Dynamics of *Enterobacteriaceae* and lactobacilli in model sourdoughs are driven by pH and concentrations of sucrose and ferulic acid

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Abstract

This study aimed to assess, in model sourdough fermentations, the relevance of physico-chemical features of flour for the interactions between *Enterobacteriaceae* and lactobacilli, and for the interactions within the latter microbial group. Initially, model sourdoughs made with white wheat flour, whole wheat flour or wheat bran were inoculated with *Cronobacter sakazakii*, *Klebsiella pneumoniae*, *Lactobacillus plantarum* and *Lactobacillus sanfranciscensis*. Subsequently, white wheat sourdoughs were prepared with phosphate buffer, ferulic acid, maltose, sucrose or baker's yeast. During sourdough propagation, *C. sakazakii* and *K. pneumoniae* disappeared after few fermentation cycles in white wheat and whole wheat sourdoughs, but persisted in phosphate buffered or in wheat bran sourdoughs. Sucrose, maltose or ferulic acid did not impact on the ecological fitness of *Enterobacteriaceae*, whereas baker's yeast inhibited these bacteria. In white wheat and in whole wheat sourdoughs, *L. sanfranciscensis* outcompeted *L. plantarum*. A variation of sucrose level and the presence of ferulic acid reduced the competitiveness of *L. sanfranciscensis*, thus favoring *L. plantarum*.

This study demonstrated that the pH is key contributor to the elimination of *Enterobacteriaceae* in cereal fermentation. In addition, *L. sanfranciscensis* prevails in wheat sourdoughs, but minor perturbations of the ecosystem reduce its competitiveness.

Keywords

Sourdough; *Cronobacter sakazakii*; *Klebsiella pneumoniae*; *Lactobacillus plantarum*; *Lactobacillus sanfranciscensis.*

1. Introduction

Many cereal foods are fermented spontaneously, here, fermentation microbiota is not controlled by back-slopping (i.e., inoculation of each batch with a portion of the previous batch) or starter cultures (Gänzle, 2019). Spontaneous cereal fermentations are characterized by a succession of *Enterobacteriaceae,* followed by *Enterococcus*, *Leuconostoc, Pediococcus* and/or *Weissella* species until acid-tolerant lactobacilli dominate. This succession has been described for spontaneous wheat and rye sourdoughs (Ercolini et al., 2013) and is so reproducible at the genus level that it has been termed "the usual suspects" (Gänzle, 2019). Community assembly in spontaneous cereal fermentations is dispersal limited (Gänzle & Ripari, 2016) and fermentation microbiota are recruited from facultative anaerobic representatives of flour microbiota (Celano, De Angelis, Minervini, & Gobbetti, 2016; De Angelis, Minervini, Siragusa, Rizzello, & Gobbetti, 2018). Major components of flour microbiota that occur in spontaneous sourdoughs include *Cronobacter*, *Enterobacter* and *Klebsiella* spp., *Leuconostoc* spp. and *Lactobacillus plantarum.* On the cereal raw materials used for food fermentations, *Enterobacteriaceae* are typically the most abundant group among the facultative anaerobes while *L. plantarum* is present as minor component (Minervini et al., 2015; Pswarayi & Gänzle, 2019).

Microbiota of spontaneous sourdoughs differ from microbiota of sourdoughs that are maintained by back-slopping (Gänzle & Zheng, 2018; Minervini, De Angelis, Di Cagno, & Gobbetti, 2014). Back-slopping eliminates dispersal limitation and selects for highly competitive microbiota (Gänzle & Ripari, 2016). The microbiota of mature, back-slopped wheat and rye sourdoughs are comparable globally (Gobbetti, Minervini, Pontonio, Di Cagno, & De Angelis, 2016). Microbiota of these sourdoughs are relatively stable over time, and are dominated by host-adapted lactobacilli (Gänzle & Zheng, 2018). Flour intrinsic parameters also modulate establishment of sourdough microbiota. For instance, *L. sanfranciscensis* is found in wheat and rye sourdoughs but not in sourdoughs fermented with other substrates. Compared to white flour, whole-grain flour has a higher concentration of ash

and a higher buffering capacity, which affects organic acid production by lactobacilli (Gänzle, Ehmann, & Hammes, 1998). Whole wheat flour and bran also have a higher content of phenolic acids than white flours (Li, Shewry, & Ward, 2008).

The metabolic and physiological features of sourdough lactobacilli that contribute to their competitiveness in back-slopped sourdoughs are well-characterized, and have been validated in model fermentations with defined inocula of wild type strains or isogenic mutants (Gänzle & Zheng, 2018; Meroth, Walter, Hertel, Brandt, & Hammes, 2003). Competitiveness of *L. sanfranciscensis* in sourdoughs that are maintained as leavening agent depends on its rapid growth, which is supported by efficient metabolism of sucrose and maltose and the use of oxygen and fructose as electron acceptors (Gänzle, Vermeulen, & Vogel, 2007; Vogel et al., 2011).

Factors that determine community assembly and succession of fermentation microbiota in spontaneous sourdoughs have not been systematically studied. It was therefore the aim of this study to assess the relevance of physico-chemical features of flour for the interactions between *Enterobacteriaceae* and lactobacilli, two major representatives of the usual suspects, and for the interactions within the latter microbial group. The competitiveness of *Enterobacteriaceae* and lactobacilli was assessed by inoculation of wheat flour with *L. plantarum*, *Klebsiella pneumoniae* and *Cronobacter sakazakii* that were isolated from spontaneous cereal fermentations (Pswarayi & Gänzle, 2019), and with *L. sanfranciscensis* from traditional sourdoughs. The four species were differentially enumerated over 6 – 10 fermentation cycles. Initially, we used model sourdoughs made with white wheat flour, whole wheat flour or wheat bran. Subsequently, the impact of substrates, pH, and ferulic acid was determined by competition experiments in supplemented white wheat sourdoughs.

2. Materials and methods

2.1. Microrganisms and culture conditions

L. sanfranciscensis FUA3417, FUA3024 (isogenic to ATCC27651) and FUA3331 (isogenic to LTH2590) are isolates from traditional sourdoughs; *L. plantarum* FUA3590 is an isolate from spontaneous cereal fermentations, and *C. sakazakii* FUA10024 and *K. pneumoniae* FUA10025 are isolates from millet malt (Pswarayi & Gänzle, 2019). All strains were routinely cultured in MRS5 agar or broth (Meroth et al., 2003) at 30 °C for 48 h.

2.2. Model sourdoughs fermentation

White wheat, whole wheat flour and wheat bran were purchased from a local supermarket. For sourdoughs production, 10 g of flour were mixed with 10 mL of sterile tap water and inoculated with a mixture of *L. sanfranciscensis* FUA3417 and FUA3024, *L. plantarum*, *C. sakazakii* and *K. pneumoniae*. The inoculum was prepared by growing each strain individually in MRS5 broth at 30 °C for 24 h. Cells were harvested by centrifugation, were washed twice with sterile peptone saline containing 10 g/L peptone and 9 g/L NaCl and re-suspended in 10 mL of sterile tap water. The final cell densities in dough were approximately 5 log CFU/g for lactobacilli and 6 log CFU/g for *C. sakazakii* and *K. pneumoniae*. Doughs were fermented for 12 h at 25 °C and then propagated with 10% inoculum every 12 h for five days. Fermentations were carried out in triplicate independent experiments, each analyzed in duplicate. Aliquots of fresh samples were analyzed by plate counts at 0, 2, 4, 8, 12 h during the first fermentation and at 12 h for the following fermentations.

2.3. Fermentation of supplemented wheat sourdoughs

White wheat sourdoughs were prepared with one of the following additional ingredients: (i) 100 mmol/L sodium phosphate buffer pH 6.5, added at a final concentration of 0.871 g/kg; (ii) 0.1 g/kg ferulic acid; (iii) 170 g/kg maltose; (iv) 5 g/kg dried baker's yeast; (v) 17 g/kg sucrose. Doughs were inoculated with the bacterial mixture described above except those with added sucrose, where the two *L. sanfranciscensis* strains were replaced by the sucrose-positive strain *L. sanfranciscensis* FUA3331 (Korakli, Rossmann, Gänzle, & Vogel, 2001). The doughs were fermented as described above and propagated twice a day for three days. The additional ingredients were added at each back-slopping;

111 baker's yeast was incorporated into dough only at the first fermentation cycle. Fermentations were
112 carried out in triplicate independent experiments, analyzed in duplicate. Samples analyzed by plate
113 counts at 0 and 12 h for the first fermentation and at 12 h for the subsequent fermentations.

114 *2.4. Measurement of pH and plate count*

115 The pH of sourdoughs was measured with a glass electrode after dilution of dough with MilliQ
116 water. Viable lactic acid bacteria were quantified by surface plating of serial dilutions on MRS5 agar
117 medium supplemented with 0.1 g/L cycloheximide (Minervini, Lattanzi, De Angelis, Di Cagno, &
118 Gobbetti, 2012). Plates were incubated under modified atmosphere (76% N_2, 4% O_2, and 20% CO_2)
119 at 30 °C for 48 h. *Enterobacteriaceae* were enumerated after surface plating onto Luria Bertani and
120 Violet Red Bile agar media (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and
121 incubated aerobically at 30 °C for 48 h and at 45 °C for 24 h, respectively. Yeasts were enumerated
122 on MRS5 agar supplemented with 0.1 g/L each of erythromycin and chloramphenicol after incubation
123 at 30 °C for 48 h. Colonies of *L. sanfranciscensis* or *L. plantarum* were readily identified on the basis
124 of their morphology (Pswarayi & Gänzle, 2019). Colonies of *L. sanfranciscensis* were less than 1
125 mm of diameter and whitish, whereas those of *L. plantarum* were larger (1-2 mm diameter), had
126 entire margin and white color.

127 *2.5. Isolation of lactobacilli, DNA extraction and PCR with species or strain specific primers*

128 Lactic acid bacteria were isolated from sourdoughs, after the 1st, 3rd, 5th and 10th fermentation.
129 About 24 colonies of presumptive *L. sanfranciscensis* and *L. plantarum* per sample were cultivated
130 in MRS5. Overnight cultures were harvested by centrifugation and DNA was extracted with the
131 DNeasy Blood and Tissue Kit (Qiagen Inc., Toronto, Canada). PCR confirmation was based on
132 species-specific primers for *L. sanfranciscensis* and strain-specific primers for *L. plantarum* (Table
133 1). PCR was performed with a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA,
134 USA) in a total volume of 25 µL containing 10 × buffer, 50 mmol/L $MgCl_2$, 10 mmol/L dNTP mix
135 (0.2 mmol/L of each), 10 mmol/L of each primer, 5 U/µL of GoTaq DNA polymerase (all reagents

from Invitrogen, Waltham, USA), 18.65 µL of RNase free water and 1 µL of template DNA. To verify specific amplification of target gene/sequence, the size of the amplicons (Table 1) was determined by agarose gel electrophoresis.

2.6. Analysis of metabolites

Organic acids, alcohols and sugars were quantified using an Agilent 1200 HPLC system, which was equipped with an Aminex HPX-87 column (Bio-Rad, Mississauga, Canada) and a refractive index detector. Samples were eluted with H_2SO_4 0.49 g/L at 70 °C and a flow rate of 0.4 mL/min (Galle, Schwab, Arendt, & Gänzle, 2010). Before HPLC analysis, proteins were precipitated by mixing of samples with an equal volume of 70 g/L perchloric acid, followed by incubation at 4 °C overnight, centrifugation at 10,000×g for 10 min, and filtering through a 0.22-µM filter.

2.7. Statistical analyses

Data from the technical repeats were averaged; data from three biological replicates were analyzed by one-way analysis of variance and pairwise comparison of treatment means with Tukey's procedure using Statistica 7.0 for Windows. Significant differences were assessed at an error probability of 5% ($P < 0.05$). Permutation analysis was carried out using PermutMatrix. Spearman correlations between chemical characteristics of the sourdoughs and cell densities of bacteria inoculated in the doughs were computed using Statistica v. 7.0.

3. Results

3.1. Bacterial population dynamics in white wheat flour-, whole wheat flour- and wheat bran-based sourdoughs

Wheat, whole wheat and wheat bran sourdoughs were used to assess the competitiveness of *L. sanfranciscensis*, *L. plantarum*, *C. sakazakii* and *K. pneumoniae* (Figure 1). Differential enumeration using colony morphology and PCR confirmed that fermentations were characterized by a defined microbiota throughout 11 fermentation cycles (data not shown).

160 Initially, the sourdough pH ranged from 5.47 (white wheat) to 6.51 (Figure 1). After the 11th
161 fermentation cycle, pH of white wheat and whole wheat sourdoughs were not significantly ($P > 0.05$)
162 different and fell in the range 4.07 – 4.27 (Fig. 1). In contrast, the wheat bran-based sourdough
163 showed the highest pH value. The concentration of lactic acid was highest in whole wheat sourdoughs;
164 acetic acid concentrations were highest in whole wheat and wheat bran sourdoughs (Fig. 1).

165 *C. sakazakii* and *K. pneumoniae* grew in the first fermentation cycles to 7 (*C. sakazaki*) and 8 (*K.*
166 *pneumoniae*) log CFU/g at the end of the first fermentation cycle (Fig. 1). Afterwards, their cell
167 density decreased and cell counts were below the detection level after the 5th / 6th fermentation cycle
168 in white wheat and whole wheat sourdoughs. In both sourdoughs, the decline of *C. sakazakii* and *K.*
169 *pneumoniae* population corresponded approximately to the fermentation cycle where the pH dropped
170 to a value of less than 4.5. In wheat bran sourdough, *C. sakazakii* and *K. pneumoniae* grew to cell
171 counts of about 8 and ca. 9 log CFU/g, respectively (Figure 1C). Their cell density stayed relatively
172 stable until they disappeared at the 9th (*C. sakazakii*) and 11th (*K. pneumoniae*) fermentation cycle.

173 In white wheat sourdoughs *L. sanfranciscensis* reached higher cell counts than *L. plantarum*,
174 increasing to 9.2 log CFU/g and remaining constant afterwards (Figure 1A). In the whole wheat
175 sourdough *L. sanfranciscensis* also prevailed, but with a smaller difference in cell counts between the
176 two lactobacilli (Figure 1B). In wheat bran sourdoughs, *L. sanfranciscensis* and *L. plantarum* reached
177 the highest cell density (ca. 9.7 log CFU/g) after the 2nd and 7th fermentation cycle, respectively
178 (Figure 1C). The cell counts of *L. sanfranciscensis* and *L. plantarum* did not differ ($P > 0.05$) after
179 the 6th fermentation cycle (Fig. 1C).

180 *3.2. Determinants for selection of fermentation microbiota in wheat sourdoughs*

181 Subsequent experiments aimed to elucidate factors that determine the competitiveness of
182 *Enterobacteriaceae* and lactobacilli in sourdoughs. Initially, the substrate supply was manipulated by
183 addition of maltose, sucrose, or by addition of baker's yeast, which rapidly depletes amino acids and
184 sugar levels in sourdough (Fig. 2).

185 Addition of maltose or sucrose to white wheat sourdoughs did not alter the dynamics of the pH or
186 the concentration of lactate (Fig. 2) relative to the white wheat sourdough (Fig. 1). Growth and decline
187 of the populations of *C. sakazakii* and *K. pneumoniae* also remained unchanged by addition of
188 disaccharides (Figs. 2 and 1). Disaccharides impacted, however, the relative fitness of *L.*
189 *sanfranciscensis* and *L. plantarum*. Addition of maltose increased the cell counts of *L. plantarum*,
190 which were equivalent to *L. sanfranciscensis* after the 2nd fermentation cycle (Fig. 2A). Sucrose
191 addition reduced the ecological fitness of *L. sanfranciscensis*, which dropped to cell counts below the
192 detection limit after six fermentation cycles (Fig. 2B). Addition of baker's yeast inhibited growth of
193 *C. sakazakii* and *K. pneumoniae;* cell counts of these organisms remained below 7 log CFU/g until
194 both were eliminated after the 6th fermentation cycle. Baker's yeast also delayed growth of *L.*
195 *sanfranciscensis*, which grew to high cell counts only after yeast cell counts were reduced by more
196 than 1 log CFU/g (Figure 2C). After the 6th fermentation cycle, yeasts were eliminated from the
197 sourdoughs and the pattern of stable *L. sanfranciscensis* and declining *L. plantarum* populations (Fig.
198 2C) matched the patterns in white wheat sourdoughs (Fig. 1A).

199 The impact of the pH and phenolic acids on the microbial ecology was assessed by analysis of
200 sourdoughs that were supplemented with phosphate buffer or ferulic acid (Fig. 3). Supplementation
201 of sourdough with ferulic acid did not alter the dynamics of the pH, lactate concentration, or the
202 populations of *Enterobacteriaceae* (Figs 3B and 1A). Ferulic acid strongly impacted the
203 competitiveness of *L. sanfranciscensis*. After initial growth to about 8.5 log CFU/g, cell counts of *L.*
204 *sanfranciscensis* decreased after the fourth fermentation cycle. The addition of phosphate buffer
205 prevented acidification to values below pH 5.5 in all samples (Fig. 3A). In phosphate-supplemented
206 sourdoughs, all four organisms were maintained at cell counts of more than 7 log CFU/g throughout
207 seven fermentation cycles and *K. pneumoniae* was the most abundant species.

208 *3.3. Permutation analysis of microbiological and biochemical data*

Based on permutation analysis of microbiological and biochemical data, the sourdoughs were grouped into two main clusters (I and II), with the whole wheat sourdough as outlier (Fig. 4). With exception of the phosphate-supplemented sourdough, sourdoughs made with white wheat flour clustered together (cluster I). Phosphate-supplemented sourdough were similar to wheat bran sourdough (cluster II). Microbiological features correlated to biochemical data. The pH was positively correlated ($r > 0.46$; $P < 0.05$) with *C. sakazaki* and *K. pneumoniae*. Negative correlations were found between *L. plantarum* and *L. sanfranciscensis*.

4. Discussion

The current study investigated interactions between key members of the usual suspects, *Enterobacteriaceae* and lactobacilli, as well as between *L. sanfranciscensis* and *L. plantarum*. *L. sanfranciscensis* is the key organisms in traditional sourdoughs; *L. plantarum* typically becomes dominant in spontaneous sourdough fermentations but also occurs in association with *L. sanfranciscensis* in mature sourdoughs (Minervini et al., 2012).

Competition of *Enterobacteriaceae* and lactobacilli

At early stages of spontaneous cereal fermentations typically, the redox potential decreases (Hammes et al., 2005), favoring the growth of facultative anaerobes, including *Enterobacteriaceae*, and lactic acid bacteria. This study probed the role of pH, substrate supply and phenolic acids for the transition of *Enterobacteriaceae* to a *Lactobacillus*-dominated bacterial community that is characteristic for spontaneous fermentations. The succession of microbiota in carrot juice and sauerkraut, and the competition of lactobacilli and *Enterobacteriaceae* in fermentations with millet malt and maize suggest that this transition is mainly regulated by the pH (Lee et al., 2005; Wuyts et al., 2018; Pswarayi & Gänzle, 2019). Back-slopping of sourdoughs, however, periodically increases the pH and persistence of individual organisms is as dependent on their growth rate as on their acid resistance (Van Kerrebroeck, Maes, & De Vuyst, 2017). In the current study *C. sakazakii* and *K. pneumoniae* were eliminated after few fermentation cycles in white wheat and whole wheat

234 sourdoughs, but persisted in buffered or wheat bran sourdoughs. Wheat bran has a higher buffering
235 capacity than flour (Clèment et al., 2018). This indicates a key role of the pH in displacement of
236 *Enterobacteriaceae* (De Angelis et al., 2018). In wheat bran sourdoughs, populations of *C. sakazakii*
237 and *K. pneumoniae* declined before the pH was reduced to levels below 5, suggesting an additional
238 role of lactate and acetate in inhibition of *Enterobacteriaceae*.

239 The ecological fitness of *Enterobacteriaceae* was not impacted by the addition of sucrose, maltose
240 or ferulic acid. Wheat and rye contain 0.5 – 1 g ferulic acid/kg; however, ferulic acid occurs
241 predominantly in bound form and less than 0.05 g/kg are present in free form or released from esters
242 during fermentation (Li et al., 2008; Ripari, Bai, & Gänzle, 2019). Ferulic acid was added at a level
243 of 0.1 g/kg, approximately matching its MIC against *Escherichia coli* (Sànchez-Maldonado, Schieber,
244 & Gänzle, 2011). The MIC of ferulic acid against *C. sakazakii*, however, is over ten-fold higher (Shi
245 et al., 2016). Baker's yeast strongly inhibited growth of *Enterobacteriaceae*. This result conforms to
246 observations that growth of *Enterobacteriaceae* is observed in spontaneous sourdoughs (Ercolini et
247 al., 2013), but not in sponge doughs that are started with baker's yeast (De Vuyst et al., 2014).

248 Because sourdoughs for use in baking are generally back-slopped multiple times prior to use, *C.*
249 *sakazakii* and *K. pneumoniae* are not relevant in baking applications. Spontaneous cereal
250 fermentations are widely used, however, in production of cereal porridges and beverages (Franz et
251 al., 2014). In these products, persistence of *Enterobacteriaceae* may result in fermentation failure
252 (Celano et al., 2016) or risks of food-borne diseases (Todorov & Holzapfel, 2015).

253 **Competition between *L. plantarum* and *L. sanfranciscensis***

254 *L. plantarum* and *L. sanfranciscensis* are the most typical representatives of the microbiota in
255 spontaneous sourdoughs and in mature sourdoughs, respectively (Gänzle & Zheng, 2018). These two
256 species also co-exist in mature sourdoughs (Corsetti et al., 2001). Non-competitive association is
257 supported by the different substrate preferences. *L. plantarum* preferentially metabolizes glucose
258 while *L. sanfranciscensis* preferentially metabolizes maltose and sucrose (Gänzle et al., 2007). In

white wheat and in whole wheat sourdoughs, *L. sanfranciscensis* outcompeted *L. plantarum*, matching prior observations that used undefined starter cultures as inoculum (Meroth et al., 2003). An increase in pH increased competitiveness of *L. plantarum* relative to *L. sanfranciscensis*. Optimum growth of *L. sanfranciscensis* is observed in the narrow range of 5.0 – 6.0 (Gänzle et al., 1998); *L. plantarum* grows over a much wider pH range and is more acid resistant than *L. sanfranciscensis* (G-Alegría et al., 2004). Surprisingly, both an increase – through addition of sucrose – and a decrease – through addition of baker's yeast – of sucrose reduced the competitiveness of *L. sanfranciscensis*. The competitiveness of *L. sanfranciscensis* in sourdoughs was also reduced by relatively minor shifts in the fermentation time or temperature (Meroth et al., 2003), or by the interruption of fermentation cycles by refrigeration (Siragusa et al., 2009). Overall, these data suggest that *L. sanfranciscensis*, an organism with a small genome size (Vogel et al., 2011), is adapted to a very narrow ecological niche.

L. plantarum is tolerant to antimicrobial phenolic acids (Sànchez-Maldonado et al., 2011). Degradation of phenolic acids by *L. plantarum* reduces their antimicrobial activity (Filannino, Di Cagno, & Gobbetti, 2018) and increases the ecological fitness of the organism in phenolic acid-rich substrates (Sekwati-Monang, Valcheva, & Gänzle, 2012; Filannino, Bai, Di Cagno, Gobbetti, & Gänzle, 2015; Ripari et al., 2019). The current study provides experimental support for the hypothesis that dominance of *L. plantarum* over *L. sanfranciscensis* in phenolic acid rich substrates relates to resistance and sensitivity of these species, respectively, to antimicrobial phenolic acids (Sànchez-Maldonado et al., 2011; Sekwati-Monang et al., 2012). Wheat and rye contain mainly bound phenolic acids that have no antimicrobial activity (Li et al., 2008) but millet and sorghum have a high concentration of free – and antimicrobially active – phenolic acids (Sekwati-Monang et al., 2012).

In conclusion, this study demonstrated that the pH and, to a lesser extent, organic acids are key contributors to the elimination of *Enterobacteriaceae* in spontaneous cereal fermentations. This adds to our understanding of "the usual suspects" as representatives of plant microbiota that are stratified

(*Enterobacteriaceae* → *Leuconostoc* and *Weissella* → *Lactobacillus* sp.) in order of decreasing abundance and increasing acid resistance.

A more complex picture was obtained with respect to the competition between *L. sanfranciscensis* and *L. plantarum*. This indicates that *L. sanfranciscensis* is sensitive to even minor perturbations of the sourdough ecosystem, and likely reflects adaptation of the organism to a very narrow ecological niche.

Author Contributions

F.D. performed the experiments and drafted the manuscript, M.D. discussed the results and revised the manuscript, F.M. performed statistical analyses on the results and wrote the manuscript, Ma.Go. revised the manuscript, and Mi.Gä. ideated the study, supported the experiments and revised the manuscript.

Conflicts of interest

The authors have no competing interests.

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Figure legends

Figure 1. Values of pH (■) and cell density (log CFU/g) of *Lactobacillus sanfranciscensis* (●), *Lactobacillus plantarum* (○), *Klebsiella pneumoniae* (▼) and *Cronobacter sakazakii* (Δ) in sourdoughs prepared with white wheat flour (A), whole wheat flour (B) or wheat bran (C). The inlet shows the concentrations of lactic acid, acetic acid, and maltose after selected fermentation cycles. Sourdoughs were propagated for 11 fermentation cycles; numbers on the x axis indicate the number of fermentation cycles and cycle 0 refers to the first dough after inoculation. Data are the means ± standard deviations of three independent experiments analyzed in duplicate. Values below the x-axis indicate values below the detection limit of 3 log CFU/g.

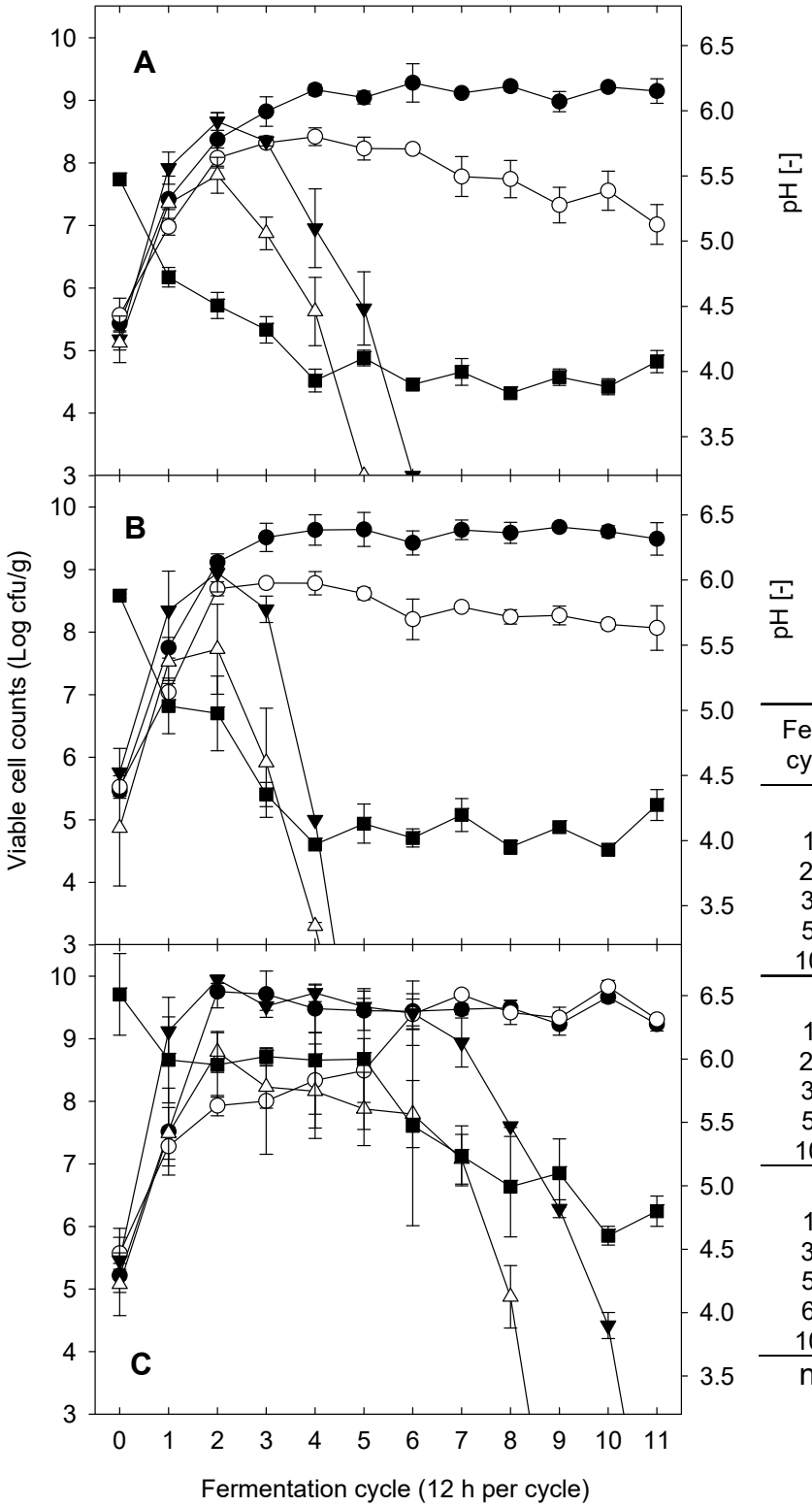
Figure 2. Values of pH (■) and cell density (log CFU/g) of *Lactobacillus sanfranciscensis* (●), *Lactobacillus plantarum* (○), *Klebsiella pneumoniae* (▼),*Cronobacter sakazakii* (Δ) and presumptive yeasts (♦) found in the sourdoughs prepared with white wheat flour and addition of maltose (A), sucrose (B), and baker's yeast (C). The inlet shows the concentrations of lactic acid, acetic acid, and maltose after selected fermentation cycles. Sourdoughs were propagated for seven fermentation cycles; numbers on the x axis indicate the number of fermentation cycles and cycle 0 refers to the first dough after inoculation. Data are the means ± standard deviations of three independent experiments analyzed in duplicate. Values below the x-axis indicate values below the detection limit of 3 log CFU/g.

Figure 3. Values of pH (■) and cell density (log CFU/g) of *Lactobacillus sanfranciscensis* (●), *Lactobacillus plantarum* (○), *Klebsiella pneumoniae* (▼) and *Cronobacter sakazakii* (Δ) in the sourdoughs prepared with white wheat flour and addition of phosphate buffer (A), and ferulic acid (B) The inlet shows the concentrations of lactic acid, acetic acid, and maltose after selected fermentation cycles. Sourdoughs were propagated for seven fermentation cycles; numbers on the x

448 axis indicate the number of fermentation cycles and cycle 0 refers to the first dough after inoculation.
449 Data are the means ± standard deviations of three independent experiments analyzed in duplicate.
450 Values below the x-axis indicate values below the detection limit of 3 log CFU/g.

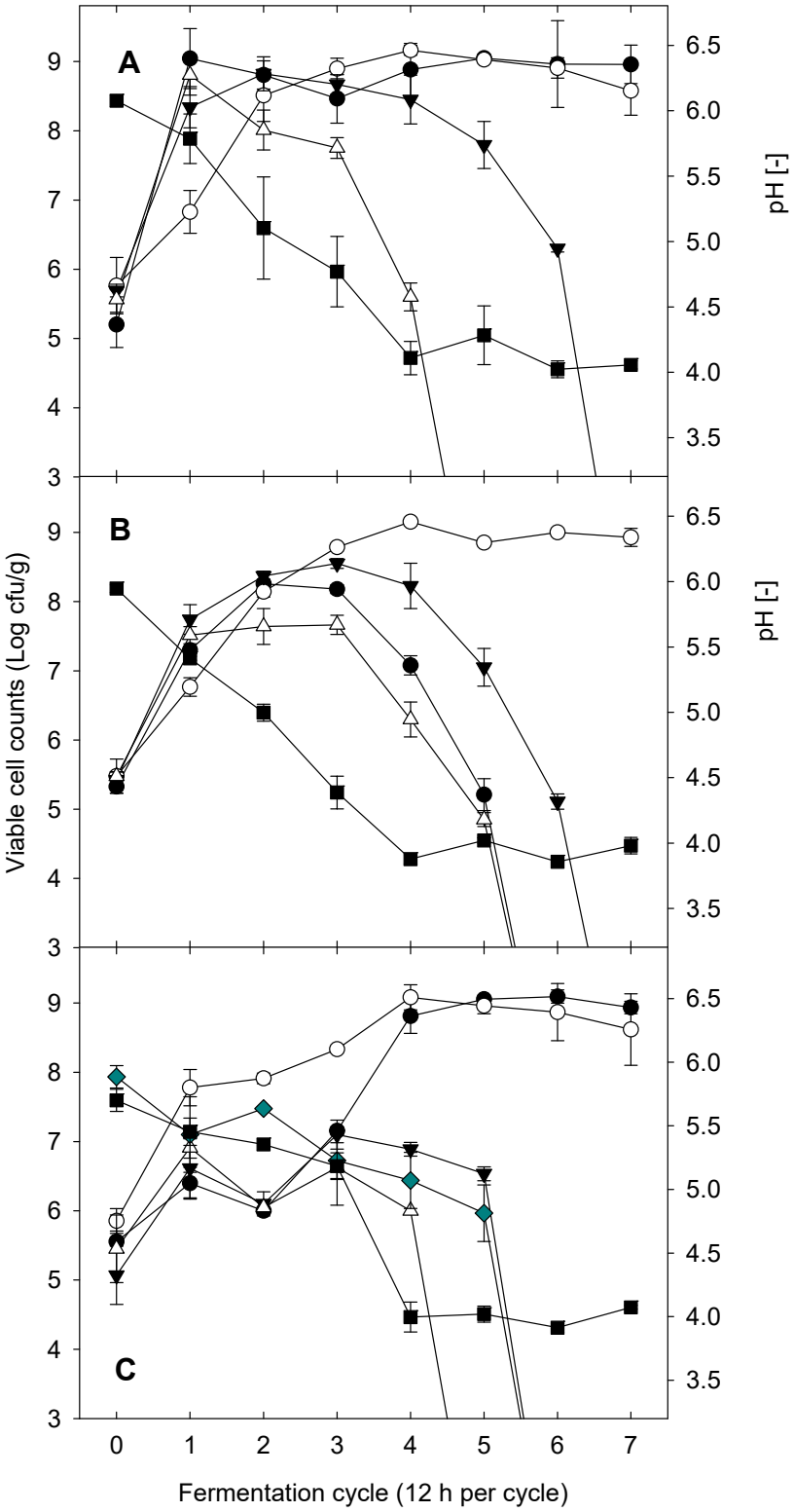
451

452 **Figure 4.** Permutation analysis summarizing the concentrations of lactic acid, acetic acid, ethanol,
453 maltose, pH and cell densities of *Lactobacillus sanfranciscensis*, *Lactobacillus plantarum*, *Klebsiella*
454 *penumoniae* and *Cronobacter sakazakii* found in the sourdoughs obtained after seven cycles of
455 propagation and prepared with white wheat flour alone or with addition of sucrose, baker's yeast,
456 phosphate buffer, maltose or ferulic acid, or prepared with whole wheat flour or wheat bran. Data are
457 average values from three independent experiments analyzed in duplicate. Euclidean distance and
458 McQuitty's criterion were used for clustering. Colours correspond to normalized mean data levels
459 from low (green) to high (red). The colour scale, in terms of units of standard deviation, is also shown.



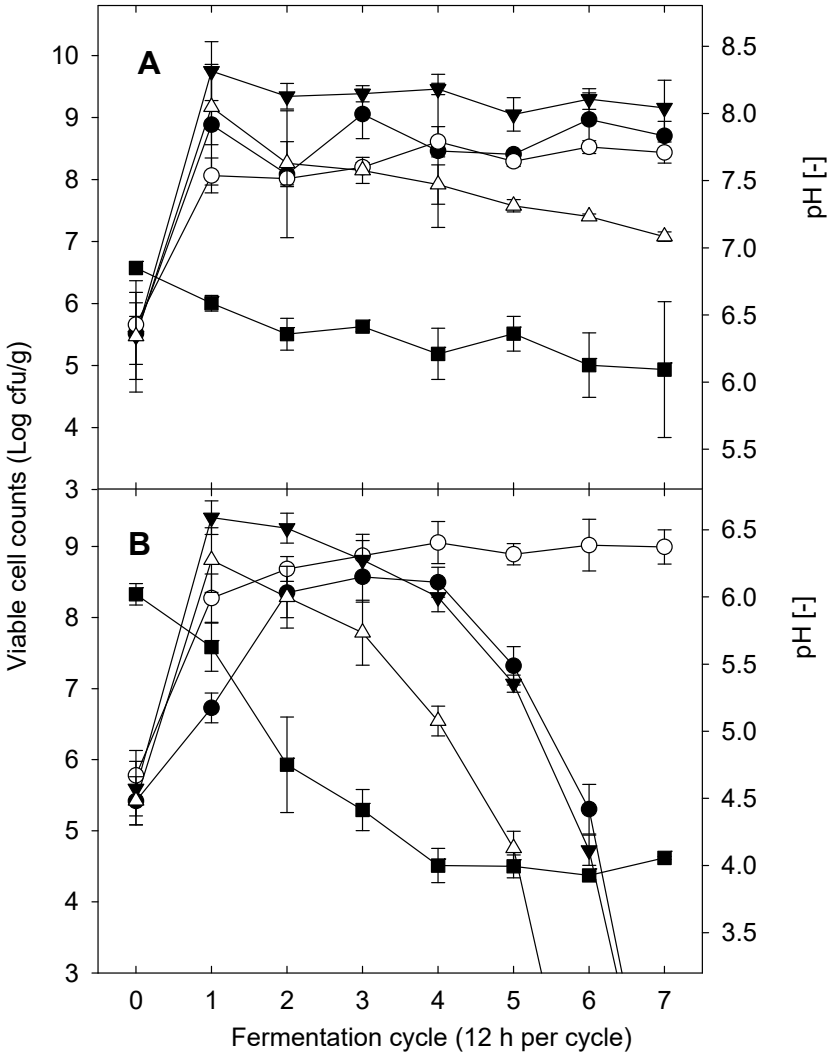
Ferm. cycle	Concentration (mmol/L)		
	Lactate	Acetate	Maltose
	White wheat sourdough		
1st	30±2	7±4	84±7
2nd	49±6	6±1	102±3
3rd	66±1	6±2	122±2
5th	62±4	6±1	77±5
10th	58±1	6±1	76±6
	Whole wheat sourdough		
1st	50±5	31±7	47±2
2nd	84±8	27±0	45±2
3rd	144±16	45±10	37±10
5th	131±21	44±9	34±0
10th	111±12	39±2	32±9
	Wheat bran sourdough		
1st	5±2	17±12	1±0
3rd	8±0	37±25	n.d.
5th	97±6	39±2	11±1
6th	31±19	26±1	n.d.
10th	69±28	40±15	4±0

n.d., not detected.

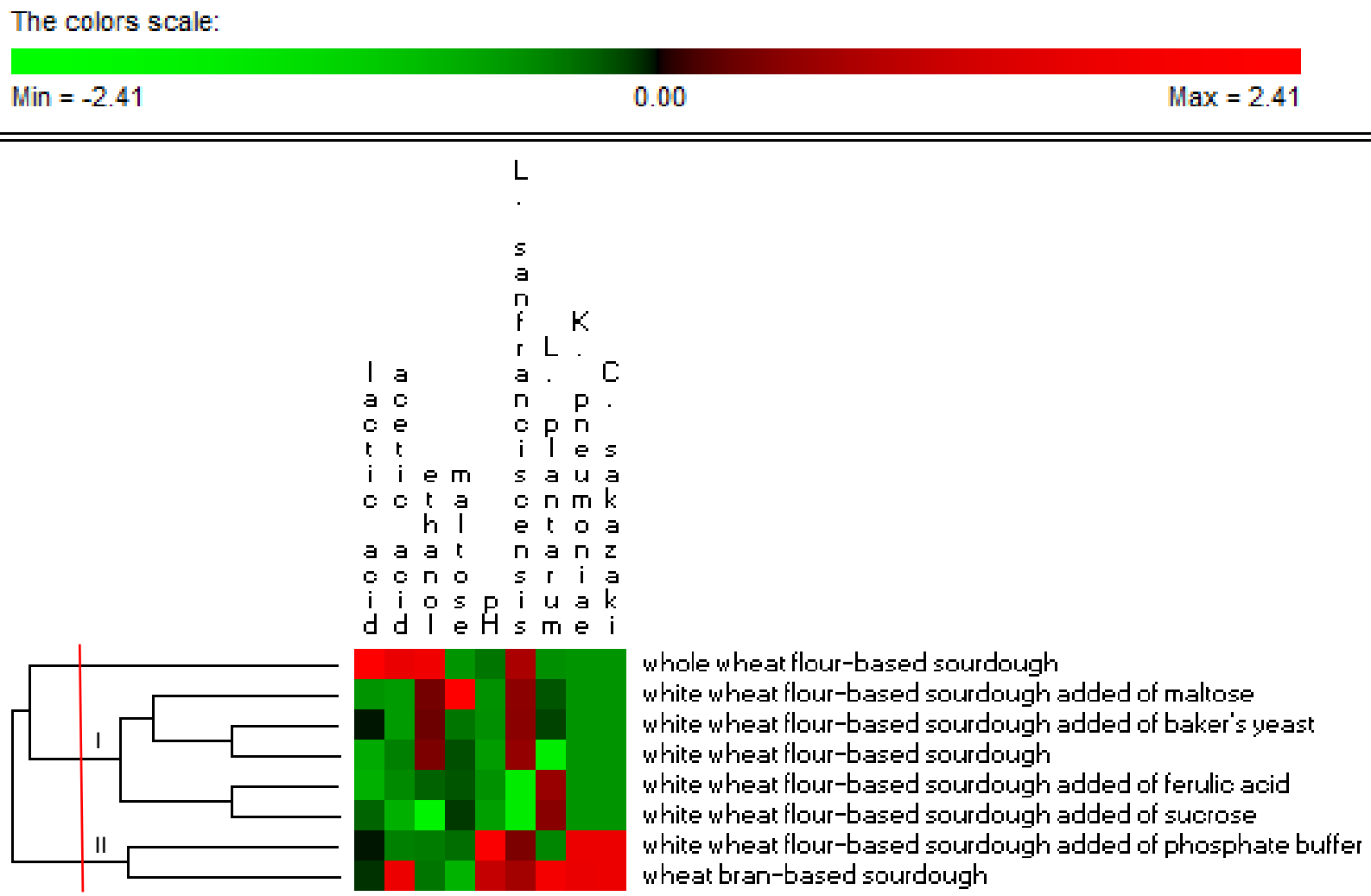


Concentration (mmol/L)		
Lactate	**Acetate**	**Maltose**
White wheat + maltose		
19±0	n.d.	400±54
19±1	n.d.	350±25
45±2	n.d.	370±40
61±3	n.d.	370±35
White wheat + sucrose		
15±0	n.d.	66±2
25±4	n.d.	62±3
55±4	n.d.	72±2
66±7	n.d.	84±8
White wheat + yeast		
22±2	n.d.	17±9
11±0	n.d.	36±5
65±5	n.d.	88±3
70±11	n.d.	55±51

not detected.



Ferm. cycle	Concentration (mmol/L)		
	Lactate	**Acetate**	**Maltose**
	White wheat + phosphate		
1st	11±0	9±2	66±3
2rd	12±0	8±1	59±5
4th	13±1	6±0	57±1
6th	13±2	6±1	60±1
	White wheat + ferulic acid		
1st	28±10	2±0	77±4
2rd	36±1	6±1	75±4
4th	60±1	0±0	77±14
6th	57±1	5±3	73±7


The colors scale:
Min = -2.41
0.00
Max = 2.41
lactic acid
acetic acid
ethanol
maltose
pH
L. sanfranciscensis
L. plantarum
K. pneumoniae
C. saki
I
II
whole wheat flour-based sourdough
white wheat flour-based sourdough added of maltose
white wheat flour-based sourdough added of baker's yeast
white wheat flour-based sourdough
white wheat flour-based sourdough added of ferulic acid
white wheat flour-based sourdough added of sucrose
white wheat flour-based sourdough added of phosphate buffer
wheat bran-based sourdough