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

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ABSTRACT

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

1. Introduction

Bottle refermentation or conditioning is a common practice in the production of Belgian specialty beers (Van Landschoot et al., 2005). Beer bottles are incubated to enable a secondary fermentation after the addition of yeast cells and an energy source during the bottling process (Vanderhaegen et al., 2003a). The yeast cells protect the beer from oxidation by scavenging oxygen and can add new flavors to the beer upon maturation (Vanderhaegen et al., 2003a). For some beers, such as Berliner Weisse beer, a starter culture of yeasts and lactic acid bacteria (LAB) is used for refermentation (Verachtert and Derdelinckx, 2005). Other beers, such as gueuze beers, are the refermented products of mixtures of spontaneously fermented lambic beers (Verachtert and Iserentant, 1995). For the production of gueuze beer, a young (typically one-year old) lambic beer with residual dextrin carbohydrates is mixed with old (typically three-years old) lambic beer, which contains the microbiota that can convert the dextrin carbohydrates to more simple fermentable carbohydrates (Verachtert and Iserentant, 1995). Once mixed, the beer referments spontaneously, without the addition of energy sources, yeast or bacterial cells (Verachtert and Iserentant, 1995). *Dekkera*/*Brettanomyces* spp. (*Dekkera* is the name of the sexual form of this yeast and will be used throughout this paper) and LAB species are the dominant microorganisms in the refermenting beer, although after 14 months of refermentation only LAB are isolated (Verachtert and Iserentant, 1995). In contrast to the storage of some wines, beer storage is

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usually considered negative for the flavor quality of the beer (Vanderhaegen et al., 2006). Nevertheless, aging of gueuze beers is a common practice in traditional lambic beer breweries and is well appreciated by the consumers. Aging may last for more than ten years and hence means an investment by the brewery in end-products and space. It is however not clear to what extent this long-lasting aging process contributes to the (flavor) quality of gueuze beers.

Lambic beers are the result of a spontaneous fermentation process that proceeds for up to three years. We previously reported the microbial succession of a traditional lambic beer fermentation process in the Belgian lambic beer brewery, Cantillon (Spitaels et al., 2014). This fermentation process consists of a succession of three phases that starts with an *Enterobacteriaceae* phase, which proceeds for up to 1 month and in which multiple *Enterobacteriaceae* species are dominant. After one month, the main fermentation phase starts, which is characterized by the dominant presence of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* until month 3. The last phase is the maturation phase, in which the beer obtains its characteristic acidity and dryness, and is characterized by the presence of *Pediococcus damnosus* as the only LAB species and *Dekkera bruxellensis* as the dominant yeast species. Both microorganisms are still cultivable in the lambic beer at the end of a two-years monitoring period (Spitaels et al., 2014). In addition, acetic acid bacteria (AAB) are isolated inconsistently throughout this period.

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

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

2. Materials and methods

2.1. Brewery

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

2.2. Sampling

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

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

2.3. PCR-DGGE

The bottled gueuze beers were homogenized by swirling and three crude beer samples (100 mL each) per bottle, *i.e.*, three replicate samples per gueuze beer bottle and thus nine replicate samples per bottling year, were centrifuged at 8000 × *g* for 10 min (4 °C). Cell pellets were stored at −20 °C until further processing. DNA was prepared from the cell pellets as described by Camu et al. (2007). The DNA concentration, purity, and integrity were determined using 1% (wt/vol) agarose gels, stained with ethidium bromide, and by optical density (OD) measurements at 234, 260, and 280 nm. Total DNA solutions were diluted to an OD_{260} of 1.0. Amplification of about 200 bp of the V3 region of the 16S rRNA genes with the F357 (with a GC clamp attached) and R518 primers, followed by DGGE analysis, and processing of the resulting fingerprints was performed as described previously (Duytschaever et al., 2011), except that DGGE gels were run for 960 min instead of 990 min. For the amplification of about 200 bp of the D1/D2 region of the 26S rRNA gene, the NL1 (with GC clamp) and LS2 primers were used as previously reported by Cocolin et al. (2000). However, PCR amplicons of both 16S rRNA and 26S rRNA genes were not consistently obtained from the gueuze beer samples and a nested PCR approach was therefore applied by means of a second PCR assay using the same primers and the products of the first PCR assay as template.

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