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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

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ABSTRACT

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

1. Introduction

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

Although lambic beers were originally only brewed in the Senne river valley and southeast of Brussels, they are now also brewed elsewhere in Belgium. In West-Flanders, the most western province of Belgium and thus outside the Senne river valley, two independent breweries produce lambic beers. In the past, both breweries obtained the necessary lambic wort and lambic beer from breweries located in the Senne river valley. One brewery filled their casks with purchased wort to be able to produce their own lambic beer. The other brewery purchased finished lambic beers to blend them and produce gueuze beers. Because of the growing interest in

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beers of spontaneous fermentation, both breweries stopped buying lambic wort or beer from traditional lambic breweries and started to brew a lambic-type of beer from fresh wort, conform to the lambic beer production process.

The production activities of American craft breweries, including American coolship ales and other types of beers, resemble those of the industrial lambic breweries (Bokulich et al., 2012). The latter breweries do not only produce lambic beers and products derived thereof, but also the more typical ales and lager beer brands. Industrial lambic breweries mostly filter, pasteurize and carbonate their spontaneously fermented beers, which are sometimes also sweetened (Van Oevelen et al., 1976). Moreover, they can brew lambic-type beers continuously, because they have the capacity to prechill the wort before its transfer into the open cooling tun and hence do not need the cold winter months to properly cool their wort in one night as traditional lambic breweries do. Also, industrial brewers generally do not use old, small wine or cognac casks for fermentation (2–6 hL). Instead, their wooden casks are usually larger and custom-made on-site (about 170–200 hL).

The present study aimed to characterize the microbial succession in an industrial lambic beer fermentation process during one year, and to compare this succession of microorganisms with that of a lambic beer fermentation in a traditional lambic brewery.

2. Materials and methods

2.1. Brewery

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

2.2. Brewing process and sampling to study the succession of the microbiota

Mash was prepared and boiled for 1.5 h in the brewery according to the brewer's recipe, which included acidification of the wort to pH 4 through the addition of lactic acid. This acidification is commonly performed in all industrial lambic beer breweries. After the acidification, the wort was prechilled to 40 °C and centrifuged to remove the hot break. The prechilled wort was then transferred into a cleaned open cooling tun and a 500-mL sample was taken aseptically. A second 500-mL sample was taken from the wort in the cooling tun after overnight cooling at the start of the wort transfer into the 170 hL cask. The transfer process required about 8 h. The cooling tun was sampled a third time shortly before the wort was completely transferred into the wooden cask. Samples were taken from this cask after the transfer of the cooled wort and after 1, 2 and 3 weeks and 1, 2, 3, 6, 9 and 12 months. Two lambic batches were analyzed. Batch A was brewed on January 4, 2011 and was sampled at all time points mentioned above. The wort temperature of batch A after overnight cooling was about 22 °C. Batch B was brewed on July 27, 2010 and was sampled at the same time points for three months only. The wort temperature of batch B after overnight cooling was about 29 °C. Two weeks after transfer of the batch A wort into the cask there was no apparent production of foam, indicating that the fermentation process did not start, hence the brewer decided to mix batch A (which is further referred to as the acceptor batch A) with a 3-months old fermenting lambic wort from another batch (further referred to as the donor batch A) to initiate the fermentation. Mixing occurred through the bottom apertures of the casks and was performed in a ratio of 5 hL to 165 hL (±3%, vol/vol). Both the donor and acceptor batches A were sampled at the time of mixing, which is referred to below as the mixing point. The acceptor batch A was sampled prior to and 15 min after mixing, enabling debris to settle.

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

2.3. Denaturing gradient gel electrophoresis (DGGE) analysis

Crude brew samples (50 mL) were centrifuged at 8000 × *g* for 10 min (4 °C) at the day of sampling and cell pellets were stored at −20 °C until further processing. DNA was prepared from the pellets as described by Camu et al. (2007). The DNA concentration, purity, and integrity were determined using 1% (wt/vol) agarose gels stained with ethidium bromide and by optical density (OD) measurements at 234, 260, and 280 nm. The quality of the DNA was assessed as good, when absorbance ratios were $OD_{260}/OD_{280} > 1.8$ and $OD_{234}/OD_{260} > 0.5$. Total DNA solutions were diluted to an OD_{260} of 1. Amplification of about 200 bp of the V3 region of the 16S rRNA genes with the F357 (with a GC clamp) and R518 primers, followed by denaturing gradient gel electrophoresis (DGGE) analysis, and processing of the resulting fingerprints was performed as described previously (Duytschaever et al., 2011), except that DGGE gels were run for 960 min instead of 990 min. For the amplification of about 200 bp of the D1 region of the LSU-rRNA genes of eukaryotic microorganisms, NL1 (with GC clamp) and LS2 primers were used, as previously reported by Cocolin et al. (2000).

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

2.4. Culture media, enumeration and isolation

Samples were serially diluted in 0.9% (wt/vol) saline and 50 μL of each dilution was plated in triplicate on multiple isolation media solidified with 1.5% agar. Bacterial isolation media [de Man-Rogosa-Sharpe (MRS) agar (Oxoid, Erembodegem, Belgium) (De Man et al., 1960), violet red bile glucose (VRBG) agar (Mossel et al., 1978, 1962) and acetic acid medium (AAM) agar (Lisdiyanti et al., 2003)] were supplemented with 5 ppm amphotericin B (Sigma–Aldrich, Bornem, Belgium) and 200 ppm cycloheximide (Sigma–Aldrich) to inhibit fungal growth and were selected as described earlier (Spitaels et al., 2014c). Inoculated MRS agar plates were incubated at 28 °C aerobically and at 20 °C anaerobically for the isolation of LAB; inoculated VRBG agar plates were incubated at 28 °C aerobically for the isolation of *Enterobacteriaceae*; and inoculated AAM