1 **Microbial ecology of sorghum sourdoughs: Effect of substrate supply and phenolic**

2 **compounds on composition of fermentation microbiota**

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4 Bonno Sekwati-Monang[a, 1], Rosica Valcheva[b], and Michael G. Gänzle[a*]

5 [a)] Department of Agricultural, Food and Nutritional Science and [b)]Centre of Excellence

6 for Gastrointestinal Inflammation and Immunity Research,

7 University of Alberta, Edmonton, AB, Canada

8

9

10 [*)] corresponding author footnote:

11 Michael Gänzle, University of Alberta, Department of Agricultural, Food and Nutritional

12 Science, 4-10 Ag/For Centre, Edmonton, AB, Canada, T6G 2P5.

13 phone, + 1 780 492 0774; fax, + 1 780 492 4265; e-mail, mgaenzle@ualberta.ca

14 [1)] present address, National Food Technology Research Centre, Private Bag 008, Kanye,

15 Botswana

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19 **Abstract (400 words)**

20 The choice of the cereal substrate determines sourdough microbiota, however, the
21 substrate-associated ecological factors for this phenomenon have not been elucidated.
22 This study investigated the competitiveness of *Lactobacillus sanfranciscensis* LTH 2590,
23 a wheat sourdough isolate, and four isolates from sorghum sourdoughs (ting),
24 *Lactobacillus casei* FUA3166*,* *Lactobacillus harbinensis* FUA3199, *Lactobacillus*
25 *parabuchneri* FUA3169, and *Lactobacillus coryniformis* FUA3307, in sorghum
26 sourdoughs, sorghum sourdoughs supplemented with maltose, or wheat sourdoughs.
27 Fermentations were characterised by determination of cell counts, pH, and quantification
28 of metabolites. Maltose was the main carbon source in wheat sourdoughs whereas
29 glucose was the major carbon source in sorghum. *L. coryniformis* and *L. parabuchneri*
30 produced 1,3- and 1,2-propanediol from glycerol and lactate, respectively, metabolites
31 that were previously not observed in sourdough. To determine the competitiveness of
32 strains, wheat and sorghum slurries were inoculated with equal cells counts of *L.*
33 *sanfranciscensis*, *L. parabuchneri*, and *L. casei* fermented at 28°C or 34°C and
34 propagated by back-slopping every 24h. Lactobacilli in sourdough were quantified by
35 plating and species-specific quantitative PCR (qPCR). Generally, sorghum and wheat
36 sourdoughs inoculated with isolates from ting gave no appreciable differences in the
37 metabolites produced during the fermentation process. *L. sanfranciscensis* grew in wheat
38 but not in sorghum sourdoughs, or sorghum sourdoughs supplemented with 2% maltose,
39 1% tryptone, 0.1 % L-cysteine and 2 % sucrose. Furthermore, *L. sanfranciscensis*
40 decreased progressively during propagation of sorghum sourdoughs but ting isolates were
41 overgrown by *L. sanfranciscensis* after three propagations in wheat sourdoughs

42 independent of the incubation temperature. The anti-microbial activity of four different
43 types of sorghum extracts was tested against *L. sanfranciscensis*, *L. parabuchneri*, and *L.*
44 *casei* to correlate the resistance to phenolic compounds to growth in wheat or sorghum
45 sourdoughs. *L. sanfranciscensis* was inhibited by phenolic extracts from sorghum flours
46 whereas ting isolates were resistant. In conclusion, microbiota of sorghum sourdough
47 differ from wheat and rye because sorghum contains active concentrations of
48 antimicrobial phenolic compounds, and offers glucose as major carbon source.

49

50 **Introduction**

51 Sourdough has been traditionally used in baking applications to acidify and leaven bread
52 dough (Hammes and Gänzle 1998). Lactic fermented cereals are also used for further
53 processing into beverages, crackers, gruels and porridges (Hammes et al., 2005; Vogel *et*
54 *al*., 1999). The composition of sourdough microbiota is influenced by the fermentation
55 process, particularly time, temperature, and dough yield (De Vuyst and Vancanneyt,
56 2007; Meroth et al., 2003; Vogel et al., 1999). Additionally, endogenous factors such as
57 flour carbohydrates, enzymes, and microbial interaction impact microbial growth and
58 metabolic activity (Gobbetti, 1998; Hammes and Gänzle, 1998; Meroth et al., 2003; Van
59 der Meulen et al., 2007). Microbiota of sourdoughs propagated in wheat or rye flours do
60 not exhibit characteristic differences (De Vuyst and Vancanneyt; 2007). However, the use
61 of other cereal flours or pseudocereals select for fermentation microbiota that differ from
62 wheat and rye sourdoughs (Hammes et al., 2005; Moroni et al., 2011; Vogelmann et al.,
63 2009).

64 Traditional fermentations of millet, sorghum, maize, pseudocereals, and pulses are
65 carried out in Africa and Asia (Nout, 2009). One example is ting, a Botswana
66 traditionally fermented sorghum product produced from sorghum flour, water and lactic
67 fermentation (Sekwati-Monang and Gänzle , 2011). Microbiota of back-slopped sorghum
68 sourdoughs only partially overlap with the microbiota of wheat and rye sourdoughs. *L.*
69 *reuteri*, *L. plantarum*, and *L. fermentum* were isolated from sorghum as well as wheat and
70 rye sourdoughs. *L. casei*, *L. coryniformis*, *L. parabuchneri*, *L. harbinensis,* however, were
71 among dominant ting microbiota but are not frequently found in wheat- or rye
72 sourdoughs. *L. sanfranciscensis*, a key organism in traditionally produced wheat and rye

73 sourdoughs, was particularly absent in ting, kisra, or model sorghum sourdoughs
74 performed at the laboratory scale (De Vuyst and Vancanneyt, 2007; Hammad et al., 1991;
75 Sekwati Monang and Gänzle, 2011; Vogelmann et al., 2009). Moreover, ting isolates
76 exhibited usual metabolic activities, including lactate conversion to 1,2-propanediol,
77 glycerol conversion to 1,3-propanediol (Sekwati-Monang and Gänzle, 2011), and
78 metabolism of phenolic acids (Svensson et al., 2010).

79 In analogy to the selection of starter cultures for use in wheat and rye sourdough
80 fermentations, the selection of cultures for sorghum sourdough fermentation requires
81 strains that are highly adapted to the cereal substrate. Moreover, novel metabolites from
82 sorghum-adapted fermentation microbiota allow novel applications of sourdough starter
83 cultures (Gänzle et al., 2009). However, the substrate-associated ecological factors
84 selecting for specific microbiota remain unknown. It was therefore the aim of this
85 investigation to identify factors that determine the composition of fermentation
86 microbiota in sorghum sourdoughs. Growth and metabolism of the ting isolates *L. casei*
87 FUA3166*, L. harbinensis* FUA3199, *L. parabuchneri* FUA3169, and *L. coryniformis*
88 FUA3307 in wheat and sorghum sourdoughs was compared to growth and metabolism of
89 *L. sanfranciscensis.*

90 **2. Materials and Methods**

91 2.1. Bacterial strains and growth conditions

92 *L. sanfranciscensis* LTH2590, a wheat sourdough isolate (Böcker et al., 1995), and four
93 isolates from sorghum sourdoughs (ting) produced in Botswana, *L. casei* FUA3166*, L.*
94 *harbinensis* FUA3199, *L. parabuchneri* FUA3169, and *L. coryniformis* FUA3307
95 (Sekwati-Monang and Gänzle, 2011), were cultivated in modified MRS medium

96 (mMRS) (Stolz et al., 1995). Strains were grown overnight at 30°C without agitation
97 unless otherwise specified. To obtain defined inoculum for sourdough fermentations,
98 cells from 10 mL overnight culture were harvested by centrifugation at 4000 x g for 5
99 min at 15ºC, washed twice with sterile phosphate (PBS) buffer, and re-suspended in 10
100 mL of sterile tap water.

101 2.2. Model sourdough fermentations

102 White sorghum flour and whole wheat flour were obtained from a local supermarket.
103 Pure sorghum cultivars (PAN 3860, Town and Segaolane) were obtained from the
104 National Food Technology Research Centre, Kanye, Botswana, and ground in an Ultra
105 CentrifugalMillZM200 (Retsch, Burlington, Canada) to a particle size of 0.5 mm or less.
106 Ten g of flour were mixed with tap water and cell suspensions in tap water of one of the 5
107 lactobacilli to achieve an initial cell count of approximately 10^7 cfu / g, and incubated at
108 34°C (ting isolates) or 28°C (*L. sanfranciscensis*). Acid aseptic doughs were used as
109 controls; acid aseptic doughs were acidified to a pH of 4.0 with a mixture of lactic and
110 acetic acids (4:1) (v/v). Samples were taken every 24h for characterization of cell counts,
111 pH, and the quantification of metabolites by HPLC as described below. Growth of *L.*
112 *sanfranciscensis* was additionally monitored in sorghum sourdoughs supplemented with
113 2% maltose, 2% sucrose, 0.1% L-cysteine, or 1% tryptone. Fermentations were carried
114 out in triplicate independent experiments.

115 To determine the competitiveness of strains, wheat and sorghum sourdoughs were
116 inoculated with a strain cocktail consisting of *L. sanfranciscensis*, *L. parabuchneri*, and
117 *L. casei.* Inocula were prepared by growing the three strains individually in mMRS broth
118 at 34°C or 28ºC for 18 – 24 h. Cell pellets harvested by centrifugation at 4000 x g for 5

119 min at 15ºC, washed twice with sterile phosphate (PBS) buffer, and re-suspended in 10
120 ml of sterile tap water. Sourdoughs were inoculated with the three strains to achieve
121 approximately equal cell counts of 10^7 cfu / g, and fermented at 28°C or 34°C for 24h.
122 After 24 h of incubation, sourdoughs were propagated by using ripe sourdough to
123 inoculate a new batch of sourdough with a 5 % inoculum (1 g ripe sourdough, 10 g flour,
124 10 g sterile tap water). The propagation of sourdoughs was continued over four
125 fermentation cycles corresponding to four days. Fermentations were carried out in
126 triplicate independent experiments.

127 2.3. Determination of bacterial counts and quantification of substrates and metabolites.

128 The pH of sourdoughs was measured with a glass electrode after dilution of sourdough
129 samples with MilliQ water. Cell counts were enumerated after plating of serial 10-fold
130 dilutions of sourdough samples on mMRS agar. In samples from sourdoughs inoculated
131 with strain cocktails, colonies representing *L. sanfranciscensis* were readily differentiated
132 from colonies representing *L. parabuchneri* or *L. casei* on the basis of the colony
133 morphology. Differential cell counts for *L. sanfranciscensis* and (*L. parabuchneri* +
134 *L. casei*) are reported. Sugars, organic acids and glycerol were quantified by HPLC using
135 Aminex HPX-87 column, 300mm x 7.8mm (BioRad, Torrance, California, USA). For
136 HPLC analyses, sourdoughs were diluted with 5 volumes of milliQ water and centrifuged
137 to remove solids. Proteins were precipitated by addition of 50 µL of 70% perchloric acid
138 to 1 mL sample, overnight incubation at 4ºC, and centrifugation to remove solids.
139 Samples were eluted with 5 mmol / L H_2SO_4 at a flow rate of 0.4 mL / min and 80°C.
140 The injection volume was 10 µL and quantification was based on refractive index
141 detection. Lactate was additionally quantified by UV detection at 210 nm to avoid

142 interference with glycerol. Concentration of maltose, glucose, lactate, acetate, ethanol,

143 glycerol, 1,2-propanediol and 1,3-propanediol were determined using external standards.

144 2.4. DNA extraction from bacterial cultures and sourdough.

145 For extraction of DNA from bacterial cultures, cells of *L. casei*, *L. parabuchneri* and *L.*

146 *sanfranciscensis* were harvested from overnight cultures, DNA was extracted using

147 DNeasy Blood and Tissue Kit (Qiagen Inc, Toronto, Ontario, Canada).

148 For extraction of total DNA from sourdough samples, 10 g of sourdough was mixed with

149 90 mL of sterile saline-tryptone (8.5 g NaCl and 1 g tryptone per L). An aliquot of 50 mL

150 was centrifuged at 4°C for 5 min at 500 x g to remove solids. Cells were harvested by

151 centrifugation 15 min at 5000 x g and cell pellets were stored at -20°C until use. Frozen

152 cell pellets were thawed, washed three times with 1 mL of sterile phosphate- buffered

153 saline (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per L, pH 7.4), and DNA

154 was extracted using DNeasy Blood and Tissue Kit (Qiagen). DNA was purified by

155 adding an equal volume of phenol to the DNA solution, mixing, and recovery of the

156 aqueous phase after centrifugation at 2000 x g, 5 min. The aqueous phase was mixed with

157 an equal amount of 24:1 (v/v) chloroform-isoamyl acohol, and DNA was precipitated by

158 adding 0.1 volume of 3M sodium acetate (pH 5) and 2 volumes of ethanol, and

159 incubation at -20°C overnight. The precipitated DNA was recovered by centrifugation,

160 dried at 50°C for 5 min, and resuspended in 100 µL of sterile water. Quantity and quality

161 of DNA was checked on a Nanodrop spectrophotometer system ND- 1000, version 3.3.0

162 (Thermo Fisher Scientific Inc, Wilmington, DE, USA).

163 2.5. PCR and quantitative PCR analyses.

164 PCR quantification of lactobacilli was based on primers specific for *L. sanfranciscensis*,
165 *L. parabuchneri*, or the *L. casei*-group (Table 1). The oligonucleotide primers were
166 purchased from Invitrogen (Burlington, ON, Canada). PCR was performed with a Gene
167 Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) in a total volume
168 50 µL containing 5X buffer (10 µL), 1.5 µL of each deoxynucleotide triphosphate, 1 µL
169 of each primer, 0.25 µL of GoTaq DNA polymerase (all reagents from Promega
170 Corporation, Madison, USA), 34.25 µL sterile Milli-Q water and 2 µl of template DNA.
171 DNA was amplified over 35 cycles with annealing temperatures shown in Table 1. To
172 verify specific amplification of target DNA, the size of the amplicons (Table 1) was
173 determined by agarose gel electrophoresis. PCR products were purified using QIAquick
174 PCR purification kit (Qiagen) and the concentration of purified amplicons was
175 determined in a Nanodrop spectrophotometer for use as external standard in quantitative
176 PCR reactions.

177 Quantitative PCR (qPCR) was performed on a 7500 Fast Real-Time PCR System
178 (Applied Biosystems). The PCR mixture contained 10 µL 2x QuantiFast SYBR Green
179 master mix (Qiagen), 1 µL (0.5 µM) of each primer (Table 1), 2 µL (app. 10 g / L) of
180 template DNA and 8 µL sterile Milli-Q water to a final volume of 20 µL. Negative
181 controls contained no template DNA, positive controls contained genomic DNA isolated
182 from the respective bacterial cultures. The specificity of each primer pair (Table 1) was
183 verified in PCR reactions with template DNA from *L. sanfranciscensis*, *L. parabuchneri*
184 or *L. casei,* as well as with qPCR-generated melting curves (data not shown). Standard
185 curves for absolute quantification of the targeted microorganisms in the sourdoughs were
186 generated by preparing 10 fold dilutions of purified and quantified PCR product for each

187 individual strain. DNA from sourdough samples were analysed in duplicate in microAmp
188 fast optical 96 well plates (Applied Biosystems) and the copy number of target DNA was
189 calculated by using a standard curve generated in the same 96 well plate as the samples.
190 The gene copy numbers for individual strains were expressed as % of the calculated total
191 gene copy numbers to normalize for losses of bacterial DNA during DNA isolation from
192 sourdough. Results are reported as means ± standard deviation of triplicate independent
193 experiments analysed in duplicate.

194 2.6. Sensitivity of *L. sanfranciscensis*, *L. casei*, and *L. parabuchneri* to antimicrobial
195 phenolic compounds in sorghum.

196 Phenolic compounds from sorghum flours were extracted with aqueous methanol
197 followed by liquid-liquid extraction with ethyl acetate as described (Svensson *et al*.,
198 2010). Extracts were evaporated to dryness and re-dissolved in methanol. Their
199 antimicrobial activity was determined by a critical dilution assay performed on 96 well
200 microtiter plates as described (Gänzle et al., 1996). Prior to inoculation with the indicator
201 strains, microtiter plates were incubated for 2 h to evaporate solvents. Overnight cultures
202 of *L. sanfranciscensis* LTH2590, *L. parabuchneri* FUA3169 and *L. casei* FUA3166 in
203 mMRS were diluted 1:10 and used as indicator strains. The plates were incubated at 34ºC
204 (*L. parabuchneri* and *L. casei*) and 28ºC (*L. sanfranciscensis*) overnight and examined
205 visually for growth of the three strains. The highest dilution factor of sorghum extracts
206 inhibiting bacterial growth was expressed as Arbitrary Units (AU); results of three
207 independent determinations are shown.

208 **3. Results**

209 3.1. Quantification of substrates and metabolites during sourdough fermentations

210 Efficient maltose and sucrose metabolism contribute to the competitiveness of sourdough
211 lactobacilli (Gänzle et al., 2007; Gobbetti, 1998). To determine whether differences in the
212 carbohydrates supply of wheat and sorghum sourdoughs select for different fermentation
213 microbiota, maltose and glucose concentrations were determined in chemically acidified
214 doughs and in sourdoughs. Maltose was the major carbon source in wheat sourdoughs. In
215 sorghum sourdoughs, glucose was the dominant carbon source but the low levels of
216 maltose were depleted during fermentation (Figure 1).

217 3.2. Quantification of metabolites during sourdough fermentations

218 Metabolites of sourdough lactobacilli were quantified in wheat and sorghum sourdough
219 to evaluate whether the fermentation substrate determines the formation of
220 1,2-propanediol by *L. parabuchneri* or the formation of 1,3-propanediol by
221 *L. coryniformis*. In keeping with the respective metabolic pathways for hexose
222 fermentation, lactate was the major metabolite of *L. harbinensis, L. casei*, and
223 *L. coryniformis* whereas *L. parabuchneri* produced lactate, acetate, and ethanol (Figure
224 2). In sourdoughs fermented with *L. parabuchneri* and *L. coryniformis,* 1,2- and
225 1,3 propanediol were detected as minor metabolites, respectively (Figure 2). Metabolite
226 patterns were not different between wheat sourdoughs, sorghum sourdoughs, or sorghum
227 sourdoughs supplemented with maltose. To determine whether the availability of glycerol
228 in the fermentation substrate influences the formation of 1,2-propanediol or
229 1,3-propanediol, sourdough fermentations were carried out with four different sorghum
230 varieties; white sorghum flour, PAN 3860, Town, and Segaolane. Sorghum flours contain
231 glycerol esters of phenolic acids, which are hydrolysed to release glycerol during lactic
232 fermentation (Svensson et al., 2010). The glycerol content of unfermented sorghum

233 varieties was quantified by HPLC. The sample pretreatment with perchloric acid
234 hydrolyses glycerol esters of phenolic acids. The (potential) glycerol content of sorghum
235 varieties ranged from 1.5 to 12 mmol / L. White sorghum varieties (commercial white
236 sorghum flour, Segaolane) contained more glycerol than red varieties (PAN3860, Town)
237 and the highest glycerol concentration was measured in commercial white sorghum flour
238 (Table 2). Glycerol was metabolized by *L. coryniformis* but not by any of the other
239 lactobacilli (data not shown), confirming that its presence in sorghum acts as substrate for
240 1,3-propanediol. Moreover, the sorghum varieties with the highest (potential) glycerol
241 content also supported formation of the highest concentrations of 1,3-propanediol by *L.*
242 *coryniformis* (Table 2). However, the concentration of 1,3-propanediol formed by *L.*
243 *coryniformis* did not correspond to the initial concentration of glycerol (Table 2), and
244 1,3-propanediol was also formed in wheat sourdoughs in which glycerol was not detected
245 (Figure 2).

246 3.3. Evaluation of growth and competitiveness of lactobacilli in wheat and sorghum
247 sourdoughs.

248 To compare the competitiveness of lactobacilli in wheat and sorghum sourdoughs,
249 sourdoughs were inoculated with a strain cocktail consisting of *L. casei*, *L. parabuchneri*,
250 and *L. sanfranciscensis*. Sourdoughs were fermented at 28°C and 34°C to match typical
251 fermentation temperatures for wheat sourdoughs and ting, respectively, and maintained
252 by back-slopping every 24h over four cycles of fermentation. Fermentation microbiota
253 were monitored by plate counts and differential enumeration of ting and wheat isolates,
254 and by qPCR with species-specific primers. The differential enumeration of ting isolates
255 (*L. casei* and *L. parabuchneri*) and the wheat sourdough isolate *L. sanfranciscensis*

256 demonstrated that cell counts of *L. sanfranciscensis* was below detection level in all
257 sorghum sourdoughs, independent of the fermentation temperature (Figure 3D). Ting
258 isolates persisted in high cell counts in sorghum sourdoughs propagated by continuous
259 back-slopping (Figure 3B). However, *L. sanfranciscensis* displaced the ting isolates in
260 wheat sourdoughs independent of the temperature (Figure 3B).

261 Culture-dependent analyses were confirmed by qPCR. The identity and relative quantity
262 of strains was assessed by primers for specific for *L. sanfranciscensis*, *L. parabuchneri*,
263 or for the *L. casei* group. Absolute levels of gene copy numbers in sourdough samples
264 ranged from 1 – 10% of total plate counts (data not shown). Quantitative PCR detected
265 all three strains used in strain cocktail in all samples (Figure 3A and 3C). In wheat
266 sourdoughs, gene copy numbers for *L. sanfranciscensis* were high throughout
267 fermentation and accounted for more than 90% of bacterial gene copy numbers after the
268 fourth fermentation cycle (Figure 3A). In sorghum sourdoughs, gene copy numbers of *L.*
269 *sanfranciscensis* declined by about 1 log with each fermentation cycle, corresponding to
270 the dilution by a factor of 20 with each back-slopping step (Figure 3C). In contrast, gene
271 copy numbers of *L. parabuchneri* and *L. casei* increased after the first two cycles of
272 fermentation of sorghum sourdoughs, and remained stable throughout subsequent
273 fermentation cycles. Dominance of *L. sanfranciscensis* in wheat sourdoughs was
274 established faster at 28°C, while dominance of *L. parabuchneri* and *L. casei* was
275 established faster at 34ºC (Figure 3).

276 3.4. Ecological determinants for selection of fermentation microbiota in sorghum
277 sourdoughs.

278 The failure of *L. sanfranciscensis* to grow in sorghum sourdoughs contrasts the
279 competitiveness of this species in wheat sourdoughs. To determine whether the failure to
280 grow in sorghum is attributable to an inadequate supply of carbohydrates, amino acids, or
281 peptides, growth of *L. sanfranciscensis* was analysed in sorghum sourdoughs
282 supplemented with 1% tryptone, 0.1 % L-cysteine, 2 % sucrose, or 2 % maltose. Whole
283 wheat sourdough was used as a positive control and sourdoughs were incubated for 24h.
284 *L. sanfranciscensis* did not grow in any of the supplemented sorghum sourdoughs and its
285 failure to grow is thus not attributable to the lack of maltose, sucrose, amino acids,
286 peptides, or low molecular weight thiols.

287 Sorghum flour contain phenolic compounds with antimicrobial activity (Svensson et al.,
288 2010) which are absent in wheat. The sensitivity of *L. sanfranciscensis* to a crude extract
289 of sorghum phenolics was compared to the sensitivity of *L. parabuchneri* and *L. casei*
290 (Table 4). Extracts from four different sorghum flours were evaluated; whole grain flour
291 was used for the pure cultivar grains PAN 3860, Town, and Segaolane, whereas the
292 commercial white sorghum flour was prepared from decorticated grains. The
293 antimicrobial activity of extracts from whole grains of the PAN 3860, Town, and
294 Segaolane varieties against *L. sanfranciscensis* was 100 – 200 fold higher in comparison
295 to extracts from commercial white flour (Table 3). The ting isolates *L. parabuchneri* and
296 *L. casei* were much more resistant to phenolic compounds from sorghum flours; these
297 strains were not inhibited by extracts from commercial white flours. It should be noted
298 that flours from PAN3860, Segaolane, and town supported growth of *L. parabuchneri*
299 and *L. casei* despite the presence of phenolic compounds with antimicrobial activity (1.3

300 – 5.3 AU) while commercial white sorghum flour did not support growth of *L.*
301 *sanfranciscensis* (1.3 AU).

302 **4. Discussion**

303 Although wheat and rye sourdoughs do not exhibit characteristic differences with respect
304 to their fermentation microbiota, the cereal substrate was shown to have a decisive
305 influence on sourdough microbiota (De Vuyst and Vancanneyt, 2007; Moroni et al.,
306 2011; Vogel et al., 1999; Vogelmann et al., 2009). The knowledge on factors determining
307 the microbial ecology of wheat and rye sourdoughs can thus not be transferred to cereal
308 fermentations in Africa and Asia, which predominantly employ maize, millet, or sorghum
309 (Nout, 2009), or to the development of sourdoughs for production of gluten-free bread
310 (Moroni et al., 2009).

311 This study evaluated the influence of substrate supply, temperature, and the presence of
312 antimicrobial compounds on the fermentation microbiota in sorghum sourdoughs.
313 Conventional analyses of sourdough microbiota and their metabolites were combined
314 with qPCR analysis. qPCR analysis represented only 1 – 10% of bacterial cell counts. A
315 comparable discrepancy between cell counts in sourdough and 16S rRNA gene copy
316 numbers was previously reported and attributed to the incomplete recovery of bacterial
317 DNA from the matrix (Scheierlinck et al., 2009; Wischebrock et al., 2011). The
318 expression of qPCR results to relative gene copy numbers (Su et al., 2011; this study)
319 compensates for the inaccurate representation of bacterial counts by qPCR.

320 Effective metabolism of maltose by sourdough lactobacilli is a key feature of their
321 competitiveness (Gobbetti, 1998; Stolz *et al*., 1993). *L. sanfranciscensis* grows rapidly on
322 maltose, which is metabolised by maltose phosphorylase, and metabolized preferentially

323 over glucose (Stolz et al., 1993, 1996). Maltose is a major carbon source in wheat
324 sourdoughs and remains available throughout fermentation. In contrast, maltose levels in
325 sorghum sourdoughs are low due to the absence of β-amylase activity in ungerminated
326 sorghum grains (Taylor et al., 2006). The low maltose concentration likely contributes to
327 the reduced competitiveness of *L. sanfranciscensis*. However, supplementation of
328 sorghum flour with maltose, sucrose or peptides did not support growth of *L.*
329 *sanfranciscensis*, indicating that different substrate supply is not the major factor
330 selecting for fermentation microbiota in sorghum.

331 The ting isolates *L. coryniformis* and *L.parabuchneri* produce 1,3-and 1,2-propanediol,
332 respectively, during sourdough fermentation, metabolites that were not previously
333 reported in cereal fermentations (Zhang et al., 2010, Sekwati-Monang and Gänzle, 2011).
334 Glycerol conversion to 1,3-propanediol directs hexose metabolism to the alternative
335 metabolite acetate (Veiga da Cunha and Foster, 1992) but also may support accumulation
336 of the antimicrobial intermediate β-hydroxypropionaldehyde (reuterin, Vollenweider and
337 Lacroix, 2004). The comparison of four different sorghum flours differing in their content
338 of glycerol esters of phenolic acids demonstrated that glycerol liberated from glycerol
339 esters of hydroxyl-cinnamic acids (Svensson et al., 2010) supports 1,3-propanediol
340 formation by *L. coryniformis*. Reduction of the glycolytic intermediate glyceraldehyde-3-
341 phosphate to glycerol (Gänzle et al., 2007) may provide an alternative substrate for
342 1,3-propanediol formation in sourdough.

343 This study employed two fermentation temperatures, 28°C, representing the fermentation
344 temperature of traditional sourdough fermentations, and 34°C, representing the
345 fermentation temperature of ting fermentations in Botswana. The difference of 6°C

346 exerted a small but noticeable influence on the competitiveness of *L. sanfranciscensis* and
347 ting isolates, in keeping with prior reports that fermentation temperature of less than 30°C
348 favour *L. sanfranciscensis* (Meroth et al., 2003).

349 Sorghum is a rich source of phytochemical, including phenolic acids, flavonoids, and
350 tannins (Awika and Rooney, 2004; McGrath et al., 1982; Taylor et al., 2006). Phenolic
351 acids and tannins are the main polyphenols in cereals, whilst flavonoids are encountered
352 in low concentrations (Subba Rao and Muralikrishna, 2002). Phenolic compounds from
353 sorghum, particularly phenolic acids and tannins exhibit antimicrobial activity (Ramos-
354 Nino et al., 1996; Scalbert, 1991; Soetan et al., 2006; Svensson et al., 2010). Crude
355 extracts of phenolic compounds from PAN3860 were previously characterized (Svensson
356 et al., 2010) and contained predominantly phenolic acids, phenolic acid esters, and
357 flavonoid glucosides, thus including compounds with known antimicrobial activity. This
358 study demonstrated that antimicrobially active phenolic compounds in sorghum select
359 against *L. sanfranciscensis*. The resistance of ting isolates to phenolic compounds is more
360 than 10-fold higher when compared to *L. sanfranciscensis* (this study). Ting isolates also
361 converted phenolic acids to decarboxylated or reduced metabolites (Svensson et al., 2010,
362 Sanchez-Maldonado et al., 2011). Metabolites formed by decarboxylation of hydroxyl-
363 benzoic or hydroxyl-cinnamic acids, or by reduction of hydroxyl-cinnamic acids have a
364 lower antimicrobial activity compared to the substrates (Sanchez-Maldonado et al.,
365 2011). The metabolism of phenolic acids by lactic acid bacteria, including lactobacilli
366 isolated from ting, was thus considered a mechanism of detoxification of inhibitory
367 compounds in plants (Sanchez-Maldonado et al., 2011).

368 In conclusion, this study elucidated properties of sorghum sourdoughs that select for
369 different fermentation microbiota when compared to wheat sourdoughs. Sorghum
370 sourdough contains active concentrations of antimicrobial phenolic compounds, and
371 offers glucose as major carbon source. In contrast, wheat sourdough does not contain
372 antimicrobial compounds and offers maltose as a major carbon source. These results
373 facilitate the selection of starter cultures in developing countries, which not only
374 necessitates consideration of the different economical and societal environment, but also
375 the use of different cereals or pseudocereals as substrate (Holzapfel, 2002; Nout, 2009).
376 Owing to the high content of phenolic compounds with antioxidant properties, and the
377 lack of gluten proteins with toxicity to celiac patients, sorghum and millet are of
378 increasing importance in developed countries (Taylor et al., 2006). The adaptation to
379 substrates rich in phenolic compounds makes lactobacilli from traditional sorghum
380 fermentations also a suitable source for competitive starter cultures in other cereals or
381 pseudocereals which are rich in phenolic compounds, e.g. millet or buckwheat (Moroni et
382 al., 2009).

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505

506 **Figure legends**

507 **Figure 1:** Maltose (Panel A and B) and glucose (Panel C and D) concentrations during
508 sourdough fermentation with sorghum (Panel A, C) and whole wheat (Panel B, D).
509 Doughs were fermented with *L. parabuchneri* (●), *L. harbinensis* (■), *L. coryniformis*
510 (▲), *L. casei* (▼) and or incubated after chemical acidification (❑). Results are shown as
511 means ± standard deviation of triplicate independent experiments.

512 **Figure 2.** Metabolite formation during growth of *L.harbinensis*, *L.casei*, *L. parabuchneri*
513 or *L. coryniformis*, after 24 h fermentation at 34°C in sorghum sourdoughs, sorghum
514 sourdough supplemented with 2% maltose, and whole wheat sourdoughs. 1.2 Prop,
515 1,2-propanediol; 1.3 Prop, 1,3-propanediol. Results are shown as means ± standard
516 deviation of triplicate independent experiments.

517 **Figure 3:** Persistence of lactobacilli in wheat (Panel A and B) and sorghum (Panel C and
518 D) fermentations. Sourdoughs were inoculated with approximately 10^6 cfu/g of each of
519 the three strains, incubated at 28°C (closed symbols) or 34°C (open symbols) and back-
520 slopped every 24h with 10% inoculum. The contribution of the three strains to the total
521 bacterial counts was estimated by qPCR with species-specific primers (Panel A and C) or
522 by determination of cell counts and differential enumeration on the basis of the colony
523 morphology (Panel B and D). Symbols indicate *L. sanfranciscensis* (▲, Δ),
524 *L. parabuchneri* (●, ❍), *L.casei* (■, ❐), or cell counts of (*L. parabuchneri* + *L. casei*)
525 (◆, ◊), which could not reliably be differentiated on the basis of their colony
526 morphology. Results are shown as means ± standard deviation of triplicate independent

527 experiments analysed in duplicate. Lines dropping below the x-axis indicate values below

528 detection limit [1% of total cell counts or -2.5 log (% of total gene copy numbers)].

529

530 **Table 1:** Primer sequences used for species-specific quantitative PCR

Target organism (ref)	**Primer name and sequence (5'-3')**		**Tm (°C) / size (bp) of amplicon**
L. casei group (1)	LCgF: LCgR:	ATCATGGAATTGATGGATACCA TAGACTTGATAACATCTGGCTT	55 / 202
L. parabuchneri (2)	L.paraF: 23S-7R:	GCACAGACCGGAGTAACA GGTACTTAGATGTTTCAGTTC	63 / 480
L. sanfranciscensis (3)	L.sanF: 23S-7R:	GTCGGTTTTGAATATTAT GGTACTTAGATGTTTCAGTTC	63 / 411

531 (1) Sheu et al., 2009; (2) Coton et al., 2008; (3) Valcheva et al., 2007
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533 **Table 2:** Glycerol concentration in sorghum flours after sample pretreatment, and 1,2-
534 propanediol and 1,3-propanediol concentration in sorghum sourdoughs after fermentation
535 with *L. coryniformis* or *L. parabuchneri*.

Sorghum variety	Substrate /metabolite concentration (mmol / L)		
	Glycerol	1,3 propanediol[a)]	1,2 propanediol[b)]
Canadian	12.1±0.8	5.8 ± 0.3	4.8 ± 0.3
PAN3860	2.0±0.4	4.1 ± 0.2	3.4 ± 0.6
Segaolane	2.6±0.3	4.4 ± 0.2	3.6 ± 0.1
Town	1.5±0.3	4.1 ± 0.2	2.7 ± 0.3

536 [a)] Fermentation for 24h with *L. coryniformis*
537 [b)] Fermentation for 24h with *L. parabuchneri*
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539 **Table 3:** Minimum inhibitory concentration (MIC) of extracts of phenolic compounds
540 from four sorghum varieties. *L. sanfranciscensis, L.parabucheri,* and *L.casei* were used
541 as indicator strains.

Sorghum varieties	Strains	Arbitrary Unit(AU)[a)]
Canadian white	*L. sanfranciscensis*	1.3 ± 0.5
	L. parabuchneri	NI
	L. casei	NI
PAN3860	*L. sanfranciscensis*	270 ± 120
	L. parabuchneri	4.3 ± 1.8
	L. casei	5.3 ± 1.8
Segaolane	*L. sanfranciscensis*	140 ± 60
	L. parabuchneri	1.3 ± 5
	L. casei	1.9 ± 1.2
Town	*L. sanfranciscensis*	170 ± 60
	L. parabuchneri	5.3 ± 1.8
	L. casei	5.3 ± 1.8

542 [a)] Antimicrobial activity of sorghum extracts is expressed as arbitrary units (AU),
543 corresponding to the highest dilution factor of extracts inhibiting bacterial growth.
544 NI, no inhibition
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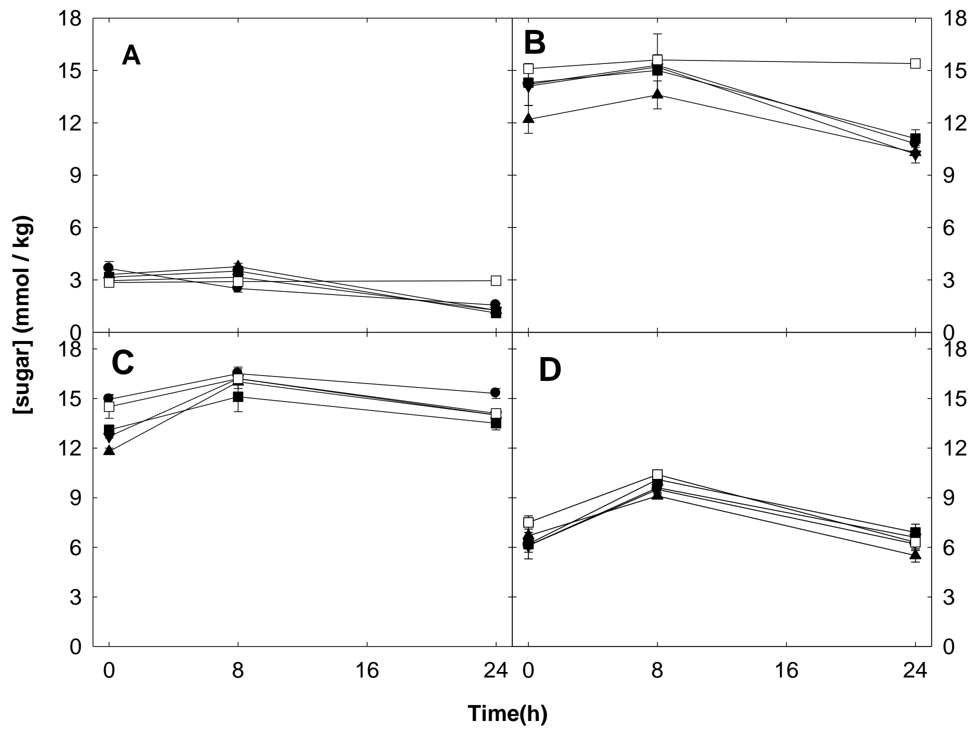

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557 **Figure 1.** Sekwati-Monang et al.
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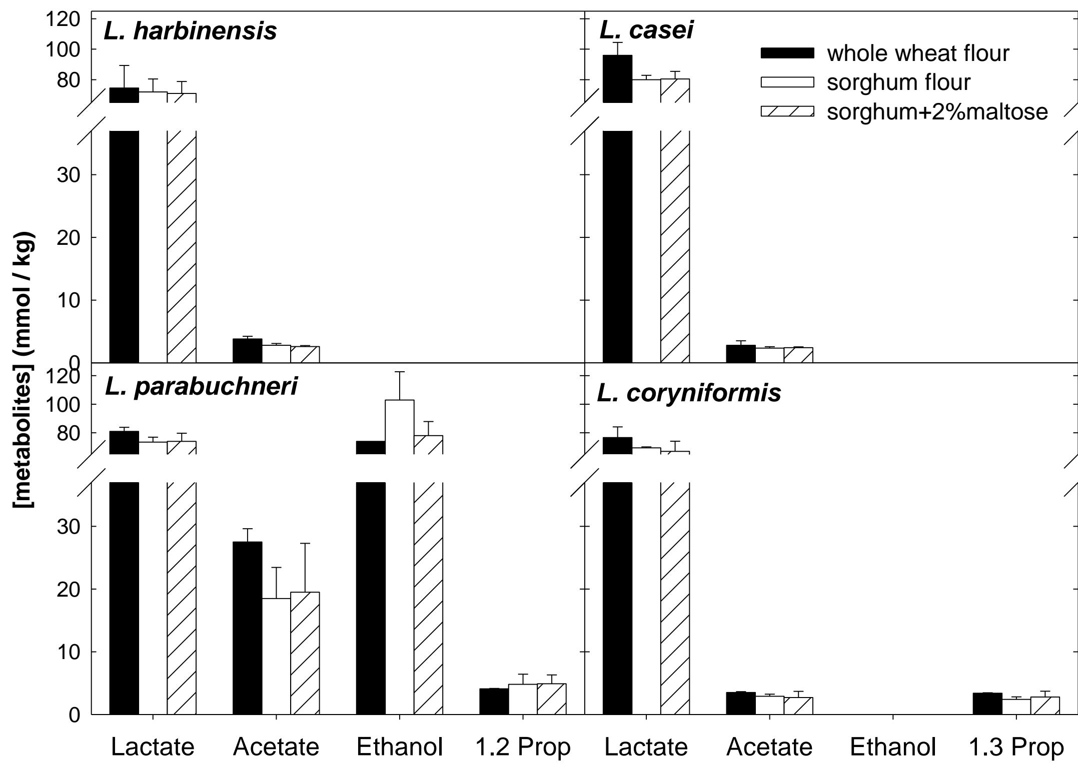

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576 **Figure 2.** Sekwati-Monang et al.
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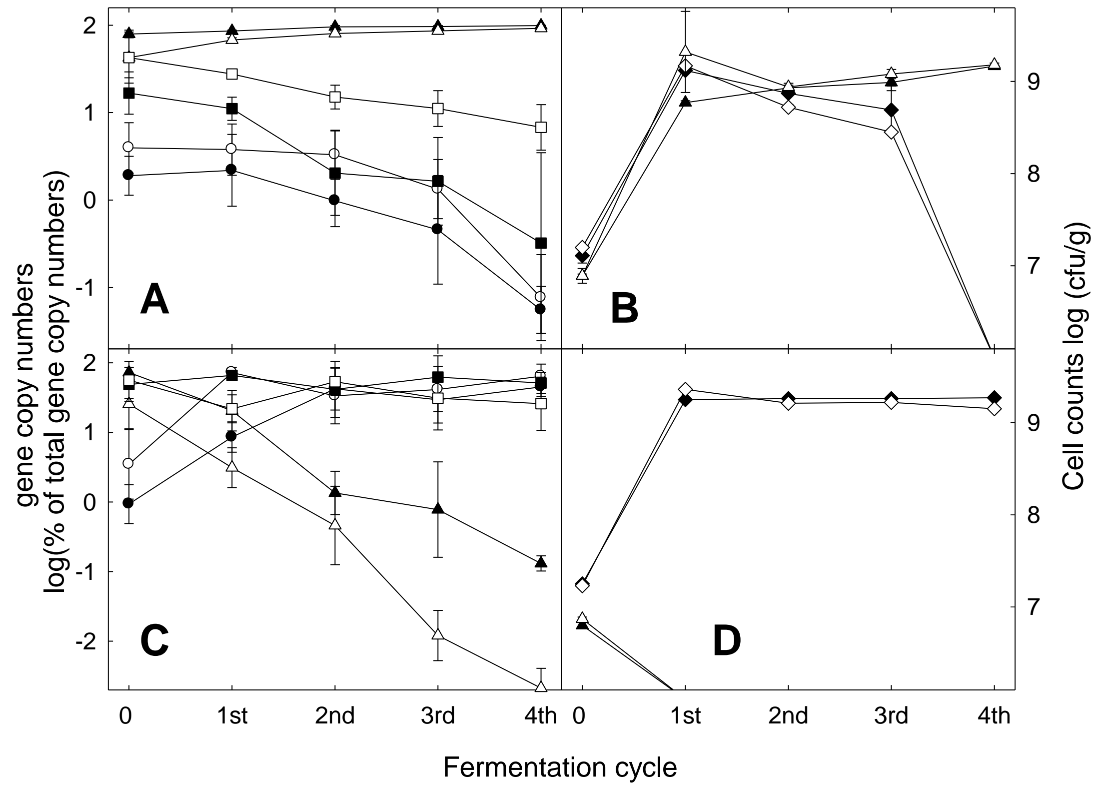

595 **Figure 3.** Sekwati-Monang *et al.*

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