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

Valery Ripari [a,b], Michael G. Gänzle [b,*], Enrico Berardi [a]

[a] Università Politecnica delle Marche, Dipartimento di Scienze Agrarie, Alimentari e Ambientali, Ancona 62010, Italy
[b] University of Alberta, Dept. of Agricultural, Food and Nutritional Science, Edmonton, AB T6G 2P5, Canada

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.

© 2016 Published by Elsevier B.V.

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

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 $500 \times g$ 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 MgCl₂, 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

Table 1
Identity of the species isolated from traditional sourdoughs.

Sourdough sample	Origin[a]	Initial starter	Identification
CA	Ancona	–[b]	*Lactobacillus plantarum* group[c], *Pediococcus pentosaceus*, *Saccharomyces cerevisiae*
SP	Camerino	–[b]	*L. plantarum* group, *Leuconostoc holzapfelii*, *S. cerevisiae*
PeL	Montelupone	–[b]	*L. brevis*, *Pc. pentosaceus*, *S. cerevisiae*
M	Civitanova Alta	–[b]	*L. plantarum* group, *Pc. pentosaceus*, *S. cerevisiae*
AC	Belforte	Apple	*Acetobacter cerevisiae*, *L. sakei*, *L. graminis*, *L. brevis*, *S. cerevisiae*
UPRF	Porto Recanati	Apple	*A. cerevisiae*, *L. plantarum* group, *S. cerevisiae*, *S. unisporus*
LM	Camerino	Apple pulp	*A. cerevisiae*, *L. sakei*, *L. plantarum* group, *Pc. pentosaceus*, *S. cerevisiae*
G	Ancona	Fruits	*A. cerevisiae*, *L. brevis*, *L. rossiae*, *L. plantarum* group, *Leuconostoc holzapfelii*, *Leuconostoc pseudomesenteroides*, *Leuconostoc kimchi*, *Weissella cibaria*, *S. cerevisiae*
MA	Filottrano	Cow manure	*L. plantarum*, *L. sanfranciscensis*, *S. cerevisiae*
AA	Ancona	Honey	*L. plantarum* group, *Pc. pentosaceus*, *W. cibaria*, *S. cerevisiae*, *Wickerhamomyces anomalus*
EC	Morrovalle	Honey	*L. graminis*, *Pc. pentosaceus*, *S. cerevisiae*
FA	Ancona	Honey	*L. brevis*, *Pc. pentosaceus*, *S. cerevisiae*
GF	Altidona	Honey	*L. graminis*, *Pc. pentosaceus*, *S. cerevisiae*, *S. barnettii*
PA	Agugliano	Honey	*L. sanfranciscensis*, *Lc. holzapfelii*, *Lc. kimchi*, *Lc. mesenteroides*, *W. confusa*, *S. cerevisiae*
FLM	Pollenza	Yoghurt	*W. cibaria*, *Pc. pentosaceus*, *Lc. holzapfelii*, *Lc. mesenteroides*, *S. cerevisiae*
VM	Macerata	Yoghurt	*L. sanfranciscensis*, *W. cibaria*, *Lc. holzapfelii*, *S. cerevisiae*
AV	Strettura (Umbria)	Yoghurt	*L. rossiae*, *Pc. pentosaceus*, *L. paracasei*, *Lc. mesenteroides*, *L. plantarum* group, *Lc. holzapfelii*, *S. cerevisiae*
CP	Fermo	Water and flour	*L. plantarum* group, *L. spicheri*, *Lc. holzapfelii*, *S. cerevisiae*
PM	Moresco	Vinegar	*L. rossiae*, *L. plantarum* group, *Lc. mesenteroides*, *S. cerevisiae*

[a] Unless otherwise noted, samples were collected in the Marche region.
[b] Sourdoughs Ca, SP, Pel and M have been propagated for more than 100 years and reliable information on the inoculum was not available.
[c] *L. plantarum* was identified on the basis of 16S rRNA sequences and thus not differentiated from *L. paraplantarum* or *L. pentosus*.

Rotor Gene-Q (Qiagen, USA) using Type-it HRM PCR Kit (Qiagen, USA). Bacterial DNA was amplified with primers 5′-TCC TAC GGG AGG CAG CAG T-3′ and 5′-GGA CTA CCA GGG TAT CTA ATC CTG TT-3′ targeting all bacterial 16S rRNA genes. The assay was calibrated by amplification of DNA isolated from defined bacterial strains. The HRM-qPCR methodology was extended to analyse yeast populations in sourdough. Yeast DNA was amplified with Yeast-r/Yeast-f primers (Park et al., 2009) and NL1/LS2 primers (O'Donnell, 1993).

The PCR conditions were: denaturation 5 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 10 s. At the final HRM analysis was performed using a temperature increasing from 70 °C to 90 °C at 0.1 °C/step with 2 s holding time at each step. All HRM-qPCR analyses were carried out in technical replicates.

3. Results

3.1. Microbiota of traditional sourdoughs started with different inocula

The assessment whether the use of inoculum of plant or animal origin influences sourdough microbiota was initially based on the identification of bacteria and yeasts from 19 sourdoughs (Table 1). The sourdoughs were initially started with flour and water only, or with flour, water and the single use of an initial starter as indicated in Table 1. Almost all of the isolated yeasts were strains of *Saccharomyces cerevisiae*. The composition of bacterial microbiota in the 19 sourdoughs

Table 2
Plant material used as inoculum for sourdough fermentations and bacterial and yeast cell counts in *de novo* sourdough samples at the 4th refreshment.

Inoculum	Bacterial cell count (cfu/g)	Yeast cell count (cfu/g)
Malus domestica (apple) flowers	1.0×10^9	2.0×10^7
Sinapis alba (mustard) flowers	5.0×10^9	5.0×10^7
Veronica persica (speedwell) flowers	4.0×10^9	1.0×10^6
Myrtus communis (myrtle) berries	5.8×10^8	8.7×10^7
Crataegus monogyna (hawthorn) berries	2.1×10^9	6.6×10^7
Punica granatum (pomegranate) fruit	1.2×10^9	1.4×10^7
mother of vinegar	8.4×10^9	1.2×10^7

did not correlate to the initial starter used (Table 1). However, the exceptional presence of *Acetobacter cerevisiae* was noted only in sourdoughs that were initially inoculated with apples, apple pulp, or fruits (Table 1).

3.2. Microbiota of de novo sourdoughs started with different inocula in the laboratory

To further explore the use of plant inocula on the development of sourdough microbiota, *de novo* sourdoughs were inoculated with plant material as indicated in Table 2 and propagated over 10 fermentation cycles with wheat flour. All sourdoughs exhibited high cell counts of lactic acid bacteria and yeasts already after 4 fermentation cycles (Table 2). The ratio of lactic acid bacteria to yeasts after 4 refreshments was 100:1.

Identification of bacteria isolates was performed after 1, 4, and 10 fermentation cycles; yeasts were identified after 4 fermentation cycles only (Table 3). Generally, the diversity of species in the samples decreased with an increasing number of fermentation cycles (Table 3). After 10 fermentation cycles, only one or two bacterial species were isolated from each sourdough. In two sourdoughs, *L. sanfranciscensis* was detected after only one fermentation cycle (Table 3). Remarkably, *Enterobacteriaceae*, bacilli, or staphylococci were not detected as dominant members of sourdough microbiota at any stage of development. After 10 fermentation cycles, sourdoughs contained *L. sanfranciscensis* (4 sourdoughs), organisms of the *L. plantarum* group (2 sourdoughs); *L. rossiae*, *L. graminis*, and *Gluconacetobacter cerinus* were identified in one sourdough each (Table 3). Yeasts isolated from sourdoughs were identified as *Wickerhamomyces anomalus*, *Saccharomyces cerevisiae* and *Aureobasidium proteae*.

3.3. Validation of HRM-qPCR assays for qualitative analysis of sourdough microbiota

To achieve a more comprehensive analysis of the development of microbiota in *de novo* sourdoughs, a HRM-qPCR assay developed previously (Lin and Gänzle, 2014a) was validated for all bacterial species isolated in *de novo* sourdoughs (Table 4). The HRM-qPCR differentiated amplicons of bacterial 16S rDNA if the difference of the melting

Table 3
Identification of bacterial isolates in *de novo* sourdoughs after 1, 4, and 10 fermentation cycles.

Sourdough sample	1st fermentation cycle	4th fermentation cycle	10th fermentation cycle
Apple flowers	*Lc mesenteroides, L. plantarum* group, *L. graminis*	*L. plantarum* group, *L. rossiae*	*L. plantarum* group, *L. rossiae*
Mustard flowers	*Gluconobacter cerinus, L. sakei, L. graminis, Leuconostoc mesenteroides*	*L. rossiae, L. brevis, L. graminis*	*L. graminis*
Veronica persica flowers	*L. sanfranciscensis, L. plantarum* group, *G. cerinus L. rossiae*	*L. sanfranciscensis, L. plantarum* group	*L. sanfranciscensis, G. cerinus*
Hawthorn berries	*L. sakei, L. curvatus, L. graminis, Lc. mesenteroides, L. sanfranciscensis*	*L. sanfranciscensis, L. plantarum* group, *L. curvatus, L. graminis*	*L. sanfranciscensis*
Myrtle berries	*Lc. holzapfelii, Lc. mesenteroides, Enterococcus hirae, Acetobacter tropicalis*	*Lc. mesenteroides, Lc. holzapfelii*	*L. sanfranciscensis*
Pomegranate	*Lc. holzapfelii, L. plantarum* group	*Pediococcus pentosaceus, Lc. holzapfelii*	*L. plantarum* group
Mother of vinegar	*A. cibinongensis, G. cerinus*	*L. sanfranciscensis, Lc. holzapfelii, L. sakei, L. brevis*	*L. sanfranciscensis*

temperatures was greater than 0.1 °C, and differentiated 9 of the 14 bacterial species (Table 4). With exception of *Lactobacillus sakei, Lactobacillus curvatus*, and *L. graminis*, all species occurring in the same sample were differentiated (Table 3 and Table 4).

Two primer pairs were evaluated to establish a HRM-qPCR assay for differentiation of yeasts isolated in this study (Table 5). The melting temperature of amplicons obtained with primers Yeast-r/Yeast-f differed only by 0.2 °C; these primers were thus not suitable for the differentiation of yeasts in the *de novo* sourdoughs (Table 5). The melting temperatures of amplicons obtained with primers NLS1/LS2 ranged from 81 °C to 82.7 °C and differentiated between the three yeast species that were identified in the samples (Table 5).

3.4. Use of culture-independent HRM-qPCR to characterize the development of microbiota in de novo sourdoughs

HRM-qPCR was employed to characterize the development of bacterial and yeast sourdough microbiota after each fermentation cycle (Figs. 1 and 2). Comparable to culture-dependent analysis, which accounts for strains comprising at least 1% of the total bacterial and yeast microbiota, respectively, HRM-qPCR analysis detects organisms if they account for 0.1–1% of the bacterial and yeast population, respectively (Lin and Gänzle, 2014a). Melting temperatures of specific amplicons after amplification of DNA isolated from sourdough differed from melting temperatures of the same amplicon obtained from pure strains. The difference was up to 0.1 °C for lactic acid bacteria, and up to 0.2 °C for acetic acid bacteria (Compare Fig. 1 and Table 4). This difference is explained by preferential binding of the dye to GC rich amplicons (Gudnason et al., 2007).

Table 4
Melting temperatures of 16rRNA PCR amplicons of bacterial reference strains.

Reference organism	T_m (°C)[a]
L. rossiae apple3B	83.6 ± 0.0
Lc. mesenteroides SA1PSC2	83.7 ± 0.05
Lc. holzapfelii MG 1PSB	83.8 ± 0.0
L. sanfranciscensis AM1OPSB	84.1 ± 0.03
Pc. pentosaceus MG2B	84.4 ± 0.02
L. plantarum appleB	84.4 ± 0.07
L. brevis SA3B	84.4 ± 0.07
L. graminis SA1PSA[b]	84.5
L. sakei SA1PSB	84.6 ± 0.0
L. curvatus BIANC5B	84.6 ± 0.02
Enterococcus hirae MYRT1 SB	85.0 ± 0.02
Gluconobacter cerinus AM1PSD	85.8 ± 0.0
Acetobacter tropicalis MG1PSA1	86.0 ± 0.0
Acetobacter cibinongensis AM1PSC	86.3 ± 0.0

[a] Left/right borders of the cells are drawn to connect melting peaks of 16S rRNA amplicons that could not be differentiated.
[b] *L. graminis* was indistinguishable by HMR-qPCR from either *Pc. pentosaceus*, *L. plantarum* and *L. brevis* or *L. sakei* and *L. curvatus*.

To facilitate display of the experimental results, HRM-qPCR melting curves are omitted from Figs. 1 or 2 if they are comparable to melting curves obtained with the subsequent sample (data not shown). Melting curves obtained after the first fermentation cycle were dominated by peaks representing acetic acid bacteria (Fig. 1), in keeping with the isolation of *Gluconobacter* and *Acetobacter* in most samples obtained after the first fermentation cycle (Table 3). Bacterial populations as analysed by HRM-qPCR rapidly stabilized and remained unchanged after 4–8 fermentation cycles (Fig. 1 and data not shown). Major melting peaks observed after 8 or 10 fermentation cycles matched in most cases the species that was predominant after 10 fermentation cycles (Fig. 1 and Table 3). Doughs started with hawthorn, myrtle berries or mother of vinegar yielded melting curves with a dominant peak at 84.1 °C (Fig. 1), matching the melting temperature of amplicons of *L. sanfranciscensis*, the dominant species in these samples (Table 3). Melting peaks in samples from sourdoughs started with mustard or apple flowers matched *L. graminis, L. plantarum*, and *L. rossiae*, again confirming the identification of isolates from these samples (Fig. 1). Melting peaks in samples from sourdoughs started with speedwell flowers also matched melting peak of bacterial species that were identified by culture dependent analysis (Table 3 and data not shown). Culture dependent analysis of sourdough started with pomegranate identified *L. plantarum* after 10 fermentation cycles; HRM-qPCR analysis confirmed presence of *L. plantarum* and additionally indicated the presence of *L. sanfranciscensis* (Fig. 1).

HRM-qPCR analyses of yeast microbiota in *de novo* sourdoughs are shown in Fig. 2. Because yeast isolates were obtained and characterized only after 4 fermentation cycles, not all melting peaks observed in HRM-qPCR analysis (Fig. 2) could be matched to the melting temperature of amplicons obtained from yeast isolates (Table 5). Yeast microbiota in *de novo* sourdoughs inoculated with apple flowers, myrtle berries, pomegranate fruits, or vinegar stabilized after less than 4 fermentation cycles and were characterized by a peak with a melting temperature of 83 °C (Fig. 2), in keeping with the presence of *S. cerevisiae* as only or dominant yeast species after 4 fermentation cycles. HRM-qPCR analysis also confirmed the presence of *S. cerevisiae* in *de novo* sourdoughs inoculated with mustard flowers and hawthorn berries (Fig 2), however, in these sourdoughs, *S. cerevisiae* was replaced by unknown yeasts after 10 fermentation cycles.

Table 5
Melting temperatures of PCR amplicons of reference strains representing selected species of yeasts using yeast-R/yeast-F primers or using NLS1/LS2 primers.

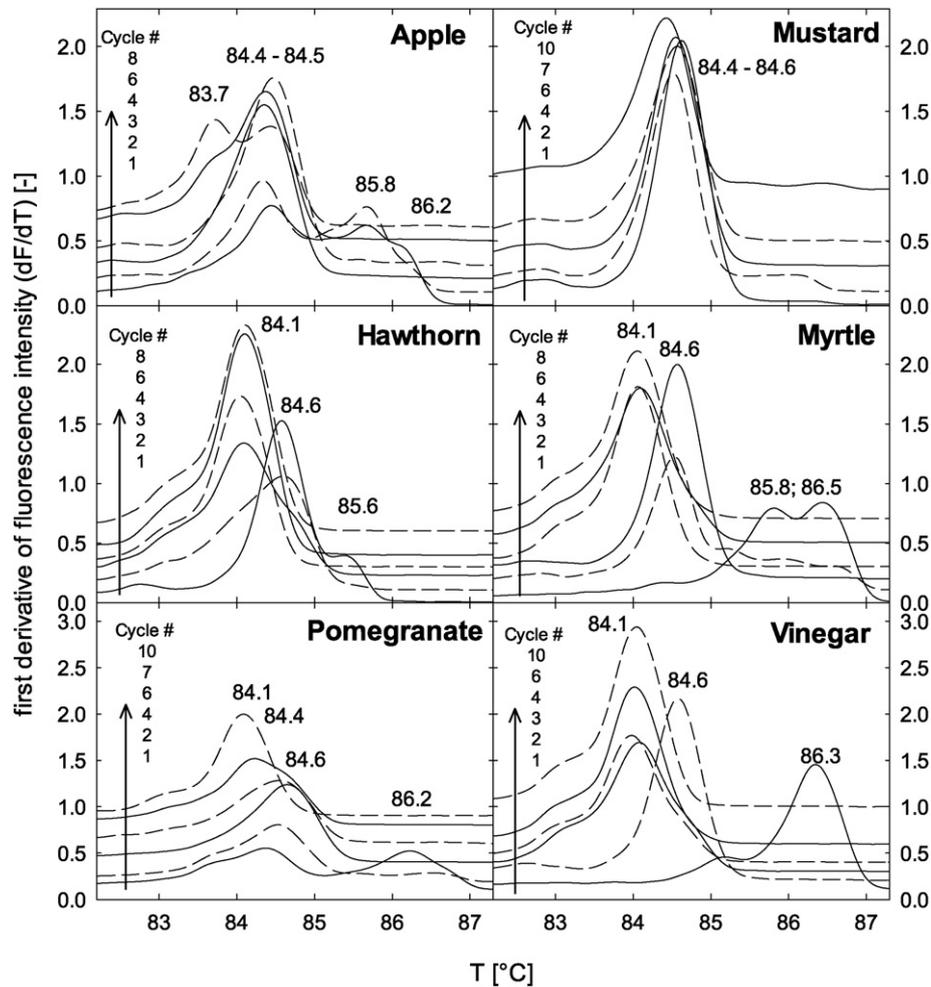| Reference organism	T_m (°C) of product from yeast-R/yeast-F primers	T_m (°C) of product from NLS1/LS2 primers
S. cerevisiae AM53y	78.5 ± 0.03	82.7 ± 0.04
A. proteae MG1PSC2	78.3 ± 0.05	81.9 ± 0.03
W. anomalus CB41y	78.5 ± 0.02	81.0 ± 0.01

Fig. 1. HRM-qPCR melting curves of bacterial 16S rDNA amplicons that were obtained with community DNA isolated from *de novo* sourdoughs that were propagated over 10 consecutive fermentation cycles. At the first fermentation cycle, sourdoughs were inoculated with the materials as indicated in the panels: apple flowers, mustard flowers, hawthorn berries; myrtle berries, pomegranate, mother of vinegar. Melting curves were offset by 0.1 dF/dT per fermentation cycle and are shown by alternating solid and dashed lines. The sequence of cycles shown is indicated in the individual panels. The melting temperature of major peaks is also indicated for the individual panels, the corresponding bacterial species are shown in Table 4.

3.5. Relative ecological fitness of lactobacilli isolated from de novo sourdoughs

Inoculation of *de novo* sourdoughs with different plant material resulted in dominance of different species of lactic acid bacteria after 10 fermentation cycles (Table 3 and Fig. 1). These differences may result from the presence or absence of individual species in the raw material used to start the fermentation, reflecting dispersal limitation, and / or may result from strain-specific differences in the ecological fitness of isolates, reflecting selection. To distinguish between dispersal limitation and selection, the relative ecological fitness of *L. rossiae*, *L. plantarum*, *L. sanfranciscensis*, and *L. graminis* was analysed by competition experiments in three different flours. Sourdoughs were propagated and fermentation microbiota were analysed by HRM-qPCR (Fig. 3). Melting curves obtained after the first fermentation cycle conform to the presence of all four strains in the three sourdoughs. After only 4 fermentation cycles, however, *L. sanfranciscensis* displaced the other species and prevailed as dominant representative of the bacterial microbiota. This result indicates that the presence of *L. plantarum*, *L. graminis*, *L. rossiae* in *de novo* sourdoughs started with apple and mustard flowers or pomegranate fruits reflects the absence of *L. sanfranciscensis* in the inoculum rather than superior fitness of the strains.

4. Discussion

The present study assessed the relevance of selection and dispersal in the development of sourdough microbiota. The experimental design used various plant materials to inoculate sourdoughs at the first fermentation cycle, reflecting a practice that is employed by artisanal bakers when establishing *de novo* sourdough fermentation. The use of plant material resulted in rapid establishment of a stable consortium of yeasts and lactobacilli. *L. sanfranciscensis* was rapidly established as dominant microorganism in 4 of the 7 sourdoughs. *L. sanfranciscensis* is considered a key species in those sourdoughs that used as sole leavening agent; however, its presence in laboratory-made sourdoughs is unprecedented (De Vuyst et al., 2014; Harth et al., 2016; Lönner et al., 1986; Rizzello et al., 2015; Van der Meulen et al., 2007). The absence of *L. sanfranciscensis* in three of the 7 sourdoughs excludes its presence as a laboratory-derived contaminant, and indicates that the carryover of *L. sanfranciscensis* is not common to all plants.

Analysis of sourdough microbiota requires a combination of culture-dependent and culture-independent methods (De Vuyst et al., 2014). Culture-independent methods often fail to account for all members of sourdough microbiota (Meroth et al., 2003; Van der Meulen et al., 2007). Indeed, the yield of bacterial DNA from sourdough fermented with different strains of the same species may differ up to 100,000fold
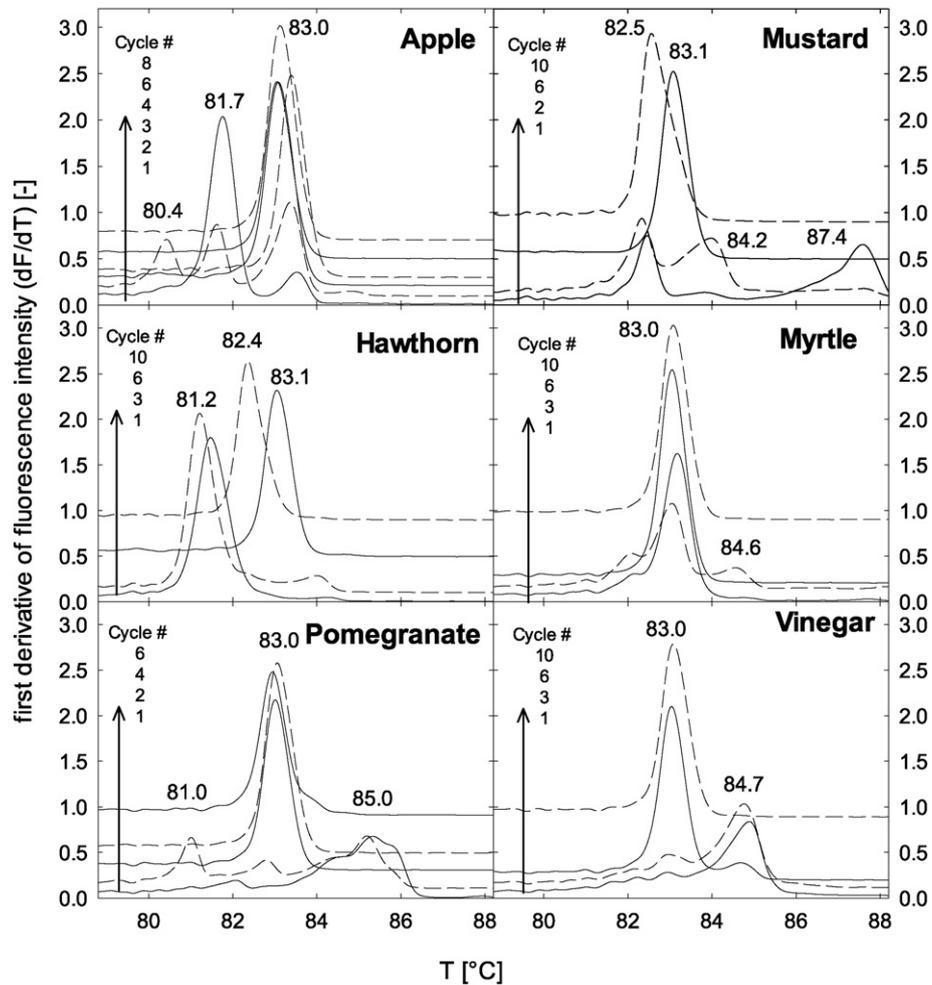
Fig. 2. HRM-qPCR melting curves of yeast DNA amplicons that were obtained with community DNA isolated from *de novo* sourdoughs that were propagated over 10 consecutive fermentation cycles. At the first fermentation cycle, sourdoughs were inoculated with the materials as indicated in the panels: apple flowers, mustard flowers, hawthorn berries; myrtle berries, pomegranate, mother of vinegar. Melting curves were offset by 0.1 dF/dT per fermentation cycle and are shown by alternating solid and dashed lines. The sequence of cycles shown is indicated in the individual panels. The melting temperature of major peaks is also indicated in the individual panels; the corresponding yeast species are shown in Table 4.
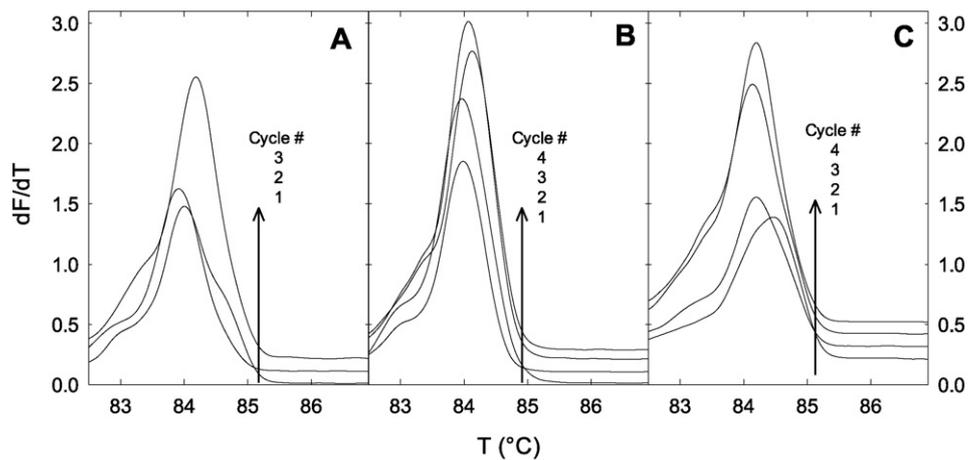

Fig. 3. HRM-qPCR melting curves of bacterial 16S rDNA amplicons that were obtained with community DNA isolated from sourdoughs prepared from white wheat flour (Panel A), whole wheat flour (Panel B), or rye malt flour (Panel C). Sourdoughs were inculated with approximately 10^7 cfu/g of each of *L. rossiae*, *L. plantarum*, *L. sanfranciscensis* and *L. graminis* and propagated over 4 consecutive fermentation cycles. Melting curves were offset by 0.1 dF/dT per fermentation cycles and the sequence of cycles shown is indicated in the individual panels. The melting peak of 84.1°C matches the melting peak of 16S rDNA amplicons from *L. sanfranciscensis*. Results are representative for two independent experiments analysed in duplicate.

even if the organisms are present at the same cell counts (Scheirlinck et al., 2009; Zheng et al., 2015b).

HRM-qPCR was recently established for culture-independent analysis of sourdough microbiota (Lin and Gänzle, 2014a). In comparison to approaches using high-throughput sequencing of 16S rRNA gene sequences, HRM-qPCR has a lower resolution, however, in samples containing only few bacterial genotypes, HRM-qPCR is a highly suitable method for rapid high-throughput analysis of multiple samples (Lin and Gänzle, 2014a). The present study confirmed the suitability of HRM-qPCR for qualitative assessment of dominant bacterial species in sourdough (Table 3 and Fig. 1); only one of the seven sourdoughs harboured an organism that was detected by HRM-qPCR but not by culture. This discrepancy may reflect the lower limit of detection of the HRM-qPCR assay (Lin and Gänzle, 2014a), or failure of the specific strain of L. sanfranciscensis to grow on the mMRS (Minervini et al., 2012).

This study indicates that HRM-qPCR analyses are also suitable for characterization of yeast microbiota. HRM-qPCR primers targeted 28S rRNA genes or internal transcribed spacer regions which are commonly used in fungal taxonomy (Sandhu et al., 1995; Iwen et al., 2002). This study identified W. anomalus, A. proteae, and S. cerevisiae in sourdough microbiota. The yeast population was established rapidly and the ratio of bacteria to yeasts after four fermentation cycles matched the ratio observed in mature sourdoughs (Ottogalli et al., 1996). S. cerevisiae is associated with food fermentations or with diverse plant material (Wang et al., 2012). W. anomalus are isolated from fruits (Lee et al., 2011), citrus fruits peels (Martos et al., 2013), tree exudates, soil, frass (Kurtzman et al., 1998). Aureobasidium sp. were isolated from plant leaf surfaces (Pollock et al., 1992), grape (Barata et al., 2012), berries (Tournas and Katsoudas, 2005), and apples (Kurtzman et al., 1998). Remarkably, Candida humilis and Kazachstania exigua, typical members of type I sourdough microbiota (de Vuyst et al., 2014), were not identified.

The development of sourdough microbiota during de novo fermentation under laboratory conditions is well documented in multiple studies. If flour is the only source of bacterial contaminants, sourdough microbiota consistently develop in three stages that are characterized by dominance of Enterobacteriaceae in the first fermentation cycle, followed by growth of adventitious lactic acid bacteria including enterococci and streptococci. Lactic acid bacteria representing typical sourdough microbiota are usually present only after about 10 fermentation cycles (Ercolini et al., 2013; Lönner et al., 1986; Rizzello et al., 2015; Van der Meulen et al., 2007; for a review, see De Vuyst et al., 2014). The development of microbiota in sourdoughs started with diverse plant material differs in several aspects from this well-described succession of fermentation microbiota. (i) Enterobacteriaceae were absent at any stage. (ii) Adventitious lactic acid bacteria including Enterococcus spp. were present only after the first fermentation cycle but replaced by sourdough-adapted lactic acid bacteria already after four fermentation cycles. (iii) Acetic acid bacteria were present in 4 of the 7 sourdoughs. While acetic acid bacteria are not considered typical members of sourdough microbiota, their occasional presence in sourdough was also confirmed by analysis of traditional sourdoughs maintained in bakeries (Table 1). In 4 of the 7 sourdoughs, L. sanfranciscensis became established as dominant member of fermentation microbiota already after 1–4 fermentation cycles. This result conforms to the composition of microbiota of traditional sourdoughs but contrasts the absence of this species in de novo sourdoughs that were inoculated only with flour. Flowers or berries are thus a more suitable source for typical sourdough microbiota when compared to flour. The observation that diverse plant material resulted in a comparable development of microbiota in de novo sourdoughs may point to the relevance of plant or flower associated insects (Wong et al., 2011). The fruit fly Drosophila melanogaster harbours Lactobacillus brevis, Lactobacillus fructivorans and L. plantarum as dominant members of intestinal microbiota (Wong et al., 2011). Our observations concur with the hypothesis that flowers or flower-associated insects harbour L. sanfranciscensis (Groenewald et al., 2006; Zheng et al., 2015b). The rapid development of typical sourdough microbiota

in de novo sourdoughs started with plant material is also reflected by the rapid establishment of yeasts. However, the evolution of yeast microbiota in de novo sourdoughs was not as well documented in the present study and elsewhere as the development of bacterial microbiota (De Vuyst et al., 2014).

Selection and dispersal shape the composition and function of sourdough microbiota (Di Cagno et al., 2014; Lin and Gänzle, 2014b; Meroth et al., 2003; Su et al., 2012; Vrancken et al., 2010). This study demonstrates that the development of microbiota in de novo sourdoughs is dispersal limited unless inocula of plant or animal origin are used to transfer typical sourdough microbiota. Dispersal limitation was confirmed by the finding that L. sanfranciscensis exhibits superior ecological fitness when competing against other sourdough isolates. The comparison of de novo sourdoughs with traditional sourdoughs that were propagated for several month or years, however, indicates that microbiota of sourdoughs maintained in bakeries are mainly shaped by selection for highly adapted microorganisms. The contamination or inoculation of sourdoughs with plant (insect)- or animal associated microorganisms is unlikely in the laboratory; however, plant- or animal associated microorganisms contaminate sourdoughs that are maintained in bakeries by continuous propagation over long periods of time (Su et al., 2012).

In conclusion, our study provides scientific support for the artisanal practice to inoculate de novo sourdoughs with flowers, berries, or related plant material. The use of manure or intestinal tissue to inoculate de novo sourdoughs favours establishment of representatives of animal microbiota as dominant fermentation organisms in sourdoughs (Su et al., 2012). This study contributes to improved control of sourdough fermentations in artisanal and industrial bakeries based on the improved knowledge of the community assembly in sourdoughs.

Acknowledgements

References

Barata, A., Malfeito-Ferreira, M., Loureiro, V., 2012. The microbial ecology of wine grape berries. Int. J. Food Microbiol. 153, 243–259.

Brandt, M.J., 2007. Sourdough products for convenient use in baking. Food Microbiol. 24, 161–164.

De Vuyst, L., Van Kerrebroeck, S., Harth, H., Huys, G., Daniel, H.-M., Weckx, S., 2014. Microbial ecology of sourdough fermentations: diverse or uniform? Food Microbiol. 37, 11–29.

Di Cagno, R., Pontonio, E., Buchin, S., De Angelis, M., Lattanzi, A., Valerio, F., Gobbetti, M., Calasso, M., 2014. Diversity of the lactic acid bacterium and yeast microbiota in the switch from firm- to liquid-sourdough fermentation. Appl. Environ. Microbiol. 80, 3161–3172.

Ercolini, D., Pontonio, E., De Filippis, F., Minervini, F., La Storia, A., Gobbetti, M., Di Cagno, R., 2013. Microbial ecology dynamics during rye and wheat sourdough preparation. Appl. Environ. Microbiol. 79, 7827–7836.

Gänzle, M.G., 2014. Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiol. 37, 2–10.

Gänzle, M.G., Ehmann, M., Hammes, W.P., 1998. Modeling of growth of Lactobacillus sanfranciscensis and Candida milleri in response to process parameters of sourdough fermentation. Appl. Environ. Microbiol. 64, 2616–2623.

Gänzle, M.G., Vermeulen, N., Vogel, R.F., 2007. Carbohydrate, peptide and lipid metabolism of lactobacilli in sourdough. Food Microbiol. 24, 128–138.

Groenewald, W.H., Van Reenen, C.A., Todorov, S.D., Du Toit, M., Witthuhn, R., Holzapfel, W.H., Dicks, L.M.T., 2006. Identification of lactic acid bacteria from vinegar flies based on phenotypic and genotypic characteristics. Am. J. Enol. Vitic. 57, 519–525.

Gudnason, H., Dufva, M., Bang, D.D., Wolff, A., 2007. Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. Nucleic Acids Res. 35, e127.

Hammes, W.P., Gänzle, M.G., 1998. Sourdough breads and related products. In: Wood, B.J.B. (Ed.), Microbiology of Fermented Food. Chapman and Hall, London, pp. 199–216.

Harth, H., Van Kerrebroeck, S., De Vuyst, L., 2016. Community dynamics and metabolite target analysis of spontaneous, backslopped barley sourdough fermentations under laboratory and bakery conditions. Int. J. Food Microbiol. 228, 22–32.

Huey, B., Hall, J., 1989. Hypervariable DNA fingerprinting in E. coli minisatellite probe from bacteriophage M13. J. Bacteriol. 171, 2528–2532.

Iwen, P.C., Hinrichs, S.H., Rupp, M.E., 2002. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med. Mycol. 40, 87–109.

Kurtzman, C.P., Fell, J.W., Boekhout, R., 1998. The Yeasts - A correct Taxonomic Study. fifth ed. Elsevier (Eds), Amsterdam.

Lee, Y.J., Choi, Y.-R., Lee, S.Y., Park, J.T., Shim, J.H., Park, K.H., 2011. Screening wild yeast strains for alcohol fermentation from various fruits. Mycobiology 39, 33–39.

Lin, X.B., Gänzle, M.G., 2014a. Quantitative high-resolution melting PCR analysis for monitoring of fermentation microbiota in sourdough. Int. J. Food Microbiol. 186, 42–48.

Lin, X.B., Gänzle, M.G., 2014b. Effect of lineage-specific metabolic traits of *Lactobacillus reuteri* on sourdough microbial ecology. Appl. Environ. Microbiol. 80, 5782–5789.

Lönner, C., Welander, T., Molin, N., Dostàlek, M., Blickstad, E., 1986. The microflora in a sour dough started spontaneously on typical Swedish rye meal. Food Microbiol. 3, 3–12.

Martos, M.A., Zubreski, E.R., Garro, O.A., Hours, R.A., 2013. Production of pectinolytic enzymes by the yeast *Wickerhanomyces anomalus* isolated from citrus fruits peels. Biotechnology Research International 2013, 435154.

Meroth, C.B., Walter, J., Hertel, C., Brandt, M.J., Hammes, W.P., 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 69, 475–482.

Minervini, F., Di Cagno, R., Lattanzi, A., De Angelis, M., Antonielli, L., Cardinali, G., Cappelle, S., Gobbetti, M., 2012. Lactic acid bacterium and yeast microbiotas of 19 sourdoughs used for traditional/typical Italian breads: interactions between ingredients and microbial species diversity. 78, 1251–1264.

Minervini, F., Celano, G., Lattanzi, A., Tedone, L., De Mastro, G., Gobbetti, M., De Angelis, M., 2015. Lactic acid bacteria in durum wheat flour are endophytic components of the plant during its entire life cycle. Applied and Environmental Microbiology 81, 6736–6748.

Nemergut, D.R., Schmidt, S.K., Fukami, T., O'Neill, S.P., Bilinski, T.M., Stanish, L.F., Knelman, J.E., Darcy, J.L., Lynch, R.C., Wickey, P., Ferrenberg, S., 2013. Patterns and processes of microbial community assembly. Microbiol. Mol. Biol. Rev. 77, 342–356.

O'Donnell, K., 1993. *Fusarium* and its near relatives. In: Reynolds, D.R., Taylor, J.W. (Eds.), The Fungal Anamorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics. CAB International, Wallingford, pp. 225–233.

Park, E.J., Chang, H.W., Kim, K.H., Nam, Y.D., Roh, S.W., Bae, J.W., 2009. Application of quantitative real-time PCR for enumeration of total bacterial, archaeal, and yeast populations in kimchi. J. Microbiol. 47, 682–685.

Picard, C., Di Cello, P.C., Ventura, F., Fani, M.R., Guckert, A., 2000. Frequency and biodiversity of 2,4-diacetylphloroglucinol-producing bacteria isolated from the maize rhizosphere at different stages of plant growth. Applied and Environmental Microbiology 66, 948–955.

Pollock, T.J., Thorne, L., Armentrout, R.W., 1992. Isolation of new *Aureobasidium* strains that produce high-molecular-weight pullulan with reduced pigmentation. Appl. Environ. Microbiol. 58, 877–883.

Rizzello, C.G., Cavoski, I., Turk, J., Ercolini, D., Nionelli, L., Pontonio, E., De Angelis, M., De Filippis, F., Gobbetti, M., Di Cagno, R., 2015. Organic cultivation of *Triticum turgidum*

subsp. *durum* is reflected in the flour-sourdough fermentation-bread axis. Appl. Environ. Microbiol. 81, 3192–3204.

Sandhu, G.S., Kline, B.C., Stockman, L., Roberts, G.D., 1995. Molecular probes for diagnosis of fungal infections. J. Clin. Microbiol. 33, 2913–2919.

Scheirlinck, I., Van der Meulen, R., De Vuyst, L., Vandamme, P., Huys, G., 2009. Molecular source tracking of predominant lactic acid bacteria in traditional Belgian sourdoughs and their production environments. J. Appl. Microbiol. 106, 1081–1092.

Sekwati-Monang, B., Valcheva, R., Gänzle, M.G., 2012. Microbial ecology of sorghum sourdoughs: Effect of substrate supply and phenolic compounds on composition of fermentation microbiota. Int. J. Food Microbiol. 159, 240–246.

Siragusa, S., Di Cagno, R., Ercolini, D., Minervini, F., Gobbetti, M., De Angelis, M., 2009. Taxonomic structure and monitoring of the dominant population of lactic acid bacteria during wheat flour sourdough type I propagation using *Lactobacillus sanfranciscensis* starters. Appl. Environ. Microbiol. 75, 1099–1109.

Stolz, P., 1999. Mikrobiologie des Sauerteiges. In: Spicher, G., Stephan, H. (Eds.), Handbuch Sauerteig: Biologie, Biochemie, Technologie, fifth ed., pp. 35–60.

Su, M.S.-W., Oh, P.S., Walter, J., Gänzle, M.G., 2012. Intestinal origin of sourdough *Lactobacillus reuteri* isolates as revealed by phylogenetic, genetic, and physiological analysis. Appl. Environ. Microbiol. 78, 6777–6780.

Tournas, V.H., Katsoudas, E., 2005. Mould and yeast flora in fresh berries, grapes and citrus fruits. Int. J. Food Microbiol. 105, 11–17.

Van der Meulen, R., Scheirlinck, I., Van Schoor, A., Huys, G., Vancanneyt, M., Vandamme, P., De Vuyst, L., 2007. Population dynamics and metabolite target analysis of lactic acid bacteria during laboratory fermentations of wheat and spelt sourdoughs. Appl. Environ. Microbiol. 73, 4741–4750.

Vellend, M., 2010. Conceptual synthesis in community ecology. Q. Rev. Biol. 85, 183–206.

Vogelmann, S.A., Seitter, M., Singer, U., Brandt, M.J., Hertel, C., 2009. Adaptability of lactic acid bacteria and yeasts to sourdoughs prepared from cereals, pseudocereals and cassava and use of competitive strains as starters. Int. J. Food Microbiol. 130, 205–212.

Vrancken, G., De Vuyst, L., Van Der Meulen, R., Huys, G., Vandamme, P., Daniel, H.M., 2010. Yeast species composition differs between artisan bakery and spontaneous laboratory sourdoughs. FEMS Yeast Res. 10, 471–481.

Wang, Q.M., Liu, W.Q., Liti, G., Wang, S.A., Bai, F.Y., 2012. Surprisingly diverged populations of *Saccharomyces cerevisiae* in natural environments remote from human activity. Mol. Ecol. 21, 5404–5417.

Wong, C.N., Ng, P., Douglas, A.E., 2011. Low-diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. Environ. Microbiol. 13, 1889–1900.

Zheng, J., Zhao, X., Lin, X.B., Gänzle, M.G., 2015a. Comparative genomics of *Lactobacillus reuteri* from sourdough reveals adaptation of an intestinal symbiont to food fermentations. Sci. Report. 5, 18234.

Zheng, J., Ruan, L., Sun, M., Gänzle, M.G., 2015b. Genomic analysis of lactobacilli and pediococci demonstrates that phylogeny matches ecology and physiology. Appl. Environ. Microbiol. 81, 7233–7243.