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Impact of wood species on microbial community composition, beer chemistry and sensory characteristics during barrel-ageing of beer

Running title: Impact of wood species on barrel-aged beer

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Abstract

Barrel-ageing of conventionally fermented beers is becoming increasingly popular in recent years, but only very little is known about the underlying process. In this study, we show that wood species significantly affects the bacterial community composition, beer chemistry and sensory characteristics throughout 38 weeks of barrel-ageing. Whereas the microbial communities of oak- and acacia-aged beer became dominated by *Pediococcus damnosus* and *Brettanomyces bruxellensis*, beer aged in oak barrels also contained a large fraction of *Acetobacter* sp. (29.34%) and to a lesser extent *Paenibacillus* sp. (2.74%) that were almost undetected in acacia-aged beer. Oak barrels also imparted substantial concentrations of eugenol, lactones and vanillin, while acacia-aged beer contained high concentrations of total polyphenols and β-glucan, which also translated into different sensory perceptions. Altogether, our results provide novel insights into the barrel-ageing process of beer, and may pave the way for a new generation of beers with a noteworthy flavour complexity.

Key words: Acacia; Bacteria; Beer; Oak; Wood; Yeast

Abbreviations: NA

Introduction

For centuries, wooden barrels have been a key factor in the production of alcoholic beverages like (port)wine, whisky and cider (Cantwell and Bouckaert, 2016). While originally serving only as a means of storage and transportation, nowadays wooden barrels are predominantly used for flavour formation by extracting flavour-active wood compounds such as (mono)phenolics and tannins (De Rosso *et al*., 2009; Fernández De Simón *et al*., 2014). Wooden barrels also play a crucial role in the production of traditional sour beers like Belgian lambic beers and acidic red ales (Bossaert *et al*., 2019; De Roos and De Vuyst, 2019b; Snauwaert *et al*., 2016). Here, the wooden barrels are not used for extraction of wood compounds, but predominantly for their capability to allow oxygen influx and to harbour and sustain microorganisms like lactic acid bacteria (LAB), acetic acid bacteria (AAB) and *Brettanomyces* yeasts, giving these beers their unique flavour profile (Bossaert *et al*., 2019; De Roos and De Vuyst, 2019b). In recent years, a trend has emerged in the craft brewing industry to age conventionally fermented beers in wooden barrels to add flavour and complexity to the beers, including wood-derived flavours (e.g. toasted wood, smoke, spice and vanilla notes), aromas from the previously matured beverage, and/or sourness resulting from microbial activity (Bossaert *et al*., 2019). This allows brewers to produce novel beers of a remarkable flavour complexity without adjusting their brewing scheme (Bossaert *et al*., 2019). Although the use of wood alternatives like wood shavings, cubes, extracts or chips also adds woody notes to the beer, long-term ageing in wooden barrels (often up to one or more years) generates a more complex flavour profile (Cantwell and Bouckaert, 2016; Tonsmeire, 2014). The resulting sensory perception of the wood-aged beers largely depends on a number of factors, including the wood species, the geographic origin of the wood, the toasting level, the size and history of the barrel, the duration of the maturation, the environment in which the beers are matured, and intrinsic beer parameters like alcohol level, bitterness and pH (Bossaert *et al*., 2021; De Rosso *et al*., 2009; Fan *et al*., 2006; Sterckx *et al*., 2012).

Wooden barrels also represent a suitable habitat for microorganisms, including fungi and bacteria, which may affect the chemical and sensory profile of the beers. These microorganisms may originate from the brewing or maturation environment (Bokulich *et al*., 2012), or may have developed and remained in the barrels after previous usage and sanitation, and inoculate the next batch of maturing beer (De Roos *et al*., 2019a; Kocijan *et al*., 2021). During the course of maturation, microorganisms like LAB, AAB and *Brettanomyces* spp. produce a diverse range of metabolites, including organic acids and volatile phenols, resulting in beers with a noteworthy sourness and complex character (Bossaert *et al*., 2019; 2021; De Roos and De Vuyst, 2019b). On the other hand, barrel-inhabiting microorganisms may also be harmful and unwanted, particularly if they are associated with production of off-flavours and spoilage

(Guzzon *et al*., 2011; Stadler and Fischer, 2020). Additionally, barrel-residing microbes may interact with the barrels and with wood-derived compounds, as the release of wood compounds may also provide substrates for microbial growth (de Revel *et al.*, 2005; Gollihue *et al*., 2018) or compounds with antimicrobial properties (Smailagić *et al*., 2020). Therefore, it is reasonable to assume that barrel-associated factors like wood species, barrel age and toasting degree not only affect formation of wood compounds directly, but also have the potential to affect microbial community composition and influence the chemical and sensory properties of the beer indirectly. In previous research, it has been shown that the chemistry of oak barrel-aged, conventionally fermented beers is strongly dependent on the initial beer characteristics (such as alcohol content and pH), the duration of maturation and the oak wood, while the microbiology was only affected by the beer properties (like alcohol content and bitterness level), and the duration of the maturation process and not the different types of oak wood tested (Bossaert *et al*., 2021). Nevertheless, as only oak barrels were investigated, the effect of wood species on the microbial community composition and beer chemistry during barrel-ageing of conventionally fermented beers still remains to be investigated.

The goal of this study was to test the hypothesis that wood species affects the microbial community composition, beer chemistry and sensory characteristics during barrel-ageing of conventionally fermented beer. To this end, two different wood species were investigated, including European oak (*Quercus petraea* and *Quercus robur*) and acacia (*Robininia pseudoacacia*). Changes in the composition of the microbial community and its density were assessed by deep sequencing of amplicons of the V4 region of bacterial 16S ribosomal RNA (rRNA) gene and the fungal internal transcribed spacer 1 (ITS1) region, and quantitative PCR (qPCR), respectively.

Materials and methods

Experimental design and sample collection

Experiments were performed using new (unused) 225-liter oak and acacia barrels from the cooperage Garbellotto Spa (Pordenone, Italy) that had exactly the same dimensions. We specifically focussed on oak and acacia as oak is by far the most commonly used wood species for barrel-ageing of beer, while acacia barrels are becoming increasingly popular and are already regularly used for (white) wine maturation (Cerezo *et al*., 2009; Delia *et al*., 2017). Oak barrels belonged to the cooperage's near infrared (NIR) aroma category 'Sweet', which is described by the cooperage as oak that contains high contents of furfural and vanillin, introducing a sweet aroma into the beverage ageing inside. No such NIR profile was available for the acacia barrels. The beer used in this study was a top-fermented blond beer with 10.31% alcohol by

volume (ABV) and an intermediate bitterness that corresponds to a concentration of 14.07 ppm iso-α-acids. Although hop compounds like iso-α-acids and β-acids are known to have antimicrobial properties (Schurr *et al*., 2015), previous research suggests that an iso-α-acids concentration as the one in the studied beer still allows the establishment of a diverse microbial community (Bossaert *et al*., 2021). The beer was used immediately after primary fermentation without a prior filtration or pasteurization step. Before transferring the beer into the barrels, barrels were filled with a mixture of 0.1% citric acid in water for five days to saturate the wood and thus avoid leakage, and to extract the most pungent tannins. Barrels were then disinfected by burning sulphurous paper, i.e. the most common disinfection method used for cleaning wooden barrels (Kocijan *et al*., 2021), to mimic the level of disinfection applied in industrial conditions. Nevertheless, as microbes may penetrate the wood pores up to 1.25 cm below the wood surface, a complete sterilization of the wood is generally not achieved (Stadler and Fischer, 2020; Swaffield and Scott, 1995), leaving microorganisms behind that may inoculate the beer (De Roos *et al*., 2019a). After flushing with carbon dioxide to remove oxygen and filling the barrels with beer completely, barrels were sealed off with a water lock to allow pressure equalization, yet prevent microbial contamination and minimize exposure to oxygen. Subsequently, the barrels were stored for 38 weeks, side by side in a controlled laboratory environment at a temperature of 22.4°C ± 1.4°C and 49.4% ± 7.9% relative humidity. To compensate for beer losses due to evaporation and wood saturation, and to avoid beer oxidation through contact with the gradually increasing headspace, the barrels were topped-off after 19 weeks. This was done using beer from the same brewing batch that had been stored separately in stainless steel kegs next to the wooden barrels. Prior to topping-off the barrels, the beer stored in kegs was subjected to microbial and chemical analyses to ensure that the beer still sufficiently resembled to original beer and that no microbial contamination had occurred. The topping-off was performed by removing the water lock, carefully placing a sterilized tube via the bunghole half-way into the beer volume and slowly pumping new beer into the barrel until it was full again, avoiding any disturbance of potential biofilms formed in the barrels. The experiment was performed using two barrels per treatment. Beer samples were taken shortly before transfer to the wooden barrels (reference beer, week 0) and after 2, 12 and 38 weeks of barrel-ageing. The 38-week time frame was chosen as it is an industrially relevant time period for the production of sour beers via barrel-ageing of conventionally fermented beer and it allowed sufficient time to monitor beer acidification and the extraction of wood compounds. Samples were taken by removing the water lock and sampling through the bunghole, as described previously (Bossaert *et al*., 2021). In short, sampling was conducted at three different heights in the barrels (bottom, middle and top) using sterile jumbo pipettes (Bürkle, Munich, Germany) and by applying flame sterilization, while minimizing any disturbance of potential biofilms formed in the barrels. Samples of 50 ml were taken at

each height and combined to obtain a total sample volume of 150 ml per barrel. After centrifugation of the samples (3,500 × g for 15 min at 4°C), obtained cell pellets and supernatants were preserved at -20°C for microbiological, and chemical and sensory analyses, respectively.

Microbiological analyses

Microbial communities were investigated by Illumina MiSeq amplicon sequencing and qPCR. These techniques have major advantages in comparison to more conventional culture-dependent methods, particularly higher sensitivity and higher accuracy, including detection and quantification of otherwise unculturable microbes (estimated to represent more than 98-99% of the microorganisms in a sample) (Gupta *et al*., 2019; Puspita *et al*., 2012; Steen *et al*., 2019). For the sequencing approach, genomic DNA was extracted from 500 µl of the cell pellet via the phenol-chlorophorm DNA extraction protocol described by Lievens *et al.* (2003). A negative DNA extraction control was included in which the cell pellet was substituted by DNA-free water. Following ten-fold dilution of the DNA, PCR amplification was carried out targeting the hypervariable V4 region of the bacterial 16S rRNA gene and the fungal ITS1 region, using barcoded versions of primers 515F and 806R and BITS and B58S3, respectively (Bokulich and Mills, 2013; Caporaso *et al*., 2011). Barcoded primers were designed according to Kozich *et al.* (2013) (dual index sequencing strategy) (Tables S1 and S2, Supporting Information). A negative PCR control in which template DNA was replaced by DNA-free water was included in each PCR run, as well as a bacterial and fungal mock community DNA sample. Mock communities were composed of a number of species that are relevant to the beer environment (Bossaert *et al*., 2021; Table S3, Supporting Information). PCR amplification, library preparation, sequencing and bioinformatics analysis were performed as described previously (Bossaert *et al.*, 2021). Correspondingly, as Illumina sequencing works best when amplicons have a similar length, fungal amplicons were divided over two libraries, containing DNA fragments with a length between 200 and 400 bp and ranging from 400 to 550 bp (further referred to as $ITS1_{200\text{-}400}$ and $ITS1_{400\text{-}550}$, respectively). As a result, three distinct sequencing runs were performed on our samples together with a number of samples from other experiments, including one for bacteria and two for fungi. Negative DNA extraction controls and PCR controls gave only few sequences, and were therefore removed from the datasets. Bacterial sequences were clustered into zero-radius operational taxonomic units (zOTUs, also known as amplicon sequence variants (ASVs)) (Callahan *et al*., 2017; Edgar, 2016), while fungal sequences were classified into operational taxonomic units (OTUs) according to a 3% sequence dissimilarity cut-off (Edgar, 2013). 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 show intraspecific variation in their ITS (Zhao *et al*., 2015), fungal diversity

is still commonly assessed by the use of 97% OTUs as fungal species proxies (Sielaff *et al*., 2019). For each sample, the total number of 16S rRNA gene and $ITS1_{200\text{-}400}$ and $ITS1_{400\text{-}550}$ sequence reads was rarefied to 2,500 reads (Tables S4 – S6, Supporting Information). 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 UNITE database (v6, for fungi). Further, a BLAST search against type materials in GenBank was executed to verify the identity of the most important (z)OTUs. To assess bacterial and fungal density, 16S rRNA gene and ITS1 copy numbers were determined using qPCR (in duplicate) with the same primers as those used for the sequencing approach, but without barcodes (Bossaert *et al*., 2021).

Chemical and sensory analyses

Twenty wood-related aroma compounds, thirteen higher alcohols and esters, five organic acids, pH, and three polyphenolic fractions were measured in duplicate, as described in Bossaert *et al.* (2021). In short, wood compounds were determined via headspace solid phase micro extraction in combination with gas chromatography-mass spectrometry (HS-SPME-GC-MS), higher alcohols and esters were measured in a separate HS-SPME-GC-MS run with different settings, and organic acids were determined via high-performance liquid chromatography (HPLC) (Tables S7 and S8, Supporting Information). Further, polyphenols were assessed according to EBC-protocols: total polyphenols: 9.11 and flavonoid content: 9.12. The proanthocyanidins content was quantified according to Bate-Smith (1973). Additionally, D-glucose, D-fructose, sucrose and β-glucan were measured with a Gallery Plus Beermaster (Thermo Fisher Scientific, Austin, USA) and the Alcolyzer beer ME (Anton Paar GmbH, Graz, Austria) was used to monitor the ethanol content (Table S9, Supporting Information). Furthermore, quantitative descriptive sensory evaluation was performed for the following descriptors: astringency, smoky, woody, whisky, bitterness, spicy, phenolic, esters, and acetic. To this end, an in-house tasting panel with experience in sensory evaluation of beers was trained for detecting and scoring of the previously mentioned attributes on a scale from 0 (not detected) to 8 (high intensity), using reference flavour standards dissolved in a standard base beer (FlavorActiV, Thame, UK; The Siebel Institute of Technology, Chicago, USA). Throughout the training, panel performance was evaluated and corrected, and assessors were only selected for the tasting panel when they were able to correctly score at least 80% (in total) of all attributes at all intensity levels. After training and evaluation, the panel was composed of ten assessors, including six males and four females ranging in age from 20 to 50 years. Beer samples were evaluated during several sessions, at 11h00 in separate odourless tasting booths, and each assessor received 10 ml of the respective beer samples at a temperature of 12°C in a black beer tasting glass for sensory analysis. Sensory scores were

only considered reliable when the standard deviation over all panel members was lower than 1.5 for each sensory attribute separately. Median values of attribute scores were calculated providing a good representation of the sensory perception of the beers, even when data were not symmetrically distributed or when there were some extreme values at the high or low end of the sensory attribute scale.

Data visualization and statistical analyses

For each sample in each of the three data sets first a rarefaction curve was constructed via the Phyloseq package in R (v3.6.1) (McMurdie and Holmes, 2013) to visualize whether our sampling was adequate to capture the microbial diversity. Next, alpha diversity was calculated as the observed number of (z)OTUs and as Simpson's index of diversity (1-D). This index is a metric that characterizes diversity in a sample, taking into account the number of unique (z)OTUs and their relative abundance. The index varies between 0 and 1, indicating low and high diversity, respectively (Simpson, 1949). To check whether gene copy numbers, (z)OTU richness and Simpson's index of diversity are significantly different across wood species and/or maturation time, a Kruskal-Wallis rank sum test was performed using the stats package in R (R Core Team, 2019). To test for significant differences in microbial community composition (beta diversity) across the two wood species investigated and sampling points, perMANOVA was carried out on the Bray-Curtis distances of Hellinger-transformed relative abundance data with the adonis function (vegan package) using 1000 permutations (Oksanen *et al.*, 2019). To test whether beer chemistry was significantly different across both wood species investigated and maturation time, perMANOVA with 1000 permutations was performed on Euclidean distances of the normalized chemical data. Additionally, the scaled chemical data set was subjected to principal component analysis (PCA) (stats package in R; R Core Team, 2019) to visualize similarities in chemical composition between samples in a two-dimensional space.

Results

Bacterial and fungal communities

Analysis of the mock communities revealed that all taxa present in the mocks were found and that no single contaminant passed the quality filtering and decontamination steps, indicating the robustness of our analysis (Tables S4 – S6, Supporting Information). Furthermore, microbial diversity appeared to be sufficiently covered by the applied sequencing depth, as illustrated by the rarefaction curves, which generally approached or tended to approach saturation (Fig. S1, Supporting Information). In total, 276 bacterial zOTUs and 144 fungal OTUs were retrieved, among which 143 fungal OTUs in the $ITS1_{200-400}$ data set and one OTU in the $ITS1_{400-550}$ data set (Tables S4 – S6, Supporting Information). As microbiome

sequencing data sets typically represent relative abundances and the $ITS1_{400-550}$ data set only contained one OTU, which corresponded to *S. cerevisiae* employed for the primary fermentation and which was found in all samples investigated, further diversity analyses were only performed for the remaining two data sets, i.e. bacterial zOTU data and $ITS1_{200-400}$ data (Tables S10 – S11, Supporting Information). Agarose gel electrophoresis, however, suggested that the fungal community at the beginning of the maturation process was dominated by *S. cerevisiae*, whereas wild yeasts became dominant after 12 weeks of maturation. However, it should be noted that our qPCR analysis targeting the ITS1 fragment did not make a distinction between both groups of fungi.

A Kruskal-Wallis test revealed that wood species did not significantly affect bacterial and fungal density nor the observed (z)OTU richness and Simpson's index of diversity (Table 1). By contrast, maturation time significantly affected bacterial abundance and both alpha diversity metrics (Table 1). Bacterial 16S rRNA gene copy numbers increased from 3.79 log 16S rRNA gene copies per µl DNA at the start of the maturation to an average of 5.86 ± 0.24 (standard error of the mean, SEM) at week 12 and decreased again to 4.36 ± 0.02 log 16S rRNA gene copies per µl DNA at week 38 (Fig. 1A). Fungal ITS1 copy numbers did not change significantly throughout maturation, although the observed fungal OTU richness and Simpson's index of diversity were significantly affected (Table 1). Nevertheless, as can be seen from Fig. 1B, fungal ITS1 copy numbers were highest at the start of the maturation (6.03 log ITS1 copies per µl DNA), and declined after two weeks of maturation after which population growth stagnated (Fig. 1B). Observed bacterial and fungal richness was relatively high in the reference beer (97 bacterial zOTUs, 28 fungal OTUs) and during the first weeks of the maturation, reaching an average of 138 ± 14.0 bacterial zOTUs and 46 ± 2.8 fungal OTUs after two weeks of maturation (Fig. 1C-D). Subsequently, bacterial and fungal richness dropped, reaching 29.8 ± 18.8 bacterial zOTUs and 20.8 ± 7.7 fungal OTUs at week 12. While the fungal richness decreased further to 2.3 ± 0.2 OTUs after 38 weeks of maturation, the bacterial richness was found to increase again to reach 60.3 ± 8.8 zOTUs (Fig. 1C-D). Likewise, Simpson's index of diversity (1-D) was found to follow a similar pattern (Fig. 1E-F). Plating of a subset of samples on common growth media for bacteria, fungi, cycloheximide-resistant fungi, LAB and AAB confirmed these trends. However, it was also clear from these efforts that not all microbes present could be detected by the plating method (data not shown).

PerMANOVA comparing microbial community composition among samples showed that maturation time played a significant role in the bacterial and fungal community composition, whereas the effect of wood species was only significant for the composition of the bacterial community (Table 2). While the bacterial communities in acacia barrels progressed from a large fraction of low-abundant zOTUs

(grouped together in 'Others') to a bacterial community strongly dominated by *Pediococcus damnosus* (zOTU 1, up to 96.5% relative abundance at week 38), the oak barrels also contained a substantial fraction of *Acetobacter* sp. (zOTU 6) (100% match in Genbank with both *Acetobacter malorum* and *Acetobacter cerevisae*) and *Paenibacillus* sp. (zOTU 17), reaching a relative abundance up to 45.2 and 4.4%, respectively (Fig. 2; Table S4, Supporting Information). In line with the bacterial communities, fungal communities also evolved to a dominance of a few species. In the first weeks of maturation, samples were generally characterized by a considerably high relative abundance of *Penicillium* sp. (OTU 3) in addition to a large fraction of low-abundant fungi (grouped together in 'Others') (Fig. 3; Table S5, Supporting Information). When maturation progressed, fungal communities could be clearly distinguished from the initial community (Fig. 3), and all became dominated by *Brettanomyces bruxellensis* (OTU 1), reaching an average relative abundance of 99.4% ± 0.2% after 38 weeks of maturation (Fig. 3; Table S5, Supporting Information). Interestingly, whereas *B. bruxellensis* became the dominant fungal species in three out of the four investigated barrels after already 12 weeks of maturation (average relative abundance in these three barrels: 84.8% ± 6.1%), in one of the oak barrels the fungal community at week 12 was for 96.2% occupied by the related species *Brettanomyces anomalus* (OTU 2) (Fig. 3; Table S5, Supporting Information). This OTU was also detected in the other oak barrel at week 12, albeit at a relative abundance of only 3.0%. In the acacia barrels it was not found, with the exception of one sample taken at week 2 (Table S5, Supporting Information).

Beer chemistry and sensory characteristics

PerMANOVA revealed that both wood species and maturation time significantly influenced the chemical composition of the beer (Table 3; Table S7, Supporting Information). Accordingly, PCA ordination clearly separated samples taken at the different time points (Fig. 4). Specifically, samples from different time points were mainly separated by PC1 (explaining 33.3% of the variation), whereas PC2 (explaining 20.5% of the variation) allowed distinction between wood species, especially at the end of the maturation (Fig. 4). For all barrels, pH gradually decreased throughout maturation, from 3.97 to 3.84 ± 0.02 (Fig. 5A). Concomitantly, the concentration of organic acids (lactic acid and acetic acid) increased over time (Fig. 5B-C). Additionally, the concentration of a number of wood-derived flavour compounds increased throughout the maturation process (Fig. 5D-H). For these compounds, a clear distinction can be made between the oak and acacia barrels. In particular, during the course of maturation, the concentrations of eugenol, cis-3-methyl-4-octanolide and trans-3-methyl-4-octanolide in oak barrels substantially increased from 9.33 to 34.66 ± 4.43 ppb, from 0.00 to 96.01 ± 33.24 ppb, and from 2.38 to 154.75 ± 9.01 ppb, respectively, while concentrations after 38 weeks of ageing in the acacia barrels were much lower (8.98 ±

2.67 ppb, 12.16 ± 1.52 ppb and 14.89 ± 1.20 ppb, respectively) (Fig. 5D-F). A similar trend is observed for vanillin, which increased from 24.29 to 1376.60 ± 27.95 ppb in oak barrels and to 472.53 ± 3.37 ppb in acacia barrels after 38 weeks of maturation (Fig. 5G). In contrast, the concentration of total polyphenols doubled in the acacia barrels (from 249.34 to 522.57 ± 48.00 ppm), whereas a slight decrease was observed in the oak barrels (from 249.34 to 231.86 ± 0.29 ppm) (Fig. 5H). The acacia barrels could also be distinguished from oak barrels based on the concentrations of ethyl acetate and β-glucans. Specifically, the concentration of ethyl acetate decreased from 48.55 to 39.04 ± 1.28 ppm in acacia barrels and increased to 64.49 ± 2.21 ppm in oak barrels after maturation period of 38 weeks (Fig. 5I), whereas β-glucan levels increased from 1.76 to 11.73 ± 1.43 mg/l in the acacia barrels, and to 4.55 ± 1.76 mg/l in the oak barrels (Fig. 5J). Notably, samples taken at the start of the maturation generally contained more higher alcohols and esters than samples taken later in the process. For example, the concentration of isoamyl acetate decreased from 3.47 to 0.68 ± 0.34 ppm after 38 weeks of maturation (Fig. 5K). In addition, the concentration of 4-vinyl guaiacol decreased from 815.08 to 43.33 ± 0.45 ppb (Fig. 5L).

Besides a chemical analysis of the beer samples, also their sensory perceptions were evaluated (Fig. 6; Fig. S2 and Table S12, Supplementary Information). In this respect, a number of general trends could be observed. Sensory attributes described as ‘astringency’ (Fig. 6A), ‘smoky’ (Fig. 6B), ‘woody’ (Fig. 6C) and ‘whisky’ (Fig. 6D) increased until week 12, followed by a decrease towards the end of the maturation. In contrast, beer bitterness followed the opposite trend, i.e. a decrease until week 12 and an increase or stabilization towards week 38 (Fig. 6E). Further, sensory descriptors ‘spicy’ (Fig. 6F) and ‘phenolic’ (Fig. 6G) reached a minimum score at week 2, whereas the sensory scores for ‘esters’ varied across wood species and time points (Fig. 6H) and scores for ‘acetic’ mainly increased towards the end of the maturation (Fig. 6I). Additionally, the tasting panel was able to recognise which beer samples were aged in oak and which in acacia barrels. More specifically, acacia barrels mainly provided a pungent and astringent mouthfeel to the beer, especially at the first weeks of the maturation, whereas beer aged in oak barrels was generally described as more sweet and less pungent. Finally, panel members indicated that the complex acidity detected at the end of the maturation fitted nicely with the flavour profile of the oak barrels, while it was not considered agreeable in combination with sharp, astringent flavour notes as brought by acacia barrels.

Discussion

Our results show that wood species significantly affected bacterial community composition, beer chemistry and sensory characteristics during barrel-ageing of conventionally fermented beer. Although

the bacterial community composition in all barrels significantly changed from a diverse community to a dominance of *P. damnosus* after 38 weeks of maturation, beer ageing in oak barrels also coincided with a relatively large fraction of *Acetobacter* sp. (most probably *A. cerevisiae* or *A. malorum*; on average 29.34% relative abundance) and a lower amount of *Paenibacillus* sp. (on average 2.74%), while they were almost not detected in acacia barrels (average relative abundance of 0.04% and 0.64%, respectively). These bacteria were already found at low relative abundances in the original beer prior to wood maturation, suggesting that wood species had an important role in their proliferation. Indeed, as both wood species differ in porosity and chemical composition, the amount of oxygen and the concentration and type of substrates available for microbial growth in the maturing beer are most likely different (de Revel *et al*., 2005; Gollihue *et* al., 2018; Torija *et al*., 2009), generating differences in the microbial community composition. Furthermore, also extractable antimicrobial compounds may differ across wood species. For instance, Smailagić *et al*. (2020) assessed the antimicrobial properties of wood extracts from oak and acacia and found that the acacia extracts displayed highest antimicrobial activity, which may explain our findings. Nevertheless, it has also been shown that their impact can vary across microorganisms, as well as the type, structure and concentration of the compounds (Alañón *et al*., 2015; Smailagić *et al*. 2020).

Besides the factors mentioned above, the microorganisms themselves may also play an active role in the selection of strains developing in the beer. In line with previous studies (Bossaert *et al*., 2021), we found that, from week 12 onwards, the microbial communities were dominated by *P. damnosus* and *B. bruxellensis*. Considering the high intraspecific conservatism of the 16S rRNA gene and the ITS region in *P. damnosus* and *B. bruxellensis*, respectively, and the short read lengths used, identifications of these taxa were performed down to the species level. As such, it remains to be investigated whether the different microbial communities evolved to a dominance of the same or different strains of these species. Strikingly, both species are often found together (Bossaert *et al*., 2019; 2021; De Roos and De Vuyst, 2019b; Tonsmeire, 2014), suggesting that they might benefit from each other or have complementary nutrient assimilation profiles. Due to their glucosidase activity, *Brettanomyces* spp. manage to breakdown and consume complex carbohydrates like maltotetraose and maltopentaose that remain after primary fermentation (Menoncin and Bonatto, 2019), and make more simple sugars available for other microbes like *P. damnosus* (De Roos and De Vuyst, 2019b; Kumara and Verachtert, 1991). In fact, this property is of utmost importance in wood-ageing, as it also allows *Brettanomyces* yeasts to convert wood (hemi)cellulose into simple sugars, hence boosting microbial growth in these conditions (Colomer *et al*., 2019; Spear *et al*., 1993). Moreover, their β-glucosidase activity is not only an important feature in the release of fermentable carbohydrates, but it could also set free antimicrobial phenolic compounds,

affecting the community structure (Guld *et al*., 2019; Kuo *et al*., 2018). Furthermore, factors like initial beer properties and brewery environment have been identified as key factors in the establishment of microbial communities in wood-aged beer (Bossaert *et al*., 2021). The beer used in this study had a high alcohol content (10.31% ABV) and intermediate bitterness (14.07 ppm iso-α-acids), and thus provided rather stringent conditions for microbial growth and possibly restricted the variety of species that could survive in these conditions. (Sanchez-Gonzalez *et al*., 2009; Schurr *et al*., 2015).

In addition to differences in microbiology, we also found significant differences in the chemistry of oak- and acacia-aged beer. Specifically, beer aged in oak barrels contained higher concentrations of eugenol, cis- and trans-3-methyl-4-octanolide (also called 'oak lactones'), vanillin, and ethyl acetate than beer aged in acacia barrels. On the contrary, acacia-aged beer contained more total polyphenols and β-glucan than oak-aged beer. These differences can be attributed to many factors. For example, the differences in beer chemistry may be explained by initial differences in the wood's chemical composition and the extraction rate from the wood. Overall, European oak is known to contain substantial quantities of lactones, eugenol, vanillin, and hydrolysable tannins, imparting a rich and balanced flavour palette to the beverage, whereas acacia is characterized by higher contents of guaiacol, 4-vinyl guaiacol, syringol and flavonoid polyphenols than European oak, generating more subtle, spicy notes in combination with a round mouthfeel, without the sweetness and bigger flavours of oak (Alañón *et al*., 2018; Culleré *et al*., 2013; Fernández de Simón *et al*., 2014). In fact, acacia generally has a higher phenolic content and a higher antioxidant capacity than European oak (Smailagić *et al*., 2019). These findings were also confirmed by the tasting panel involved in this study. Panel members indicated that oak-aged beer samples had a nice, soft and complex flavour that balanced well with the sourness, whereas acacia-aged beer was perceived as spicy, with sharp tannins and did not pair off as nicely with the sourness. However, it should be noted that there is discrepancy between the concentrations of chemical compounds and the sensory attribute scores determined for the same beer samples. This is most likely due to differences in the beer matrix that can have a substantial impact on the perception of sensory attributes, including the enhancement of certain attributes while others are masked by the beer matrix (Castro and Ross, 2018; Sterckx *et al*., 2011). Furthermore, the differences in beer chemistry can also be caused by the dissimilarities in the microbial community composition, but additional research is needed to link the chemical profiles with the microbial community composition and microbial activity in the barrels. Furthermore, several other factors like geographical origin, wood seasoning, toasting, maturation time, and initial beer parameters like pH and alcohol content may affect the chemistry of barrel-aged beers (Cadahía *et al*., 2003; Fernández de Simón *et al*., 2014). Therefore, future studies should focus on the

interactions between beer characteristics, wood characteristics and the microorganisms residing in the beer or wood, to better understand how each of these factors affect beer chemistry and quality, e.g. through the use of *in-vitro* systems mimicking wood maturation (Wolfe and Dutton, 2015).

Conclusion

Wood species significantly affected the bacterial community composition, beer chemistry and sensory characteristics during barrel-ageing of conventionally fermented beer. Both in oak and acacia barrels, the bacterial and fungal community composition shifted from a diverse community to a dominance of *P. damnosus* and *B. bruxellensis*, respectively. However, in oak barrels, also large fractions of *Acetobacter* sp. and, to a lesser extent, of *Paenibacillus* sp. were found. Further, beer chemistry significantly changed over time and across wood species. More specifically, oak barrels imparted substantial concentrations of eugenol, lactones and vanillin, while acacia-aged beer was characterized by high concentrations of total polyphenols and β-glucan, which also translated into different sensory perceptions. These differences could originate from the extraction of different chemical compounds from both wood species, directly affecting beer chemistry, or from the extraction of different microbial substrates or antimicrobial compounds, affecting the microbial community structure and hence beer chemistry. Further research is needed to better understand the mechanisms involved in this process. Such knowledge will be the basis of a new generation of beers with a complex aroma character.

Declaration of competing interest

The authors declare that they do not have any known competing financial interests or personal relationships that could have affected the work reported in this study.

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Data availability

The sequence data can be found in the Sequence Read Archive under BioProject accession number PRJNA758797 (BioSample accession numbers SAMN21035756 – SAMN21035768, SAMN21036548 – SAMN21036548, SAMN21036747 – SAMN21036750). (z)OTU tables and chemical data are available in Supplementary Information.

Ethical guidelines

Ethics approval was not required for this research.

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 $ITS1_{200\text{-}400}$ dataset according to the UNITE v6 database and distribution over the investigated samples

Table S6: Identification of operational taxonomic units (OTUs) in the $ITS1_{400\text{-}550}$ 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 and bacterial 16S rRNA gene copy numbers

Table S11: Diversity metrics for the fungal communities and fungal ITS1 copy numbers

Table S12: Sensory evaluation scores

Figure S1: Rarefaction curves for (A) the bacterial V4 dataset, (B) the fungal $ITS1_{200\text{-}400}$ dataset, and (C) the fungal $ITS1_{400\text{-}550}$ dataset. Beer samples were taken at week 0 (reference), and after 2, 12 and 38 weeks of ageing in oak and acacia barrels. Rarefaction curves indicate that the applied sequencing depth was sufficient to cover the microbial diversity, as the curves for all samples approached saturation or tended to approach saturation.

Figure S2: Sensory evaluation scores presented as radar plots

References

1. Alañón, M.E., García-Ruiz, A., Díaz-Maroto, M.C., Pérez-Coello, M.S. and Moreno-Arribas, M.V., 2015. Antimicrobial and antioxidant activity of pressurized liquid extracts from oenological woods. Food Control, 50, pp.581-588.

2. Alañón, M.E., Marchante, L., Alarcón, M., Díaz-Maroto, I.J., Pérez-Coello, S. and Díaz-Maroto, M.C., 2018. Fingerprints of acacia aging treatments by barrels or chips based on volatile profile, sensorial properties, and multivariate analysis. Journal of the Science of Food and Agriculture, 98(15), pp.5795-5806.
3. Bate-Smith, E.C., 1973. Haemanalysis of tannins: the concept of relative astringency. Phytochemistry 12(4), pp.907–912.
4. Bokulich, N.A., Bamforth, C.W. and Mills, D.A., 2012. Brewhouse-resident microbiota are responsible for multi-stage fermentation of American coolship ale. PloS one, 7(4), p.e35507.
5. Bokulich, N.A. and Mills, D.A., 2013. Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. Applied and environmental microbiology, 79(8), pp.2519-2526.
6. Bossaert, S., Crauwels, S., De Rouck, G. and Lievens, B., 2019. The power of sour – A review: Old traditions, new opportunities. BrewingScience 72(3-4), pp.78-88.

Annotation of [6]: This review article presents a comprehensive overview of the different methods used for sour beer production, including traditional methods for the production of lambic beer, Flanders' red ale, among others, and novel souring methods ranging from kettle souring to the use of non-conventional yeast species to acidify beer. Furthermore, the key microbial species involved in these processes are discussed, as well as their most important characteristics and their impact on beer. This aspect is especially relevant for the current work as many of the microorganisms found in this study (lactic acid bacteria, acetic acid bacteria, *Brettanomyces* yeasts, …) are common sour beer microbes.

7. Bossaert, S., Winne, V., Van Opstaele, F., Buyse, J., Verreth, C., Herrera-Malaver, B., Van Geel, M., Verstrepen, K.J., Crauwels, S., De Rouck, G. and Lievens, B., 2021. Description of the temporal dynamics in microbial community composition and beer chemistry in sour beer production via barrel ageing of finished beers. International Journal of Food Microbiology, 339, p.109030.

Annotation [7]: This study represents one of the first studies describing the temporal dynamics in microbial community composition, beer chemistry and sensory characteristics during the ageing of conventionally fermented beers in oak barrels. Results 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.

8. Cadahía, E., Fernández de Simón, B. and Jalocha, J., 2003. Volatile compounds in Spanish, French, and American oak woods after natural seasoning and toasting. Journal of agricultural and food chemistry, 51(20), pp.5923-5932.
9. Callahan, B.J., McMurdie, P.J. and Holmes, S.P., 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. The ISME Journal, 11(12), pp.2639-2643.
10. Cantwell, D. and Bouckaert, P., 2016. Wood & Beer: A Brewer's Guide. Brewers Publications, Colorado, USA.
11. Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J. and Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the national academy of sciences, 108(S1), pp.4516-4522.
12. Castro, L.F. and Ross, C.F., 2018. Correlation between sensory descriptive analysis and volatile composition of beer using multivariate analysis: The effect of the nonvolatile matrix on the sensory perception and volatile fraction behavior. Journal of the American Society of Brewing Chemists, 76(2), pp.86-95.
13. Cerezo, A.B., Espartero, J.L., Winterhalter, P., Garcia-Parrilla, M.C. and Troncoso, A.M., 2009. (+)-Dihydrorobinetin: a marker of vinegar aging in acacia (Robinia pseudoacacia) wood. Journal of agricultural and food chemistry, 57(20), pp.9551-9554.
14. Colomer, M.S., Funch, B. and Forster, J., 2019. The raise of *Brettanomyces* yeast species for beer production. Current opinion in biotechnology, 56, pp.30-35.
15. Culleré, L., Fernández de Simón, B.F., Cadahía, E., Ferreira, V., Hernández-Orte, P. and Cacho, J., 2013. Characterization by gas chromatography–olfactometry of the most odor-active compounds in extracts prepared from acacia, chestnut, cherry, ash and oak woods. LWT-Food Science and Technology, 53(1), pp.240-248.
16. Delia, L., Jordão, A.M. and Ricardo-da-Silva, J.M., 2017. Influence of different wood chips species (oak, acacia and cherry) used in a short period of aging on the quality of 'Encruzado'white wines. Mitteilungen Klosterneuburg, 67, pp.84-96.
17. de Revel, G., Bloem, A., Augustin, M., Lonvaud-Funel, A. and Bertrand, A., 2005. Interaction of *Oenococcus oeni* and oak wood compounds. Food Microbiology, 22(6), pp.569-575.
18. De Roos, J., Van der Veken, D. and De Vuyst, L., 2019a. The interior surfaces of wooden barrels are an additional microbial inoculation source for lambic beer production. Applied and environmental microbiology, 85(1), pp. e02226-18.

Annotation of [18]: In this study it is shown that wooden barrels are an important source of microbes for the production of lambic beers and thereby affect the microbial communities formed throughout wood maturation. Furthermore, the study provided indications that the composition of the microbial community could possibly reflect different characteristics of the lambic barrels in terms of age, wood thickness and wood porosity.

19. De Roos, J. and De Vuyst, L., 2019b. Microbial acidification, alcoholization and aroma production during spontaneous lambic beer production. Journal of the Science of Food and Agriculture, 99(1), pp.25-38.
20. De Rosso, M., Panighel, A., Dalla Vedova, A., Stella, L. and Flamini, R., 2009. Changes in chemical composition of a red wine aged in acacia, cherry, chestnut, mulberry, and oak wood barrels. Journal of Agricultural and Food Chemistry, 57(5), pp.1915-1920.
21. Edgar, R.C., 2013. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nature methods, 10(10), pp.996-998.
22. Edgar, R.C., 2016. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. BioRxiv, p.081257.
23. Fan, W., Xu, Y. and Yu, A., 2006. Influence of oak chips geographical origin, toast level, dosage and aging time on volatile compounds of apple cider. Journal of the Institute of Brewing, 112(3), pp.255-263.
24. Fernández de Simón, B., Martínez, J., Sanz, M., Cadahía, E., Esteruelas, E. and Muñoz, A.M., 2014. Volatile compounds and sensorial characterisation of red wine aged in cherry, chestnut, false acacia, ash and oak wood barrels. Food chemistry, 147, pp.346-356.
25. Gollihue, J., Richmond, M., Wheatley, H., Pook, V.G., Nair, M., Kagan, I.A. and DeBolt, S., 2018. Liberation of recalcitrant cell wall sugars from oak barrels into bourbon whiskey during aging. Scientific reports, 8(1), pp.1-12.
26. Guld, Z., Rácz, A., Tima, H., Kállay, M. and Sárdy, D.N., 2019. Effects of aging in oak barrels on the trans-resveratrol and anthocyanin concentration of red wines from Hungary. Acta alimentaria, 48(3), pp.349-357.
27. Gupta, S., Mortensen, M.S., Schjørring, S., Trivedi, U., Vestergaard, G., Stokholm, J., Bisgaard, H., Krogfelt, K.A. and Sørensen, S.J., 2019. Amplicon sequencing provides more accurate microbiome information in healthy children compared to culturing. Communications biology, 2(1), pp.1-7.

28. Guzzon, R., Widmann, G., Malacarne, M., Nardin, T., Nicolini, G. and Larcher, R., 2011. Survey of the yeast population inside wine barrels and the effects of certain techniques in preventing microbiological spoilage. European Food Research and Technology, 233(2), pp.285-291.
29. Kocijan, T., Bossaert, S., Van Boeckel, G., De Rouck, G., Lievens, B. and Crauwels, S., 2021. Sanitation of wooden barrels for ageing beer-a review. BrewingScience, 74(3-4), pp.51-62.
30. Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. and 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. Applied and environmental microbiology, 79(17), pp.5112-5120.
31. Kumara, H.S. and Verachtert, H., 1991. Identification of lambic superattenuating micro-organisms by the use of selective antibiotics. Journal of the Institute of Brewing, 97(3), pp.181-185.
32. Kuo, H.P., Wang, R., Huang, C.Y., Lai, J.T., Lo, Y.C. and Huang, S.T., 2018. Characterization of an extracellular β-glucosidase from *Dekkera bruxellensis* for resveratrol production. Journal of food and drug analysis, 26(1), pp.163-171.
33. 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 Microbiology Letters, 223(1), pp.113-122.
34. McMurdie, P.J. and Holmes, S., 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS one, 8(4), p.e61217.
35. Menoncin, M. and Bonatto, D., 2019. Molecular and biochemical aspects of *Brettanomyces* in brewing. Journal of the Institute of Brewing, 125(4), pp.402-411.
36. 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. and Wagner, H., 2019. vegan: Community Ecology Package. R package version 2.5-6.
37. Puspita, I.D., Kamagata, Y., Tanaka, M., Asano, K. and Nakatsu, C.H., 2012. Are uncultivated bacteria really uncultivable? Microbes and environments, p.ME12092.
38. R Core Team, 2019. R: A Language and Environment for Statistical Computing (Version 3.5. 2, R Foundation for Statistical Computing, Vienna, Austria, 2018).
39. Sanchez-Gonzalez, Y., Cameleyre, X., Molina-Jouve, C., Goma, G. and Alfenore, S., 2009. Dynamic microbial response under ethanol stress to monitor *Saccharomyces cerevisiae* activity in different initial physiological states. Bioprocess and biosystems engineering, 32(4), pp.459-466.

40. Schurr, B.C., Hahne, H., Kuster, B., Behr, J. and Vogel, R.F., 2015. Molecular mechanisms behind the antimicrobial activity of hop iso-α-acids in *Lactobacillus brevis*. Food microbiology, 46, pp.553-563.
41. 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. and Karouia, F., 2019. Characterization of the total and viable bacterial and fungal communities associated with the International Space Station surfaces. Microbiome, 7(1), pp.1-21.
42. Simpson, E.H., 1949. Measurement of diversity. Nature, 163(4148), pp.688-688.
43. Smailagić, A., Veljović, S., Gašić, U., Zagorac, D.D., Stanković, M., Radotić, K. and Natić, M., 2019. Phenolic profile, chromatic parameters and fluorescence of different woods used in Balkan cooperage. Industrial Crops and Products, 132, pp.156-167.
44. Smailagić, A., Ristivojević, P., Dimkić, I., Pavlović, T., Dabić Zagorac, D., Veljović, S., Fotirić Akšić, M., Meland, M. and Natić, M., 2020. Radical scavenging and antimicrobial properties of polyphenol rich waste wood extracts. Foods, 9(3), p.319.

Annotation of [44]: Smailagić *et al*. (2020) have assessed the radical scavenging and antimicrobial activities of various wood extracts, including oak and acacia extracts. They demonstrated that the minimum inhibitory concentration (MIC) against *Staphylococcus aureus* was lower for acacia extracts than for oak extracts, thus indicating that extracts prepared from both wood species show differences in their antimicrobial properties, which may explain some of the results obtained in our study.

45. Snauwaert, I., Roels, S.P., Van Nieuwerburgh, F., Van Landschoot, A., De Vuyst, L. and Vandamme, P., 2016. Microbial diversity and metabolite composition of Belgian red-brown acidic ales. International Journal of Food Microbiology, 221, pp.1-11.
46. Spear, L., Gallagher, J., McHale, L. and McHale, A.P., 1993. Production of cellulase and β-glucosidase activities following growth of *Streptomyces hygroscopicus* on cellulose containing media. Biotechnology letters, 15(12), pp.1265-1268.
47. Stadler, E. and Fischer, U., 2020. Sanitization of Oak Barrels for Wine - A Review. Journal of agricultural and food chemistry, 68(19), pp.5283-5295.
48. Steen, A.D., Crits-Christoph, A., Carini, P., DeAngelis, K.M., Fierer, N., Lloyd, K.G. and Thrash, J.C., 2019. High proportions of bacteria and archaea across most biomes remain uncultured. The ISME journal, 13(12), pp.3126-3130.

49. Sterckx, F.L., Missiaen, J., Saison, D. and Delvaux, F.R., 2011. Contribution of monophenols to beer flavour based on flavour thresholds, interactions and recombination experiments. Food chemistry, 126(4), pp.1679-1685.
50. Sterckx, F.L., Saison, D. and Delvaux, F.R., 2012. Wood Aging of Beer. Part I: Influence on Beer Flavor and Monophenol Concentrations. Journal of the American Society of Brewing Chemists, 70(1), pp.55-61.
51. Swaffield, C.H. and Scott, J.A., 1995. Existence and development of natural microbial populations in wooden storage vats used for alcoholic cider maturation. Journal of the American Society of Brewing Chemists, 53(3), pp.117-120.
52. Tonsmeire, M., 2014. American sour beers: Innovative techniques for mixed fermentations. Brewers Publications, Colorado, USA.
53. Torija, M.J., Mateo, E., Vegas, C.A., Jara, C., González, A., Poblet, M., Reguant, C., Guillamon, J.M. and Mas, A., 2009. Effect of wood type and thickness on acetification kinetics in traditional vinegar production. International Journal of Wine Research, 1, pp.155-160.
54. Wolfe, B.E. and Dutton, R.J., 2015. Fermented foods as experimentally tractable microbial ecosystems. Cell, 161(1), pp.49-55.
55. Zhao, Y., Tsang, C.C., Xiao, M., Cheng, J., Xu, Y., Lau, S.K. and Woo, P.C., 2015. Intra-genomic internal transcribed spacer region sequence heterogeneity and molecular diagnosis in clinical microbiology. International journal of molecular sciences, 16(10), pp.25067-25079.

Legends to figures

Figure 1: Temporal dynamics in bacterial 16S rRNA gene copy numbers (A) and fungal ITS1 copy numbers (B), bacterial (C) and fungal (D) (z)OTU richness, and Simpon's index of diversity (1-D) (E and F) throughout wood maturation of beer in oak (▲) and acacia (●) barrels. Beer samples were taken at the start (week 0) and after 2, 12 and 38 weeks of barrel-ageing. The standard error (n = 2) is shown at each data point. ITS1 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 $ITS1_{200\text{-}400}$ data set).

Figure 2: Temporal dynamics in bacterial community composition throughout maturation of beer in oak (A) and acacia (B) barrels. Data are presented for each biological replicate separately (n = 2). Specified zOTUs had an overall relative abundance larger than 5% 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 zOTUs was retrieved through a search against the Silva v1.23 database, and confirmed with a BLAST search against type materials in GenBank. The obtained percentage of identity with the GenBank entries is indicated for each zOTU.

Figure 3: Temporal dynamics in fungal community composition throughout maturation of beer in oak (A) and acacia (B) barrels. Data are presented for each biological replicate separately (n = 2). Specified OTUs had an overall relative abundance larger than

5% 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 OTUs was retrieved through a search against the UNITE v6 database, and confirmed with a BLAST search in GenBank. The obtained percentage of identity with GenBank entries is indicated for each OTU.

Figure 4: Principal component analysis (PCA) displaying the differences in chemical composition of beer samples taken at the start (week 0) and after 2, 12 and 38 weeks of maturation in oak (▲) and acacia (●) barrels. Data are presented for each biological replicate separately (*n* = 2). Chemical variables are presented as vectors, and the ellipses represent the 95%-confidence interval for each sampling time (except at week 0), assuming a multivariate normal distribution.

Figure 5: Temporal changes in beer chemistry throughout maturation in oak (▲) and acacia (●) barrels. Beer samples were taken at the start (week 0) and after 2, 12, and 38 weeks of barrel-ageing. Data are presented for each biological replicate separately (*n* = 2). Displayed parameters: (A) pH, (B) lactic acid, (C) acetic acid, (D) eugenol, (E) cis-3-methyl-4-octanolide, (F) trans-3-methyl-4-octanolide, (G) vanillin, (H) total polyphenols, (I) ethyl acetate, (J) β-glucan, (K) isoamyl acetate, (L) 4-vinyl guaiacol. For a detailed overview of the different chemical parameters measured in this study, the reader is referred to Tables S7 and S9, respectively (Supporting Information).

Figure 6: Median scores of the different sensory attributes as evaluated by a trained tasting panel comprised of ten panel members. Assessed beer samples were taken at week 0 (reference), and after 2, 12 and 38 weeks of ageing in oak (▲) and acacia (●) barrels (*n* = 2). Evaluated attributes were scored on a scale from 0 (not present) to 8 (high intensity) and include (A) Astringency, (B) Smoky, (C) Woody, (D) Whisky, (E) Bitterness, (F) Spicy, (G) Phenolic, (H) Esters, and (I) Acetic.

Table 1. Results of Kruskal-Wallis test[a] assessing the impact of wood species and maturation time on bacterial and fungal density[b], observed (z)OTU richness and Simpson's index of diversity during barrel-ageing of conventionally fermented beer[c]

Factor	Density					
	Bacteria			Fungi		
	df	χ^2	*P*	df	χ^2	*P*
Wood species	2	1.131	0.568	2	4.222	0.121
Maturation time	3	9.872	**0.020**	3	2.782	0.427
Factor	**Observed (z)OTU richness**					
	Bacteria			**Fungi**		
	df	χ^2	***P***	**df**	χ^2	***P***
Wood species	2	0.484	0.785	2	0.051	0.975
Maturation time	3	9.429	**0.024**	3	9.009	**0.029**
Factor	**Simpson's index of diversity**					
	Bacteria			**Fungi**		
	df	χ^2	***P***	**df**	χ^2	***P***
Wood species	2	0.637	0.727	2	0.489	0.783
Maturation time	3	9.725	**0.021**	3	10.200	**0.017**

[a]df: degrees of freedom; χ^2: Kruskal-Wallis χ^2 statistic; *P*: *P*-value (statistically significant values are denoted in bold ($P < 0.05$)).

[b]Assessed by determination of bacterial 16S rRNA gene and fungal ITS1 copy numbers.

[c]Beer samples were taken at the start (week 0) and after 2, 12, or 38 weeks of maturation in oak and acacia barrels.

Table 2. Results of permutational multivariate analysis of variance (PerMANOVA)[a] comparing bacterial and fungal community composition during barrel-ageing of conventionally fermented beer[b]

Factor	Community composition					
	Bacteria			Fungi		
	df	***F***	***P***	**df**	***F***	***P***
Wood species	2	1.991	**0.048**	2	1.560	0.107
Maturation time	2	7.640	**0.001**	2	4.895	**0.002**

[a]df: degrees of freedom; *F*: *F* statistic; *P*: *P*-value based on 1,000 permutations (statistically significant values are denoted in bold ($P < 0.05$)).

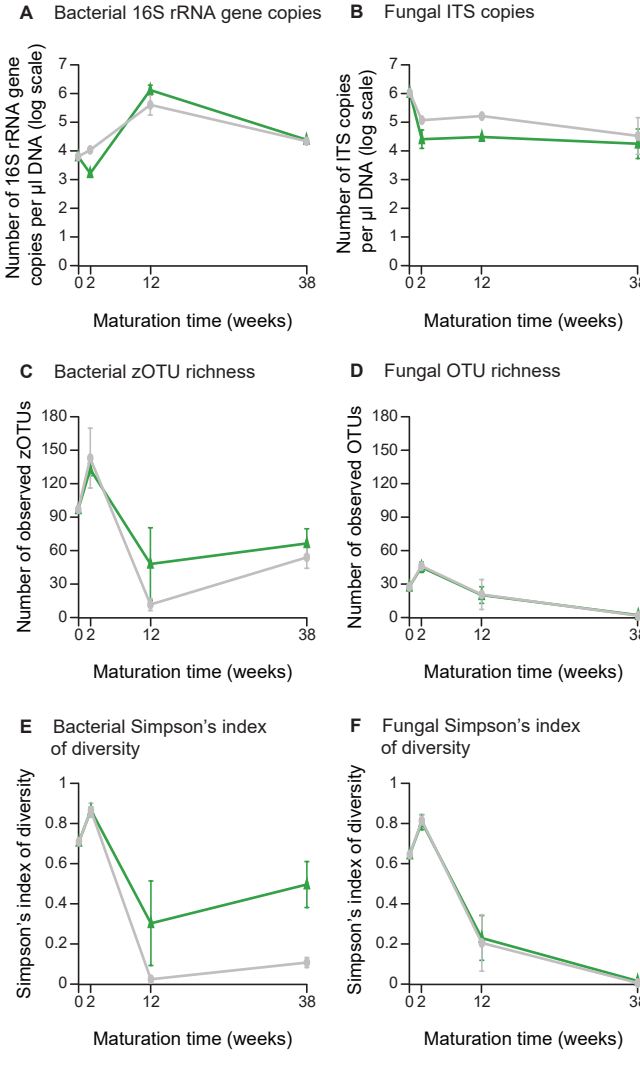
[b]Beer samples were taken at the start (week 0) and after 2, 12, or 38 weeks of maturation in oak and acacia barrels.

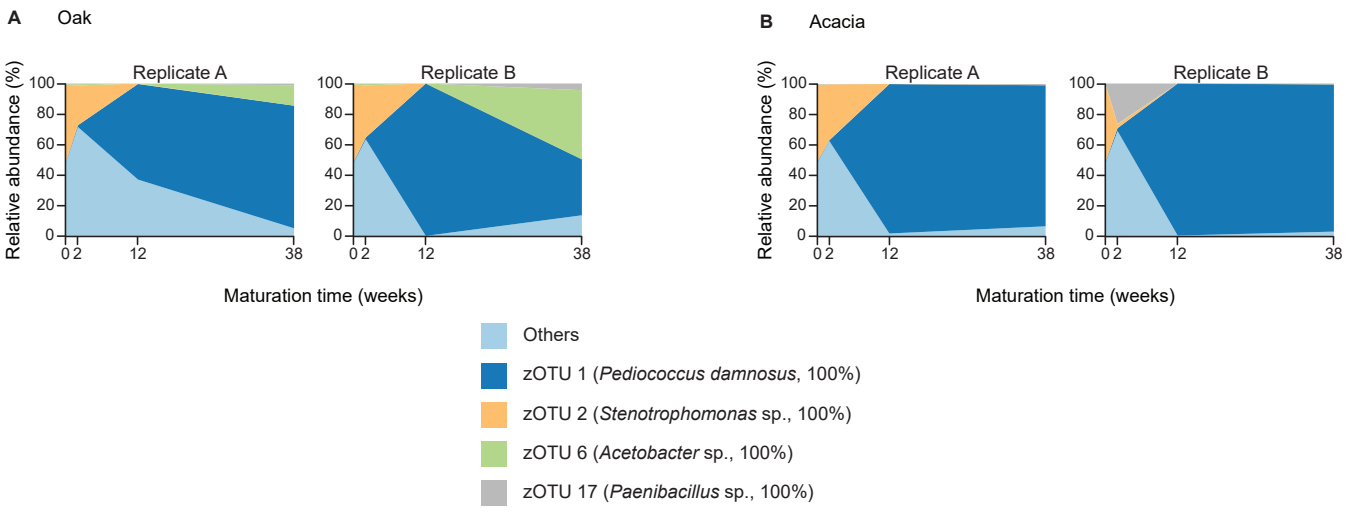
Table 3. Results of permutational multivariate analysis of variance (PerMANOVA)[a] comparing beer chemistry during barrel-ageing of conventionally fermented beer[b]

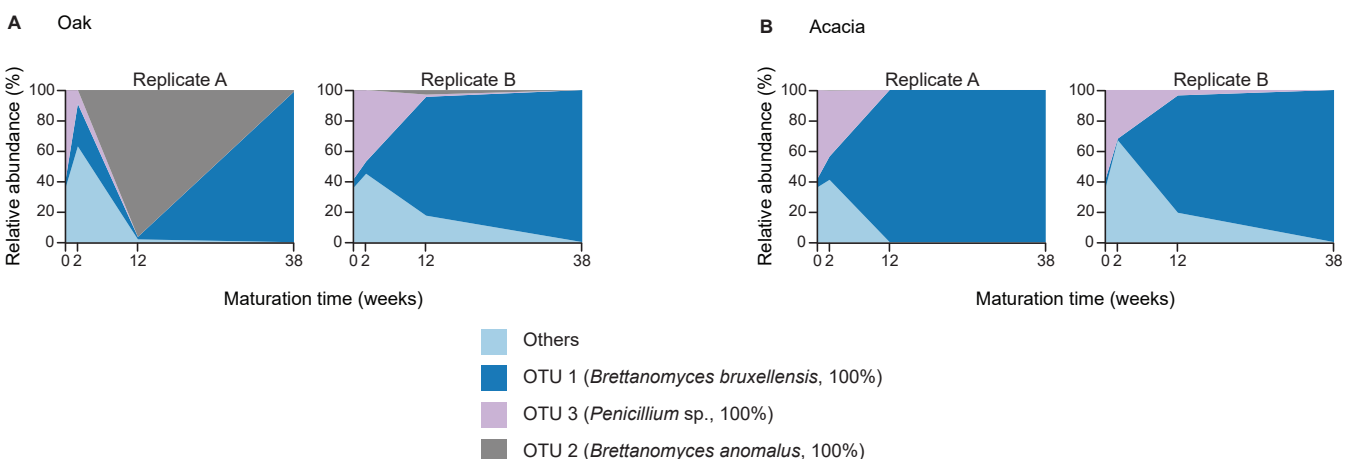
Factor	Beer chemistry		
	df	***F***	***P***
Wood species	2	3.288	**0.012**
Maturation time	2	5.159	**0.006**

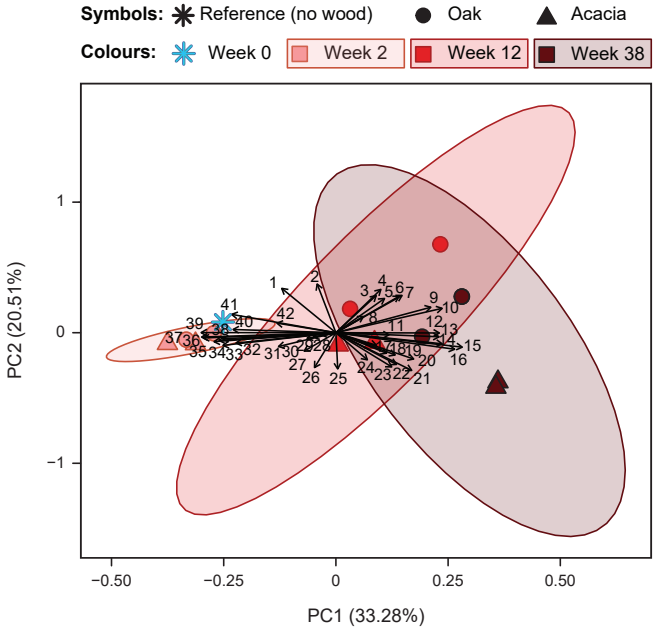
[a]df: degrees of freedom; *F*: *F* statistic; *P*: *P*-value based on 1,000 permutations (statistically significant values are denoted in bold ($P < 0.05$)).

[b]Beer samples were taken at the start (week 0) and after 2, 12, or 38 weeks of maturation in oak and acacia barrels.

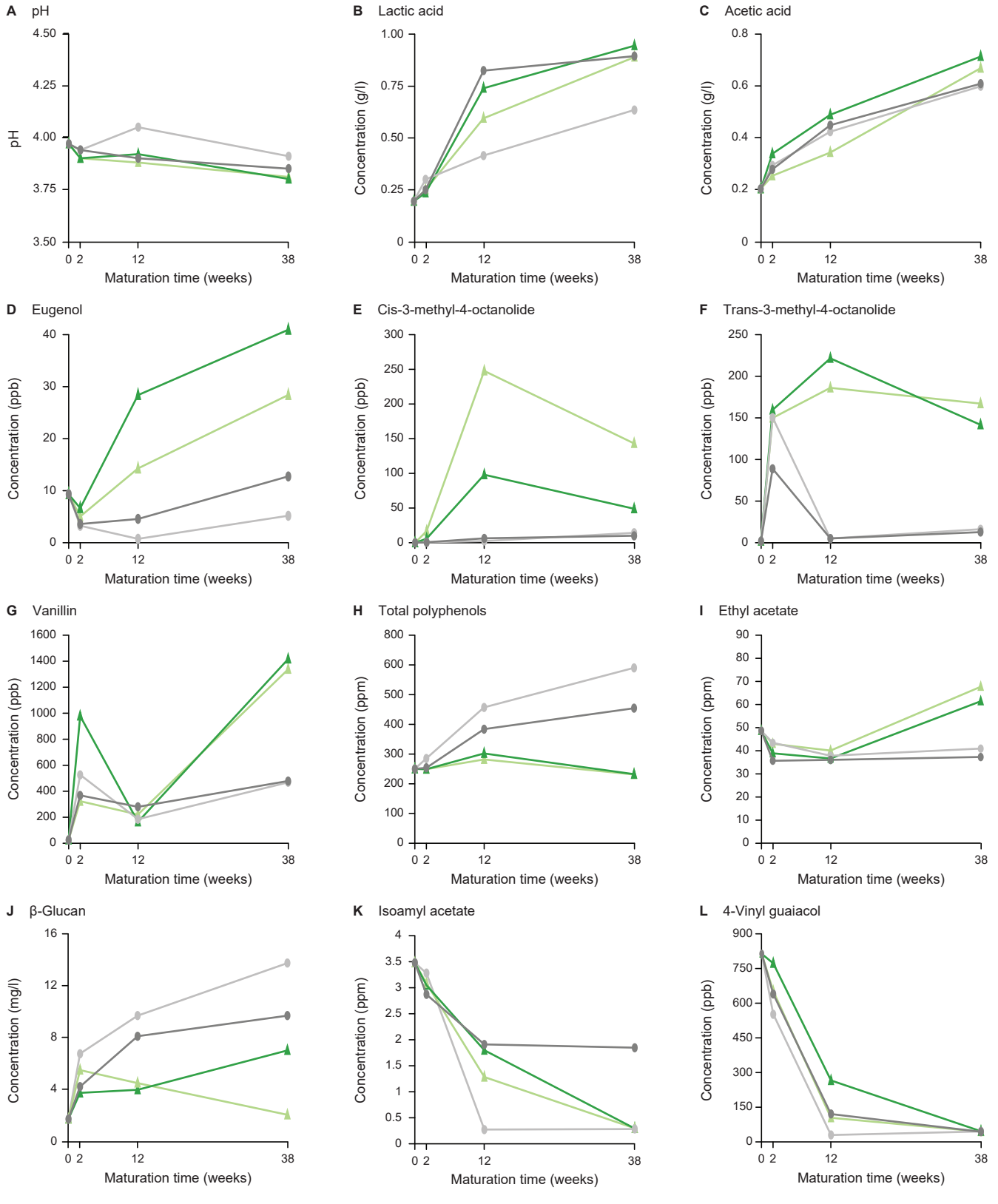

A
Bacterial 16S rRNA gene copies
Number of 16S rRNA gene copies per µl DNA (log scale)
Maturation time (weeks)
B
Fungal ITS copies
Number of ITS copies per µl DNA (log scale)
Maturation time (weeks)
C
Bacterial zOTU richness
Number of observed zOTUs
Maturation time (weeks)
D
Fungal OTU richness
Number of observed OTUs
Maturation time (weeks)
E
Bacterial Simpson's index of diversity
Simpson's index of diversity
Maturation time (weeks)
F
Fungal Simpson's index of diversity
Simpson's index of diversity
Maturation time (weeks)
Oak
Acacia

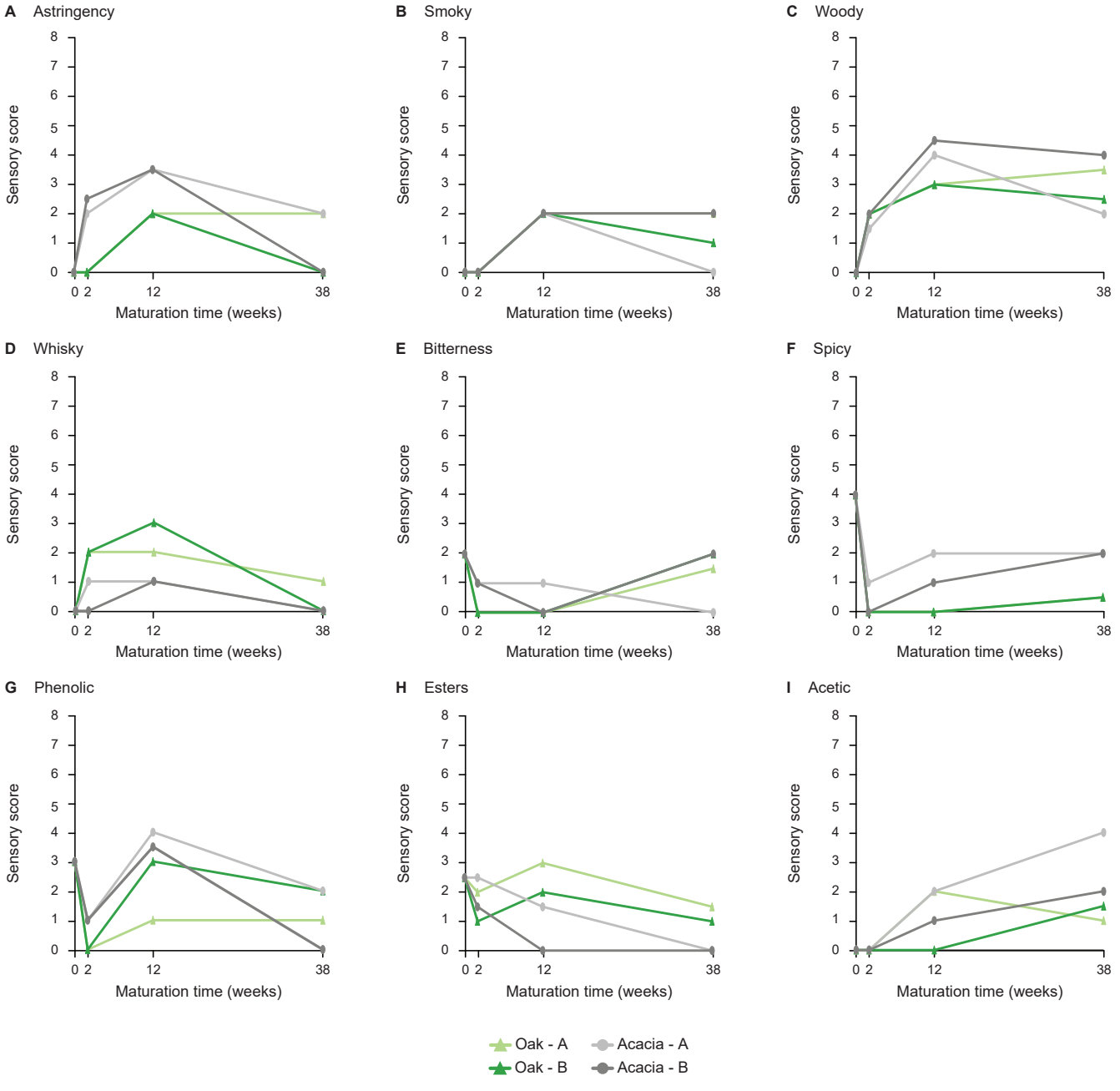

A
Oak
Replicate A
Replicate B
Relative abundance (%)
100
80
60
40
20
0
0 2
12
38
Maturation time (weeks)
B
Acacia
Replicate A
Replicate B
Relative abundance (%)
Maturation time (weeks)
Others
zOTU 1 (Pediococcus damnosus, 100%)
zOTU 2 (Stenotrophomonas sp., 100%)
zOTU 6 (Acetobacter sp., 100%)
zOTU 17 (Paenibacillus sp., 100%)


A
Oak
Replicate A
Replicate B
Relative abundance (%)
100
80
60
40
20
0
0 2
12
38
Maturation time (weeks)
B
Acacia
Replicate A
Replicate B
Relative abundance (%)
Maturation time (weeks)
Others
OTU 1 (Brettanomyces bruxellensis, 100%)
OTU 3 (Penicillium sp., 100%)
OTU 2 (Brettanomyces anomalus, 100%)



(1) pH
(2) Proanthocyanidin
(3) m-thymol
(4) Ethyl butanoate
(5) 4-methyl guaiacol
(6) Total polyphenols
(7) Ethyl hexanoate
(8) Salicyl aldehyde
(9) 4-ethyl guaiacol
(10) 4-ethyl phenol
(11) n-propanol
(12) Phenyl ethyl alcohol
(13) Succinic acid
(14) Methyl vanillate
(15) Acetic acid
(16) Lactic acid
(17) Isobutanol
(18) Cis-3-methyl-4-octanolide
(19) Ethyl vanillate
(20) Propionic acid
(21) Eugenol
(22) Ethyl acetate
(23) Vanillin
(24) Iso-eugenol
(25) Acetovanillone
(26) Trans-3-methyl-4-octanolide
(27) Ethyl decanoate
(28) Furfural
(29) Malic acid
(30) 5-methyl furfural
(31) Ethyl octanoate
(32) o-thymol
(33) 4-hydroxy benzaldehyde
(34) 2-methyl butanol
(35) Isobutyl acetate
(36) 4-vinyl guaiacol
(37) Isoamyl acetate
(38) 3-methyl butanol
(39) Phenyl ethyl acetate
(40) Guaiacol
(41) Flavanoids
(42) Syringol


A pH
pH
4.50
4.25
4.00
3.75
3.50
B Lactic acid
Concentration (g/l)
1.00
0.75
0.50
0.25
0
C Acetic acid
Concentration (g/l)
0.8
0.6
0.4
0.2
0
D Eugenol
Concentration (ppb)
40
30
20
10
0
E Cis-3-methyl-4-octanolide
Concentration (ppb)
300
250
200
150
100
50
0
F Trans-3-methyl-4-octanolide
Concentration (ppb)
250
200
150
100
50
0
G Vanillin
Concentration (ppb)
1600
1400
1200
1000
800
600
400
200
0
H Total polyphenols
Concentration (ppm)
800
600
500
400
300
200
100
0
I Ethyl acetate
Concentration (ppm)
90
80
70
60
50
40
30
20
10
0
J β-Glucan
Concentration (mg/l)
16
12
8
4
0
K Isoamyl acetate
Concentration (ppm)
4
3.5
3
2.5
2
1.5
1
0.5
0
L 4-Vinyl guaiacol
Concentration (ppb)
900
750
600
450
300
150
0
0 2 12 38
Maturation time (weeks)
Oak - A
Oak - B
Acacia - A
Acacia - B


A Astringency
B Smoky
C Woody
D Whisky
E Bitterness
F Spicy
G Phenolic
H Esters
I Acetic
Sensory score
0 1 2 3 4 5 6 7 8
0 2 12 38
Maturation time (weeks)
Oak - A
Acacia - A
Oak - B
Acacia - B