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Metabolic strategies of beer spoilage lactic acid bacteria in beer

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ABSTRACT

Beer contains only limited amounts of readily fermentable carbohydrates and amino acids. Beer spoilage lactic acid bacteria (LAB) have to come up with metabolic strategies in order to deal with selective nutrient content, high energy demand of hop tolerance mechanisms and a low pH. The metabolism of 26 LAB strains of 6 species and varying spoilage potential was investigated in order to define and compare their metabolic capabilities using multivariate statistics and outline possible metabolic strategies. Metabolic capabilities of beer spoilage LAB regarding carbohydrate and amino acids did not correlate with spoilage potential, but with fermentation type (heterofermentative/homofermentative) and species. A shift to mixed acid fermentation by homofermentative (hof) *Pediococcus claussenii* and *Lactobacillus backii* was observed as a specific feature of their growth in beer. For heterofermentative (hef) LAB a mostly versatile carbohydrate metabolism could be demonstrated, supplementing the known relevance of organic acids for their growth in beer. For hef LAB a distinct amino acid metabolism, resulting in biogenic amine production, was observed, presumably contributing to energy supply and pH homeostasis.

1. Introduction

In general beer represents a harsh environment for bacteria to cope with, as various hurdles have to be taken in order to grow (Vriesekoop et al., 2012). The high microbiological stability of beer results from the presence of ethanol (0.5–10% *w*/*w*), high carbon dioxide content (ca. 0.5% *w*/*v*) and the presence of hops (about 17–55 ppm of iso-α-acids). In addition, beer is characterized by a low pH (3.8–4.7) and, as a consequence of the fermentation by yeast, a selective nutrient content insufficient for growth of many microorganisms (Suzuki, 2011). Energy generation even becomes more difficult, as hops act as pH dependent proton ionophores (Behr and Vogel, 2009; Simpson, 1993), which in consequence inhibit the proton motive force (*pmf*) dependent uptake of nutrients and essential enzyme reactions (Suzuki, 2011). Nevertheless, lactic acid bacteria (LAB) of the genera *Lactobacillus* (*L.*) and *Pediococcus* (*P.*) are capable to grow in and spoil beer. Spoilage by these bacteria leads to visible turbidity, sediment formation, acidification, off-flavors and ropiness, depending on species and strain (Suzuki, 2011).

In general various metabolic adaptions to unfavored conditions are described for LAB (van de Guchte et al., 2002). In presence of alternative electron acceptors or in case of substrate limitation the pyruvate metabolism can be affected, resulting in the production of alternative end products like diacetyl, acetoin or formate (Holzapfel and Wood, 2014). Homofermentative (hof) LAB can switch to mixed acid fermentation as a consequence of carbon source limitation or a low pH, potentially generating more ATP and producing less acidic end products (Mozzi et al., 2010). Organic acids can be fermented or used as precursors of alternative electron acceptors. LAB are also able to catabolize amino acids to generate additional energy and counteract acid stress, preferably at the end of fermentations when carbon and energy sources are scarce and the pH is low. This can be done by amino acid decarboxylation coupled with electrogenic transport resulting in biogenic amines or by using systems like the arginine deiminase pathway (ADI), which leads to the alkalization of the cell and additional ATP production. All these metabolic adaptions to unfavored conditions may be complemented by energy efficient transport mechanism, as electrogenic precursor-product exchange or phosphoenolpyruvate-sugar phosphotransferase systems (PTS) (Holzapfel and Wood, 2014; van de Guchte et al., 2002). In case of beer, a positive relationship between the beer spoilage ability of lactobacilli and their metabolic versatility was observed by Dolezil and Kirsop (1980). Fernandez and Simpson (1993) did not find a correlation of hop resistance to metabolic products, sugar profile or pH tolerance, while later a significant relationship of spoilage susceptibility of beer to 8 parameters, including pH, the content of free amino nitrogen, total soluble nitrogen content as well as the concentrations of various individual amino acids and maltotriose was identified (Fernandez and Simpson, 1995). Suzuki et al. (2005) investigated the metabolism of important heterofermentative (hef) beer spoilage LAB species *L. brevis*, *L. lindneri* and *L. paracollinoides*. Organic acid metabolism, as malolactic fermentation and citrate utilization, as well as the ADI system to use arginine were suggested to be important for beer spoilage LAB (Suzuki et al., 2005).

The purpose of this study was to determine metabolic strategies and features of 6 important beer spoilage species in beer and to compare

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their metabolism. The beer spoilage potential of 26 strains was determined, followed by a comprehensive HPLC analysis of carbohydrate, amino acid and organic acid conversion after growth in lager beers with 2 different pH values. Metabolic capabilities of all strains, from no to strong spoilage potential, were investigated in lager beer with elevated pH and therefore reduced antibacterial properties ($lager_{pH5.0}$). Metabolic data were also collected from the same lager beer with a pH value of 4.3, which prevents the growth of strains with no spoilage potential and reveals the actual metabolism of true beer spoilage strains in a typical (pH) lager beer. The relation of spoilage potential, fermentation type (hof/hef) and species to the metabolic capabilities ($lager_{pH5.0}$), was further investigated using multivariate statistics.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

26 strains of 6 species were used (Table 1). The selection of strains was based on a prescreening using a high-throughput method described by Preissler et al. (2010) to estimate the spoilage potential of 60 strains in order to get a broad spectrum of spoilage potential for each species. For all experiments microorganisms were precultured at 25°C using a modified MRS medium ($mMRS_1$) with a pH of 6.2 as previously described by Schurr et al. (2013). Cells were stored at −80°C with a final glycerol concentration of 40%. Before storage all strains were propagated 4 times on $mMRS_1$ to get them into a comparable physiological state. All strains were identified (validated) on the species level using Matrix-Assisted-Laser-Desorption/Ionization-Time-Of-Flight Mass (MALDI-TOF MS) Spectrometry as described by Kern et al. (2013).

2.2. Beer spoilage test

In order to determine the beer spoilage potential, strains were tested using a system similar to the one described by Suzuki et al. (2005). All beers were degassed using vacuum and sterile filtered (0.2 μm, Nalgene™ Rapid-Flow™ Filters, Thermo Scientific, Waltham, USA) before usage. Test beers were purchased from the same brewery having the following parameters: Lager beer with 18 international bitterness units (IBU), pH 4.3, gravity 11.5 wt.%; alcohol 5.1 vol.%, wheat beer with 14 IBU, pH 4.4, gravity 12.5 wt.%, alcohol 5.5 vol.%; pilsner beer with 29 IBU, pH 4.4, gravity 11.5 wt.%, alcohol 5.1 vol.%. Lager beer, adjusted to pH 5.0 ($lager_{pH5.0}$) with 6 M NaOH (Carl Roth, Karlsruhe, Germany), was inoculated with 2% of an $mMRS_1$ pre-culture, which was checked for contamination (species-level) using MALDI-TOF MS, and incubated at 25°C. After visible growth in $lager_{pH5.0}$, 10 ml degassed test beers were inoculated with 5×10^3 cells/ml from $lager_{pH5.0}$ and incubated at 25°C for 60 days. Tubes were examined for visible growth (= beer spoilage) every second day. The test was performed using 3 biological replicates. As controls triplicate approaches of all test beers without inoculation were carried out. Based on their growth behavior (visible growth), strains were classified into 4 beer spoilage potential groups, from strong potential (SB – growth in pilsner beer) to no potential (NB – no growth in test beers). After 60 days the optical density at 590 nm as well as the pH were determined for all samples.

2.3. HPLC analysis

Samples for high performance liquid chromatography (HPLC) analysis were taken from $lager_{pH5.0}$ and $lager_{pH4.3}$ after 60 days of incubation within the beer spoilage test. Two biological replicates were analyzed for each strain and beer as well as for the not inoculated control beers. For the determination of the sugar profile of lager beer, samples of 5 different batches were taken from degassed lager beer within one year. All samples were centrifuged at 15,000×*g* for 5 min and supernatant was subjected to protein precipitation. This was either done using perchloric acid (amino acid, organic acid analysis) or zinc sulfate (carbohydrate analysis). For the former method 50 μl of a 70% (*v*/*v*) perchloric acid (Sigma-Aldrich, St. Louis, USA) were mixed with 1 ml of sample, followed by incubation at 4°C overnight. The precipitate was removed by centrifugation at 15,000×*g* for 30 min, while the supernatant was used for HPLC analysis. For the analysis of carbohydrates 750 μl of sample were mixed with 450 μl of 10% zinc sulfate (Sigma-Aldrich, St. Louis, USA)

Table 1
Strains, alternative identifiers (e.g. DSMZ, ATCC, JCM) and source of isolation. TMW = Technische Mikrobiologie Weihenstephan.

Species	Strain	Alternative identifiers	Source
P. claussenii	TMW 2.54		brewery environment
	TMW 2.340	DSM 14800 T/ATCC BAA-344 T	spoiled beer
	TMW 2.53		brewery environment
	TMW 2.1531		brewery environment
P. damnosus	TMW 2.1533		bottled beer
	TMW 2.1535		brewery environment
	TMW 2.1532		brewing yeast sample
	TMW 2.1534		pilsner beer
	TMW 2.1536		wine
L. backii	TMW 1.1430		spoiled beer
	TMW 1.1988		wheat beer
	TMW 1.1989		bottled beer
	TMW 1.1991		brewery environment
	TMW 1.1992		brewery environment
L. paracollinoides	TMW 1.1994		brewery environment
	TMW 1.1995		pilsner beer
	TMW 1.696	DSM 15502 T [WB]/JCM 11969 T [WB]	brewery environment
	TMW 1.1979	DSM 20197/ATCC 8291	beer
L. lindneri	TMW 1.481		brewery environment
	TMW 1.1285	DSM 20692 T	spoiled beer
	TMW 1.1433		spoiled beer
	TMW 1.1993		beer
L. brevis	TMW 1.313		brewery environment
	TMW 1.465		brewery environment
	TMW 1.6	DSM 20054 T	feces
	TMW 1.1369		honey

[WB] = variant lacking spoilage associated hop tolerance genes with weak spoilage potential.
[DSMZ] = German Collection of Microorganisms and Cell Cultures.
[ATCC] = American Type Culture Collection.
[JCM] = Japan Collection of Microorganisms.