Metabolism of phenolic acids in whole wheat and rye malt sourdoughs

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18 **Abstract.**

19 This work aimed to study the phenolic acid metabolism of sourdough lactic acid bacteria (LAB) in
20 laboratory media, and in sourdough fermentation with single cultures and in co-fermentations.
21 Lactobacilli were selected from isolates obtained from 35 sourdough samples. Isolates (114 strains)
22 were screened for phenolic acid decarboxylase gene *pdc* and EPS production. Ferulic acid metabolism
23 of the 18 *pdc* positive strains was evaluated in mMRS; all *pcd* positive strains converted ferulic acid
24 by decarboxylation and / or reduction. Single whole wheat and rye malt dough fermentation
25 fermented with lactobacilli or yeasts were characterized with respect to free, conjugated, or bound
26 phenolic acids. Concentrations of free, conjugated, or bound phenolic acids were not altered
27 substantially in chemically acidified sourdoughs, or in yeast fermented doughs. *L. plantarum*
28 metabolized free ferulic acid in wheat and rye malt sourdoughs; *L. hammesii* DSM 16381 metabolized
29 syringic and vanillic acids and reduced levels of bound ferulic acid in wheat sourdoughs. Co-
30 fermentation of *L. hammesii* and *L. plantarum* achieved release of bound ferulic acid and conversion
31 of the resultant free ferulic acid to dihydroferulic acid and volatile metabolites. Phenolic acid
32 metabolism in sourdoughs was enhanced by co-fermentation with strains exhibiting complementary
33 metabolic activities. Results may enable improvement of bread quality by targeted conversion of
34 phenolic acids during sourdough fermentation.

35 **Keywords**

36 Phenolic acids, ferulic acid, phenolic acid decarboxylase, sourdough, pdc gene, Lactobacillus

37

38 **1. Introduction.**

39 Phenolic compounds are secondary metabolites in plants that provide protection against pathogens
40 and ultraviolet radiation (Beckmann, 2000). Phenolic compounds have been considered nutritionally
41 undesirable because some phenolic compounds precipitate proteins, inhibit digestive enzymes and
42 thus inhibit nutrient absorption (McSweeney et al., 2001). A reduced rate of nutrient absorption,
43 however, also reduces the glycemic index of foods and can be considered health-beneficial (Ding et
44 al., 2013; Chung et al., 1998). In particular, dietary phenolic acids have antidiabetic effects
45 (Vinayagam et al., 2015). The beneficial effects of phenolic compounds thus depend on their quantity
46 and bioavailability (Chung et al., 1998; Lodovici et al., 2001), and on the nutritional status of the
47 consumer.

48 Phenolic acids are the major class of phenolic compounds in cereals (Shahidi & Naczk, 2000; Shewry
49 et al., 2010). Wheat and rye contain 0.5 – 1 g /kg phenolic acids; these predominantly occur in
50 conjugated form (0.1 – 0.2 g / kg), or bound to cell wall polysaccharides 0.4 – 0.9 g / kg) with ferulic
51 acid typically accounting for more than 50% of total phenolic acids (Li et al., 2008; Shewry et al.,
52 2010). Cross-linking of cell wall polysaccharides and proteins by phenolic acids influences the bread-
53 making quality of wheat and rye flours. The solubilization of arabinoxylans during fermentation
54 improves the water binding capacity and the baking quality of rye flour and, to a lesser extent, of
55 wheat flour (Gänzle, 2014). Moreover, release of bound phenolic acids increases their bioavailability
56 (Gänzle, 2014; Katina et al., 2012**.** Moreover, microbial conversion of phenolic acids generates
57 volatile phenolic compounds (Rodriguez et al., 2009) which impact bread flavor (Czerny and
58 Schieberle, 2002).

59 Phenolic acids in wheat and rye include hydroxycinnamic acids (C6-C3 compounds) and
60 hydroxybenzoic acids (C6-C1 compounds). Both classes of compounds have antibacterial activity
61 (Sanchez-Maldonado et al., 2011). Lactic acid bacteria have a high tolerance to antimicrobial
62 phenolic acids; their resistance is partially dependent on their capacity to convert phenolic acids to
63 metabolites with reduced metabolic activity (Sanchez-Maldonado et al., 2011). Hydroxy-benzoic

64 acids are metabolized by decarboxylation to volatile phenolic compounds (for review, see Rodriguez
65 et al., 2009; Gänzle, 2014). Hydroxy-benzoic acids are metabolized by decarboxylation to the
66 corresponding vinyl-derivatives, by reduction of the double bond in the C3 side chain, or by
67 sequential activity of both enzymes (Rodriguez et al., 2009). An example, ferulic acid is reduced to
68 dihydroferulic acid, decarboxylated to 4-vinyl-2-methoxyphenol (vinyl-guaiacol), or decarboxylated
69 and reduced to 4-ethyl-2-methoxyphenol (ethyl-guaiacol, Beek & Priest, 2000). Specific lactobacilli
70 also hydrolyse esters of phenolic acids by ferulic acid esterase activity (Hole et al., 2012).
71 Studies on metabolism of phenolic compounds were conducted mainly with *L. plantarum*. *L.*
72 *plantarum* occurs in intestinal ecosystems and in insects, in association with plants, and in many food
73 fermentations (Martino et al., 2016). The origin of strains of *L. plantarum* is unrelated to either the
74 phylogenetic position or the metabolic potential, demonstrating that strains of *L. plantarum* frequently
75 transition from one niche to another (Duar et al., 2017; Martino et al., 2016). This lifestyle has been
76 termed "nomadic" and is associated with a relatively large genome size, corresponding to a broad
77 metabolic diversity (Duar et al., 2017). *L. plantarum* frequently occurs in fermentation of plant foods
78 rich in phenolic compounds including table olives, sauerkraut and cucumbers (Ruiz-Barba &
79 Jimenez-Diaz, 1994; Plengvidhya et al., 2007; Costilow et al., 1956). The phenolic acid
80 decarboxylase Pdc/PadA of *L. plantarum* decarboxylates hydroxycinnamic acids including p-
81 coumaric and caffeic acids (Cavin et al., 1997). Mutational disruption of *pdc* in *L. plantarum* revealed
82 the presence of a second, uncharacterized phenolic acid decarboxylase which is induced by ferulic
83 acid (Barthelmebs et al, 2000). *L. plantarum* expresses phenolic acid and vinylphenol reductases as
84 an alternative pathway for metabolism of hydroxycimmanic acids (Santamaria et al., 2018a and
85 2018b). In addition, hydroxybenzoic acid decarboxylases exist in *L. plantarum* and some other
86 lactobacilli (De Las Rivas et al., 2009; Filannino et al., 2015).
87 The conversion of phenolic acids in sourdough affects nutritional and technological properties of
88 bread (Gänzle, 2014), however, data on conversion of phenolic acids in rye is limited to simulated
89 rye doughs without microbial activity (Boskov Hansen et al., 2002) or yeast-fermented rye dough

90 with uncharacterized bacterial microbiota (Katina et al., 2012). This study therefore aimed to assess
91 conversion of phenolic acids in wheat and rye sourdoughs. Lactic acid bacteria were screened for
92 genes coding for phenolic acid decarboxylases (*pdc*) and phenolic acid conversion by *pdc*-positive
93 isolates was verified by metabolite analysis. Selected isolates and two reference strains with well-
94 characterized metabolism of phenolic acids (Sanchez-Maldonado et al., 2011) were studied with
95 respect to their impact on phenolic acid compounds in whole wheat and rye malt sourdoughs. Free,
96 conjugated, and bound phenolic acids were quantified by LC-MS/MS (Li et al., 2008).

97 **2. Materials and Methods.**

98 **2.1. Strains and growth conditions.**

99 The strains analyzed in this study were isolated from Italian sourdoughs; *Lactobacillus plantarum* TMW 1460
100 and *Lactobacillus hammesii* DSM 16381 were used as reference strains known to metabolise phenolic acids
101 (Sànchez-Maldonado et al, 2011; Valcheva et al., 2005). *Candida humilis* FUA4001 and *S. cerevisiae* FA1
102 represent sourdoughs isolates obtained previously (Ripari et al, 2016). Yeasts and lactic acid bacteria were
103 cultivated in modified de Man, Rogosa Sharpe medium (mMRS, Gänzle et al., 1998) at 30°C.

104 **2.2. Isolation and identification of LABs.**

105 Lactic acid bacteria and yeasts were isolated from Italian sourdoughs as described (Ripari et al., 2016).
106 Sourdough samples were diluted in peptone water and appropriate dilutions were plated on mMRS. At least
107 ten colonies with different morphologies were purified and maintained at −80 °C with glycerol as
108 cryoprotectant. DNA was isolated from LAB using the DNeasy Blood & Tissue kit (Qiagen, Toronto, Canada)
109 with the automated extractor QIAcube (Qiagen). Isolates from sourdough were analysed by RAPD–PCR using
110 M13-5′- GAGGGTGGCGGTTCT-3′ (Huey and Hall, 1989) to eliminate clonal isolates. Bacterial isolates with
111 different RAPD profile were identified by sequencing after PCR amplification of genes coding for 16S rRNA,
112 using primers P0 (GAGAGTTTGATCCTGGCTCAG) and P6 (CTACGGCTACCTTGTTACGA) (Picard et
113 al., 2000).

114 **2.3. EPS production.**

115 Each strain was analyzed for EPS production on agar plates. Strains are transferred on mMRS agar containing
116 5 % of sucrose. Plates were incubated at 30°C for 4-5 days. EPS formation was assessed visually and by
117 assessing colonies with a sterile toothpick.

118 **2.4. Molecular screening, amplification of *pcd***

119 The *pdc* gene encoding the p-coumaric acid decarboxylase was amplified by PCR using degenerative primers
120 49 (5′- GANAAYGGNTGGGARTAYGA) targeting the Pdc sequence (D/E)NGWEYE, and primer 50 (5′-
121 GGRTANGTNGCRTAYTTYT) targeting EKY(A/E)TYP, [R= G or A; Y= G, C, or A; and N= G, A, C, or
122 T]. These degenerate primers were based on well-conserved domains approximately 100 amino acids apart of
123 the PDC proteins (De Las Rivas et al., 2009). PCR reactions were performed in a total volume of 25 μL
124 containing 2 μL of template DNA (approximately 10 ng), 1x buffer, 2.5-2 mM MgCl2, 200 μM of each dNTP,
125 1 U of AmpliTaq DNA polymerase, and 1-0.8 μM of each primer. The reactions were performed using the
126 following cycling parameters: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at
127 94 °C for 1 min, annealing at 50-60 °C for 1min, and extension at 72°C for 30s. For the final extension, 7min
128 at 72°C. At the end of the amplification the result was observed through electrophoresis in 2% agarose gel.
129 The size of the bands was estimated by comparison with a marker (1Kb plus DNA ladder GeneRuler). The
130 expected size of the amplicon was 321 bp.

131 **2.5. Fermentation in synthetic medium and phenolic acids extraction.**

132 Metabolism of ferulic acid was investigated using the protocol described in Sanchez-Maldonado et al (2011)
133 with modifications. Each strain positive for the presence of *pdc*, was inoculated in 10 ml of mMRS broth. After
134 incubation for 24h at 30 °C, 1ml of this suspension was then added to another 10 ml of mMRS and incubated
135 for 18h at 30 °C. Standards of all compounds that were quantified were obtained from SigmaAldrich
136 (Mississauga, ON, Canada) and dissolved in a solution of 50% methanol and 50% buffer, sterilized by filtration.
137 Ferulic acid concentrations are below the detection limit in standard mMRS; the final concentration of phenolic
138 acids in mMRS was 1 mmol/L. mMRS supplemented with ferulic acid was inoculated with each preculture
139 and incubated for 24h at 30 ° C. Sterile media were used as control.

140 The extraction of ferulic acid and its metabolites was performed on the supernatant, obtained by centrifugation
141 of tubes at 8000 x g for 10 min. To achieve pH 1.5, 80 µl of HCl 25% v/v in water were added. After addition
142 of ethyl acetate 500 µl, the solution was mixed for one minute every 10 min for a total of 30 min. Following,

143 centrifugation 8000 x g for 5 min. The extraction was repeated with another 500 µl of ethyl acetate. The
144 supernatants collected were placed in screwcap vials for UPLC analysis after filtration with a 0.2 µm.

145 **2.6. Model sourdoughs.**

146 Model sourdoughs were prepared in triplicate independent fermentations by mixing 10 g of whole white flour
147 or rye malt flour, with 10 ml of sterile tap water in which the cultured cells were resuspended. The initial
148 bacteria count was $1x10^8$ CFU/g, for yeasts it was $1x10^6$ CFU/g.

149 In case of co-fermentation with two strains, both strains were added to achieve approximately $1x10^8$ CFU/g
150 for each strain. The sourdoughs were placed in sterile tubes, and incubated at 30 °C for 24 hours. Doughs
151 fermented with yeast only were acidified to a pH of 4 or less by addition of a solution of lactic acid and acetic
152 acid in a molar ratio of 4: 1; acidification was carried out to prevent bacterial growth. For both types of flour,
153 acidified controls acidified with lactic and acetic acid were incubated at 24 °C under the same conditions as
154 sourdoughs to account for the activity of flour enzymes. Sourdoughs were freeze dried for analysis of phenolic
155 acids; the pH, viable cell counts, and organic acids and monosaccharides were analysed with fresh sourdoughs.

156 **2.7. Determination of pH and cell counts.**

157 After 24 h 1 g of sourdough was added to 9 mL of 18 MΩ water for pH analysis; subsequent tenfold dilutions
158 of this suspension were prepared with 0.1% peptone to assess viable cell counts by surface plating of
159 appropriate dilutions on mMRS agar, followed by incubation at 30 °C for 3 days. Plates were visually examined
160 for a uniform colony morphology matching the inoculum to exclude bacterial contamination.

161 **2.8. Organic acid and monosaccharides extraction and determination using HPLC analysis.**

162 After 24 h of fermentation at 30°C, 200-500 mg of dough were mixed with an equivalent volume (0.2 – 0.5
163 mL) of 7% percloric acid, and incubated at 4 ° C overnight to precipitate proteins. After centrifugation at
164 10,000 x g for 5 min, the supernatant was collected for analysis on a Aminex HP87X column linked to RI and
165 UV-Vis detectors. Mobile phase was 5 mM sulfuric acid (5%) in HPLC water, using 70°C as temperature and
166 0.4 ml/min as flow rate (Galle et al., 2010). The organic acids, monosaccharides and ethanol were quantified
167 by comparison with calibration curves made with the respective standard having a coefficient of correlation
168 ≥0.98. Results are shown as average of triplicate ± standard deviation.

169 **2.9. Extraction of free, conjugated + free and bound phenolic acids from sourdoughs.**

170 Extraction of free, conjugated+free and bound phenolic acids was based on the protocol established by Li et
171 al. (2008) with some modifications (Figure 1). After addition of 1 mL 80% ethanol to 0.25 g of sample, samples
172 were sonicated in a sonicator bath for 10 min and solids were removed by centrifugation at 8000 x g for 5 min
173 at 4°C. The extraction was performed for three independent fermentations for each condition.
174 To quantify free phenolic acids, phenolic acids were obtained by two consecutive extractions of 250 mg freeze
175 dried sourdough with ethanol/water (1ml of an 80:20 v/v mixture), followed by evaporation of the solvent
176 under N_2 at 40°C. Samples were re-dissolved in 500 µL of 2 % acetic acid and 2 µL of 12 M HCl and extracted
177 twice with 500 µL of ethyl acetate. The organic phase was recovered after and solvent was evaporated under
178 N_2 at 40°C. The residue was suspended in 0.1% (v/v) formic acid in methanol (100 µL) for UPLC analysis.
179 Conjugated phenolic acids from sourdoughs were extracted in in the same way; prior to extraction with ethyl
180 acetate, conjugated phenolic acids were hydrolysed with 2 M NaOH for 4 h to convert conjugated phenolic
181 acids to free phenolic acids (Figure 1).
182 Bound phenolic acids were extracted from the pellet obtained after extraction of free and conjugated phenolic
183 acids with ethanol / water. Bound phenolic acids in the pellet were hydrolyzed with 400 µL 2 M NaOH for 4
184 h. The supernatant was collected by centrifugation, acidified with 120 µL of 12 M HCl to achieve pH 2, and
185 extracted with ethyl acetate (Figure 1). The organic phase was collected by centrifugation, solvent was
186 evaporated under N_2 at 40°C, and samples were re-dissolved in 100 µL 0.1% (v/v) formic acid in methanol

187 **LC-DAAD-MS/MS analysis of phenolic acids and metabolites of phenolic acids.**

188 Phenolic acids and metabolites extracted from mMRS were analysed by UHPLC-DAAD as previously
189 described (Sanchez-Maldonado et al., 2011). Extracts were separated on a Kinetex PFP column (100·x 3.0mm,
190 2.6 µm) and quantified on a SPD-M20A Prominence diode array detector. The mobile-phase consisted of (A)
191 0.1 % (v⁄v) formic acid in water and (B) 0.1% formic acid in water:acetonitrile (10:90, v⁄v). Samples were
192 eluted with the following gradient: 0–20% B (1.5min), 20% B (4.5min), 20–90% B (7.5min), 90% B (8min).
193 The assay was calibrated with standard compounds dissolved in o.1% formic in methanol.
194 Phenolic acids and metabolites extracted from sourdough were quantified by LC-DAAD-MS/MS as described
195 by Filannino et al., (2015). MS/MS analysis was performed on a 4000 Q TRAP LC-MS/MS System (MDS
196 SCIEX, Applied Biosystems,Streetsville, ON, Canada) and phenolic acids were identified with an information-
197 dependent acquisition method with parameters identical to parameters described by Filannino et al., (2015).

198 Phenolic acids were quantified with the UV280nm signal after calibration with standard compounds
199 dissolved in 0.1% formic in methanol.

200 **3. Results.**

201 **3.1. LAB population of traditional sourdoughs: EPS and pdc screenings.**

202 Sourdough fermentations were carried out with reference strains and with isolates from 35
203 sourdoughs. Microbiota of 19 of the 35 sourdoughs was described previously (Ripari et al., 2016).
204 The 35 sourdoughs harboured diverse microbiota that were composed of species of the genera
205 *Lactobacillus, Pediococcus, Weissella, Leuconostoc,* and *Acetobacter* (Ripari et al., 2016 and Table
206 S1 of the online supplementary material). An overview on the microbiota of the 35 sourdoughs is
207 shown in Figure S1A of the online supplementary material; the most common species were
208 *Pediococcus pentosaceus* (23 isolates), isolates related to *Lactobacillus plantarum* (*L. plantarum, L.*
209 *paraplantarum,* or *L. pentosus*, 21 isolates), *Leuconostoc* spp. (18 isolates), *Lactobacillus brevis* (9
210 isolates), and *Lactobacillus sanfranciscensis* (7 isolates). Isolates were characterized with respect to
211 production of exopolysaccharides from sucrose and the presence of *pdc* coding for phenolic acid
212 decarboxylase (Fig. S2). All *Leuconostoc* spp., most *Weissella* and *Acetobacter* spp., and some *P.*
213 *pentosaceus* and *L. plantarum* produced EPS from sucrose. All strains of *Lactobacillus rossiae*, and
214 most strains of *L. brevis* and the *L. plantarum* groups harboured the gene coding for phenolic acid
215 decarboxylase (Table S1). Most of the sourdoughs contained at least one strain that was capable of
216 EPS production from sucrose but only 21 of the 35 sourdoughs contained a *pdc* positive strain (Figure
217 S2B). Because EPS production by lactic acid bacteria in sourdough is well characterised (Galle et al.,
218 2010; Ua-Arak et al., 2016), subsequent analyses focused on conversion of phenolic acids.

219 **3.2. Metabolism of ferulic acid in mMRS.**

220 To confirm that the presence of pdc gene relates to the capacity to metabolize phenolic acids,
221 fermentations of 18 *pdc*-positive strains in mMRS with ferulic acid were be performed. Metabolites

222 of ferulic acid were detected in all culture supernatants (Figure 2). Most strains and particularly strains
223 of *L. plantarum* reduced ferulic acid concentrations by more than 75%. Vinyl guaiacol was detected
224 in culture supernatants of *pdc* positive strains. Strains that reduced ferulic acid concentrations to less
225 than 0.25 mmol / L also produced dihydroferulic acid, the product of ferulic acid reductase activity.
226 In strains exhibiting both decarboxylase and reductase activities, the concentration of dihydroferulic
227 acid was higher than the concentration of vinyl-guaiacol (Figure 3). Only *L. plantarum* LA1 produced
228 ethyl guaiacol, the product of decarboxylation and reduction of ferulic acid, in low concentrations
229 (<0.1 mmol/L).

230 **3.3. Phenolic acid metabolism in whole wheat sourdoughs.**

231 To determine phenolic acid metabolism in sourdoughs, whole wheat sourdoughs were fermented with
232 *L. brevis* AS3, a carboxylase positive but reductase negative strain, *L. plantarum* MAXIII and
233 TMW1.460 with decarboxylase and reductase activities, and *L. hammesii* DSM 16381, a strain with
234 esterase activity that is not capable of ferulic acid conversion (Sanchez Maldonado et al., 2011; Figure
235 3 and data not shown). Two doughs were fermented with *S. cerevisiae* and *C. milleri* for comparison.
236 Viable cell counts, concentration of organic acids and pH are shown in Table 1. In all sourdoughs,
237 the presence of a uniform colony morphology that matched the colony morphology of the strain used
238 as inoculum demonstrated that the inoculum dominated the fermentation microbiota in all sourdoughs.
239 Doughs fermented with yeasts remained free of bacterial contaminants (Table 1 and data not shown).
240 Sourdough fermentation reduced the pH to pH 3.5 to 3.7; doughs fermented with yeasts had a pH of
241 4.10 – 4.16 and the chemically acidified dough has a pH of 3.96. The concentration of organic acids
242 in sourdough matched the fermentation type of the respective cultures (Table 1).

243 Quantification of phenolic acids differentiated between free phenolic acids, conjugated phenolic acids,
244 and bound phenolic acids (Figure 3A, 3B, and 3C). Ferulic acid was the most abundant phenolic acid
245 in all samples (Figure 3); it occurred mainly in bound form (Figure 3C). Syringic acid was the second
246 most abundant phenolic acid, it occurred in free and conjugated form but was not bound to insoluble

247 dough components (Figure 3). Vanillic and dihydroxybenzoic acids were minor components in all
248 fractions; 4-coumaric and syringic acids were minor components in free and conjugated phenolic
249 acids.

250 The content and profile of phenolic acids did not change substantially in the chemically acidified
251 control (Figure 3), indicating that cereal enzymes are not major contributors to the conversion of
252 phenolic acids during sourdough fermentation. Likewise, yeasts did not to degrade phenolic acids in
253 whole wheat sourdough, or substantially change their distribution in free, conjugated, or bound
254 fractions.

255 *L. plantarum* TMW1.460 and MAXXIII degraded most of the free ferulic acid but the concentration
256 of bound ferulic acid remained unchanged (Figure 3). Fermentation with *L. hammesii* DSM16381
257 reduced the concentration of bound ferulic acid in comparison to the chemically acidified control or
258 sourdoughs fermented with other lactobacilli; however, a corresponding increase in free or conjugated
259 phenolic acids was not observed. *L. hammesii* DSM 16381 also degraded free vanillic and syringic
260 acids, indicating that the strain decarboxylates hydroxybenzoic acids but not hydroxycinnamic acids.

261 **3.4. Phenolic acid metabolism in rye malt sourdoughs.**

262 Conversion of phenolic acids was also studied in rye malt flour to determine whether malt enzymes
263 influence conversion of phenolic acids and their distribution in free and bound fractions. Viable cell
264 counts, concentration of organic acids, and the pH values after 24 h of fermentation are shown in
265 Table 1. Rye malt sourdoughs supported formation of a higher concentration of organic acids when
266 compared to whole wheat sourdoughs, reflecting the higher buffering capacity of rye malt when
267 compared to whole wheat (Table 1).

268 The number of phenolic acids in rye malt and their concentrations were higher when compared to
269 whole wheat. Ferulic acid, vanillic acid, chlorogenic acid, sinapic acid, syringic acid, 4-coumaric acid,
270 and caffeic acid were detected (Figure 4). Ferulic acid was the most abundant compound in all

271 fractions; sinapic acid was abundant in conjugated phenolic acids. Bound phenolic acids included
272 sinapic acid, 4-coumaric acid and chlorogenic acid as well as low amounts of caffeic and cinnamic
273 acids in addition to ferulic acid (Figure 4A, 4B and 4C). The concentration of free vannilic ($p=0.032$)
274 and syringic acids ($p<0.001$) increased in chemically acidified rye malt doughs, likely reflecting
275 enzyme activities of the rye malt. *L. plantarum* MAXXIII metabolized free ferulic acid (Figure 4A).
276 Fermentation with *L. hammesii* increased the concentration of free ferulic, sinapic and 4-coumaric
277 acids but also increased recovery of ferulic acid and some other phenolic acids in the conjugated and
278 bound phenolic acids relative to the unfermented and the chemically acidified sourdoughs (Figure
279 4C). *S. cerevisiae* FA1 generally did not degrade phenolic acids of rye malt dough, or influence the
280 distribution of phenolic acids in the three fractions.

281 **3.5. Co-fermentation in whole wheat sourdoughs.**

282 *L. plantarum* and *L. hammesii* exhibited complementary activities with respect to their ability to
283 release phenolic acids from the bound fraction (*L. hammesii*), and the ability to convert hydroxy-
284 cinnamic acids (*L. plantarum*). To determine whether co-fermentation of *L. hammesii* and *L.*
285 *plantarum* increases conversion of bound and free phenolic acids, whole wheat sourdoughs were
286 inoculated with *L. hammesii* in combination with 6 different strains of *L. plantarum*. Cell counts and
287 metabolite concentrations of the sourdoughs are shown in Table 2. Acetate and ethanol concentrations
288 in these sourdoughs were higher than in sourdoughs fermented with *L. plantarum* (Table 1 vs. Table
289 2) but lower than in sourdoughs fermented with *L. hammesii* (Table 1 vs. Table 2), indicating growth
290 and metabolic activity of both strains.

291 The impact of co-fermentation on the concentration of free, conjugated and bound phenolic acids was
292 analysed by LC-DAAD-MS/MS (Figure 5). The concentration of phenolic acids in unfermented and
293 chemically acidified doughs was comparable to the controls that were prepared for sourdoughs
294 fermented with single strains of yeasts or lactobacilli (Figure 3). Irrespective of the presence of *L.*
295 *hammesii*, strains of *L. plantarum* metabolized free ferulic acid in whole wheat sourdoughs (Figure

296 3A and 5A). Co-fermentation of *L. hammesii* with *L. plantarum* LA1, CVP2, MAXXIII and M7
297 resulted in levels of conjugated and bound ferulic acid ranging between the concentration in the
298 unfermented dough and the concentration in the chemically acidified control (Fig. 5C), indicating
299 that ferulic acid esterase activity of *L. hammesii* has only a limited influence on the release of ferulic
300 acid from ester linkages in these sourdoughs. However, co-fermentation of *L. hammesii* in
301 combination with *L. plantarum* LM01, PM4, MAXXIII and M7 depleted conjugated (strain
302 MAXXIII M7) or bound (strains LM01 and PM4) ferulic acid, indicating that co-fermentation of *L.*
303 *plantarum* with a ferulic acid esterase positive strain increased conversion of this compound during
304 fermentation (Fig. 5B and 5C).

305 To assess the impact of co-fermentation on metabolism of phenolic acids, we additionally analysed
306 metabolites from ferulic acid. The analytical setup provides quantitative information on the
307 concentration of dihydroferulic acid but only qualitative information is obtained on the volatile
308 compounds vinyl guaiacol and ethyl guaiacol, because the method use for the extraction from dough
309 samples involves one evaporation step. In keeping with the metabolite patterns observed in mMRS-
310 ferulic acid (Fig. 2), all strains of *L. plantarum* produced dihyroferulic acid and vinyl-guaiacol as
311 major metabolites from ferulic acid during growth in rye malt sourdough (Fig. 5A). The highest
312 concentration of dihydroferulic acid was observed in rye malt sourdoughs fermented with *L.*
313 *hammesii* and *L. plantarum* LM01 (Fig. 5A); a high capacity for formation of dihyroferulic acid was
314 also observed in fermentation with *L. plantarum* LM01 in mMRS-ferulic acid (Figure 2). All rye malt
315 sourdoughs fermented with *L. plantarum* and *L. hammesii* also contained ethyl-guaiacol (Figure 5A),
316 a metabolite that was produced only by *L. plantarum* LA1 during growth in mMRS-ferulic acid (Fig.
317 2).

318 **4. Discussion.**

319 The present study investigated metabolism of phenolic acids of lactobacilli in sourdoughs. Strains of
320 lactobacilli for use in sourdough fermentation were selected from reference strains (Filannino et al.,

321 2015; Sanchez-Maldonado et al., 2011), and by screening 114 isolates of lactic and acetic acid
322 bacteria isolated from sourdoughs. In model sourdoughs that were inoculated with one or two defined
323 strains, the strains were differentially enumerated by culture-based differential enumeration. If
324 sourdoughs are inoculated defined strains that differ in their colony morphology, differential
325 enumeration based on colony morphology is more reliable than qPCR or sequence based
326 methodologies (Lin and Gänzle, 2014; Meroth et al., 2003; Scheirlinck et al., 2009; Sekwati-Monang
327 et al., 2012; Zheng et al., 2015b). The selection of strains for sourdough fermentation also aimed to
328 explore the potential for co-fermentation with strains exhibiting complementary enzyme activities to
329 enhance the conversion of phenolic acids.

330 The characterization of 35 sourdoughs provided 117 isolates that represented the diversity of
331 spontaneous sourdoughs and back-slopped type I sourdoughs (Gänzle and Ripari, 2016). In keeping
332 with prior report on EPS production by sourdough microbiota, EPS production of sourdough isolates
333 was mainly attributed to sucrose-dependent production of dextrans or fructans by *Weissella* spp. and
334 *Leuconostoc* spp. (Van der Meulen et al., 2007; Galle et al., 2010). In addition, sourdoughs contained
335 *Acetobacter* spp. (Ripari et al., 2016). *Acetobacter* spp. are rarely isolated from traditional sourdough
336 (Gänzle and Ripari et al., 2016) but have been employed for production of high molecular weight
337 EPS in sourdoughs (Ua-Arak et al., 2016).

338 Phenolic acid metabolism of cereal-associated lactobacilli has been investigated in isolates from
339 whiskey and sorghum sourdoughs (Beek and Priest, 2000; Svensson et al., 2010), however, a
340 screening of sourdough isolates has not been reported. Phenolic acid decarboxylase was not only
341 identified in *L. plantarum*, the species for which phenolic acid metabolism is best described
342 (Rodriguez et al., 2009; de las Rivas et al., 2009, Cavin et al., 1997) but also in *L. brevis* and *L.*
343 *rossiae*. The antibacterial activity of phenolic acids is higher when compared to the products of
344 bacterial metabolism (Sanchez-Maldonado et al., 2011). Therefore, phenolic acid metabolism by
345 lactobacilli increases the ecological fitness in substrates that contain high concentrations of phenolic

acids, e.g. sorghum (Sekwati-Monang et al., 2012). Moreover, reduction of phenolic acids by heterofermentative lactobacilli regenerates reduced co-factors and thus increases the energy yield in the phosphoketolase pathway (Filannino et al., 2016; Gänzle, 2015). All strains that tested positive in the PCR screening for presence of *pdc* also metabolised ferulic acid in mMRS (this study). However, because multiple decarboxylases with differential substrate specificity are present in genomes of lactobacilli (Rodriguez et al., 2009, Barthelmebs et al., 2000), screening for presence of *pcd* only is unlikely to accurately predict phenolic acid metabolism.

Li et al. (2008) developed a protocol for quantification of free, conjugated, and bound phenolic acids. Alkaline hydrolysis before extraction releases bound phenolic acids; alkaline hydrolysis after extraction hydrolyses phenolic acids that are conjugated to other compounds including phenolic acid dimers, glycosides, or phenolic acid esters of polyols (Li et al., 2008). Composition and concentration of phenolic acids that were determined in the present study for whole wheat flour and rye malt (Figure 5 and 6) match the composition and concentration of phenolic acids previously observed in wheat and rye (Li et al., 2008; Shewry et al., 2010). In wheat, ferulic acid was the most abundant compound in all three fractions and particularly in the bound phenolic acids; sinapic acid was most abundant in the conjugated phenolic acids and other hydroxycinnamic or hydroxybenzoic acids were minor constituents (Li et al., 2008; Figure 5). Also matching prior observations for rye varieties, the concentration and variety of phenolic acids in rye malt was greater when compared to wheat with ferulic acid as most abundant compound in all three fractions (Shewry et al., 2010; Figure 6). Of note, the alkaline hydrolysis of phenolic acid esters including chlorogenic acid also results in degradation of some compounds, particularly phenolic acids with o-dihydroxy moieties (Sanchez-Maldonado et al., 2014). For some compounds, e.g. sinapic acid and 4-coumaric acid, the recovery from fermented samples was consistently higher than the recovery of unfermented samples. This may relate to the differential degradation of phenolic acids during alkaline hydrolysis or to hydrolysis of phenolic acid esters by rye enzymes (Boskov-Hansen et al., 2002).

371 Sourdough isolates of *C. humilis* and *S. cerevisiae* used in the present study did not metabolize
372 phenolic acids or phenolic acid esters. The production of volatiles by *S. cerevisiae* is strain specific
373 and aims at attraction of insects to overcome dispersal limitation (Davis et al., 2013)**.** The metabolism
374 of phenolic acids in sourdough by lactobacilli was strain specific, matching prior reports with respect
375 to strain specific phenolic acid metabolism of lactobacilli in other substrates (Svensson et al., 2010;
376 Filannino et al., 2015; Rodrigues et al., 2009). In the present study, fermentation with *L. brevis* had
377 only a limited impact on the concentration and distribution of phenolic acids in sourdough, matching
378 the limited conversion of ferulic acid *in vitro* (Figures 4 and 5). Strains of *L. plantarum* consistently
379 metabolized free ferulic acid in wheat and rye malt sourdoughs, matching the capacity of this species
380 for ferulic acid metabolism *in vitro*. Remarkably, phenolic acids other than ferulic acid were not
381 converted. Hydroxycinnamic acid reductases and decarboxylases have differential activity with
382 ferulic acid and caffeic or 4-coumaric acid as substrates (Svensson et al., 2010; Sanchez-Maldonado
383 et al., 2011; Barthelmebs et al., 2000; Santamaria et al., 2018a). The phenolic acid decarboxylase of
384 *L. plantarum* recognizes caffeic and coumaric acids but not ferulic acid as substrate; however, the
385 homologous enzyme from *B. subtilis* decarboxylates all three hydroxycinnamic acids (Cavin et al.,
386 1997 and 1998). *L. hammesii* metabolized hydroxybenzoic acids in wheat but not in rye malt
387 sourdoughs, possibly reflecting that the fermentation substrate influences the expression of enzymes
388 active on phenolic acids (Filannino et al., 2015). This strain also increased concentrations of free
389 ferulic acid by esterase activity but did not hydrolyse chlorogenic acid. Because the genome of this
390 strain does not encode for one of the feruloyl esterases that were characterized on the biochemical
391 level (Zheng et al., 2015a; Hole et al., 2012), this activity is attributable to a previously
392 uncharacterized enzyme. Hydrolysis of phenolic acid esters by esterase positive lactobacilli increases
393 the bioavailability of dietary phenolic compounds in oats and barley (Hole et al., 2012) and reduced
394 the chlorogenic acid content in sunflower seed flour (Fritsche et al., 2016).

395 Strain-specific metabolism of phenolic acids allowed the unprecedented use of co-fermentation with
396 strains that exhibit complementary pattern of metabolism. Esterase activity of *L. hammesii* increased
397 the concentration of ferulic acid which was further converted to dihydroferulic acid and volatile
398 phenolic compounds by *L. plantarum*. Vinyl-guaiacol with a smoky flavour note and ethyl-guaiacol
399 with a clove like / spicy aroma are undesirable flavour compounds in beer or wine (Wackerbauer et
400 al, 1982; Shinohara et al., 2000), but may contribute to the typical flavour of bread.

401 In conclusion, this study characterized phenolic acid metabolism of lactic acid bacteria isolated from
402 sourdough. The phenolic acid decarboxylase pdc^+ was present mainly in *L. rossiae*, *L. brevis*, and *L.*
403 *plantarum*. Pdc positive strains metabolised ferulic acid *in vitro* and in wheat and rye sourdoughs,
404 however, Pdc positive *L. plantarum* did not metabolise other hydroxycinnamic or hydroxybenzoic
405 acids in wheat and rye malt sourdoughs. This result indicates that phenolic acid metabolism by
406 lactobacilli is dependent on multiple decaboxylases and reductases which are only partially
407 characterized. Likewise, *L. hammesii* released bound ferulic acid by an uncharacterized esterase.
408 Phenolic acid metabolism in sourdoughs was enhanced by co-fermentation with strains exhibiting
409 complementary metabolic activities. The impact of phenolic acid metabolism on bread quality and
410 on health-beneficial effects of phenolic compounds remains subject to future investigations.

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573

Figure legends.

Figure 1. Schematic overview of phenolic acid extractions from whole wheat dough and rye malt dough.

Figure 2. Concentration of ferulic acid and its metabolites after fermentation with *pdc* positive strains. Ferulic acid was added to the medium at a concentration of 1 mmol/L. White bars, ferulic acid; black bars, vinylguaiacol; dark gray bars, ethyl guaiacol; light gray bars, dihydroferulic acid. Data are shown as means ± standard deviations of three independent fermentations.

Figure 3. Concentration of free (**Panel A),** conjugated plus free (**Panel B),** and bound **(Panel C)** phenolic acids in whole wheat sourdoughs. Strains were fermented with single strain as indicated. Data represent means ± standard deviation of three independent fermentations. Values for the same compound in the same fraction are different ($P<0.005$) if they do not share a common superscript.

Figure 4. Concentration of free (**Panel A),** conjugated plus free (**Panel B),** and bound **(Panel C)** phenolic acids in rye malt sourdoughs. Strains were fermented with single strains as indicated. Data represent means ± standard deviation of three independent fermentations. Values for the same compound in the same fraction are different ($P<0.005$) if they do not share a common superscript.

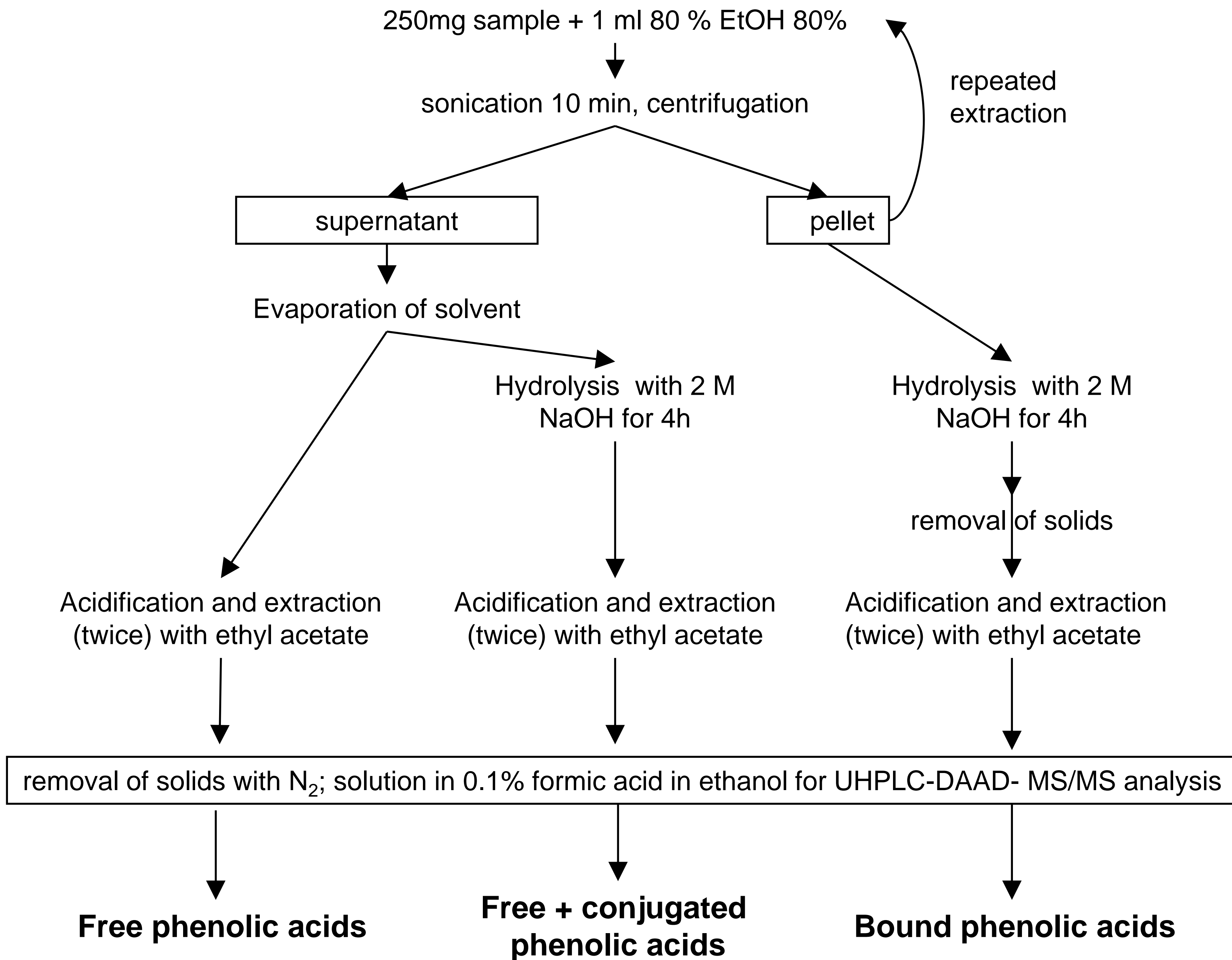
Figure 5. Concentration of free (**Panel A),** conjugated plus free (**Panel B),** and bound **(Panel C)** ferulic acid and its metabolites in whole wheat sourdoughs. Black bar, ferulic acid, light gray bars, vinyl guaiacol, white bars, ethyl guaiacol, dark dray, dihydroferulic acid. C0, unfermented control (0h); CA, chemically acidified controls; other samples were fermented with *L. hammesii* and one strain of *L. plantarum* as indicated. Data represent means ± standard deviation of three independent fermentations. Values for ferulic in the same fraction are different ($P<0.005$) if they do not share a common superscript.

Table 1. Metabolite concentrations, pH, and viable cell counts in whole wheat and rye malt sourdoughs fermented with single strains of LAB or yeast. Samples were analysed in unfermented doughs (control) or after 24h of fermentation (all other doughs). Data are shown as means ± of three independent fermentations.

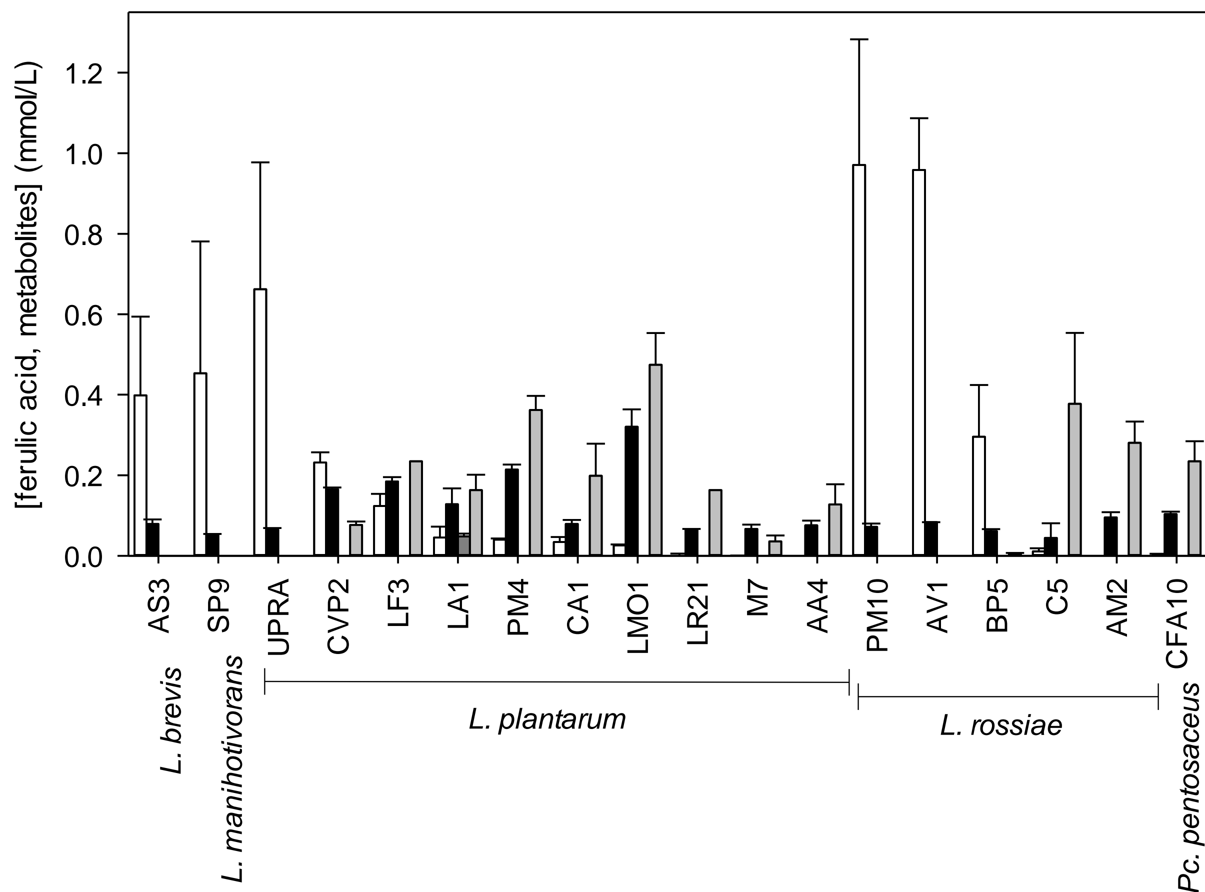
Strain	[acetate] (mmol/kg)	[lactate] (mmol/kg)	[ethanol] (mmol/kg]	pH	cell count log(cfu/g)
whole wheat sourdoughs					
Unfermented control	0.0 ± 0.0	0.00 ± 0.00	0.0 ± 0.0	6.00 ± 0.04	< 4
Chemically acidified dough	15.8 ± 0.6	37.5 ± 2.2	0.0 ± 0.0	3.96 ± 0.06	< 4
L. brevis AS3	18.2 ± 1.0	102.4 ± 4.4	54.4 ± 3.0	3.72 ± 0.02	9.9
L. plantarum MAXXIII	4.0 ± 0.3	102.7 ± 4.6	0.0 ± 0.0	3.55 ±0.02	10.1
L. plantarum TMW1460	5.1 ± 0.5	107.2 ± 3.4	0.0 ± 0.0	3.49 ± 0.03	10.0
*L. hammesii*DSM16381	20.8 ± 0.3	107.7 ± 1.6	51.9 ± 1.4	3.56 ± 0.06	10.0
S. cerevisiae FA1	13.1 ± 0. 5	39.4 ± 2.8	215.8 ± 7.2	4.16 ± 0.10	8.0
C. humilis FUA4001	12.1 ± 0.7	49.1 ± 0.6	131.4 ± 13.3	4.10 ± 0.05	7.9
Rye malt sourdoughs					
Control	0.0 ± 0.00	0.00 ± 0.00	0.0 ± 0. 0	5.54 ± 0.01	< 4
Chemically acidified	Nd	nd	Nd	3.86 ± 0.12	< 4
L. hammesii DSM16381	35.6 ± 2.0	118.7 ± 4.2	32.1 ± 1.4	3.92 ± 0.03	9.70
L. plantarum MAXXIII	3.4 ± 3.0	140.6 ± 16.8	35.2 ± 3.7	3.90 ± 0.02	9.80
S. cerevisiae FA1	20.5 ± 1.2	80.1 ± 5.9	278.7 ± 27.6	4.13 ± 0.03	7.90

Table 2 Cell counts, pH, and metabolite concentrations in whole wheat sourdoughs fermented with a combination of *L. hammesii*DSM16381 and strains of the *L. plantarum* group as indicated. Data represent means ± standard deviations of triplicate independent fermentations.

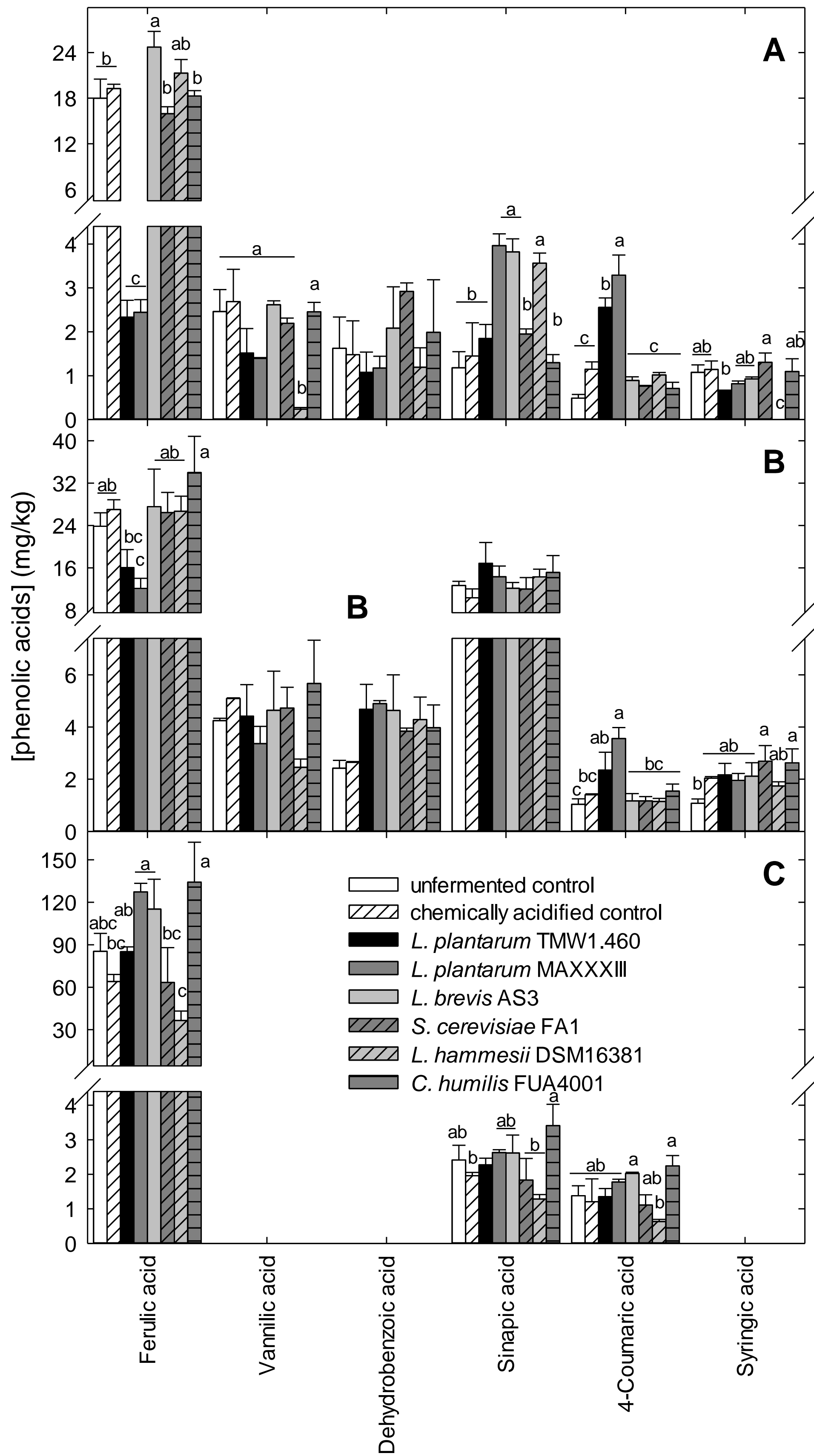
Fermentation organisms (strain numbers)	**[metabolites] (mmol/L)**					
	ethanol	**mannitol**	**acetate**	**lactate**	**cell count log(cfu/g)**	**pH**
DSM16381 + PM4	18.6 ± 2.8	6.8 ± 0.4	11.4 ± 0.4	107.9 ± 1.4	10.1	3.16 ± 0.06
DSM16381 + LM01	7.6 ± 1.0	2.6 ± 2.1	5.9 ± 5.3	76.3 ± 1.9	9.70	3.32 ± 0.06
DSM16381 + CVP2	19.8 ± 2.1	5.7 ± 0.5	10.4 ± 0.7	111.0 ± 1.2	10.1	3.4 ± 0.04
DSM16381 + M7	12.8 ± 0.6	6.3± 0.6	10.7 ± 1.0	117.8 ± 4.4	10.3	3.23 ± 0.03
DSM16381 + MAXXIII	19.7 ± 2.5	6.8 ± 0.6	11.6 ± 0.4	112.3 ± 2.9	10.2	3.35 ± 0.04
DSM16381 + LA1	14.1 ± 3.6	7.0 ± 1.0	10.8 ± 0.3	111.5 ± 0.7	10.1	3.15 ± 0.03
Chemically acidified control	0.0 ± 0.0	0.4± 0.1	18.0 ± 2.5	55.74± 1.3	< 4	3.57 ± 0.20
Unfermented control	0.0 ± 0.0	0.0± 0.0	0.0 ± 0.0	0.0 ± 0.0	< 4	5.91 ± 0.02



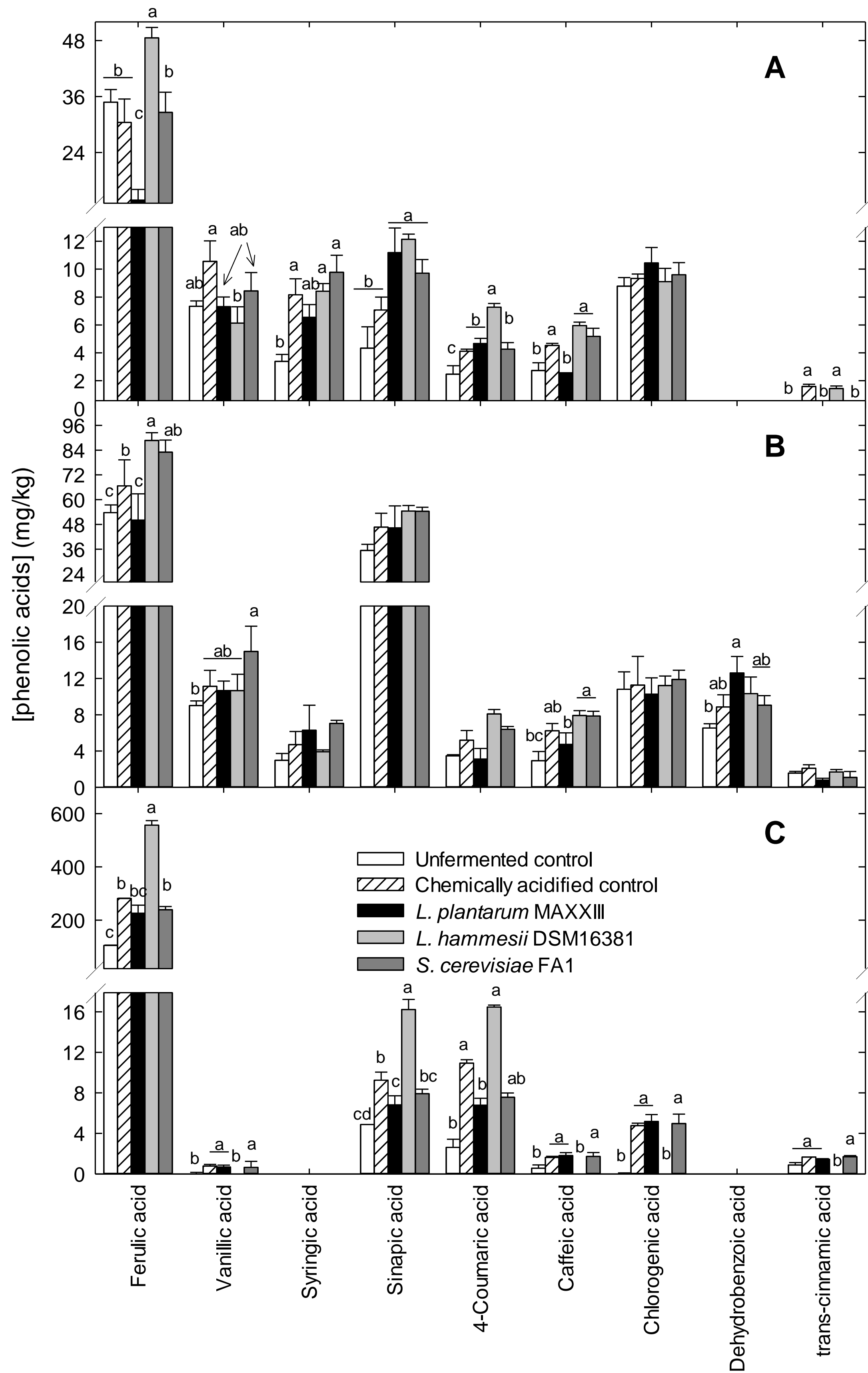
250mg sample + 1 ml 80 % EtOH 80%
repeated extraction
sonication 10 min, centrifugation
supernatant
pellet
Evaporation of solvent
Hydrolysis with 2 M NaOH for 4h
Hydrolysis with 2 M NaOH for 4h
removal of solids
Acidification and extraction (twice) with ethyl acetate
Acidification and extraction (twice) with ethyl acetate
Acidification and extraction (twice) with ethyl acetate
removal of solids with N_2; solution in 0.1% formic acid in ethanol for UHPLC-DAAD- MS/MS analysis
Free phenolic acids
Free + conjugated phenolic acids
Bound phenolic acids



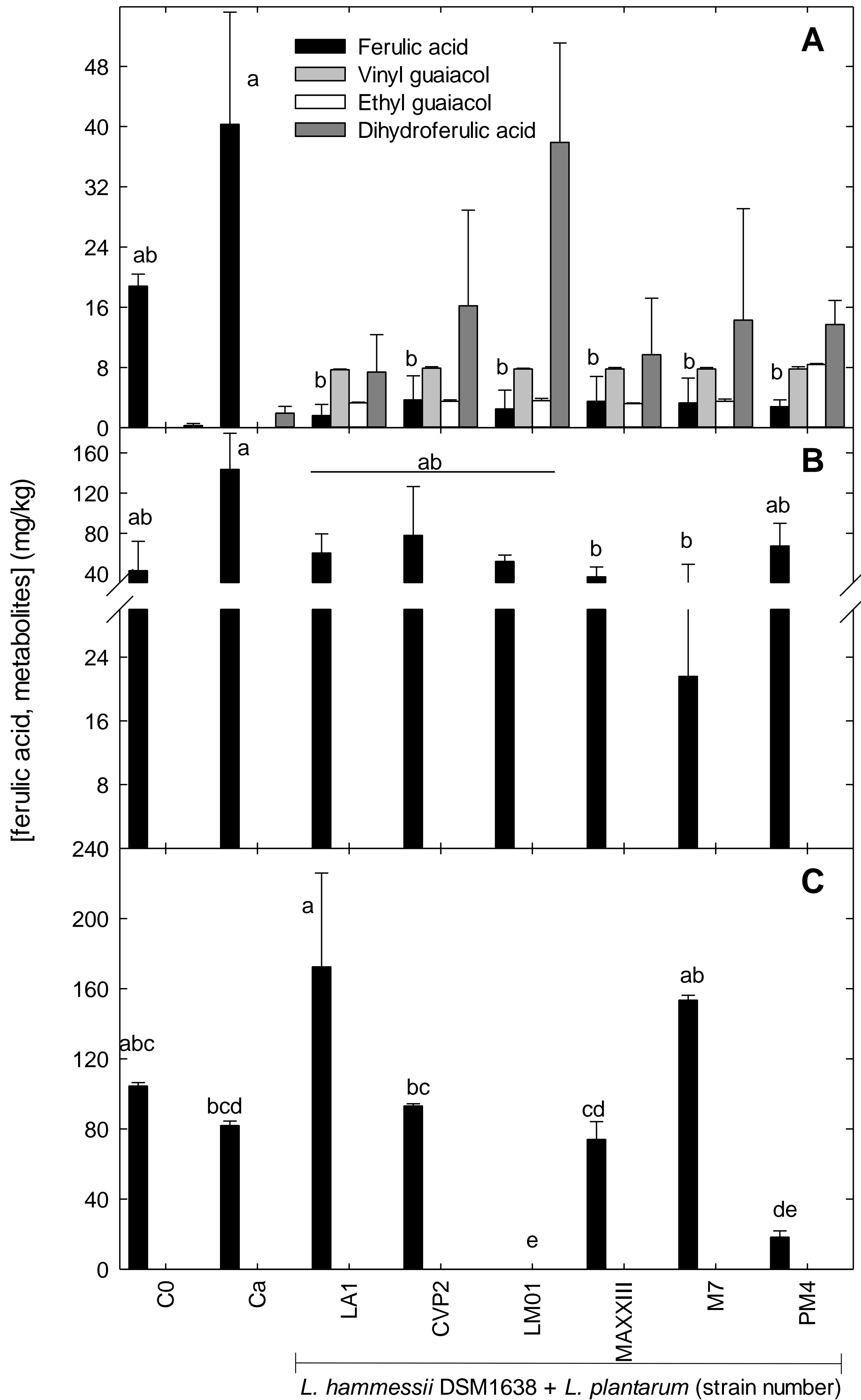
1
2 **Figure 2.** Concentration of ferulic acid and its metabolites after fermentation with *pdc* positive
3 strains. Ferulic acid was added to the medium at a concentration of 1 mmol/L. White bars, ferulic
4 acid; black bars, vinylguaiacol; dark gray bars, ethyl guaiacol; light gray bars, dihydroferulic acid.
5 Data are shown as means ± standard deviations of three independent fermentations.
6


A
B
C
[phenolic acids] (mg/kg)
unfermented control
chemically acidified control
L. plantarum TMW1.460
L. plantarum MAXXXIII
L. brevis AS3
S. cerevisiae FA1
L. hammesii DSM16381
C. humilis FUA4001
Ferulic acid
Vannilic acid
Dehydrobenzoic acid
Sinapic acid
4-Coumaric acid
Syringic acid

8 **Figure 3.** Concentration of free (**Panel A),** conjugated plus free (**Panel B),** and bound **(Panel C)**
9 phenolic acids in whole wheat sourdoughs. Strains were fermented with single strain as indicated.
10 Data represent means ± standard deviation of three independent fermentations. Values for the
11 same compound in the same fraction are different (P<0.005) if they do not share a common
12 superscript.


A
B
C
[phenolic acids] (mg/kg)
Unfermented control
Chemically acidified control
L. plantarum MAXXIII
L. hammesii DSM16381
S. cerevisiae FA1
Ferulic acid
Vanillic acid
Syringic acid
Sinapic acid
4-Coumaric acid
Caffeic acid
Chlorogenic acid
Dehydrobenzoic acid
trans-cinnamic acid

14 **Figure 4.** Concentration of free (**Panel A),** conjugated plus free (**Panel B),** and bound **(Panel C)**
15 phenolic acids in rye malt sourdoughs. Strains were fermented with single strains as indicated.
16 Data represent means ± standard deviation of three independent fermentations. Values for the
17 same compound in the same fraction are different ($P<0.005$) if they do not share a common
18 superscript.



A
B
C
Ferulic acid
Vinyl guaiacol
Ethyl guaiacol
Dihydroferulic acid
[ferulic acid, metabolites] (mg/kg)
C0
Ca
LA1
CVP2
LM01
MAXXIII
M7
PM4
L. hammessii DSM1638 + L. plantarum (strain number)

20 **Figure 5.** Concentration of free (**Panel A),** conjugated plus free (**Panel B),** and bound **(Panel C)**
21 ferulic acid and its metabolites in whole wheat sourdoughs. Black bar, ferulic acid, light gray bars,
22 vinyl guaiacol, white bars, ethyl guaiacol, dark dray, dihydroferulic acid. C0, unfermented control
23 (0h); CA, chemically acidified controls; other samples were fermented with *L. hammesii* and one
24 strain of *L. plantarum* as indicated. Data represent means ± standard deviation of three independent
25 fermentations. Values for ferulic in the same fraction are different ($P<0.005$) if they do not share
26 a common superscript.