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

Keywords: fermentation; bacterial diversity; high-throughput sequencing; lactic acid bacteria

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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

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

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

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

2. Material and methods

2.1 Sourdough propagation

Sourdoughs were made at the Bakery Pilot Plant of the Federal University of Santa Catarina following traditional protocol for sourdoughs type I. Organic refined wheat flour (Paullinia company, Marechal Cândido Rondon, Paraná, Brazil) and mineral water [1:1 (w/w)] were mixed with a resulting dough yield [(dough mass/flour mass) × 100] of 200 (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

2000, ThermoFisher Scientific Inc, Waltham, Massachusetts, EUA). DNA was diluted up to 50 ng μl^{-1} and stored at -20 ºC.

Insert Table 1 here.

2.3 Metagenomic analysis

The total DNA extracted from the flour and sourdough samples was used as template for 16S metagenomic analysis, which was performed by Neoprospecta Microbiome Technologies (Florianópolis, Brazil) using the Illumina MiSeq platform (Illumina Inc., San Diego, California). Library preparation was performed using Neoprospecta´s NGS Procotol (Christoff et al., 2017). Briefly, the V3-V4 hypervariable region of the 16S rRNA gene was amplified with primers 341F (CCTACGGGRSGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011; Wang and Qian, 2009). The PCR reaction was carried out in triplicates using Platinum Taq Polymerase (Invitrogen, USA) with the following conditions: 95 °C for 5 min, 25 cycles of 95 °C for 45 s, 55 °C for 30 s and 72 °C for 45 s and a final extension of 72 °C for 2 min. Library preparation (attachment of TruSeq adapters, purification with AMPureXP beads and qPCR quantification) was performed using Illumina 16S Library Preparation Protocol (Illumina Technical Note 15044223 Rev. B). Sequencing was performed using MiSeq Reagent Kit v3 with 2x300 bp paired-end reactions.

2.4 Bioinformatics

Sequencing data for each sample was processed on Quantitative Insights into Microbial Ecology (Qiime) software package (Caporaso et al., 2010). Initially, the sequencing output was analyzed by a read quality filter, which removed reads with an average Phred score < 20 followed by a clustering of 100% identical reads. In order to remove putative chimeric sequences, clusters with less than 5 reads were excluded from further analysis. The remaining good-quality sequences were further clustered at 97% similarity to define operational taxonomic units (OTU). Classification of OTUs was made by comparing them with a custom 16S rRNA database (NEORefDB, Neoprospecta Microbiome Technologies, Brazil). Sequences were taxonomically assigned with at least 99% identity in the reference database. In order to evaluate the microbial community shifts among samples, OTUs were summed up into the same genera and the relative abundance of each genus was compared with a heat-map on Qiime.

2.5 Length heterogeneity-PCR (LH-PCR)

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

2.6 Repetitive element palindromic-PCR (REP-PCR)

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

there was no statistical difference between counts in mMRS. In general, counts of viable microorganisms were lower in the other culture media and evolved more slowly, reaching above 7.0 log CFU.g^{-1} only from BS6 for Wheat Flour Agar Medium (WFAM) and Sourdough Agar Medium (SDAM).

Insert Table 2 here.

Clusterization by LH-PCR and REP-PCR (Table 3) of the 100 randomly selected colonies were used to classify and select those that would belong to different species and would be sequenced. Only a small part of the sourdough population could be recovered by the culture-dependent method, a quite homogeneous population, with 11 biotypes. Each biotype was taxonomically characterized through 16S rRNA gene partial sequencing. The LAB isolated belonged to the species *Lactobacillus farciminis*, *Lactobacillus brevis*, *Lactococcus lactis*, *Leuconostoc citreum* (two biotypes), *Enterobacter hormaechei*/*cloacae*, *Enterococcus gilvus*, *Enterococcus hirae*, *Enterecoccus durans*, *Enterococcus faecium* and *Enterococcus faecalis*. *Ec. faecium* and *L. brevis* were the most dominant species. While some species of *Enterococcus* were present variably, *L. brevis* was found from BS2 and persisted until the final propagation step. *Lc. lactis* was isolated from BS2 to BS4 and persisted only for SD2, until BS7. *Eb. hormaechei/cloacae* was recovered from BS1 and BS6. The first biotype of *Ln. citreum* was recovered from BS5 of SD2; the second one was isolated from BS7 and BS8 from SD2 and SD1, respectively. *L. farciminis* was isolated only in BS8 and BS9, in both SD.

Insert Table 3 here.

2.3 Metagenomic analysis

DNA extracted from the flour and sourdough samples was used as template for 16S metagenomics analysis to describe the bacterial diversity (Figure 2). The flour microbial consortium was composed of thirteen different species belonging to *Proteobacteria* phylum. *Escherichia hermannii* (relative abundance of 43.56%) was predominant, followed by *Kosakonia cowanii* (20.21%), and *Pantoea ananatis* (18.85%). *Pseudomonas rhodesiae* (5.10%), *Pseudomonas tolaasii* (2.90%), *Pantoea agglomerans* (2.42%), and *Pseudomonas fluorescens* (2.24%) were also present. After the BS1, twenty-three species were found. *Firmicutes* - *Clostridium saccharobutylicum* (29.62%),

Clostridium beijerinckii (19.55%), *Clostridium aurantibutyricum* (15.96%) and *Bacillus cereus* group (12.44%) became predominant. *E. hermannii* (7.45%), and *K. cowanii* remained representative (5.02%). *Lactobacillus curvatus* (1.17%), *Lc. lactis* (0.07%), *Ln. citreum* (0.02%) and *Pediococcus pentosaceus* (0.02%) were found, however with low relative abundance. *Pseudomonas* corresponded to 1.19%, *Enterococcus* and *Enterobacter* 0.25%. From BS2, the genus *Clostridium* was flanked by LAB. The dough was dominated by *L. curvatus* (37.46%), *C. saccharobutylicum* (25.07%), and *L. farciminis* (10.21%). *E. hermannii* (4.73%) and *K. cowanii* (3.33%) were still present. Other seven LAB species were found – *Lc. lactis* (1.11%), *Lactobacillus graminis* (0.33%), *Lactobacillus kimchiensis* (0.18%), *Lactobacillus plantarum* (0.16%), *Lactococcus garvieae* (0.12%), *L. brevis* (0.09%) and *Lactobacillus sakei* (0.02%) – as well as *Enterococcus*, *Enterobacter*, and *Pseudomonas* (0.82%, 0.25% and 0.3%, respectively).

Insert Figure 2 here.

From BS4, twenty-nine species were found. The dough was dominated by LAB. The relative abundance of *L. curvatus (*42.36%) and *L. farciminis* (44.07%) were higher compared to earlier steps. *C. saccharobutylicum* (2.51%), *E. hermannii* (2.31%) and *K. cowanii* (1.04%) were still found, but at lower relative abundance than in the previous BS. *Pseudomonas* and *Pantoea* corresponded to 0.16% and 0.59%, respectively. For BS5, SD1 was dominated by *L. farciminis* (85.39%) and *L. curvatus* (11.28%). Among the other seventeen species detected, only *Ln. citreum* (0.55%), *Pd. pentosaceus* and *E. hermannii* (0.54%) were found with relative abundance higher than 0.5%. *L. brevis* (0.10%), *L. graminis* (0.12%), *L. kimchiensis* (0.38%), *Lactobacillus nantensis* (0.08%) were present at low incidence. Among the seven species of the genus *Clostridium* present in BS2, only *C. aurantibutyricum* (0.05%) and *C. beijerinckii* (0.02%) remained. *B. cereus* group, *Enterococcus*, and *Enterobacter* were inhibited. Fifteen different species were detected at BS7, for SD1. The dough was dominated by *L. farciminis* (78.30%), followed by *L. curvatus* (16.03%), *Ln. citreum* (3.31%) and *Pd. pentosaceus* (1.11%). The same sub-dominant LAB species detected in BS5 were found in BS7 but in slightly lower proportions. The genus *Clostridium* was inhibited. *Eb. cloacae* (0.3%), *E. hermannii* (0.46%), *K. cowanii* (0.14%), *P. fluorescens* (0.06%) and two species of the genus *Pantoea* - *Pantoea vagans* and *Pa. ananatis* (both with 0.07%) were the

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

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

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

sequences reduced as the propagation evolved. For SD2, the highest number of sequences of LAB was observed from BS2 to BS7. *Enterococcus*, *Clostridium* and *Bacillus* were predominantly found from BS2 to BS4, and reduced for the subsequent BS, as well as observed for SD1.

Insert Figure 3A and 3B here

On the other hand, the genus *Pseudomonas* and the group of *Enterobacteriaceae* presented a wide distribution during the propagation, including for the final BS. The Pearson correlation coefficient (Table 4) showed a significant negative correlation between the "Lacto" group *versus* the genera *Bacillus*, *Clostridium*, *Escherichia*, and *Pseudomonas*, for SD1. These genera were found to decrease with the increasing of "Lacto" group relative abundance. For SD2, significant correlation was observed only for the six combined genera, effect that was also observed for SD1. There was no significant relationship between "Lacto" and "Entero" groups. However, the relative low number of *Enterococcus* and *Enterobacter* sequences detected from SD1 to SD4 may explain any unobserved relationship.

Insert Table 4 here.

4. Discussion

Temperature is one of the main parameters that influence the final microbiota composition of sourdoughs (Minervini et al., 2014; Vrancken et al., 2011). This is the first study to our knowledge in which spontaneous sourdough fermentation was followed in Brazil, a country with great climatic diversity. In general, the climate is warm in almost all the territory, with average temperatures above 18 ºC in all months of the year in most states (Brasil, 2002). Two common temperatures in tropical climates were selected for sourdough preparation, and had important implications on the microbial dynamics, especially for the subdominant microflora.

Low counts were found in the flour, and no LAB isolates could be retrieved. The community profiles obtained with pyrosequencing procedure did not detect LAB in the flour, in accordance with its isolates. Before fermentation, low colony counts were found; however, LAB numbers rapidly increased after the BS1. From BS5 onward, the counts

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

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

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

al., 2010b). Just one BS was able to completely turn the microbial community from *Proteobacteria* to mainly *Firmicutes*. Among the species found in the flour, only *E. hermannii* and *K. cowanii* persisted, possibly due to its ability to tolerate the biochemical changes in the matrix. Commonly, *Enterobacteriaceae* grows in the first days of propagation, and survives because of a certain tolerance for acid stress (Ercolini et al., 2013). *B. cereus* is often found in cereals and wheat flour and are well adapted to the bakery environment (Martínez Viedma et al., 2011; Oltuszak-Walczak and Walczak, 2013). Clostridia has quite efficient mechanisms in sugar uptake (Mitchell, 2016). These features, coupled with the semi-anaerobic conditions and the availability of carbohydrates certainly favored the codominance of this groups in BS1.

For BS4 the bacterial profile markedly changed, and *Lactobacilli* completely dominated the SD. There was a marked decrease in pH from BS0 (6.26 ± 0.01) to BS4 (3.79 ± 0.01). TTA increased from 1.40 ± 0.13 to 13.85 ± 0.12 (Menezes et al., 2018). Consequently, the highest concentrations of organic acids were found for these BS, as reported in our previous study (Menezes et al., 2019). Acidification is deeply linked to the assembly of the microbial consortia. The highest concentrations of organic acids coincided with the exponential growth phase of the sourdough communities and are associated with competitiveness between species (De Vuyst et al., 2014). Suppression of *Pseudomonas* (Kiymaci et al., 2018; Nakai and Siebert, 2004), enterobacteria (Skrivanova et al., 2006), *B. cereus* (Soria and Audisio, 2014) and clostridial groups (Schoster et al., 2013; Thylin et al., 1995) is correlated with organic acids synthesis and with a concomitant drop in pH. In turn, LAB are well adapted to the sourdough acid (Corsetti et al., 2007; Corsetti and Settanni, 2007). From the BS2, the dough has become more hostile to *Enterobacteriaceae* and *Clostridium* and more favorable to LAB. When fermentation begins to occur under acidic conditions, evident after the BS2, the growth of non-LAB bacteria is gradually inhibited. Thus, as the number of fermentation steps increases, the LAB becomes more adapted to environmental conditions (Minervini et al., 2014). By definition, LAB are predominant in mature sourdoughs (Gobbetti et al., 2016). LAB can overcome other contaminating microbiota mainly by thriving under in fermentation systems. Most of the LAB metabolic traits are, actually, adaptations that contribute to its competitive advantage in the sourdough environment (Gänzle and Ripari, 2016). Synthesis of bacteriocins probably contributes with a selective advantage in a microbial niche complex, such as sourdoughs (Vogel et al., 1993; Marques et al., 2017). Similarly to organic acids, an increase in mannitol production was observed from BS2 to

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

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

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

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

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

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

inhibitory effect was observed, however, only for SD1 propagated at 21 ± 1 ºC, indicating that temperature was an important factor shaping the microbial succession.

Although the SD had the same matrix until BS4, a variation in the temperature could change the composition of the final microbiota and, therefore, it would be able to modify the characteristics of the final product, as already reported in our previous study (Menezes et al., 2019). This consideration is pertinent for standardization of sensorial, nutritional and technological bread quality. The temperature of 30 °C can favor atypical bacterial groups, being inadequate for the propagation of sourdough in Brazil. This is the first study that investigated the relationship between temperature and the presence of groups of non-LAB bacteria, including potential pathogens, in wheat sourdoughs. Considering that the technology and functional fermentation performances are determined, among other factors, by the conditions of the process, as temperature, and the fermenting microbiota, the future research efforts should be dedicated to ensuring the consistent quality and safety of sourdoughs (Brandt, 2018; Gänzle and Zheng, 2018; Gobbetti et al., 2016). Evidently, to consider only one parameter at a time is not enough to fully explain the dynamics of the sourdough community. It is important not to neglect the fact that microbial growth is a result of multiple combinations of different parameters (Minervini et al., 2014), taking into account the complexity of the microbiota that composes a sourdough at different stages of propagation.

SD1 presented a lower diversity with LAB dominance. After nine BS, SD1 was metabolically and microbiologically stable. While SD2 still harboring atypical microorganisms. Supposedly, at a higher temperature, sourdough would take longer to achieve stability. Regardless of temperature, microbial diversity was markedly simplified after the BS5 for both SD. The highest bacterial diversity was detected for the first steps of propagation and gradually became lower as propagation progressed, finally reaching the lowest diversity in BS9. In general, microbial diversity tends to be simplified gradually through the BS (De Angelis et al., 2018). As the number of backslopping steps increases, the environmental conditions become more and more selective, resulting in the dominance of a few species (Celano et al., 2016).

L. farciminis was dominant from BS4 until the end of the fermentation for both SD, regardless of temperature, which indicates a close adaptation to the nutritional restrictions and highly acidic conditions. This specie has already been isolated previously in sourdough, but is often not found frequently (De Vuyst et al., 2014; Galli et al., 2018; Gobbetti et al., 2016; Liu et al., 2016). *L. farciminis* has a many carbohydrate subsystem

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

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

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

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

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

5. Conclusions

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

At 21 ± 1º C, the mature sourdough was composed exclusively by LAB, being able to inhibit the other bacterial groups as the propagation evolved. Otherwise, the temperature of 30 ± 1º C favored the persistence of atypical bacterial groups such as *Pseudomonas* and *Enterobacteriacea* in the end of backslopping steps. The Pearson correlation demonstrated that there was an antagonistic relationship between *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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