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1 **Sourdough bacterial dynamics revealed by Metagenomic analysis in Brazil**

2

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13

14 **Abstract** This study dealt with the influence of the temperature on the bacterial dynamics
15 of two spontaneously fermented wheat sourdoughs, propagated at 21 ± 1 ºC (SD1) and
16 30 ± 1 ºC (SD2), during nine backslopping steps (BS1 to BS9). *Proteobacteria* was the
17 only phylum found in flour. *Escherichia hermannii* was predominant, followed by
18 *Kosakonia cowanii,* besides species belonging to the genera *Pantoea* and *Pseudomonas.*
19 After one step of propagation, *Clostridium* and *Bacillus cereus* group became
20 predominant. *Lactobacillus curvatus* was found at low relative abundance. For the second
21 backslopping step*,* *Clostridium* was flanked by *L. curvatus* and *Lactobacillus farciminis*.
22 From BS4 (6th day) onward, lactic acid bacteria (LAB) became predominant. *L. farciminis*
23 overcame *L. curvatus* and remained dominant until the end of propagations for both
24 sourdoughs. At 21 ºC, *Bacillus*, *Clostridium*, *Pseudomonas,* and *Enterobacteriaceae* were
25 gradually inhibited. At the end of propagation, SD1 harbored only LAB. Otherwise, the
26 temperature of 30 ºC favored the persistence of atypical bacteria in SD2, as *Pseudomonas*
27 and *Enterobacteriaceae*. Therefore, the temperature of 21 ºC was more suitable for
28 sourdough propagation in Brazil. This study enhanced the knowledge of temperature's
29 influence on microbial assembly and contributed to the elucidation of sourdough
30 microbial communities in Brazil.
31

32 **Keywords**: fermentation; bacterial diversity; high-throughput sequencing; lactic acid
33 bacteria
34

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1. Introduction

Sourdough results from the fermentation of cereal flour and water, by a microbial consortium, composed mainly by lactic acid bacteria (LAB) and yeasts. The sourdough fermentation is known to contribute in several ways to the enhanced nutritional, sensorial and technological qualities of leavened bakery products, due mostly to the metabolic activity of its microbial community (De Vuyst et al., 2014; Gobbetti et al., 2018; Minervini et al., 2014). The dough is a nutrient-rich ecosystem for microbial growth. More than 80 LAB and 20 yeast species have been isolated around the world from mature sourdoughs. *Lactobacillus*, *Leuconostoc*, *Weissella*, *Saccharomyces,* and *Kazachstania* are the most frequent genera described (Gänzle and Ripari, 2016; Gobbetti et al., 2016; Van Kerrebroeck et al., 2017).

Traditional sourdoughs require continuous steps of fermentation (backslopping). The first dough prepared using flour and water is spontaneously fermented at room temperature. Posteriorly, this fermented dough will be used as inoculum for fermenting a new dough in the subsequent step. This procedure is repeated five to ten times (Minervini et al., 2014; Siepmann et al., 2018). The sourdough microbial consortia evolves from the first fermentation and through the backslopping steps, resulting in both successions of microbial populations and alteration of metabolic patterns until the microbiota becomes stable. This dynamics is affected by numerous endogenous and exogenous factors, such as flour type and origin, environmental microbiota, process parameters (*e.g.* temperature, redox potential, refreshment time, number of propagation steps) and interactions between the microbial consortium (De Vuyst et al., 2014; Gobbetti et al., 2016; Minervini et al., 2014; Van Der Meulen et al., 2007; Vogelmann and Hertel, 2011a).

The positive effects of LAB on sensorial and nutritional quality of sourdough bread has been demonstrated in many studies (Arendt et al., 2007; Corsetti and Settanni, 2007; Gänzle and Ripari, 2016; Gänzle et al., 2008, 2007; Gobbetti et al., 2014; Katina et al., 2005; Pétel et al., 2017; Poutanen et al., 2009; Torrieri et al., 2014). Beyond these aspects, research on sourdough has been advancing in order to investigate the functional features of bread. For instance, the production of nutritionally active compounds, such as γ-amino butyric acid (GABA) and potentially prebiotic exo-polysaccharides (Gobbetti et al., 2014) and the reduction of gluten immunogenicity through enzymatic degradation by microbial proteases (Curiel et al., 2013; De Angelis et al., 2010; Heredia-Sandoval et al., 2016). Moreover, the use of sourdough in bakery production has potentiality to reduce

74 the Irritable Bowel Sindrome (IBS) and the Non-Celiac Gluten Sensitivity (NCGS)
75 symptoms (Menezes et al., 2018; Muir et al., 2019). The degradation of fructan and other
76 FODMAPs (Fermentable, Oligo-, Di-, Monosaccharides and Polyols) implicated in
77 triggering the symptoms of IBS and NCGS was recently demonstrated during sourdough
78 fermentation reported in our previous study (Menezes et al., 2019).

79 Sourdough proved to be an inexhaustible source of microbial species in the
80 countries where it has been studied. Although broadly investigated in European countries,
81 USA, and most recently in Asian countries (Corsetti and Settanni, 2007; De Vuyst et al.,
82 2014; Gobbetti, 1998; Lattanzi et al., 2013; Lhomme et al., 2015; Liu et al., 2016;
83 Ventimiglia et al., 2015), the microbial diversity of sourdoughs has not yet been
84 characterized in Brazil. The geographic origin and the propagation temperature have been
85 shown to exert a strong influence on LAB diversity (Pontonio et al., 2015; Scheirlinck et
86 al., 2007). Uncovering the correlation between microbial species and their role in a
87 specific ecosystem remains one of the main objectives of microbial ecology (Morales and
88 Holben, 2011).

89 Regarding sourdough, knowledge about the fermenting microbial consortia
90 contributes to the understanding of its influence on the bread quality. The
91 interdependence between process parameters and bacterial dynamics is a field of interest
92 for the bakery industry, since standardization of bread quality is dependent on the
93 microbial community (Gobbetti et al., 2016; Menezes et al., 2019). Thus, this study aimed
94 at unraveling how temperature changes during propagation may affect the dynamics of
95 the bacterial ecosystem during the propagation of sourdoughs in Brazil. With the aim to
96 lead to the standardization of sourdough fermentation performance, allowing its safe and
97 controllable use, this research is a step forward the elucidation of the microbial succession
98 and the factors that affect it.

99

100 **2. Material and methods**

101

102 2.1 Sourdough propagation

103 Sourdoughs were made at the Bakery Pilot Plant of the Federal University of Santa
104 Catarina following traditional protocol for sourdoughs type I. Organic refined wheat flour
105 (Paullinia company, Marechal Cândido Rondon, Paraná, Brazil) and mineral water [1:1
106 (w/w)] were mixed with a resulting dough yield [(dough mass/flour mass) × 100] of 200
107 (Figure 1). The first fermentation was carried out at 24 °C for 48 h (backslopping one -

BS1). Successively, eight backslopping steps (BS2 to BS9) were carried out. In each one, a portion of the previously fermented dough (FD) was harvested and used as an inoculum for the subsequent step, mixed with wheat flour and water [FD:water:wheat flour (1:2:2 w/w)]. The mixture was incubated at 24 °C for 48 h at BS2 and 24 h at BS3 and BS4. Thereafter, the FD was fractionated in two portions; the first one was incubated at 21 ± 1 ºC (SD1) and the second one at 30 ± 1 ºC (SD2). The temperature was modified during propagation in order to evaluate how the bacterial community could be affected in case of temperature change, starting from the same sample, and considering that, the artisanal sourdough propagation is subject to temperature variations. Finally, the BS5 to BS9 were carried at 12 h intervals. The time (hours) elapsed between each backslopping was set based on the sourdough ability to double its size. At the beginning, the leavening activity was still low, so the time was longer. At the end, with high metabolic activity, the fermentation time was reduced to 12 hours. The fermentations were carried out in a Biochemical Oxygen Demand (BOD) Refrigerated Incubator (MA 403 Marconi, Piracicaba, São Paulo, Brazil) with temperature control.

Insert Figure 1 here.

2.2 Microbial enumeration and bacterial isolation

Ten grams of the flour and BS samples were homogenized by adding 90 mL 0.1% ($w.v^{-1}$) of sterile peptone solution using a vortex. A 10-fold dilution series were made and plated in the culture media presented in Table 1. The results were expressed as log $CFU.g^{-1}$. A total of 100 colonies were randomly picked from the plates, cultivated in respective broth media and re-streaked onto the same agar medium to check the purity. Posteriorly, the isolates were lyophilized (LT1000, Terroni, São Carlos, Brazil) for 24 h (90 µHg of vacuum), before fingerprinting and identification. The isolates were cultured in the respective origin medium and incubated overnight. The cultures (1 mL) were centrifuged (10,000 *g*, 10 min) and the DNA was extracted using the Genomic Wizard DNA Purification Kit (Promega Corp., Madison, WI, USA) and stored at -20 ºC. Total genomic DNA of the flour, doughs after the backslopping steps and mature sourdoughs was extracted directly from 1 g of the samples using the DNeasy Blood & Tissue kit (Qiagen, Venlo, Netherlands). DNA was eluted into DNase- and RNase-free water and concentration and purity were determined using a NanoDrop spectrophotometer (model

141 2000, ThermoFisher Scientific Inc, Waltham, Massachusetts, EUA). DNA was diluted up
142 to 50 ng μl^{-1} and stored at -20 ºC.
143
144 Insert Table 1 here.
145
146 2.3 Metagenomic analysis
147 The total DNA extracted from the flour and sourdough samples was used as
148 template for 16S metagenomic analysis, which was performed by Neoprospecta
149 Microbiome Technologies (Florianópolis, Brazil) using the Illumina MiSeq platform
150 (Illumina Inc., San Diego, California). Library preparation was performed using
151 Neoprospecta´s NGS Procotol (Christoff et al., 2017). Briefly, the V3-V4 hypervariable
152 region of the 16S rRNA gene was amplified with primers 341F
153 (CCTACGGGRSGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso
154 et al., 2011; Wang and Qian, 2009). The PCR reaction was carried out in triplicates using
155 Platinum Taq Polymerase (Invitrogen, USA) with the following conditions: 95 °C for 5
156 min, 25 cycles of 95 °C for 45 s, 55 °C for 30 s and 72 °C for 45 s and a final extension
157 of 72 °C for 2 min. Library preparation (attachment of TruSeq adapters, purification with
158 AMPureXP beads and qPCR quantification) was performed using Illumina 16S Library
159 Preparation Protocol (Illumina Technical Note 15044223 Rev. B). Sequencing was
160 performed using MiSeq Reagent Kit v3 with 2x300 bp paired-end reactions.
161
162 2.4 Bioinformatics
163 Sequencing data for each sample was processed on Quantitative Insights into
164 Microbial Ecology (Qiime) software package (Caporaso et al., 2010). Initially, the
165 sequencing output was analyzed by a read quality filter, which removed reads with an
166 average Phred score < 20 followed by a clustering of 100% identical reads. In order to
167 remove putative chimeric sequences, clusters with less than 5 reads were excluded from
168 further analysis. The remaining good-quality sequences were further clustered at 97%
169 similarity to define operational taxonomic units (OTU). Classification of OTUs was made
170 by comparing them with a custom 16S rRNA database (NEORefDB, Neoprospecta
171 Microbiome Technologies, Brazil). Sequences were taxonomically assigned with at least
172 99% identity in the reference database. In order to evaluate the microbial community
173 shifts among samples, OTUs were summed up into the same genera and the relative
174 abundance of each genus was compared with a heat-map on Qiime.

175

176 2.5 Length heterogeneity-PCR (LH-PCR)

177 Total DNA extract from the isolates and SD samples were analyzed following the
178 LH-PCR amplification as described by Savo Sardaro et al. (2018) to better understand the
179 bacterial succession ecology through the backslopping steps. Domain A of the variable
180 regions of the 16S rRNA gene from extracted DNA was amplified. The forward primer,
181 63F (5'-CAGGCCTAACACATGCAAGTC-3' was 5' end labeled with the
182 phosphoramidite dye 6-FAM and the reverse primers used were 355R (5'-GCT GCC
183 TCC CGT AGG AGT-3') (Applied Biosystems Inc., Foster City, USA). In each PCR
184 amplification, 1 µl of extracted DNA was added to 19 µl of the amplification mixture,
185 resulting in a final concentration of 1X Taq Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs,
186 0.2 mM of each primer, and 1U of Taq DNA polymerase (Promega), in a final reaction
187 volume of 20 µl. PCR conditions were as follows: an initial denaturation at 95 °C for 5
188 min, 25 cycles of denaturation at 95 °C for 30 s; different annealing temperature were
189 used (59 °C for SD and 63 °C and 65 °C bacteria strain) for 30 s; elongation at 72 °C for
190 1 min 30 s, and a final extension step at 72 °C for 7 min. PCR products amplified were
191 diluted 15 time fold for subsequent fragment analysis as described below. Capillary
192 electrophoresis (ABI Prism 310, Applied Biosystems, Foster City, USA) were performed
193 according to Bottari et al. (2010). Each peak on the electropherogram profile corresponds
194 to an amplicon with specific length (in base pairs, bp). The obtained lengths from the
195 strains were used as a reference to identify the species corresponding to single peaks in
196 the LH-PCR profile of the SD bacterial population.

197

198 2.6 Repetitive element palindromic-PCR (REP-PCR)

199 The rep-PCR was performed using DNA extracted from the 100 isolated strains.
200 PCR reactions were performed according to Perin et al. (2017), using a single primer
201 (GTG)5 (5'-GTGGTGGTGGTGGTG-3'). The PCR reactions contained 10 mL of Go
202 Taq Master Mix 2x (Promega, Madison, Wisconsin, EUA), 50 pMol of the primer, 2 mL
203 of DNA (50 ng/mL) and ultra-pure PCR water (Promega) to a final volume of 20 mL.
204 The PCR conditions were: 95 ºC for 5 min, 30 cycles at 95 ºC for 30 s; 40 ºC for 45 s; 65
205 ºC for 8 min; and final extension at 65 ºC for 16 min. The PCR products were
206 electrophoresed on agarose gels (2% w/v) in tris/borate/EDTA buffer (TBE) at constant
207 voltage (95 V) for 3 h. A 1 kb DNA ladder (Sigma-Aldrich, St. Louis, Missouri, EUA)
208 was used as a molecular size marker. Fingerprints were compared by cluster analysis

using BioNumerics 6.6 (Applied Maths, Sint-MartensLatem, Belgium). Similarities between the strains profiles were calculated using the Dice correlation coefficient and dendrograms constructed by cluster analysis (unweighted pair group method with arithmetic mean, UPGMA).

2.7 Bacterial identification

Based on rep-PCR profiles and similarities, 41 isolates were selected and subsequently identified by 16S rRNA sequencing using the primers forward 46F (GCYTAACACATGCAAGTCGA) and reverse 536R (GTATTACCGCGGCTGCTGG) (Kaplan and Kitts, 2004). The PCR reactions consisted of 10 mL of Go Taq Master Mix 2x (Promega), 10 pMol of each pair of primers, 1 mL of DNA (50 ng/mL) and ultra-pure PCR water (Promega) to a final volume of 20 mL. DNA amplification and sequencing were performed according to Perin et al. (2017), and each sequence obtained was checked manually and searched for sequence homology using the basic local alignment search tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.8 Statistical analysis

The values of bacterial enumeration in each culture media were subjected to one-way ANOVA; pair-comparison of treatment means was obtained by Tukey's procedure at $p < 0.05$, using the statistical software *Statistica* 11.0 (StatSoft Inc., Tulsa, USA). The effect of temperature incubation on SD1 and SD2 samples was evaluated independently by a Pearson correlation test. In this analysis, the absolute abundance of *Lactobacillus* and *Lactococcus* (namely “Lacto” group) in each SD was tested for correlation against genera *Bacillus, Pseudomonas*, *Clostridium*, *Escherichia*, *Enterococcus* and *Enterobacter* in that sample. A significant effect was considered on $p < 0.05$.

3. Results

3.1 Microbial enumeration and bacterial identification

The presumptive LAB counts in mMRS for flour were 3.0 ± 0.1 log CFU.g^{-1} (Table 2). After BS1, cell density of presumptive LAB in mMRS increased significantly to 5.7 ± 0.1 log CFU.g^{-1}. The counts reached 7.1 ± 0.0 log CFU.g^{-1} for BS2. For SD1, for BS5, the cell density reached 7.5 ± 0.1 log CFU.g^{-1} and stayed almost constant during the subsequent propagations, despite a slight fluctuation in BS8. For SD2, from BS5 onward

243 there was no statistical difference between counts in mMRS. In general, counts of viable
244 microorganisms were lower in the other culture media and evolved more slowly, reaching
245 above 7.0 log CFU.g^{-1} only from BS6 for Wheat Flour Agar Medium (WFAM) and
246 Sourdough Agar Medium (SDAM).
247
248 Insert Table 2 here.
249
250 Clusterization by LH-PCR and REP-PCR (Table 3) of the 100 randomly selected
251 colonies were used to classify and select those that would belong to different species and
252 would be sequenced. Only a small part of the sourdough population could be recovered
253 by the culture-dependent method, a quite homogeneous population, with 11 biotypes.
254 Each biotype was taxonomically characterized through 16S rRNA gene partial
255 sequencing. The LAB isolated belonged to the species *Lactobacillus farciminis*,
256 *Lactobacillus brevis*, *Lactococcus lactis, Leuconostoc citreum* (two biotypes),
257 *Enterobacter hormaechei/cloacae, Enterococcus gilvus, Enterococcus hirae*,
258 *Enterecoccus durans, Enterococcus faecium* and *Enterococcus faecalis*. *Ec. faecium* and
259 *L. brevis* were the most dominant species. While some species of *Enterococcus* were
260 present variably, *L. brevis* was found from BS2 and persisted until the final propagation
261 step. *Lc. lactis* was isolated from BS2 to BS4 and persisted only for SD2, until BS7. *Eb.*
262 *hormaechei/cloacae* was recovered from BS1 and BS6. The first biotype of *Ln. citreum*
263 was recovered from BS5 of SD2; the second one was isolated from BS7 and BS8 from
264 SD2 and SD1, respectively. *L. farciminis* was isolated only in BS8 and BS9, in both SD.
265
266 Insert Table 3 here.
267
268 2.3 Metagenomic analysis
269 DNA extracted from the flour and sourdough samples was used as template for
270 16S metagenomics analysis to describe the bacterial diversity (Figure 2). The flour
271 microbial consortium was composed of thirteen different species belonging to
272 *Proteobacteria* phylum. *Escherichia hermannii* (relative abundance of 43.56%) was
273 predominant, followed by *Kosakonia cowanii* (20.21%), and *Pantoea ananatis* (18.85%).
274 *Pseudomonas rhodesiae* (5.10%), *Pseudomonas tolaasii* (2.90%), *Pantoea agglomerans*
275 (2.42%), and *Pseudomonas fluorescens* (2.24%) were also present. After the BS1, twenty-
276 three species were found. *Firmicutes - Clostridium saccharobutylicum* (29.62%),

277 *Clostridium beijerinckii* (19.55%), *Clostridium aurantibutyricum* (15.96%) and *Bacillus*
278 *cereus* group (12.44%) became predominant. *E. hermannii* (7.45%), and *K. cowanii*
279 remained representative (5.02%). *Lactobacillus curvatus* (1.17%), *Lc. lactis* (0.07%), *Ln.*
280 *citreum* (0.02%) and *Pediococcus pentosaceus* (0.02%) were found, however with low
281 relative abundance. *Pseudomonas* corresponded to 1.19%, *Enterococcus* and
282 *Enterobacter* 0.25%. From BS2, the genus *Clostridium* was flanked by LAB. The dough
283 was dominated by *L. curvatus* (37.46%), *C. saccharobutylicum* (25.07%), and *L.*
284 *farciminis* (10.21%). *E. hermannii* (4.73%) and *K. cowanii* (3.33%) were still present.
285 Other seven LAB species were found – *Lc. lactis* (1.11%), *Lactobacillus graminis*
286 (0.33%), *Lactobacillus kimchiensis* (0.18%), *Lactobacillus plantarum* (0.16%),
287 *Lactococcus garvieae* (0.12%), *L. brevis* (0.09%) and *Lactobacillus sakei* (0.02%) – as
288 well as *Enterococcus*, *Enterobacter*, and *Pseudomonas* (0.82%, 0.25% and 0.3%,
289 respectively).
290
291 Insert Figure 2 here.
292
293 From BS4, twenty-nine species were found. The dough was dominated by LAB.
294 The relative abundance of *L. curvatus (*42.36%) and *L. farciminis* (44.07%) were higher
295 compared to earlier steps. *C. saccharobutylicum* (2.51%), *E. hermannii* (2.31%) and *K.*
296 *cowanii* (1.04%) were still found, but at lower relative abundance than in the previous
297 BS. *Pseudomonas* and *Pantoea* corresponded to 0.16% and 0.59%, respectively. For BS5,
298 SD1 was dominated by *L. farciminis* (85.39%) and *L. curvatus* (11.28%). Among the
299 other seventeen species detected, only *Ln. citreum* (0.55%), *Pd. pentosaceus* and *E.*
300 *hermannii* (0.54%) were found with relative abundance higher than 0.5%. *L. brevis*
301 (0.10%), *L. graminis* (0.12%), *L. kimchiensis* (0.38%), *Lactobacillus nantensis* (0.08%)
302 were present at low incidence. Among the seven species of the genus *Clostridium* present
303 in BS2, only *C. aurantibutyricum* (0.05%) and *C. beijerinckii* (0.02%) remained. *B.*
304 *cereus* group, *Enterococcus*, and *Enterobacter* were inhibited. Fifteen different species
305 were detected at BS7, for SD1. The dough was dominated by *L. farciminis* (78.30%),
306 followed by *L. curvatus* (16.03%), *Ln. citreum* (3.31%) and *Pd. pentosaceus* (1.11%).
307 The same sub-dominant LAB species detected in BS5 were found in BS7 but in slightly
308 lower proportions. The genus *Clostridium* was inhibited. *Eb. cloacae* (0.3%), *E.*
309 *hermannii* (0.46%), *K. cowanii* (0.14%), *P. fluorescens* (0.06%) and two species of the
310 genus *Pantoea* - *Pantoea vagans* and *Pa. ananatis* (both with 0.07%) were the

311 *Proteobacteria* found. At BS9, SD1 harbored eleven species. *L. farciminis* (89.39%) and
312 *L. curvatus* (8.13%) were still predominant. No *Bacillus, Pseudomonas, Enterococcus*,
313 and *Enterobacteriaceae* were found. *Ln. citreum* (0.97%), *Pd. pentosaceus* (0.52%), *L.*
314 *brevis (0.03%)*, *L. futsaii* (0.04%), *L. kimchiensis* (0.4%), *L. nantensis* (0.1%) were
315 detected at low incidence.

316 On the other hand, the higher temperature altered the microbial dynamics for SD2.
317 For the BS5, twenty-one species were found. *L. farciminis* (40.34%) and *L. curvatus*
318 (35.31%) co-dominated the dough. Other nine species were found with relative
319 abundance higher than 0.5% - *E. hermannii* (4.73%), *L. brevis* (4.55%), *K. cowaniii*
320 (2.21%), *L. graminis* (1.62%), *Pa. ananatis* (1.04%), *L. kimchiensis* (0.88%), *L.*
321 *plantarum* (1.18%), *Ln. citreum* (0.87%), and *Pd. pentosaceus* (0.50%). *L. lactis* were
322 detected at low concentrations at BS5 (0.28%) and BS7 (0.25%). *B. cereus* group,
323 *Enterococcus*, and *Enterobacteriaceae* were inhibited as for SD1. *C. aurantibutyricum*
324 and *C. beijerinckii* were inhibited at BS7 and BS9, respectively. Three species of the
325 genera *Pseudomonas* – *P. fluorescens, P. rhodesiae,* and *P. tolaasii* – and two of *Pantoea*
326 – *Pa. agglomerans,* and *Pantoea dispersa* were found at relative abundances below 0.4%.
327 For BS7, *L. farciminis* remained predominant (65.68%). However, the relative abundance
328 of *L. curvatus* was drastically reduced (7.19%), and *E. hermannii* went on to sub-
329 dominate the dough (11.16%). Other twenty-tree species were detected, including *K.*
330 *cowanii* (5.32%), *Pa. ananatis* (2.33%), *L. brevis* (1.6%), *Pa. agglomerans* (1.06%), *P.*
331 *fluorescens* (0.84%), *L. kimchiensis* (0.59%), and *Ln. citreum* (0.5%). For BS9, SD2 was
332 dominated by *L. farciminis* (64.06%) and *E. hermannii* (17.58%). Among the sub-
333 dominant LAB detected in previous steps, only *L. brevis* (2.62%), *L. kimchiensis* (0.28%),
334 and *Pd. pentosaceus* (0.28%) were found. The dough harbored thirteen different species.
335 The other species were *K. cowanii* (6.97%), *Pa. ananatis* (2.67%), *P. fluorescens*
336 (1.34%), *P. tolaasii* (1.06%), *Pa. agglomerans* (1.01%), *Erwinia persicina* (1.00%), *P.*
337 *rhodesiae* (0.61%), and *Lelliottia amnigena* (0.28%).

338 The distribution of each genus during the BS was shown in Figure 3. A total of 22
339 genera were found for SD1 (Figure 3A) and 25 for SD2 (Figure 3B), belonging to the
340 phyla *Proteobacteria*, *Firmicutes*, *Fusobacteria* and *Actinobacteria*, of which the first
341 two were the most relevant, grouped in the upper parts of each heat-map. For SD1, the
342 LAB group was distributed from BS2 to BS9. *Enterococcus*, *Clostridium*, *Bacillus*,
343 *Pseudomonas*, and the family of *Enterobacteriaceae* (*Escherichia, Kozakonia, Erwinia,*
344 *Enterobacter* and *Pantoea*) were more present from BS1 to BS4, having the numbers of

345 sequences reduced as the propagation evolved. For SD2, the highest number of sequences
346 of LAB was observed from BS2 to BS7. *Enterococcus*, *Clostridium* and *Bacillus* were
347 predominantly found from BS2 to BS4, and reduced for the subsequent BS, as well as
348 observed for SD1.
349
350 Insert Figure 3A and 3B here
351
352 On the other hand, the genus *Pseudomonas* and the group of *Enterobacteriaceae*
353 presented a wide distribution during the propagation, including for the final BS. The
354 Pearson correlation coefficient (Table 4) showed a significant negative correlation
355 between the "Lacto" group *versus* the genera *Bacillus*, *Clostridium*, *Escherichia*, and
356 *Pseudomonas*, for SD1. These genera were found to decrease with the increasing of
357 "Lacto" group relative abundance. For SD2, significant correlation was observed only for
358 the six combined genera, effect that was also observed for SD1. There was no significant
359 relationship between "Lacto" and "Entero" groups. However, the relative low number of
360 *Enterococcus* and *Enterobacter* sequences detected from SD1 to SD4 may explain any
361 unobserved relationship.
362
363 Insert Table 4 here.
364
365 **4. Discussion**
366
367 Temperature is one of the main parameters that influence the final microbiota
368 composition of sourdoughs (Minervini et al., 2014; Vrancken et al., 2011). This is the
369 first study to our knowledge in which spontaneous sourdough fermentation was followed
370 in Brazil, a country with great climatic diversity. In general, the climate is warm in almost
371 all the territory, with average temperatures above 18 ºC in all months of the year in most
372 states (Brasil, 2002). Two common temperatures in tropical climates were selected for
373 sourdough preparation, and had important implications on the microbial dynamics,
374 especially for the subdominant microflora.
375 Low counts were found in the flour, and no LAB isolates could be retrieved. The
376 community profiles obtained with pyrosequencing procedure did not detect LAB in the
377 flour, in accordance with its isolates. Before fermentation, low colony counts were found;
378 however, LAB numbers rapidly increased after the BS1. From BS5 onward, the counts

379 were stable for both SD. Other studies (Bessmeltseva et al., 2014, Coda et al., 2018;
380 Ercolini et al., 2013) also described a rapid increase in bacterial counts for the first BS,
381 followed by a relative stabilization. The largest bacterial numbers were found on mMRS,
382 WFAM and SDAM. This finding can be explained by the presence of maltose as source
383 of fermentable carbohydrate in these media. Although some statistically significant
384 differences were detected, it was not possible observing a temperature effect on the cell
385 viable numbers. The final counts for both SDs were slightly below the number usually
386 described in the literature (8 to 9 log CFU/g). However, other authors also reported counts
387 close to 7 log CFU/g for mature sourdoughs (Fujimoto et al., 2019; Liu et al., 2016;
388 Michel et al., 2016; Minervini et al., 2015). The pH and total titratable acidity (TTA)
389 values in BS9 (Menezes et al., 2018) were within the expected values for traditional
390 sourdoughs (De Vuyst and Neysens, 2005; Ventimiglia et al., 2015) indicating a good
391 progress of the fermentation.

392 *Proteobacteria* was the only phylum found in flour. Bacteria belonging to this
393 phylum usually composes the microbial community of wheat (Donn et al., 2015; Yin et
394 al., 2017). *Pseudomonas*, *Pantoea, Kozakonia*, and *Enterobacter*, commonly prevalent in
395 wheat flour worldwide (Celano et al., 2016; Ercolini et al., 2013), were isolated from
396 Brazilian wheat seeds (Stets et al., 2013). LAB were initially detected from BS1 and BS2.
397 This aspect has been also considered by Alfonso et al. (2017) that showed as lactobacilli
398 constituted the lower abundance members of the kernels, ears and semolina microbiota.
399 Monitoring LAB from field until the first step of propagation, the authors observed that
400 some strains of lactobacilli were only detected after the first fermentation. This can lead
401 the LAB to be present in the flour in concentrations below the detection limits of the
402 metagenomic analysis. Furthermore, although flour can drive the microbial diversity of
403 sourdough, along with technological parameters of production, the flour microbiota may
404 not be the main source of microorganisms. The house microbiota can also affects the
405 composition of LAB and is undoubtedly a critical parameter to establish the sourdough
406 ecosystem (Gobbetti et al., 2016; Minervini et al., 2015). LAB circulate in the bakery
407 environment, and can be found in the hands of bakers, air, and equipment. Indeed,
408 *Lactobacillus* was shown to be the genus with the highest adaptability to bakery
409 environment (Minervini et al., 2015; Scheirlinck et al., 2009).

410 Notwithstanding *Proteobacteria* is predominant in flour, this phylum is not found
411 often in mature sourdoughs (Ercolini et al., 2013). A succession between *Proteobacteria*
412 and *Firmicutes* occurs gradually from the first propagation to the second one (Weckx et

413 al., 2010b). Just one BS was able to completely turn the microbial community from
414 *Proteobacteria* to mainly *Firmicutes*. Among the species found in the flour, only *E.*
415 *hermannii* and *K. cowanii* persisted, possibly due to its ability to tolerate the biochemical
416 changes in the matrix. Commonly, *Enterobacteriaceae* grows in the first days of
417 propagation, and survives because of a certain tolerance for acid stress (Ercolini et al.,
418 2013). *B. cereus* is often found in cereals and wheat flour and are well adapted to the
419 bakery environment (Martínez Viedma et al., 2011; Oltuszak-Walczak and Walczak,
420 2013). Clostridia has quite efficient mechanisms in sugar uptake (Mitchell, 2016). These
421 features, coupled with the semi-anaerobic conditions and the availability of carbohydrates
422 certainly favored the codominance of this groups in BS1.
423 For BS4 the bacterial profile markedly changed, and *Lactobacilli* completely
424 dominated the SD. There was a marked decrease in pH from BS0 (6.26 ± 0.01) to BS4
425 (3.79 ± 0.01). TTA increased from 1.40 ± 0.13 to 13.85 ± 0.12 (Menezes et al., 2018).
426 Consequently, the highest concentrations of organic acids were found for these BS, as
427 reported in our previous study (Menezes et al., 2019). Acidification is deeply linked to
428 the assembly of the microbial consortia. The highest concentrations of organic acids
429 coincided with the exponential growth phase of the sourdough communities and are
430 associated with competitiveness between species (De Vuyst et al., 2014). Suppression of
431 *Pseudomonas* (Kiymaci et al., 2018; Nakai and Siebert, 2004), enterobacteria
432 (Skrivanova et al., 2006), *B. cereus* (Soria and Audisio, 2014) and clostridial groups
433 (Schoster et al., 2013; Thylin et al., 1995) is correlated with organic acids synthesis and
434 with a concomitant drop in pH. In turn, LAB are well adapted to the sourdough acid
435 (Corsetti et al., 2007; Corsetti and Settanni, 2007). From the BS2, the dough has become
436 more hostile to *Enterobacteriaceae* and *Clostridium* and more favorable to LAB. When
437 fermentation begins to occur under acidic conditions, evident after the BS2, the growth
438 of non-LAB bacteria is gradually inhibited. Thus, as the number of fermentation steps
439 increases, the LAB becomes more adapted to environmental conditions (Minervini et al.,
440 2014). By definition, LAB are predominant in mature sourdoughs (Gobbetti et al., 2016).
441 LAB can overcome other contaminating microbiota mainly by thriving under in
442 fermentation systems. Most of the LAB metabolic traits are, actually, adaptations that
443 contribute to its competitive advantage in the sourdough environment (Gänzle and Ripari,
444 2016). Synthesis of bacteriocins probably contributes with a selective advantage in a
445 microbial niche complex, such as sourdoughs (Vogel et al., 1993; Marques et al., 2017).
446 Similarly to organic acids, an increase in mannitol production was observed from BS2 to

447 BS4 (Menezes et al., 2019). Among LAB, only heterofermentative species are known to
448 convert fructose into mannitol, including *L. curvatus* and *Ln. citreum* (Otgonbayar et al.,
449 2011). The use of mannitol as external electron acceptors from fructose metabolism may
450 lead to an efficient equilibration of the redox balance enhanced energy generation. Their
451 production at the highest level during the first four to five days of propagation indicates
452 their contribution to the strains' competitiveness when the ecosystem was still being
453 established (Weckx et al., 2010a, 2010b).

454 The ecological concept of r- (copiotrophs) and K- (oligotrophs) selection can be
455 applied to the kinetics of a microbial population (Koch, 2001; Pianka, 1970).
456 Microorganisms classified as r-strategist show fast growth in environments with abundant
457 nutrients, which are rapidly exploited, in its turn, k-strategists grow more slowly but using
458 the limited resources more efficiently, are capable of surviving long periods of starvation
459 (Fierer et al., 2007). Gram-negative bacteria and *Proteobacteria* are within the
460 copiotrophic category, while Gram-positive bacteria are oligotrophic (Zhou et al., 2017).
461 As for soil (Bastian et al., 2009; De Vries and Shade, 2013), the microbial communities
462 in sourdoughs would be dominated by copiotrophic (r-strategists) in the early stages,
463 while oligotrophs (K-strategists) increasing as the amount of substrate decreases in the
464 final backslopping steps. K-strategists are presumably more efficient users of
465 environmental resources that would be more competitive (Yang and Lou, 2011), and r-
466 strategists would be expected to be dominant under low-stress conditions (Vasileiadis et
467 al., 2015). This theory fits the dynamic observed on sourdough, with P*roteobacteria* and
468 Gram-negative as *Enterobacteriaceae* being overcome by LAB through BS as the
469 depletion of carbon sources, acidification, and redox potential make sourdough a stressful
470 environment.

471 The temperature plays a key role for the sourdough ecosystem assembly and
472 metabolite kinetics (Decock and Cappelle, 2005; Minervini et al., 2014; Vogelmann and
473 Hertel, 2011b; Vrancken et al., 2011). Vrancken et al. (2011) demonstrated that microbial
474 succession and the final composition of the microflora were different for temperature
475 variations between 23 and 30 ºC. Viiard et al. (2016) observed that the ratio of bacterial
476 species in rye sourdoughs propagated without temperature control was affected by the
477 seasonal temperature fluctuations. Notably, the bacterial community between SD1 and
478 SD2 differed over the final propagation steps. For SD1, LAB predominated while
479 *Clostridium*, *Pseudomonas, and Enterobacteriaceae (Pantoea, Enterococcus,*
480 *Enterobacter, K. cowanii* and *E. hermannii*) were gradually inhibited. From BS5 onward,

481 only minor changes on microbiota were observed, indicating achievement of a stable
482 microbial consortium, in agreement with the stabilization of the number of viable cells,
483 TTA and pH (Menezes et al., 2019). In contrast, although the number of viable cells was
484 stable from BS5 onward, the microbial community of SD2 remained unstable until BS9.
485 Therefore, the stabilization of the counts, for temperature of 30 ºC, can not be the only
486 parameter taken into account to predict that the microbial community is stable. Other
487 authors (Minervini et al., 2012; Weckx et al., 2010b) found that the composition of
488 sourdoughs microflora was always fluctuating, although bacterial and yeast counts and
489 physical-chemical parameters were stable.

490 As for SD1, *Clostridium, Enterococcus*, and *Enterobacter* were inhibited at the
491 final BS of SD2. However, *K. cowanii* and *E. hermannii* had increased their relative
492 abundances and overcame *L. curvatus. L. farciminis* was reduced although remained
493 predominant. *Pantoea* and *Pseudomonas* which had been reduced in BS4, increased in
494 BS7 and BS9. These groups have the optimal growth temperature in the range of 30 to 37
495 ºC (Donnarumma et al., 2010; Rezzonico et al., 2009; Rogers et al., 2015) and are able to
496 grow at a pH 4.0 (Rogers et al., 2015). As these groups were predominant in flour, and at
497 each BS, they were again added to the sourdough. In SD2, they found favorable
498 temperature for growth. Bessmeltseva et al. (2014) described a similar evolution for the
499 microbial community for rye sourdough propagated at 20 and 30 ± 1 ºC. The rye flour
500 was predominantly composed of *Proteobacteria*. After 24 h of fermentation,
501 *Enterobacteriaceae* had dominated the dough, but LAB had already increased their
502 relative abundance. After the third BS, enterobacteria were totally replaced by the LAB
503 species for SD propagated at 20 ºC. On the other hand, enterobacteria were still present
504 in low numbers within sourdoughs fermented at 30 °C after the BS7.

505 The “Lacto” group had a significant negative correlation with the genera *Bacilllus*,
506 *Clostridium*, *Escherichia*, and *Pseudomonas* for SD1. As the relative abundance of
507 “Lacto” group increased, the other genera had their relative abundance significantly
508 reduced, confirming the antagonistic relationship between these genera. This inhibitory
509 effect comprises and has already been observed in other microbial communities, as the
510 human intestinal tract (Anand et al., 2018; Aoundia et al., 2016; Lei, Hsieh, Tsai, 2009;
511 O’connor et al., 2015; Servin et al., 2004; Spinler, Ross, Savidge, 2016). It is an important
512 tool that bases the biopreservation, applied in food systems to inhibit pathogenic and
513 deteriorating microorganisms (Abdel-Rahman et al., 2019; Costa et al., 2018). The

514 inhibitory effect was observed, however, only for SD1 propagated at 21 ± 1 ºC, indicating
515 that temperature was an important factor shaping the microbial succession.
516 Although the SD had the same matrix until BS4, a variation in the temperature
517 could change the composition of the final microbiota and, therefore, it would be able to
518 modify the characteristics of the final product, as already reported in our previous study
519 (Menezes et al., 2019). This consideration is pertinent for standardization of sensorial,
520 nutritional and technological bread quality. The temperature of 30 °C can favor atypical
521 bacterial groups, being inadequate for the propagation of sourdough in Brazil. This is the
522 first study that investigated the relationship between temperature and the presence of
523 groups of non-LAB bacteria, including potential pathogens, in wheat sourdoughs.
524 Considering that the technology and functional fermentation performances are
525 determined, among other factors, by the conditions of the process, as temperature, and
526 the fermenting microbiota, the future research efforts should be dedicated to ensuring the
527 consistent quality and safety of sourdoughs (Brandt, 2018; Gänzle and Zheng, 2018;
528 Gobbetti et al., 2016). Evidently, to consider only one parameter at a time is not enough
529 to fully explain the dynamics of the sourdough community. It is important not to neglect
530 the fact that microbial growth is a result of multiple combinations of different parameters
531 (Minervini et al., 2014), taking into account the complexity of the microbiota that
532 composes a sourdough at different stages of propagation.
533 SD1 presented a lower diversity with LAB dominance. After nine BS, SD1 was
534 metabolically and microbiologically stable. While SD2 still harboring atypical
535 microorganisms. Supposedly, at a higher temperature, sourdough would take longer to
536 achieve stability. Regardless of temperature, microbial diversity was markedly simplified
537 after the BS5 for both SD. The highest bacterial diversity was detected for the first steps
538 of propagation and gradually became lower as propagation progressed, finally reaching
539 the lowest diversity in BS9. In general, microbial diversity tends to be simplified
540 gradually through the BS (De Angelis et al., 2018). As the number of backslopping steps
541 increases, the environmental conditions become more and more selective, resulting in the
542 dominance of a few species (Celano et al., 2016).
543 *L. farciminis* was dominant from BS4 until the end of the fermentation for both
544 SD, regardless of temperature, which indicates a close adaptation to the nutritional
545 restrictions and highly acidic conditions. This specie has already been isolated previously
546 in sourdough, but is often not found frequently (De Vuyst et al., 2014; Galli et al., 2018;
547 Gobbetti et al., 2016; Liu et al., 2016). *L. farciminis* has a many carbohydrate subsystem

548 features (Nam et al., 2011), including the Carbon Catabolite Repression (CCR), a major
549 determining factor of growth rate and competitive success in natural ecosystems (Chen et
550 al., 2018; Ganzle and Gobbetti, 2012). Furthermore, *L. farciminis* has multiple abilities
551 to metabolize aminoacids, among them, the ADI-pathway, that contributes to production
552 of ATP (Chiou et al. 2016), pH-homeostasis and acid tolerance (Fernández and Zúñiga,
553 2006). Galli et al. (2019) observed that, among five species of *Lactobacilli*, *L. farciminis*
554 was the most competitive strain, increasing the cell numbers for the final BS, which
555 reinforces the K-strategist concept.

556 Regarding microbial succession, the classic three-phase evolution (Ercolini et al.,
557 2013; Van Der Meulen et al., 2007; Weckx et al., 2010b) was observed only for SD1.
558 Atypical species for mature sourdoughs were detected only from BS1 to BS4. As the
559 propagation steps evolved, more acidic conditions favored *Lactobacillus* over other LAB,
560 that are species expected to be present for the initial steps of propagation, as they are more
561 sensitive to acid stress (Van Der Meulen et al., 2007). On the other hand, For SD2,
562 atypical bacteria were found to increase in the final BS. The presence of non-LAB
563 bacteria in sourdoughs in previous studies might have been underestimated, since most
564 research on sourdough microbial communities encopasses only LAB (Dertli et al., 2016;
565 Lhomme et al, 2015; Liu et al., 2016; 2018; Scheirlinck et al., 2007; Van Der Meulen et
566 al., 2007). Some recent studies have applied metagenetics to describe the populations,
567 revealing the presence of persisting subpopulations, mainly *Enterobacteriaceae*
568 (Bessmeltseva et al., 2014; Ercolini et al., 2013).

569 More than 50 species were detected from flour to BS9. When the microbial
570 succession was studied by the culture-dependent approach, the number of isolated species
571 was much lower. Discrepancies have been found between the results obtained by
572 metagenomic analysis and isolate identification, whereby metagenomics tends to suggest
573 a greater bacterial diversity (Michel et al., 2016). The culture-dependent approach alone
574 does not allow to detect all the bacteria present in complex matrices due to inherent
575 limitations (Alfonzo et al., 2017). The number of isolates was probably not sufficient to
576 completely describe the species and strain diversity; this also demonstrates a weakness of
577 the culture-dependent approach. Microbial communities are highly diverse, community
578 composition can change rapidly, and the vast majority of microbial taxa cannot be
579 identified using standard culture-based methodologies. Metagenetics has the potential of
580 giving a more detailed view on the micro-ecosystem composition, which will allow the
581 expansion of classical models of ecological succession, as sourdough. Although it is not

582 possible to distinguish intra-species variations, pyrosequencing enables the description of
583 subdominant populations, which could hardly be studied through culture-dependent
584 approaches. The subdominant population slightly affects the dough features, however, its
585 effect should not be omitted (Van Der Meulen et al., 2007).

586 For further studies, it is suggested that some cell treatment should be performed
587 prior to amplification to ensure the distinction between viable and unviable cells, such as
588 the inclusion of a pre-enrichment step or propidium monoazide treatment, although it is
589 well known that these methodologies also have limitations. The use of RNA instead of
590 DNA is also subject to false positives, since some findings suggest that transcripts can
591 persist for extended lengths of time after cell death. In addition, RNA is more sensitive,
592 less stable, and its extraction is more laborious. Thus, the use of RNA may result in data
593 loss (Ju et al., 2016). It is also also recommended to follow the dynamics of yeasts, since
594 yeast population influences and is influenced by the LAB population, insofar
595 relationships of competitiveness and association are established, as already described by
596 other authors (Vrancken et al., 2010; De Vuyst et al., 2016).

597

598 **5. Conclusions**

599

600 The bacterial community of sourdoughs is showed to be affected by the
601 temperature of propagation. *L. farciminis* is prevalent in both conditions tested; however,
602 the temperature variation changed the subdominant populations. *L. farciminis* is not
603 among the microorganisms most commonly found in European sourdoughs, however it
604 was predominant in this study. As LAB were detected only after the first step of
605 propagation, they were possibly present in the flour, but below the detection limits. The
606 different processing conditions (temperature, flour origin) influenced the composition and
607 dynamics of the microbial community, demonstrating the importance of studying
608 sourdough in different parts of the world, as a source of microorganisms with new
609 fermentative potentialities.

610 At 21 ± 1° C, the mature sourdough was composed exclusively by LAB, being
611 able to inhibit the other bacterial groups as the propagation evolved. Otherwise, the
612 temperature of 30 ± 1° C favored the persistence of atypical bacterial groups such as
613 *Pseudomonas* and *Enterobacteriacea* in the end of backslopping steps. The Pearson
614 correlation demonstrated that there was an antagonistic relationship between
615 *Lactobacillus* and *Lactococcus* and the genera *Bacillus*, *Clostridium*, *Escherichia, and*

Pseudomonas. This effect was observed only at 21 ºC. Therefore, the temperature of 21 ± 1º C can be considered more suitable for the propagation of sourdoughs in Brazil, since the role of non-LAB in sourdough metabolic activity is not yet well understood.

Most research has focused on identifying only LAB in sourdough. Hence, the presence of other groups and their putative contribuition on fermentation has been neglected. Studies regarding microbial community dynamics of sourdoughs should advance the investigation into the presence of atypical microrganisms, including potentially pathogenic bacterial groups in mature sourdoughs and the consequent implications for baking, such as the production of metabolites and cross contamination in the bakery environment. In conclusion, the results emphasize the role of temperature control in i) driving the growth of LAB instead of atypical microorganisms and ii) ensuring the overall quality and safety of sourdough bread by inhibiting pathogens.

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