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Multivariate analysis of buckwheat sourdough fermentations for metabolic screening of starter cultures

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ABSTRACT

This study investigated the metabolic activity of 35 strains of lactic acid bacteria (LAB), which were able to grow in buckwheat sourdoughs and delivers a detailed explanation of LAB metabolism in that environment. To interpret the high-dimensional dataset, descriptive statistics and linear discriminant analysis (LDA) were used. Heterofermentative LAB showed a clear different metabolism than facultative (f.) heterofermentative and homofermentative LAB, which were more similar. Heterofermentative LAB were mainly characterized by high free SH groups and acetic acid production; they were also able to consume arabinose and glucose. Homofermenters were mainly characterized by lower free amino nitrogen content and they did not show a good capacity to consume arabinose and fructose. Except for the heterofermentative *Weissella cibaria* strain, only homofermentative strains showed high ornithine yields. Some f. heterofermentative strains differed from homofermentative due to the high lactic acid production as well as low glucose and arginine consumption. LAB containing more genes encoding peptidase activities and genes involved in aroma production showed a high consumption of free amino acids. Strain-dependent activities could be clearly distinguished from group dependent ones (homofermentative, f. heterofermentative and heterofermentative), e.g., some *Lactobacillus paracasei* and *Lactobacillus plantarum* strains showed the highest carbohydrate consumption. However, some microbial activities were more strain-dependent than group-dependent. Multivariate analysis of raw data delivered a detailed and clear explanation of LAB metabolism in buckwheat sourdough fermentations.

1. Introduction

In the last four decades, the microbial activity of lactic acid bacteria (LAB) was intensively investigated especially in wheat and rye sourdough fermentations. Carbohydrate, peptide and lipid metabolism of LAB has been studied as well as LAB–yeast interaction (Cagno et al., 2002; Corsetti et al., 2001; Corsetti and Settanni, 2007; De Vuyst and Vancanneyt, 2007; Gänzle et al., 2007; Gobbetti, 1998; Häggman and Salovaara, 2008; Hammes et al., 2005; Venturi et al., 2012). Since the 2000s, the investigation of microbiota of gluten-free (GF) sourdoughs increased and further information was delivered (Zannini et al., 2012). It was observed that some typical wheat and rye starter cultures were not suitable for GF sourdoughs because their growth is substrate-specific in some cereals and pseudocereals (Moroni et al., 2010b; Vogelmann et al., 2009). Moreover, several studies showed that *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus gallinarum*, *Lactobacillus paralimentarius*, *Lactobacillus graminis*, *Lactobacillus brevis*, *Lactobacillus sakei*, and *Pediococcus pentosaceus* were frequently isolated from several GF sourdoughs (rice, oats, buckwheat, teff, amaranth, maize, millet, and quinoa) (Edema and Sanni, 2008; Hüttner et al., 2010; Meroth et al., 2004; Moroni et al., 2011a, 2010a; Vogelmann et al., 2009).

In wheat and rye doughs, it was demonstrated that microbial metabolism strongly influenced the functional and nutritional properties of sourdoughs and consequently the quality of bakery goods (Gänzle et al., 2008, 2007). Proteolysis is a main factor, which influences dough quality during wheat and rye fermentations (Gänzle et al., 2008). This activity is mainly supported by flour endogenous proteases (aspartic proteases), which are activated by microbial acidification (Gänzle et al., 2008). Moreover, protein breakdown goes ahead through peptidase activity of LAB (Thiele et al., 2003; Vermeulen et al., 2005). Normally, gluten depolymerization is increased by reducing agents, such as glutathione, during sourdough fermentation. Indeed, heterofermentative LAB displayed glutathione reductase activity, which keeps the level of free SH groups constant (Gänzle et al., 2008; Jänsch et al., 2007). Furthermore, some LAB are able to convert arginine to ornithine; the latter is a precursor of 2-acetyl-1-pyrroline, which normally occurs during the baking process and gives the characteristic flavor of baked wheat bread crust (De Vuyst et al., 2009).

Buckwheat is a pseudocereal, which has interesting functional and nutritional characteristics for GF formulations. In fact, buckwheat flour

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contains proteins of high nutritional value and a wide range of free amino acids (FAA), whose composition is nutritionally superior to that of cereal grains (Hager et al., 2012; Pomeranz, 1983; Wijngaard and Arendt, 2006). Moreover, addition of buckwheat flour showed an improvement of the baking performances and nutritional values of GF bread as well as in wheat bread (Mariotti et al., 2013; Moroni et al., 2012).

In GF breads, it has been observed that protein breakdown had positive effects on bread volume and crumb structure (Moroni et al., 2011b; Renzetti and Arendt, 2009a, 2009b; Renzetti et al., 2010; Schober et al., 2007). Recently, it has been demonstrated that proteolysis was mainly affected by flour endogenous proteases through microbial acidification even in buckwheat sourdoughs (Capuani et al., 2013). However, it has been observed that the oxidation-reduction potential (ORP) do not affect positively the proteolysis increase. Indeed, addition of glutathione showed a decrease of proteolysis in buckwheat sourdoughs (Capuani et al., 2013). Moreover, it has been shown that the increase of free thiol content was correlated with the decrease of ORP (Capuani et al., 2013). However, the microbial activity of only few strains was monitored in buckwheat sourdough, e.g., proteolysis, reducing activity, metabolite production, sugar consumption and amino acid metabolism (Capuani et al., 2013, 2012).

Therefore, the aim of this study was to extend the investigation of microbial activity (considering more variables than only organic acid production) of several heterofermentative (*hetero*), facultative heterofermentative (*f. hetero*) and homofermentative (*homo*) lactic acid bacteria in buckwheat sourdoughs. The understanding of microbial activity was supported by multivariate statistical analysis, which was used to observe relevant correlations between variables (metabolite production and substrate consumption) and metabolic groups (*hetero*, *f. hetero* and *homo*). Moreover, possible correlations between metabolic activity and a set of genes were investigated.

2. Materials and methods

2.1. Microbial strains and culture conditions

35 LAB strains (*hetero*, *f. hetero* and *homo*) were employed to carry out buckwheat sourdough fermentations. A full list of used strains is displayed in Table 1. For sourdough preparation, hulled organic buckwheat grains (Ziegler & Co. GmbH, Germany) were milled and the obtained flour was utilized. All the strains were cultivated overnight at 30° (except for *E. faecalis* TMW 2.630 which was cultivated at 37 °C) in 2 mL Spicher medium (Capuani et al., 2012). *E. faecalis* TMW 2.630 was employed as reference proteolytic strain, as previously showed (Capuani et al., 2013).

2.2. Sourdough fermentations

Overnight cultures were centrifuged for 5 min at 6500 rpm and washed two times using a Ringer solution (Merck, Germany). Subsequently, the cell pellet was resuspended in 100 μL ringer solution. Dough with a dough yield of 500 was prepared adding 100 parts buckwheat flour and 400 parts water (BONAQUA) and finally mixed using a handmixer. 1.9 mL dough was added to a 2 mL tube containing the resuspended culture, obtaining a start inoculation of ca. 10^8 CFU/mL dough. The tubes were incubated at 30° and 37 °C (for *E. faecalis* TMW 2.630) for 8 h. Each fermentation was carried out in three independent replicates (n = 3). After fermentation, the pH of sourdoughs was measured. Fermentations showing pH outliers were not considered for statistical analysis.

2.3. Chemical analysis

2.3.1. Free amino nitrogen (FAN) content

Proteolysis was determined performing ninhydrin test as described by Thiele et al. (2002). Measurements were performed with a FLUOstar Omega at a wavelength of 570 nm (BMG-LABTECH, Germany), analyzing three independent replicates (n = 3).

2.3.2. Analysis of free SH groups of SDS-soluble fraction

Free thiol groups were measured according to a modified method of Jänsch at al. (2007). Sample preparation was executed according to Capuani et al. (2012). Measurements were performed using a FLUOstar Omega (BMG-LABTECH, Germany) at a wavelength of 412 nm, analyzing three independent replicates (n = 3).

2.3.3. Analysis of organic acids, ethanol and carbohydrates

Organic acids, ethanol and carbohydrates (inositol, mannitol, rhamnose, arabinose, glucose, fructose, sucrose and maltose) were analyzed as previously described (Capuani et al., 2012) using an IEC dual analysis system ICS-5000 (Dionex, USA).

Organic acids were detected using a ReproGel-H 9 μm combined with a conductivity detector and suppressor, while for ethanol detection a RI-101 detector (Shodex, Germany) was used. Carbohydrates were analyzed using a CarboPac PA20 (Dionex, USA) and an electrochemical detector ICS-5000 (Dionex, USA).

2.3.4. Analysis of free amino acids (FAA)

Sample preparation was performed as previously described (Capuani et al., 2013). FAA were determined using reversed phase high performance liquid chromatography (RP-HPLC on an UltiMate 3000 HPLC system, Dionex, Germany) as described by Schurr et al. (2013). Amino acids were quantified using external standards.

2.3.5. DNA extraction, primer design and PCR conditions

For DNA isolation, the E.Z.N.A.® Bacterial DNA Kit (Norcross, USA) was used according to the protocol of the supplier with a few adaptations. DNA was solved in TE buffer and the concentration was measured with Nanodrop® ND-1000 (Thermo Scientific, USA). The DNA concentration was adjusted to 50 ng/μL for each strain.

The primer design was done manually from annotated DNA and amino acid sequences of different *Lactobacillus* strains. Genes for primer design were chosen, according to NCBI (http://www.ncbi.nlm.nih.gov/) annotations and literature information, and were classified into the following groups: known “redox”, peptidase, dehydrogenase, electron transport chain (ETC) and “aroma”. DNA and protein multiple sequence alignments were realized with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Conserved regions were used for primer design. Melting temperature, GC content, and formation of possible primary and secondary structures were checked with multiple primer analyzer tool (http://www.thermoscientificbio.com/webtools/multipleprimer/).

Table S1 (electronic Supplementary materials) lists the degenerated PCR primers used in this study. PCR amplification was performed by using a 25 μL total volume mixture containing 1.25 U of Taq DNA polymerase (MP Biomedicals, Canada), 10× buffer with 15 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 0.4 μM of each primer and 100 ng of the extracted DNA. The optimum $MgCl_2$ concentration was tested with the addition of 25 mM $MgCl_2$ to the mastermix resulting in end concentrations of 2.5 mM and 3 mM. The thermal cycle involved 3-min activation of the polymerase at 94 °C before 30 cycles of dissociation (45-s at 93 °C), annealing (30-s, variable) and elongation (1-min, 72 °C) followed. The annealing temperature was subsequently determined with gradient-PCR and DNA of different strains gaining different positive controls. Finally, a 3–5 minute extension at 72 °C was performed. The end concentration of the Taq polymerase as well as the thermal profile for the PCR of the Fae primer varied slightly. The final concentration of the Taq polymerase in a 50 μL scale was 1.5 U. The initial dissociation was 5-min at 94 °C before the dissociation step at 94 °C for 30 s followed. The other steps are equal to the thermal profile mentioned above. The amplification of PCR products of the proper size was confirmed by electrophoresis through a 1% agarose gel (Biozym, Germany) in TBE or TAE buffer (Sambrook et al.,