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Evolution of sourdough microbiota in spontaneous sourdoughs started with different plant materials

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ABSTRACT

The preparation of sourdough in bakeries may include the use of inocula, e.g. fruits, flowers or rumen cuts to accelerate the process of selection of suitable microorganisms. The aim of this work was to investigate the effect of these inocula on the microbial evolution in sourdoughs. First, the microbiota of nineteen traditional sourdoughs that were initially started with diverse inocula was identified. Second, *de novo* sourdoughs were started with plant materials and the evolution of sourdough microbiota was investigated by culture, and by high-resolution melting curve quantitative PCR (HRM-qPCR). This study developed a new protocol for HRM-qPCR analysis of yeast microbiota in sourdough, and indicates this independent culture method suitable for characterization of yeasts. Microbiota of traditional sourdoughs were largely independent from the use of inoculum, however, *Acetobacter* spp. were identified only in sourdoughs started with apple flowers or apple pulp. In *de novo* sourdoughs started with plant materials, microbiota rapidly stabilized, and were characterized by *Lactobacillus sanfranciscensis*, *Lactobacillus plantarum*, *Lactobacillus graminis*, or *Lactobacillus rossiae*, and *Saccharomyces cerevisiae* as dominant species. Competition experiments revealed that the ecological fitness of *L. plantarum*, *L. graminis*, and *L. rossiae* in wheat or rye malt sourdoughs was lower when compared to *L. sanfranciscensis*, demonstrating that their presence in *de novo* sourdoughs reflects dispersal limitation. In conclusion, establishment of microbiota in *de novo* sourdoughs is dispersal limited. This study provides scientific support for the artisanal practice to inoculate *de novo* sourdoughs with flowers, berries, or related plant material.

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1. Introduction

Sourdough is employed in production of baked cereal products to achieve leavening, to improve bread quality, or to replace additives by "clean label" ingredients (Hammes and Gänzle, 1998; Brandt, 2007). Sourdough microbiota comprises lactic acid bacteria and yeasts (De Vuyst et al., 2014); their composition and activity determines the influence of sourdough on bread quality (Gänzle, 2014). Starter cultures for direct use in baking remain largely unavailable (Brandt, 2007); bakeries therefore typically maintain sourdoughs by continuous propagation using the previous batch as inoculum. Fermentation control is achieved by selection of raw materials for fermentation (Meroth et al., 2003; Vogelmann et al., 2009) and by selection of fermentation parameters (Di Cagno et al., 2014; Meroth et al., 2003; Stolz, 1999; Vrancken et al., 2010). Empirical control of fermentation, however, often fails to achieve consistent fermentation microbiota and product quality (Brandt, 2007). The control of sourdough fermentations thus necessitates an improved knowledge of the community assembly in sourdoughs.

The assembly of microbial communities is shaped by selection, dispersal, drift, and speciation (Nemergut et al., 2013; Vellend, 2010). Evidence for drift and speciation on assembly of sourdough microbiota is inconclusive. The role of selection for the assembly of sourdough microbiota is increasingly understood. Indeed, the competitiveness of lactobacilli in sourdough is strain- and species-specific (Siragusa et al., 2009), and depends on the process conditions (Meroth et al., 2003). The growth rate in specific fermentation substrates and the effect of pH and temperature on growth account for the effect of process conditions (Gänzle et al., 1998; Lin and Gänzle, 2014a; Meroth et al., 2003; Sekwati-Monang et al., 2012). Metabolic properties known to contribute to competitiveness of lactobacilli in sourdough include effective utilization of maltose and sucrose, the use of additional electron acceptors, and acid resistance (Gänzle et al., 2007; Lin and Gänzle, 2014b).

The term "dispersal" describes the spatial movement of organisms (Vellend, 2010); the role of dispersal on community assembly of sourdough microbiota is less well understood. *De novo* fermentation of sourdoughs under dispersal-limited laboratory conditions results in sourdough microbiota that differ from microbiota of sourdoughs in bakeries (De Vuyst et al., 2014; Minervini et al., 2012; Van der Meulen et al., 2007). *Lactobacillus plantarum* and other lactobacilli may originate from plant microbiota (Minervini et al., 2015) while sourdough strains of

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Lactobacillus reuteri are of human and animal intestinal origin (Su et al., 2012; Zheng et al., 2015a). The origin of *Lactobacillus sanfranciscensis* and other key sourdough lactobacilli, however, remains unknown (De Vuyst et al., 2014).

Analyses of laboratory-made *de novo* sourdoughs have focused on the development of microbiota in sourdoughs started with flour as the only non-sterile ingredient (De Vuyst et al., 2014). *De novo* preparation of sourdoughs in bakeries, however, may involve the use of inoculum to accelerate the establishment of suitable fermentation microbiota (De Vuyst et al., 2014). Fruits, vegetables, yoghurt, rumen cuts and even manure were reportedly used to start sourdough. The effect of these inocula on the development of *de novo* sourdoughs, however, has not been described. It was therefore the aim of this study to characterize the microbiota of sourdoughs started with different inocula. Our experimental approach included analysis of sourdoughs that were started with different inocula in bakeries, followed by propagation over several months or years; the analysis of laboratory-prepared sourdoughs inoculated with different plant materials, and competition experiments with selected isolates. Sourdough microbiota were analysed by culture, and by high resolution melting curve quantitative PCR (HRM-qPCR), a recently developed method (Lin and Gänzle, 2014a) that was extended to allow identification of sourdough yeasts.

2. Materials and methods

2.1. Collection of traditional sourdoughs

Sourdoughs were collected from bakeries of the Marche region in Italy which initiated, propagated, and used these sourdoughs in a traditional manner to produce bread without addition of baker's yeast. At sampling, it was inquired whether or not material other than flour and water was used to start the sourdoughs. At the time of sampling the age of these sourdoughs ranged from 2 months to one hundred years. The composition of microbiota in these sourdoughs was evaluated as outlined below.

2.2. De novo sourdough preparation and sampling

To prepare *de novo* sourdoughs, *Malus domestica* (apple) flowers, *Sinapis alba* (mustard) flowers, *Veronica persica* (speedwell) flowers, *Crataegus monogyna* (hawthorn) berries, *Myrtus communis* (murtle) berries, *Punica granatum* (pomegranate) fruits, and mother of vinegar were used as inoculum in addition to wheat flour. The 1st batch of dough was prepared using water, white wheat flour and 20% of the inoculum to a final dough yield of 200. The doughs were incubated for 48 h without temperature control at ambient temperature (20–25 °C). Sourdoughs were refreshed every 48 h with wheat flour, 20% of the previous batch of sourdough as inoculum, and sterile water added to achieve a dough yield of 200. Fermentations were carried out in sterile containers. Fresh samples were analysed by culture-dependent methods after the 1st, 4th, and 10th fermentation cycles. Samples taken after each fermentation cycle were conserved at −80 °C for culture-independent analysis by HRM-qPCR.

2.3. Isolation of bacteria and yeasts

Isolation of bacteria and yeast populations was carried out for both traditional samples and *de novo* sourdoughs. Bacteria in *de novo* sourdoughs were isolated and identified after the 1st, 4th, and 10th fermentation cycle; sourdough yeasts were isolated and identified after the 4th fermentation cycle. Sourdough samples were diluted in peptone water, appropriate dilutions were plated on modified de Man, Rogosa, Sharpe medium (Minervini et al., 2012) or on acidified yeast extract peptone dextrose (1% yeast extract, 2% peptone, 2% dextrose, pH 4.5) agar for isolation bacteria and yeasts. About 10 colonies per sample were selected to represent different colony morphologies, and purified by repetitive dilution streaks and maintained at −80 °C with glycerol as cryoprotectant.

2.4. Competition in experimental sourdoughs

Overnight cultures of *Lactobacillus rossiae* apple3B, *L. plantarum* appleB, *L. sanfranciscensis* AM10PSB, and *Lactobacillus graminis* SA1PSA in mMRS broth were washed with sterile tap water and re-suspended in an equal volume of sterile tap water. Doughs were prepared with sterile tap water and flour to obtain a dough yield of 200 and inoculated with the bacterial cultures to a cell count of approximately 10^7 cfu/g of each of the four species. Three different sourdoughs were prepared with material differing in buffering capacity and enzymatic activity. One sourdough was prepared with white wheat flour, a second with whole wheat flour, and a third with rye malt flour. The doughs were incubated at ambient temperature (20 °C) for 48 h and propagated with 20% inoculum for four fermentation cycles. Competition experiments with the three different flours were carried out in duplicate independent experiments analysed in duplicate.

2.5. DNA extraction

DNA was isolated from LAB and yeasts using the DNeasy Blood & Tissue kit (Qiagen, Toronto, Canada) with the automated extractor QIAcube (Qiagen). To enable DNA extraction from doughs samples, dough solids were removed by centrifugation at 500 ×*g* prior to extraction with the DNeasy Blood & Tissue kit.

2.6. RAPD-PCR analysis

Isolates from sourdough were analysed by RAPD–PCR using M13-5′-GAGGGTGGCGGTTCT-3′(Huey and Hall, 1989) to eliminate clonal isolates from the same sample. PCR reactions were performed with 200 μM dNTP, 1 μM of M13 primer, 1× buffer, 3.5 mM $MgCl_2$, 1 U of Taq polymerase, 3 μl of DNA, and sterile water in a final volume to 25 μl. Each amplification consisted of an initial denaturation time of 5 min at 94 °C followed by 40 cycles of amplification comprising a denaturation step at 94 °C for 60 s, annealing at 45 °C for 20 s, and extension at 72 °C for 2 min. Reactions were completed with 5 min elongation at 72 °C followed by cooling to 10 °C. The amplification products were separated by electrophoresis on 2.5% (*w*/*v*) agarose gel in TAE buffer, stained with SYBR®Safe DNA gel stain, and visualized by UV transillumination.

2.7. Molecular identification

Bacterial isolates were identified by partial sequencing of genes coding for 16S rRNA. PCR amplification was performed using primers P0 (59-GAGAGTTTGATCCTGGCTCAG) and P6 (59-CTACGGCTACCTTGTTACGA) according to Picard et al. (2000). Yeasts were identified by partial sequencing of 28S rRNA genes after PCR amplification with primers P1 (ATCAATAAGCGGAGGAAAAG) and P2 (CTCTGGCTTCACCCTATTC) (Sandhu et al., 1995). PCR amplicons were purified with High Pure PCR Product Purification kit (Qiagen) and sent to Macrogen (USA) for sequencing. The identification of bacterial isolates was based on comparison to sequences of bacterial type strains deposited in the ribosomal database project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp); yeast isolates were identified by nucleotide BLAST with sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/).

2.8. High resolution melting – quantitative PCR (HRM-qPCR)

HRM-qPCR was used as described (Lin and Gänzle, 2014a) to achieve the culture-independent detection of bacterial species with DNA isolated from sourdough as template. The HRM-qPCR was performed on a

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