number of (z)OTUs and their relative abundance. High scores (close to 1) indicate high diversity, low scores (close to 0) low diversity (Simpson, 1949). Further, samples were grouped according to beer type and time of sampling and significant differences between groups were determined with a pairwise Wilcoxon rank sum test with Bonferroni correction, using the *stats* package in R (v3.6.1) (R Core Team, 2019). The similarity in community composition between samples was visualized in a non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis similarities of Hellinger-transformed relative abundance data, constructed using the *MDS* function of the *vegan* package in R (v3.6.1) (Oksanen *et al.*, 2019). In addition, *perMANOVA* was performed to assess significant differences in beer chemistry between the different beer types, wood types and time of sampling. The analysis was again carried out with the *adonis* function using 1,000 permutations and was based on Euclidean distances of the normalized chemical data. Next, as chemical parameters are often related, a principal component analysis (PCA) was performed to reduce the number of variables using the *prcomp* function of the *stats* R-package (R Core Team, 2019) on the scaled chemical parameters. Redundancy analysis (RDA) was used to test the relationship between microbiological community data and beer chemistry data. RDA is the multivariate analogue of a linear model and allows to test whether the chemistry data significantly explains any variation in the microbiological community data. For this purpose, R-packages *vegan*, *ade4* and *maptools* were used (Dray and Dufour, 2007; Lewin-Koh *et al.*, 2011; Oksanen *et al.*, 2019). Additionally, a Hellinger transformation was applied to the microbiological data, the chemical data was scaled and a forward selection step was used to select the chemical parameters that showed a statistically significant contribution in explaining data variation. Finally, the fit of the computed regression model was estimated by R_{adj} , which was in turn used to calculate the amount of variation explained by the plotted RDA axes.

3. Results

3.1 Temporal dynamics of the bacterial and fungal communities

Sequence analysis revealed a total of 148 bacterial zOTUs and 332 fungal OTUs, including 330 OTUs in the ITS1₂₀₀₋₄₀₀ data set and two OTUs in the ITS1₄₀₀₋₅₅₀ data set (Tables S4-S6, Supporting information). The rarefaction curves for all samples approached saturation or tended to approach saturation, indicating that the sequencing depths were sufficient to cover the microbial diversity (Fig. S1, Supporting information). Within the ITS1₄₀₀₋₅₅₀ data set, the majority of the sequences (98 %) corresponded to the industrial brewer's yeast *S. cerevisiae* that was used for primary fermentation and which occurred in all samples analysed. Further, the remaining sequences matched with *Saccharomyces kudriavzevii* and could only be attributed to samples of beer 2 (Table S6, Supporting information). Due to the limited diversity in this data set, further diversity analyses were only performed on the V4 and ITS1₂₀₀₋₄₀₀ data sets. Band intensity on agarose gels, however, suggested that the fungal community of the unfiltered beers 1 and 3 at the beginning of the maturation process was dominated by *S. cerevisiae*. Bands corresponding to wild fungal strains (200-400 bp) became more intense than the *S. cerevisiae* band after 12 weeks and 38 weeks of maturation in the case of beer 1 and 3, respectively, suggesting that from then on these fungi became dominant. For the filtered beer 2, intensity of the band corresponding to the wild fungal strains was generally stronger than that of the *Saccharomyces* band (results not shown).

PerMANOVA revealed significant differences in the bacterial and fungal community composition of the three beers (bacteria: $F = 20.68$, $p < 0.001$ and fungi: $F = 13.65$, $p < 0.001$) and across different time points (bacteria: $F = 8.28$, $p < 0.001$ and fungi: $F = 4.36$, $p < 0.001$). In contrast, the bacterial and fungal community compositions did not significantly differ between the wood types (bacteria: $F = 0.58$, $p = 0.80$ and fungi: $F = 1.39$, $p = 0.12$). For this reason, data from the different wood types were combined when presenting the results of the diversity metrics (Fig. 1). At the start of the study, bacterial and fungal richness ranged from 57 (beer 2) to 70 zOTUs (beer 3), and from 4 (beer 1)

to 46 OTUs (beer 3), respectively. During maturation of beer 2 and 3 bacterial richness significantly (p
 < 0.05) decreased after 12 weeks of barrel ageing, while the bacterial richness of beer 1 remained
 constant throughout the entire maturation (Fig. 1A). Likewise, the Simpson's diversity index of beer 3
 significantly decreased after 12 weeks of maturation, whereas this decrease was found not to be
 significant for beer 2 (Fig. 1C). In addition, the observed richness of the fungal communities in all beers
 was significantly lower at the end of the maturation, in comparison to the richness at week 2 (Fig. 1B).
 Moreover, the fungal richness of beer 1 was already significantly lower after 12 weeks of maturation.
 On the contrary, in terms of the Simpson's diversity index of the fungal communities, the only
 significant effect was observed for beer 2 when comparing the index for week 2 and week 38 (Fig. 1D).
 In addition to changes in microbial community composition, also 16S rRNA gene and ITS1 copy
 numbers changed throughout the maturation process. 16S rRNA gene copy numbers were very low at
 the start of the experiment, especially in beers 1 and 2 where they did not exceed the threshold of
 detection (5.89×10^2 copies per μl DNA) (Fig. 1E). This suggests that, despite the fact that several
 species were detected (Fig. 1A), bacterial densities were very low in the base beers. When beers were
 subjected to maturation, 16S rRNA gene copy numbers substantially increased in beer 2 and 3,
 reaching an average of 1.2×10^7 and 0.8×10^6 16S rRNA gene copy numbers, respectively, while they
 remained low in beer 1 (Fig. 1E). Likewise, in beer 2 fungal ITS1 copies were found to increase, but
 tended to remain stable after 12 weeks of maturation. For beer 1 and 3, fungi were already abundantly
 present from the start of the maturation (Fig 1F). It has to be noted that the ITS copy numbers
 measured represented the whole fungal community, including both the primary fermentation yeast
 and any other occurring fungi, while the diversity analyses only represented the wild fungi (i.e. the
 ITS1₂₀₀₋₄₀₀ data set). In contrast to beer 2, beers 1 and 3 were not filtered prior to filling the barrels,
 and still contained yeast cells from the primary fermentation (Fig. 1F). Plating a subset of samples on
 common growth media, like 2% YPD (supplemented with 100 ppm chloramphenicol to inhibit bacterial
 growth), confirmed these trends (results not shown). However, it was also clear from these results

that not all microbes present could be cultivated and remained undetected by the plating method, while they were detected using the molecular approaches.

NMDS ordination of the Bray-Curtis distances of Hellinger-transformed relative abundance data (stress bacteria = 0.106 and stress fungi = 0.188) showed a clear separation of the different beers for both the bacterial and fungal communities (Fig. 2). Specifically, in both plots NMDS2 separated the three beers, while NMDS1 indicated substantial temporal changes in the communities. Nevertheless, no significant shifts were observed in the bacterial community composition of beer 1 during the course of maturation (Fig. 2A). Indeed, bacterial communities in beer 1 were consistently dominated by three zOTUs, corresponding to *Pediococcus damnosus* (zOTU 1), *Acetobacter* sp. (zOTU 4) and a member of the family Enterobacteriaceae (zOTU 5) (Fig. 3A). By contrast, more variation is observed in beer 2, in which the bacterial community shifted from a dominance of *Acetobacter* sp. (zOTU 2) to a dominance of *P. damnosus* (zOTU 1), accounting for 86.7 ± 18.5 (SD; amount of variability is consistently expressed as standard deviation (SD) in this study) % of the sequences at week 38 (Fig. 3B). In beer 3, the bacterial community evolved from a highly diverse community to a community dominated by a few species, including *P. damnosus* (zOTU 1), *Lactobacillus brevis* (zOTU 3) and *Acetobacter* sp. (zOTU 2) (Fig. 3C). Likewise, strong shifts were observed in the fungal communities during the course of maturation (Fig. 2B). While beer 1 was dominated by *Pichia membranifaciens* (OTU 2; 98.1 % of all sequences) at the start of the maturation, the community rapidly shifted to a dominance of *Brettanomyces* yeasts (OTU 1 (*Brettanomyces bruxellensis*) and OTU 5 (*Brettanomyces* sp.), together accounting for 76.8 ± 17.6 % of the sequences after 38 weeks of maturation) (Fig. 4A). An opposite trend was observed for beer 3 for which the fungal community was initially dominated by *B. bruxellensis* (OTU 1, 51.3 %), while it became dominated by *P. membranifaciens* (OTU 2) or *Debaryomyces* sp. (OTU 3) throughout the maturation process (Fig. 4C). In contrast to the other beers, no clear trends in fungal community shifts could be observed for beer 2. Moreover, unlike the other beers, substantial differences in fungal communities were observed across replicate barrels for beer 2 (Fig. 4B).

3.2 Temporal dynamics in beer chemistry and flavours

For a detailed overview of the chemical data for each of the samples investigated in this study the reader is referred to Tables S7 and S9 (Supporting information). PCA ordination of the beer chemistry during barrel ageing (Fig. 5) shows three distinct groups representing each of the three beers. Within each group of beer samples, the different time points at which samples were taken can be clearly distinguished, in particular for beer 1 and 3. It is also clear from this plot that beer chemistry for the three beers was already substantially different at the start of the maturation and that the changes in beer chemistry throughout maturation were different for each beer type (Fig. 5). PerMANOVA revealed significant differences in beer chemistry across the different beer styles ($F = 20.38$, $p < 0.001$), wood types ($F = 12.97$, $p < 0.001$) and time points during maturation ($F = 8.71$, $p < 0.001$).

Although the initial pH of the three beers was similar (Fig. 6A), the extent, rate and manner of acidification was different for all beers throughout the maturation process. More specifically, the pH of beer 1 decreased from an initial pH of 4.42 to 4.22 ± 0.03 after 38 weeks of maturation, where the most significant decrease occurred between 12 and 38 weeks of maturation. In contrast, the decrease in pH of beer 2 and 3 was larger and occurred more rapidly. Specifically, for beer 2 the pH decreased from 4.44 to 3.78 ± 0.09 and for beer 3 from 4.48 to 3.90 ± 0.07 at week 38. Furthermore, the barrels that were previously used for wine maturation caused a quicker decrease in pH than the new oak barrels (Fig. 6A). Likewise, also the composition of the organic acids differed across the beers. For example, the increase in concentration of lactic acid was limited for beer 1 (from 0 g/l to 0.26 ± 0.03 g/l) and was larger for beer 2 (from 0.27 g/l to 0.99 ± 0.29 g/l). The largest increase in lactic acid was observed for beer 3 (from 0.24 g/l to 1.39 ± 0.19 g/l) (Fig. 6B). In contrast, the increase in concentration of acetic acid was the largest for beer 1 (from 0.27 g/l to 1.41 ± 0.18 g/l after 38 weeks of maturation), was considerable for beer 3 (from 0.26 g/l to 0.88 ± 0.13 g/l) and low in the case of beer 2 (0.26 g/l to 0.34 ± 0.07 g/l) (Fig. 6C). The concentration of malic acid remained rather constant for beers 1 and 2, around 1.32 ± 0.29 g/l and 0.32 ± 0.10 g/l, respectively, whereas a large decrease was observed for

beer 3, from 1.38 g/l at the start to 0.59 ± 0.05 g/l at the end of the maturation. In general, the concentration of malic acid was higher in the wine barrels than in the new barrels (Fig. 6D).

Among the wood-related aroma compounds, eugenol and oak lactones *cis*-3-methyl-4-octanolide and *trans*-3-methyl-4-octanolide were found to increase over time for all beers, although the effect was most prominent in beer 1 and 3 and the concentration of these compounds in the wine barrels was lower than in the new oak barrels (Fig. 6E-G). In addition, vanillin was also found to increase over time in all beers, with the largest increase found in beer 3 (from 8.56 ppb to 818.21 ± 367.92 ppb after 38 weeks of maturation) and the smallest increase in beer 1 (from 152.47 ppb to 173.37 ± 62.36 ppb) (Fig. 6H). The concentrations of furfural and 5-methyl furfural remained constant in beers 1 and 3, yet changes were observed in the concentrations of these compounds throughout the maturation of beer 2 (Fig. 6I-J). Moreover, the observed concentrations of furfural and 5-methyl furfural were consistently larger in the new oak barrels in comparison to the barrels that were previously used for red wine maturation. For example, after 38 weeks of maturation of beer 2 the concentration of furfural in the new barrels increased from 1154.19 ppb to 4715.80 ppb, whereas the concentration of furfural in the wine barrels had decreased to 68.27 ± 6.87 ppb (Fig. 6I-J). Results also revealed that the concentration of 4-vinyl guaiacol was higher in beer 2, although this effect seems mainly due to its elevated concentration in the reference beer at the start of the maturation (Fig. 6K). In contrast, monophenolic compounds like 4-hydroxybenzaldehyde, guaiacol, 4-ethyl phenol and 4-ethyl guaiacol (Fig. 6L) were more concentrated in beer 1 than in the other beers (Table S7, Supporting Information).

Concentrations of the higher alcohols and esters in beer 2 were low at the start of the maturation, and remained constant throughout the process. In contrast, at the start of the maturation, beer 3 contained elevated concentrations of various esters like isobutyl acetate, isoamyl acetate, ethyl butanoate, ethyl octanoate, ethyl decanoate and phenylethyl acetate, whose concentration had decreased by the end of the maturation (Table S7, Supporting Information), e.g. the concentration of

isoamyl acetate decreased from 9.38 ppm to 0.43 ± 0.34 ppm (Fig. 6M). At the same time, diverse higher alcohols and esters were found to increase during the maturation of beer 1, e.g. ethyl acetate (Fig. 6N), ethyl hexanoate, ethyl octanoate, ethyl decanoate and phenylethyl alcohol (Table S7, Supporting Information). It is also clear from our results that the concentrations of proanthocyanidins, flavonoids and total polyphenols were higher in beer 1. Nevertheless, these compounds were already found in higher concentrations in beer 1 at the start of the maturation, and these elevated concentrations remained constant throughout the process (Fig. 6O; Table S7, Supporting Information). In contrast, the total polyphenol content was found to increase over time in case of beer 2 (from 149.16 ppm to 215.10 ± 15.39 ppm) and beer 3 (from 231.86 ppm to 302.70 ± 23.49 ppm) (Fig. 6O).

The ethanol concentration of all three beers was slightly higher after 38 weeks of wood maturation in comparison to the reference beer at week 0. In beer 1 the ethanol concentration increased from 9.64 % ABV to 10.08 ± 0.27 % ABV, in beer 2 from 3.12 % ABV to 3.88 ± 0.22 % ABV and in beer 3 from 8.88 to 8.98 ± 0.10 % ABV (Table S9, Supporting Information). Additionally, perMANOVA revealed significant differences in sugar concentrations across the different beers ($F = 130.232$, $p < 0.001$), wood types ($F = 2.719$, $p < 0.05$) and time points ($F = 33.730$, $p < 0.001$). In general, concentration of D-glucose, D-fructose and sucrose decreased over the maturation period (Fig. 6P-R; Table S9, Supporting Information). Effects were most pronounced in beer 3, which contained a substantially higher amount of these carbohydrates at the start of the maturation (D-glucose: 0.097 g/l, D-fructose: 0.273 g/l, and sucrose: 0.110 g/l vs 0.019 g/l, 0.044 g/l and 0.031 g/l, and 0.029 g/l, 0.060 g/l and 0.042 g/l for beers 1 and 2, respectively). At the end of the maturation, similar amounts of these sugars were found in all beers. Furthermore, different β -glucan levels and dynamics were observed for the different beers. The concentration of β -glucan was the highest in beer 1 (up to 0.105 g/l), intermediate in beer 3 (range: 0.040 g/l - 0.082 g/l) and low in beer 2 (down to 0.003 g/l). In general, β -glucan levels remained fairly constant over the maturation process, except for beer 3 where different values were recorded for different wood types (Fig. 6S; Table S9, Supporting Information).

When subjecting the beer samples to a tasting panel, wood-related flavours described as 'smoky', 'phenolic', 'woody' and 'whisky' were found to increase during the first 12 weeks of maturation entailing harsh woody flavour notes and astringency (Fig. S2, Supporting Information). Towards the end of the maturation, the beer's sourness increased while the harsh woody flavours decreased. Whereas the combined acidity of lactic acid and acetic acid was considered palatable at the end of the wood maturation of beers 2 and 3, panel members indicated that the sharp flavour notes of acetic acid in combination with the high bitterness of beer 1 was less pleasant. Furthermore, the tasting panel detected relatively high concentrations of esters in the reference of beers 2 and 3, and identified a decrease in ester concentration for beer 3 and a variable ester concentration throughout wood maturation of beer 2. In contrast, the attribute 'esters' was scored zero for beer 1 throughout the entire maturation process (Fig. S2, Supporting Information).

3.3 Relationship between beer chemistry and microbial community composition

Canonical redundancy analysis (RDA) was used to assess the relationship between beer chemistry and the microbial community composition throughout the wood maturation process (Fig. 7). In concordance with the NMDS and PCA ordination plots shown previously, beer samples were distributed across the RDA plot according to their origin and maturation time. Whereas PCA ordination was performed with all chemical variables, here a forward selection step was implemented to identify the compounds with a significant contribution to the regression model ($p < 0.05$). The model explained 56.8 % of the variation ($R^2_{adj} = 0.5676$), and the two RDA axes explained 19.91 % ($p = 0.001$) and 10.98 % ($p = 0.001$) of the data variation, respectively. The three beer types were clearly separated in the RDA plot and within each of the beer groups, samples clustered according to maturation time (Fig. 7). While beer 1 was mainly characterized by 2-methyl butanol, 3-methyl butanol, flavonoids and a high pH, beer 2 scored high on 5-methyl furfural and beer 3 contained high concentrations of ethyl butanoate, isobutyl acetate, isoamyl acetate, ethyl vanillate, 4-methyl guaiacol, trans-3-methyl-4-octanolide, propionic acid and lactic acid. High relative abundances of *P. damnosus* (zOTU 1) and

Acetobacter sp. (zOTU 2) could be linked with beer samples that contained high concentrations of 5-methyl furfural. Together with *L. brevis* (zOTU 3) and *Debaryomyces* sp. (OTU 3) these microorganisms were also found in high relative abundances in beer samples with elevated concentrations of lactic acid. In addition, the highest relative abundance of *L. brevis* (zOTU 3) and *Debaryomyces* sp. (OTU 3) was found in samples that contained high concentrations of ethyl butanoate, isobutyl acetate, isoamyl acetate, trans-3-methyl-4-octanolide, ethyl vanillate, propionic acid and 4-methyl guaiacol. Furthermore, *B. bruxellensis* (OTU 1), *Acetobacter* sp. (zOTU 4), and a member of the family Enterobacteriaceae (zOTU 5) were associated with beer samples that had a higher pH value and contained 2- and 3-methyl butanol and flavonoids (Fig. 7).

4. Discussion

4.1 Initial beer properties and wood type may affect the chemical profile of wood matured beers

Our results show that microbial community composition, beer chemistry and sensory characteristics substantially change throughout the maturation process. Furthermore, the initial beer properties may have affected the outcome of wood maturation. This can be seen from the NMDS and PCA plots, showing the dynamics in microbial composition and beer chemistry respectively, where samples primarily clustered per beer type, and secondly by sampling time. The samples investigated in this study did not converge to a similar composition in microbial community or chemical profile, notwithstanding that each of the beers encountered a decrease in pH over time. Strikingly, differences in microbial community composition already existed at the start of the maturation process. Indeed, although the studied beers were fermented with a monoculture of *S. cerevisiae*, base beers were found to contain several wild bacterial and fungal species, but at very low densities. This suggests that the beers may have become contaminated with environmental microbes or microbes associated with the brewing environment before filling the barrels. Subsequently, strains adapted to thrive in the beers during maturation may have started to grow abundantly and dominate the community (Fukami, 2015). Substrate analysis suggested that the microorganisms found thrived on common carbohydrates

such as glucose, fructose and sucrose (as can be seen for beer 3). Obviously, the beers also contained other substrates that can be used for microbial growth, such as organic acids, ethanol etc. Further research is needed to unravel the metabolic properties of the microorganisms involved in barrel ageing of finished beers.

In contrast to the effect of beer type and maturation time, the bacterial and fungal community composition did not differ between the wood types, while wood type significantly affected the beer chemistry. This was unexpected as the wine barrels were assumed to result in a different microbial community due to previous microbial colonization of the interior surfaces of the barrels while maturing the wine. It seems reasonable to assume that the applied disinfection method may have destroyed the majority of the barrel microbes, thereby reducing or minimizing their effects. Further research is needed to confirm this scenario. Beers aged in wine barrels were found to contain more malic acid, less furfural and 5-methyl furfural, less lactones (cis- and trans-3-methyl-4-octanolide), less eugenol and their pH was found to decrease more quickly. In theory, the decrease in pH may have been caused by the higher concentrations of malic acid, and/or by a higher concentration of dissolved oxygen as re-used wood tends to have a higher oxygen permeability (del Alamo-Sanza and Nevares, 2018), and/or by certain microorganisms inhabiting the wood. Further, the lower concentration of wood compounds in the beers aged in wine barrels may have been caused by a reduced amount of extractable wood compounds as a previous extraction has occurred during the preceding maturation of wine inside these barrels (Navarro *et al.*, 2016). In contrast, no clear differences were identified between the new oak barrels of category 'sweet' and 'structure'. Altogether, it can be concluded that the initial beer properties and wood type are potential key factors in determining the final chemical profile of wood matured beers.

The resulting concentrations of wood compounds may also be influenced by the microorganisms involved in barrel ageing. For example, de Revel *et al.* (2005) suggested that the vanillin extracted from wood is propitious for the viability of *Oenococcus oeni*, although other wood

compounds could be involved as well. In addition, higher concentrations of volatile wood compounds (in particular vanillin and isoeugenol) were found when malolactic fermentation of wine by *O. oeni* was performed in contact with wood (de Revel *et al.*, 2005). Likewise, our study hints towards specific interactions between the extraction of wood compounds and particular microbes. For example, a significant positive correlation (Spearman $R = 0.45$, $p < 0.001$) was found between the relative abundance of *P. damnosus* and the concentration of vanillin. However, further research using controlled lab experiments is needed to unravel the interactions between wood compounds and microbial species involved in the maturation process.

4.2 Beer bitterness may restrain the bacterial community composition and may influence beer souring

Deep amplicon sequencing of the bacterial communities that develop throughout the course of wood maturation suggested that bitterness plays an important role in the community composition. Results showed that the composition of the bacterial community of the high bitterness beer (beer 1) remained fairly constant throughout the process, while the bacterial community composition of the intermediate-bitterness beers (beers 2 and 3) changed drastically. Specifically, at the end of the maturation these beers were mainly dominated by LAB like *P. damnosus* and *L. brevis* and to a lesser extent by *Acetobacter* sp. The low relative abundance of LAB throughout the maturation of beer 1 can potentially be explained by the antibacterial effect of hop resins like hop β -acids and iso- α -acids on gram positive bacteria like LAB (Sakamoto and Konings, 2003; Schurr *et al.*, 2015). Hop resins act as ionophores which dissipate the pH gradient across the cytoplasmic membrane and disturb the proton motive force, thereby obstructing associated nutrient uptake, eventually resulting in cell death (Sakamoto and Konings, 2003). Despite the low relative abundance of LAB in beer 1, its pH value was still found to decrease, albeit at a later stage of the maturation in comparison to the other beers. This drop in pH could be attributed to other acids like acetic acid rather than lactic acid. Indeed, the elevated concentrations of acetic acid, 4-ethyl guaiacol and 4-ethyl phenol in combination with a

substantial relative abundance of *Acetobacter* sp. and *B. bruxellensis* suggest that these microorganisms are presumably the responsible souring agents in beer 1. In comparison, souring of beers 2 and 3 occurred more quickly, and was mainly caused by increasing concentrations of lactic acid and to a lesser extent by acetic acid. In addition, the concentrations of 4-ethyl guaiacol and 4-ethyl phenol were lower in these beers, as was the relative abundance of *Brettanomyces* spp. As a result, beer bitterness can possibly be considered an important factor in the establishment of the microbial communities occurring throughout wood maturation, and therefore also in determining the sour flavour notes at the end of the maturation. However, further research using well-controlled lab experiments is still needed to confirm this scenario.

As mentioned previously, the antibacterial activity of hop resins only targets gram positive bacteria, leaving gram negative bacteria, among which Enterobacteriaceae, unharmed. The presence of Enterobacteriaceae could potentially be hazardous for human health as certain members of this family (like *Enterobacter aerogenes* and *Klebsiella oxytoca*) are able to produce biogenic amines, although this activity is influenced by many factors including the maturation temperature (Gardini *et al.*, 2016). In our study, the fraction of Enterobacteriaceae was only substantial in beer 1, while they rapidly disappeared throughout the maturation of beers 2 and 3. The latter could be due to increasing cell densities of competing, dominating bacterial species in beers 2 and 3, while cell densities in beer 1 remained consistently low throughout the process. The decreasing relative abundance of Enterobacteriaceae in beers 2 and 3 could also be attributed to the quick drop in pH and the increasing concentrations of lactic acid, which is known to inhibit Enterobacteriaceae (Vivijs, 2015).

4.3 Alcohol content may affect the extraction of wood compounds and the fungal community composition

Our results indicate that alcohol content may have had a strong effect on the extraction of wood compounds. Wood compounds like cis-3-methyl-4-octanolide, trans-3-methyl-4-octanolide, eugenol and total polyphenols occurred at higher concentrations in the beers with a high alcohol content

(beers 1 and 3) in comparison to the low alcohol beer (beer 2). These findings are in line with Sterckx *et al.* (2012), who also suggested that a low pH increases the extraction of wood monophenols. Although beer 2 was low in alcohol, its pH value also decreased rapidly, possibly reducing the effect of the low alcohol level on the extraction of wood compounds. Indeed, for other wood monophenols, the increase in concentration in beer 2 was comparable to the other maturing beers.

In addition, alcohol level may have affected the composition of the non-*Saccharomyces* fungal community throughout the process of wood maturation as well. Whereas the non-*Saccharomyces* fungal communities of the high alcohol beers (beers 1 and 3) were mainly composed of yeasts that are frequently identified as spoilage organisms in the beer industry, the fungal community of the low alcohol beer (beer 2) contained many species that are not often associated with beer, including several *Penicillium* spp. and *Candida* spp. It is hypothesized that such fungal species are only found in beer 2 because it provides a less stringent environment in comparison to the beers that have a higher alcohol and/or bitterness level. However, it is also possible that the filtration step in the production process of beer 2 has played a role here, by which competing *Saccharomyces* spp. has been removed from the beer.

4.4 Wood maturation of conventionally fermented beers shares trends in microbial successions with traditional sours

There are several traditional sour beer styles that involve fermentation and/or maturation in wooden barrels, including lambic beers and Belgian red ales (Bossaert *et al.*, 2019). In contrast to the production of lambic beers, the beers investigated in this study had undergone a primary fermentation before wood maturation, whereas spontaneously infected wort is fermented and matured inside wooden barrels harbouring the desired microorganisms in the case of lambic beers (De Roos and De Vuyst, 2019a). Lambic production is characterized by a microbial succession consisting of four phases: an enterobacterial phase (unless the wort is sufficiently soured before transferring to the coolship), a main fermentation phase performed by *Saccharomyces* yeasts, an acidification phase due to lactic and

acetic acid bacteria, and finally a maturation phase containing wild yeasts like *Brettanomyces* spp. and LAB (De Roos and De Vuyst, 2019a). Similar trends were observed for the beers studied here, especially for the intermediate-bitterness beers 2 and 3. Both beers showed a substantial bacterial diversity at the start of the process, followed by an increasing dominance of LAB and AAB. Moreover, typical bacterial species occurring in lambic brewing like *P. damnosus*, *L. brevis* and *Acetobacter* sp. (De Roos *et al.*, 2019b) also occurred in these beers at high relative abundance. By contrast, no such homology in bacterial community dynamics could be observed for the high bitterness beer (beer 1). Possibly this can be explained by the fact that lambic production typically uses lower amounts of hop to avoid inhibition of LAB (De Roos and De Vuyst, 2019a), while beer 1 was significantly hopped compared to the other beers studied. However, on the contrary, with respect to the fungi, beer 1 showed the highest similarity to lambic beer production as *Brettanomyces* species took up a large and increasing share of the fungal community during the maturation process. The similarity between beers in this study and lambic beers could even be extended to American coolship ales which are also found to share trends in microbial successions with lambic beers (Bossaert *et al.*, 2019; De Roos and De Vuyst, 2019a).

5. Conclusion

In conclusion, this study clearly showed temporal changes in the microbial community composition, beer chemistry and sensory characteristics throughout the wood maturation process of finished, conventionally fermented beers. We also found that the initial beer characteristics and wood type are most likely key factors in determining the chemical profile of the end product. More specifically, beer bitterness may restrain the bacterial community composition, thereby having an impact on beer souring. Additionally, the initial alcohol content might affect the extraction of wood compounds and the fungal community composition. Whereas this study clearly increases our understanding of the maturation of finished beers, more in-depth research using controlled experiments in which only one or a few parameters are varied (Wolfe and Dutton, 2015) is necessary to confirm the observations

made in this study, as well as to gain further insights into the mechanisms behind microbial community assembly and functioning, and the resulting effects on beer chemistry and sensory characteristics. Future research could also focus on studying how these microorganisms are thriving in the barrels, and how their metabolism can be linked to flavour development. In particular, linking specific chemical compounds, microbial taxa and/or microbial functions with sensory data will allow the identification of (bio-)markers that can be used to predict the souring process and the quality of the end product. Such knowledge will be the basis of a new variety of sour beers, with a balanced mix of acidity and wood compounds.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This work was supported by VLAIO (Flanders Innovation and Entrepreneurship) [project HBC.2017.0031] and the Flemish Research Foundation (FWO) [project 1SC3220N].

Acknowledgements

We thank all breweries involved in this research. Further, we are grateful to H. Jacquemyn for advise on the statistical analyses and to G. Van Boeckel for the isolation and identification of microbial strains used in the mock communities.

Appendix A. Supplementary data

Table S1: Primer design and sample-specific barcodes for the bacterial V4 region of the 16S rRNA gene

Table S2: Primer design and sample-specific barcodes for the fungal ITS1 region

Table S3: Composition of mock communities

Table S4: Identification of zero radius operational taxonomic units (zOTUs) according to the Silva v1.23 database and distribution over the investigated samples.

Table S5: Identification of operational taxonomic units (OTUs) in the ITS1₂₀₀₋₄₀₀ dataset according to the UNITE v6 database and distribution over the investigated samples.

Table S6: Identification of operational taxonomic units (OTUs) in the ITS1₄₀₀₋₅₅₀ dataset according to the UNITE v6 database and distribution over the investigated samples.

Table S7: Chemical data

Table S8: Chemical analyses protocols

Table S9: Carbohydrates and ethanol concentration

Table S10: Diversity metrics for the bacterial communities

Table S11: Diversity metrics for the fungal communities

Figure S1: Rarefaction curves for the different datasets used in this study, i.e. the bacterial V4 dataset (A), the fungal ITS1₂₀₀₋₄₀₀ dataset (B), and the fungal ITS1₄₀₀₋₅₅₀ dataset (C). Rarefaction curves are presented for each of the studied beers. Samples were taken at different time points throughout the process of wood maturation, i.e. at week 0 (reference), week 2, week 12 and week 38. Rarefaction curves for all samples approached saturation or tended to approach saturation, indicating that the sequencing depths were sufficient to cover the microbial diversity.

Figure S2: Temporal changes in sensory attributes throughout the wood maturation process of the three beers investigated in this study. Attribute scores were assigned by a trained tasting panel (10 panel members) on a scale from 0 (not present) to 8 (high intensity). Data are presented for each replicate. A different sensory attribute is presented in each panel of the figure: (A) smoky, (B) phenolic, (C) bitter, (D) woody, (E) whisky, (F) astringency, (G) esters.

References

1. Bokulich, N.A., Mills, D.A., 2013. Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. Appl. Environ. Microbiol. 79, 2519-2526.

2. Borremans, A., Crauwels, S., Vandeweyer, D., Smets, R., Verreth, C., Van Der Borght, M., Lievens, B., Van Campenhout, L., 2019. Comparison of Six Commercial Meat Starter Cultures for the Fermentation of Yellow Mealworm (*Tenebrio molitor*) Paste. *Microorganisms*. 7, 540.
3. Bossaert, S., Crauwels, S., De Rouck, G., Lievens, B., 2019. The power of sour – A review: Old traditions, new opportunities. *BrSc*. 72, 78-88.
4. Bronner, I.F., Quail, M.A., Turner, D.J. and Swerdlow, H., 2013. Improved protocols for illumina sequencing. *Curr Protoc Hum Genet*. 79, 18-2.
5. Callahan, B.J., McMurdie, P.J., Holmes, S.P., 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J*. 11, 2639.
6. Cantwell, D., Bouckaert, P., 2016. *Wood & Beer: A Brewer's Guide*. Brewers Publications, Colorado, USA.
7. Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. USA*. 108, 4516–4522.
8. Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A., Callahan, B.J., 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*. 6, 226.
9. De Filippis, F., Laiola, M., Blaiotta, G., Ercolini, D., 2017. Different amplicon targets for sequencing-based studies of fungal diversity. *Appl. Environ. Microbiol*. 83, e00905-17.
10. De Keersmaecker, J., 1996. The mystery of lambic beer. *Sci. Am*. 275, 74-80.
11. de Revel, G., Bloem, A., Augustin, M., Lonvaud-Funel, A., Bertrand, A., 2005. Interaction of *Oenococcus oeni* and oak wood compounds. *Food Microbiol*. 22, 569-575.
12. De Roos, J., De Vuyst, L., 2019a. Microbial acidification, alcoholization and aroma production during spontaneous lambic beer production. *J. Sci. Food Agric*. 99, 25-38.

13. De Roos, J., Van der Veken, D., De Vuyst, L., 2019b. The interior surfaces of wooden barrels are an additional microbial inoculation source for lambic beer production. *Appl. Environ. Microbiol.* 85, e02226-18.
14. De Roos, J., Verce, M., Aerts, M., Vandamme, P., De Vuyst, L., 2018. Temporal and spatial distribution of the acetic acid bacterium communities throughout the wooden casks used for the fermentation and maturation of lambic beer underlines their functional role. *Appl. Environ. Microbiol.* 84, e02846-17.
15. De Rosso, M., Panighel, A., Dalla Vedova, A., Stella, L., Flamini, R., 2009. Changes in chemical composition of a red wine aged in acacia, cherry, chestnut, mulberry, and oak wood barrels. *J. Agric. Food Chem.* 57, 1915-1920.
16. De Souza, R.S.C., Okura, V.K., Armanhi, J.S.L., Jorrín, B., Lozano, N., Da Silva, M.J., González-Guerrero, M., De Araújo, L.M., Verza, N.C., Bagheri, H.C., Imperial, J., 2016. Unlocking the bacterial and fungal communities assemblages of sugarcane microbiome. *Sci. Rep.* 6, 28774.
17. del Alamo-Sanza, M., Nevares, I., 2018. Oak wine barrel as an active vessel: a critical review of past and current knowledge. *Crit Rev Food Sci Nutr.* 58, 2711-2726.
18. Dray, S., Dufour, A.B., 2007. The ade4 package: implementing the duality diagram for ecologists. *J. Stat. Softw.* 22, 1-20.
19. Dzialo, M.C., Park, R., Steensels, J., Lievens, B., Verstrepen, K.J., 2017. Physiology, ecology and industrial applications of aroma formation in yeast. *FEMS Microbiol. Rev.* 41, S95-S128.
20. Edgar, R.C., 2013. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods.* 10, 996-998.
21. Edgar, R.C., 2016. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv.* 081257.
22. Edgar, R.C., Flyvbjerg, H., 2015. Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics.* 31, 3476-3482.

23. Fan, W., Xu, Y., Yu, A., 2006. Influence of oak chips geographical origin, toast level, dosage and aging time on volatile compounds of apple cider. *J. Inst. Brew.* 112, 255-263.
24. Fukami, T., 2015. Historical contingency in community assembly: integrating niches, species pools, and priority effects. *Annu. Rev. Ecol. Evol. Syst.*, 46, 1-23.
25. Gardini, F., Özogul, Y., Suzzi, G., Tabanelli, G., Özogul, F., 2016. Technological factors affecting biogenic amine content in foods: a review. *Front Microbiol.* 7, 1218.
26. Gollihue, J., Richmond, M., Wheatley, H., Pook, V.G., Nair, M., Kagan, I.A., DeBolt, S., 2018. Liberation of recalcitrant cell wall sugars from oak barrels into bourbon whiskey during aging. *Sci. Rep.*, 8, 1-12.
27. Justé, A., Thomma, B.P.H.J., Lievens, B., 2008. Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. *Food Microbiol.*, 25, 745-761.
28. Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112-20.
29. Lewin-Koh, N.J., Bivand, R., Pebesma, J., Archer, E., Baddeley, A., Giraudoux, D.G., Rubio, V.G., Hausmann, P., Hufthammer, K.O., Jagger, T., Sebastian, P., 2011. Package 'maptools'. URL: <http://cran.r-project.org/web/packages/maptools/maptools>. Pdf. Last accessed: 06.07.2020.
30. Lievens, B., Brouwer, M., Vanachter, A.C., Lévesque, C.A., Cammue, B.P. and Thomma, B.P., 2003. Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens. *FEMS Microbiol. Lett.* 223, 113-122.
31. McMurdie, P.J., Holmes, S., 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE.* 8, e61217.

32. Navarro, M., Kontoudakis, N., Gómez-Alonso, S., García-Romero, E., Canals, J.M., Hermosín-Gutiérrez, I., Zamora, F., 2016. Influence of the botanical origin and toasting level on the ellagitannin content of wines aged in new and used oak barrels. *Food Res. Int.* 87, 197-203.
33. Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2019. *vegan: Community Ecology Package*. R package version 2.5-6.
34. Oulas, A., Pavludi, C., Polymenakou, P., Pavlopoulos, G.A., Papanikolaou, N., Kotoulas, G., Arvanitidis, C., Iliopoulos, L., 2015. Metagenomics: tools and insights for analyzing next-generation sequencing data derived from biodiversity studies. *Bioinform. Biol. Insights*, 9, BBI-S12462.
35. R Core Team, 2019. *R: A Language and Environment for Statistical Computing* (Version 3.5. 2, R Foundation for Statistical Computing, Vienna, Austria, 2018).
36. Sakamoto, K., Konings, W.N., 2003. Beer spoilage bacteria and hop resistance. *Int. J. Food Microbiol.* 89, 105-124.
37. Schurr, B.C., Hahne, H., Kuster, B., Behr, J., Vogel, R.F., 2015. Molecular mechanisms behind the antimicrobial activity of hop iso- α -acids in *Lactobacillus brevis*. *Food Microbiol.* 46, 553-563.
38. Shayeitz, A., Harrison, K., Curtin, C.D., 2020. Barrel-Induced Variation in the Microbiome and Mycobiome of Aged Sour Ale and Imperial Porter Beer. *J Am Soc Brew Chem*, 1-8.
39. Sielaff, A.C., Urbaniak, C., Mohan, G.B.M., Stepanov, V.G., Tran, Q., Wood, J.M., Minich, J., McDonald, D., Mayer, T., Knight, R., Karouia, F., 2019. Characterization of the total and viable bacterial and fungal communities associated with the International Space Station surfaces. *Microbiome*. 7, 50.
40. Simpson, E.H., 1949. Measurement of diversity. *Nature*, 163, 688-688.

41. Snauwaert, I., Roels, S.P., Van Nieuwerburg, F., Van Landschoot, A., De Vuyst, L., Vandamme, P., 2016. Microbial diversity and metabolite composition of Belgian red-brown acidic ales. *Int. J. Food Microbiol.* 221, 1-11.
42. Spitaels, F., Wieme, A.D., Janssens, M., Aerts, M., Van Landschoot, A., De Vuyst, L., Vandamme, P., 2015. The microbial diversity of an industrially produced lambic beer shares members of a traditionally produced one and reveals a core microbiota for lambic beer fermentation. *Food Microbiol.* 49, 23-32.
43. Stadler, E., Fischer, U., 2020. Sanitization of Oak Barrels for Wine - A Review. *J. Agric. Food Chem.* 68, 5283-5295.
44. Sterckx, F.L., 2011. Volatile monophenols in beer: identification, influence on beer flavour and origin from raw materials and wood aging. Doctoral dissertation, KU Leuven, Belgium.
45. Sterckx, F.L., Saison, D., Delvaux, F.R., 2012. Wood Aging of Beer. Part II: Influence of Wood Aging Parameters on Monophenol Concentrations. *J Am Soc Brew Chem.* 70, 62-69.
46. Swaffield, C.H., Scott, J.A., 1995. Existence and development of natural microbial populations in wooden storage vats used for alcoholic cider maturation. *J Am Soc Brew Chem.* 53, 117-120.
47. Tonsmeire, M., 2014. American sour beers: Innovative techniques for mixed fermentations. Brewers Publications, Colorado, USA.
48. Vivijis, B., 2015. Adaptation mechanisms for growth under acid stress in Enterobacteriaceae. Doctoral dissertation, KU Leuven, Leuven, Belgium.
49. Wang, D.M., Yao, Y.J., 2005. Intrastrain internal transcribed spacer heterogeneity in *Ganoderma* species. *Can. J. Microbiol.* 51, 113-121.
50. Wolfe, B.E., Dutton, R.J., 2015. Fermented foods as experimentally tractable microbial ecosystems. *Cell*, 161, 49-55.

51. Wuyts, S., Van Beeck, W., Oerlemans, E.F., Wittouck, S., Claes, I.J., De Boeck, I., Weckx, S., Lievens, B., De Vuyst, L., Lebeer, S., 2018. Carrot juice fermentations as man-made microbial ecosystems dominated by lactic acid bacteria. *Appl. Environ. Microbiol.* 84, e00134-18.
52. Zhao, Y., Tsang, C.C., Xiao, M., Cheng, J., Xu, Y., Lau, S.K., Woo, P.C., 2015. Intra-genomic internal transcribed spacer region sequence heterogeneity and molecular diagnosis in clinical microbiology. *Int J Mol Sci.* 16, 25067-25079.

Figure captions

Figure 1: Temporal dynamics of bacterial (A) and fungal (B) (z)OTU richness, Simpson's index of diversity (1-D) (panels C and D), and 16S rRNA gene (E) or ITS1 copy numbers (F) during the wood maturation process of the three beers investigated in this study. Beers were matured in three barrel types ($n = 2$) and data were combined per time point. The situation before transfer of the beer to the wooden barrels (week 0) is indicated by a star (reference). The lower and upper hinges of the box plots correspond to the 25th and 75th percentiles, the whiskers indicate variability outside these quartiles, while the bar inside the box corresponds with the median value. Significant differences between week 2, 12 and 38 ($p < 0.05$) are indicated above the boxplots. The limits of detection of the qPCR assays were equal to 5.89×10^2 copies of the 16S rRNA gene per μl DNA (E) and 2.95×10^2 copies of ITS1 per μl DNA (F). When copy numbers did not exceed the threshold limit in any of the samples tested per time point, this is indicated as "not detected" (ND) at the height of the detection limit. The number of positive samples used in the calculations is presented between brackets, and varied between 1 and 6 positive samples. ITS copy numbers represented the whole fungal community, including both the primary fermentation yeast and any other occurring fungi, while the diversity analyses performed were only for the wild fungi (i.e. the ITS1₂₀₀₋₄₀₀ data set).

Figure 2: Non-metric multidimensional scaling (NMDS) ordination plots based on relative abundance data of the bacterial communities (stress = 0.106) (A) and fungal communities (stress = 0.188) (B) of beer samples taken at different time points throughout the wood maturation process of the three beers investigated in this study ($n = 2$). The greater the distance between two data points, the more dissimilar the microbial communities. (z)OTUs are presented onto the NMDS plots as small circles, and the most abundant (z)OTUs are indicated with their respective identifier.

Figure 3: Temporal dynamics in bacterial community composition throughout the wood maturation process of the three beers investigated in this study. Beers were matured in three barrel types, with two replicates. Data are presented for each biological replicate separately. Specified zOTUs had an overall relative abundance larger than 5 % in at least one beer and/or a relative abundance larger than 25 % in at least one sample. All other zOTUs are grouped in the fraction 'Others'. The identity

of the most important zOTUs was retrieved through a BLAST search against type materials in GenBank and the obtained percentage of identity is indicated for each zOTU.

Figure 4: Temporal dynamics in fungal community composition throughout the wood maturation process of the three beers investigated in this study. Beers were matured in three barrel types, with two replicates. Data are presented for each biological replicate separately. Specified OTUs had an overall relative abundance larger than 5 % in at least one beer and/or a relative abundance larger than 25 % in at least one sample. All other OTUs are grouped in the fraction 'Others'. The identity of the most important OTUs was retrieved through a BLAST search against type materials in GenBank and the obtained percentage of identity is indicated for each OTU.

Figure 5: Principal component analysis (PCA) displaying the differences in chemical composition of beer samples taken at different time points throughout the wood maturation process of the three beers investigated in this study. Beers were matured in three barrel types, with two biological replicates, resulting in six chemical profiles per beer per time point (except for the reference). The chemical variables are presented as vectors, and the ellipses represent the 95 % confidence interval for each beer type, assuming a multivariate normal distribution.

Figure 6: Temporal changes in beer chemistry and carbohydrate concentration throughout the wood maturation process of the three beers investigated in this study. Beers were matured in three barrel types, with two replicates. Data are presented for each biological replicate separately. Displayed parameters: (A) pH, (B) lactic acid, (C) acetic acid, (D) malic acid, (E) eugenol, (F) cis-3-methyl-4-octanolide, (G) trans-3-methyl-4-octanolide, (H) vanillin, (I) furfural, (J), 5-methyl furfural, (K) 4-vinyl guaiacol, (L) 4-ethyl guaiacol, (M) isoamyl acetate, (N) ethyl acetate, (O) total polyphenols, (P) D-glucose, (Q) D-fructose, (R) sucrose, and (S) β -glucan. For a detailed overview of the different chemical parameters and carbohydrate concentrations measured in this study, the reader is referred to Tables S7 and S8, respectively (Supporting Information).

Figure 7: Canonical redundancy analysis (RDA) of beer samples from different time points throughout the wood maturation process of the three beers investigated in the study. Beers were matured in three barrel types, with two biological replicates, resulting in six microbiological profiles per beer per time point (except for the reference). The chemical variables, presented as vectors in the triplot were identified by forward selection as the compounds with a significant contribution to the regression model ($p < 0.05$). The small circles in the triplot represent bacterial zOTUs (black) and fungal OTUs (gray), and the most abundant (z)OTUs are indicated with their respective identifier. The scaling of the RDA was set to optimally describe the relationship among chemical variables, yielding a correlation triplot. The interpretation of such triplots can be explained as follows: (1) (z)OTUs plotted closely together presumably have similar relative frequencies along the samples, (2) an orthogonal projection of a (z)OTU onto a chemical vector line indicates the position of the maximum value of the (z)OTU along that chemical variable, and (3) the cosine of the angle between chemical vectors approximates the correlation between

860 the corresponding variables. Samples were plotted as the weighted sums of site scores, indicating that sample coordinates
861 were assigned by an ordination in the space of the original chemical variables.

A Bacterial zOTU richness

B Fungal OTU richness

C Bacterial simpson's index of diversity

D Fungal simpson's index of diversity

E Bacterial 16S rRNA gene copies

F Fungal ITS copies

zOTU 1: *Pediococcus damnosus* (100%)
zOTU 2: *Acetobacter* sp. (100%)
zOTU 3: *Lactobacillus brevis* (100%)
zOTU 4: *Acetobacter* sp. (100%)

zOTU 5: Enterobacteriaceae (100%)
zOTU 6: *Stenotrophomonas* sp. (100%)
zOTU 14: *Acinetobacter* sp. (99.6%)

OTU 1: *Brettanomyces bruxellensis* (100%)
OTU 2: *Pichia membranifaciens* (100%)
OTU 3: *Debaryomyces* sp. (100%)
OTU 4: *Kregervanrija fluxuum* (96.84%)
OTU 5: *Brettanomyces custerianus* (91.67%)

OTU 7: *Penicillium* sp. (100%)
OTU 8: *Candida norvegica* (100%)
OTU 9: *Candida atlantica* (100%)
OTU 10: *Candida friedrichii* (100%)
OTU 11: *Penicillium roquefortii* (100%)

A pH

B Lactic acid

C Acetic acid

D Malic acid

Sweet A Structure A Wine A
 Sweet B Structure B Wine B

E Eugenol

F Cis-3-methyl-4-octanolide

G Trans-3-methyl-4-octanolide

H Vanillin

Sweet A Structure A Wine A
 Sweet B Structure B Wine B

I Furfural

J 5-Methyl furfural

K 4-Vinyl guaiacol

L 4-Ethyl guaiacol

Sweet A Structure A Wine A
 Sweet B Structure B Wine B

M Isoamyl acetate

N Ethyl acetate

O Total polyphenols

P D-glucose

Q D-Fructose

R Sucrose

S β -glucan

▲ Sweet A ■ Structure A × Wine A
▲ Sweet B ■ Structure B × Wine B

Beer 1

Beer 2

Beer 3

* Week 0

● Week 2

▲ Week 12

■ Week 38

Bacterial zOTUs

zOTU 1: *Pediococcus damnosus* (100%)zOTU 2: *Acetobacter* sp. (100%)zOTU 3: *Lactobacillus brevis* (100%)zOTU 4: *Acetobacter* sp. (100%)

zOTU 5: Enterobacteriaceae (100%)

zOTU 6: *Stenotrophomonas* sp. (100%)zOTU 14: *Acinetobacter* sp. (99.6%)

Fungal OTUs

OTU 1: *Brettanomyces bruxellensis* (100%)OTU 2: *Pichia membranifaciens* (100%)OTU 3: *Debaryomyces* sp. (100%)OTU 4: *Kregervanrija fluxuum* (96.84%)OTU 5: *Brettanomyces custerianus* (91.67%)OTU 7: *Penicillium* sp. (100%)OTU 8: *Candida norvegica* (100%)OTU 9: *Candida atlantica* (100%)OTU 10: *Candida friedrichii* (100%)OTU 11: *Penicillium roquefortii* (100%)

A Bacteria **B Fungi (200 - 400 bp)** **C Fungi (400 - 550 bp)**