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Bacterial community dynamics in spontaneous sourdoughs made from wheat, spelt, and rye wholemeal flour

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Abstract

Sourdough fermentation is a traditional process that is used to improve bread quality. A spontaneous sourdough ecosystem consists of a mixture of flour and water that is fermented by endogenous lactic acid bacteria (LAB) and yeasts. The aim of this study was to identify bacterial diversity during backslopping of spontaneous sourdoughs prepared from wheat, spelt, or rye wholemeal flour. Culture-dependent analyses showed that the number of LAB (10^9 CFU/ml) was higher by three orders of magnitude than the number of yeasts (10^6 CFU/ml), irrespective of the flour type. These results were complemented by next-generation sequencing of the 16S rDNA V3 and V4 variable regions. The dominant phylum in all sourdough samples was *Firmicutes*, which was represented exclusively by the *Lactobacillales* order. The two remaining and less abundant phyla were *Proteobacteria* and *Bacteroidetes*. The culture-independent approach allowed us to detect changes in microbial ecology during the 72-hr fermentation period. *Weissella* sp. was the most abundant genus after 24 hr of fermentation of the rye sourdough, but as the process progressed, its abundance decreased in favor of the *Lactobacillus* genus similarly as in wheat and spelt sourdoughs. The *Lactobacillus* genus was dominant in all sourdoughs after 72 hr, which was consistent with our results obtained using culture-dependent analyses. This work was carried out to determine the microbial biodiversity of sourdoughs that are made from wheat, spelt, and rye wholemeal flour and can be used as a source of strains for specific starter cultures to produce functional bread.

KEYWORDS

bacterial community dynamics, biodiversity, bread, lactic acid bacteria, sourdough, wholemeal flour

1 | INTRODUCTION

According to nutrition guidelines, a healthy human diet should provide more than 25 g per day of fiber from fruits, vegetables, and wholegrain foods (EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA), 2010). This recommendation is based on evidence linking high intake of dietary fiber with health benefits, for example, reduced risk of coronary heart disease and type 2 diabetes as well as improved weight maintenance. Cereals, one of the major sources of dietary fiber, make up approximately 50% of the fiber intake in Western countries and 41.5% in Poland (Królak, Jeżewska-Zychowicz, Sajdakowska, & Gębski, 2017). Cereal products, especially bread, play an essential role in the human diet worldwide. The demand for wholegrain bread has recently increased due to its high content of dietary fiber and other bioactive components. Cereal processing can influence the levels of bioactive components in grains and can also modify the bioavailability of these components (Dewettinck et al., 2008).

The quality and nutritional value of bread, particularly of bread produced from wholegrain flour, can be increased by sourdough fermentation. The use of sourdough for traditional breadmaking is one of the oldest biotechnological processes in cereal food production. Sourdough bread is prepared from a mixture of flour (usually rye or wheat) and water that is fermented with lactic acid bacteria (LAB) and yeasts. Sourdough fermentation processes can be classified into three types based on the method of inoculation: (a) spontaneous fermentation with refreshment (also called backslopping); (b) fermentation initiated by the addition of a starter culture; and (c) a mixed process in which sourdough is initiated with a starter culture and propagated by traditional backslopping (De Vuyst et al., 2014). The type I of sourdough fermentation process is usually conducted at room temperature (20–30°C) and leads to production of a firm dough (Siepmann, Ripari, Waszczynskyj, & Spier, 2018). The diversity and stability of the microbial consortia in sourdoughs are influenced by the microbial and chemical compositions of the raw materials, by interactions between microorganisms, and by various fermentation parameters, including temperature, inoculum size, dough yield, and fermentation time (Bessmeltseva, Viiard, Simm, Paalme, & Sarand, 2014). Sourdough ecosystems can be inhabited by simple or extremely complex microbial consortia represented by distinct LAB and yeast species and/or strains (De Vuyst et al., 2014). The most prevalent LAB species belong to the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella*, whereas the most prevalent yeast species belong to the *Kazachstania* and *Saccharomyces* clades (for the latest reviews, see De Vuyst, Van Kerrebroeck, & Leroy, 2017; De Vuyst, Harth, Van Kerrebroeck, & Leroy, 2016, Gänzle & Ripari, 2016, Gobbetti, Minervini, Pontonio, Di Cagno, & De Angelis, 2016 and Minervini, Celano, Lattanzi, De Angelis, & Gobbetti, 2016). Stable sourdough ecosystems harbor mostly heterofermentative LAB, in particular lactobacilli (Huys, Daniel, & Vuyst, 2013). A great variety of *Lactobacillus* sp. representing homofermentative and heterofermentative species have been isolated from sourdoughs. Species belonging to *Weissella* (*Weissella cibaria*, *Weissella confusa*), pediococci (*Pediococcus acidilactici*, *Pediococcus pentosaceus*), and leuconostocs (*Leuconostoc mesenteroides*, *Leuconostoc citreum*) are less predominant in sourdoughs, whereas lactococci, enterococci, and streptococci were found to be subdominant (De Vuyst et al., 2014). A recent meta-analysis of 583 backslopped sourdoughs performed by De Vuyst and coworkers (Van Kerrebroeck, Maes, & De Vuyst, 2017) revealed that the two most prevalent groups of LAB were the *Lactobacillus fructivorans*, represented solely by *Lactobacillus sanfranciscensis* (present in 47% of the sourdoughs), and the *Lactobacillus plantarum* group (present in 44% of the sourdoughs). These were followed by the *Lactobacillus brevis* group (22%), the *Lactobacillus reuteri* group (19%), and the *Lactobacillus alimentarius* group (18%). *Leuconostoc*, *Pediococcus*, and *Weissella* species were identified in 18%, 15%, and 15% of the examined sourdoughs, respectively. According to this analysis, other species of LAB (*Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus rossiae*, *Lactobacillus sakei*, other lactobacilli, and enterococci/lactococci/streptococci) were present in 10% or less of the sourdoughs.

Previous studies of the dynamics of microbial communities in spontaneously started sourdoughs have shown that the stability of the microbial consortium is reached within backslopping cycles. The stabilization occurs through a three-phase evolution that is characterized consecutively by the predominance of sourdough-atypical LAB, sourdough-typical LAB, and highly adapted sourdough-typical LAB (De Vuyst et al., 2014; Van der Meulen et al., 2007; Weckx et al., 2010).

The present work is part of a project designed to characterize the biological and technological effects of LAB in sourdough and to develop wheat, spelt, and rye bakery made from wholemeal flour. A quality assessment of wholegrain flours and of spontaneous sourdoughs prepared from these flours has already been published (Litwinek, Buksa, Gambuś, Kowalczyk, & Boreczek, 2017). These sourdoughs were used in the production of bread (Litwinek et al., 2018) and in the isolation of LAB strains for developing starter cultures with optimal technological properties allowing to obtain bakery products from wholemeal flour (data unpublished). The main goal of this study was to use culture-dependent and culture-independent approaches to characterize the bacterial community dynamics in spontaneous sourdoughs made from common wheat, spelt wheat, and rye during 3 days of backslopping. The novelty of this work lays in conducting the research on an industrial scale and the use of wholegrain flour which is expected to influence the levels of bioactive components and thus the microbial diversity in sourdoughs.

2 | MATERIALS AND METHODS

2.1 | Sourdough fermentation and sampling

Spontaneous sourdoughs were prepared in duplicates from the same batches of flours using three types of wholemeal flour: wheat, spelt,

and rye. Sourdoughs were manufactured in the bakery by mixing 20 kg of flour and 30 kg of water, by means of fermentation at 30°C in a BIO-FM-Ż 400 bioreactor (Biostar) and subsequent daily backslopping at 30°C, including 2 refreshments over 24 hr. After 24 hr of fermentation, each sourdough was refreshed by adding 20 kg of the appropriate flour and 30 kg of water. The acidity (pH) was measured every 10 min using the iCINAC system (AMS), and total titratable acidity (TTA) was calculated at the end of each fermentation cycle prior to renewal and sourdough sample collection. Samples obtained at various time points (24 hr, 48 hr, and 72 hr) were stored in sterile vials and conserved at 4°C for culture-dependent analyses; the samples were then frozen at −20°C for subsequent microbial analysis using a culture-independent approach.

2.2 | Microbial analysis by culturing

Ten gram samples of sourdoughs were brought to a volume of 100 ml with sterile 0.9% NaCl solution (PS) at room temperature (RT) and homogenized in a laboratory homogenizer (H500 Pol-Eko-Aparatura; Wodzisław Śląski, Poland) for 5 min at 24,000 rpm. Tenfold dilutions in PS were then plated in duplicates on rich and selective media, and colony-forming units (CFU) of different microbial groups were counted. Total aerobic and anaerobic bacteria were counted on plate count agar containing 0.1% skim milk (PCM; Merck) supplemented with 0.1 g/L cycloheximide (Sigma-Aldrich) after 72-hr incubation at 30°C under appropriate conditions. Incubation under anaerobic conditions was performed in 2.5-liter jars containing GENbox anaer sachets (bioMérieux). Thermophilic and mesophilic LAB were grown on de Man, Rogosa, and Sharpe agar (MRS; Merck) adjusted to pH 5.4, and colonies were counted after 72 hr of incubation under anaerobic conditions at 42 and 30°C, respectively. This medium has been used taking into account that typical lactobacilli for sourdoughs backslopped at artisanal or industrial bakeries which do not grow on MRS are absent in spontaneous sourdoughs even after 10 backslopping cycles. Blichfeldt medium containing 2.5 g/L yeast extract, 10 g/L peptone, 10 g/L glucose, 10 g/L lactose, 5 g/L $CaCO_3$, 15 g/L agar; pH 6.7 supplemented with 0.1 g/L cycloheximide (Sigma-Aldrich) was used for the enumeration of acidifying cocci after incubation at 30°C for 72 hr under aerobic conditions. In addition, spore-forming bacteria were counted after plating sourdough samples treated at 65°C for 30 min on LB agar medium and subsequent incubation at 30°C for 48 hr under aerobic conditions. Finally, dilutions of the samples were plated on yeast extract-peptone-dextrose (YPD) agar medium containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 0.1 g/L chloramphenicol, and yeasts and filamentous molds were independently counted after 5 days of incubation at 25°C under aerobic conditions. For isolation of strains producing exopolysaccharides, we used also MRS without dextrose (Conda) agar medium supplemented with 10% sucrose and 0.1 g/L cycloheximide (SMRS). Bacteria were isolated from this selective medium after 72-hr incubation under aerobic conditions at 30°C.

2.3 | Identification of bacterial isolates

Bacteria were isolated from the plates used for counting different microbial groups, except for LB and YPD media as well as from SMRS. Bacterial isolates were picked up after plating sourdough samples at various dilutions ranging from 10^3 to 10^6 depending on the medium. For each medium, single colonies of different morphologies were randomly selected for identification. The isolates were purified, stored at −80°C, and identified by 16S rRNA gene sequencing as described previously (Alegría, Szczesny, Mayo, Bardowski, & Kowalczyk, 2012) with slight modifications. Amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and sequenced on the ABI3730/xl Genetic Analyzer (Applied Biosystems) using primers 27F (5′ AGAGTTTGATYMTGGCTCAG 3′) and 1492R (5′ GGTTACCTTGTTACGACTT 3′). The obtained nucleotide sequences were compared with sequences in the NCBI Reference RNA sequences (refseq_rna) database using the online BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were assigned to a given species if they showed at least 98.65% of similarity to the sequence of that species. Isolates identified as belonging to the *Lb. plantarum* group were differentiated by *recA* sequence analysis using multiplex PCR assay with the following primers: paraF (5′ GTCACAGGCATTACGAAAAC 3′), pentF (5′ CAGTGGCGCGGTTGATATC 3′), planF (5′ CCG TTTATGCGGAACACCTA 3′), and pREV (5′ TCGGGATTACCA AACATCAC 3′), according to the previously described method (Torriani, Felis, & Dellaglio, 2001).

2.4 | Extraction of total bacterial DNA

For culture-independent analyses, 10 g of each sourdough sample was homogenized for 5 min at 24,000 rpm in a final volume of 100-ml sterile PS using a laboratory homogenizer (H500 Pol-Eko-Aparatura). This suspension was then centrifuged at 4°C for 5 min at 200 *g*. The supernatant was collected and centrifuged at 4°C for 15 min at 5,000 *g* (Bessmeltseva et al., 2014). The cell pellet was frozen in liquid nitrogen and stored at −20°C until further use. The frozen pellet was thawed on ice and washed with 1 ml of TES buffer (25 mM Tris, 10 mM EDTA, 50 mM sucrose).

Prior to DNA extraction, bacteria were lysed enzymatically at 37°C during a 1-hr preincubation step in a solution containing 300 µl of TES-lysozyme buffer and 15 µl of mutanolysin at 1 U/µl as previously described for kefir grains (lysis C) (Kowalczyk, Kolakowski, Radziwill-Bienkowska, Szmytkowska, & Bardowski, 2012) or oscypek samples (Alegría et al., 2012). After the preincubation step, 3 µl of DEPC and 150 µl of 20% SDS were added; then, the samples were incubated at RT for 5 min and centrifuged for 1 min at 13,000 *g*. The collected supernatant was mixed with 4.5 µl of proteinase K (20 mg/ml) (cat. no. 124568·0100, Merck) and incubated for 30 min at 37°C. DNA extraction was performed using the phenol/chloroform method followed by incubation with RNase A (Sigma-Aldrich).

2.5 | Sequencing of 16S rRNA gene amplicons

After isolation, DNA quality was checked by electrophoresis of the sample on 0.8% agarose gels. Amplification of the conserved bacterial 16S rRNA gene fragment covering the V3 and V4 regions was performed in triplicate using the gene-specific primers: 16S_V3-F 341-357F (5′ TCGTCGGCAGCGTCA GATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3′) and 16S_V4-R 785-805R (5′ GTCTCGTGGGCTCGGAGATGTGTAT AAGAGACAGGACTACHVGGGTATCTAATCC 3′) (Klindworth et al., 2013). Amplicons of ca. 450 bp (16S) were detected on 1% agarose gels and purified using Ampure XP magnetic beads (Beckman). The amplicon libraries were pooled in equimolar ratios and indexed by PCR according to the Nextera indexing strategy (Illumina). Sample indexing allowed pooling of amplicons for sequencing runs and further extraction of the sample sequence reads from large batches of sequencing data. The amplicons were sequenced on a MiSeq sequencer in the DNA Sequencing and Oligonucleotide Synthesis Laboratory of IBB PAS in paired-end mode using a 600-cycle v3 chemistry kit (Illumina). The Illumina sequencing raw data have been deposited in the NCBI Sequence Read Archive database (BioProject PRJNA541497). The sequence reads were filtered by quality using the FastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and data quality was checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

All 16S amplicon biodiversity analysis, from the processing of raw reads to the production of figures, was conducted with Mothulity (https://github.com/dizak/mothulity), a tool for facilitating work with Mothur (https://www.mothur.org/) using the Silva.nr_v119 database (http://www.mothur.org/w/images/2/27/Silva.nr_v119.tgz). The analysis parameters were based on MiSeq SOP (standard operational procedure, https://mothur.org/wiki/MiSeq_SOP). The graphical representation of statistical analyses is a standard output of Mothulity.

3 | RESULTS

3.1 | Dynamics of LAB and yeast counts

The number of colony-forming units (CFU) in sourdough samples obtained at three time points (24 hr, 48 hr, and 72 hr) was determined by plate counting. The total numbers of aerobic and anaerobic bacteria, thermophilic and mesophilic LAB, acid-producing bacteria, spore-forming bacteria, and yeasts were determined (Figure 1).

There were no significant differences in the numbers of aerobic or anaerobic bacteria present in wheat, spelt, and rye flour. In all samples, the number of mesophilic LAB did not change during the 72-hr fermentation and ranged from 6.8×10^8 to 3.1×10^9 CFU/ml. Counts of LAB at 42°C (thermophilic LAB) and at 30°C (mesophilic LAB) showed no statistically significant differences and corresponded to those obtained for bacteria cultivated under anaerobic conditions. In all samples, the number of acid-producing cocci present after the 24-hr fermentation (4.8×10^8 – 1.4×10^9 CFU/ml) was higher by one order of magnitude than the number present after 48 and 72 hr of fermentation (7×10^7–1.3×10^8 and 4×10^7–5.7×10^8 CFU/ml, respectively). The analysis also showed that the number of LAB (almost 10^9 CFU/ml) was higher by three orders of magnitude than the number of yeasts (approx. 10^6 CFU/ml), irrespectively of the flour type.

3.2 | Species diversity of LAB

Microorganisms were isolated from the wheat, spelt, and rye sourdough samples by culturing on rich medium, including PCM and MRS, as well as on semiselective media under both aerobic and anaerobic conditions. The purified colonies were identified by amplification of the 16S RNA gene, DNA sequencing, and sequence comparison. The results of identification of the 239 bacterial isolates obtained from the sourdough samples are summarized in Table 1. Among the bacterial isolates, 217 were identified as LAB. In all sourdough samples obtained after 48 hr and 72 hr, the dominant population of LAB was lactobacilli and consisted of the two most frequently isolated types of bacteria, *Lb. plantarum* phylogenetic group with genetically related members not distinguishable by 16S rDNA sequencing (Wuyts et al., 2019) and *Lb. brevis* species. Isolates belonging to *Lb. plantarum* group were further analyzed using species-specific *recA* gene-derived PCR primers. The size of the amplicons (318 bp) permitted classification of the isolates to *Lb. plantarum* species. For each sourdough type, the highest number of *Weissella* and *Leuconostoc* bacteria was isolated from samples collected after 24 hr of fermentation. The number of *Leuconostocaceae* isolates decreased during backslopping; thus, after 72 hr, this type of LAB was not isolated from wheat, spelt, or rye sourdoughs. Bacteria belonging to *Enterococcus* sp., two *Pediococcus* species, *Lactococcus lactis*, other distinct *Lactobacillus* species, and *Streptococcus thermophilus* were also detected among the isolated LAB.

3.3 | Dynamics of the sourdough bacterial community based on culture-independent analysis

In addition to classical microbiological analysis, 16S rRNA-based next-generation sequencing, representing a culture-independent approach, was performed to analyze the bacterial community dynamics in sourdoughs made from wheat, spelt, and rye wholemeal flours during 3 days of backslopping. Primers specific for the V3-V4 region were used to distinguish different bacterial species. Pooled 16S rRNA gene amplicons of bacterial communities from nine sourdough samples (sourdoughs from three types of flour, each collected at three time points) were sequenced on a MiSeq sequencer (Illumina). Restrictive filtration and normalization techniques for sequence quality were applied using Mothulity software, yielding good quality sequences for further analysis. With the use of similar amounts of DNA for sequencing, we obtained comparable numbers of good quality reads from each sourdough sample (Table 2). Considering the highest values of Good's

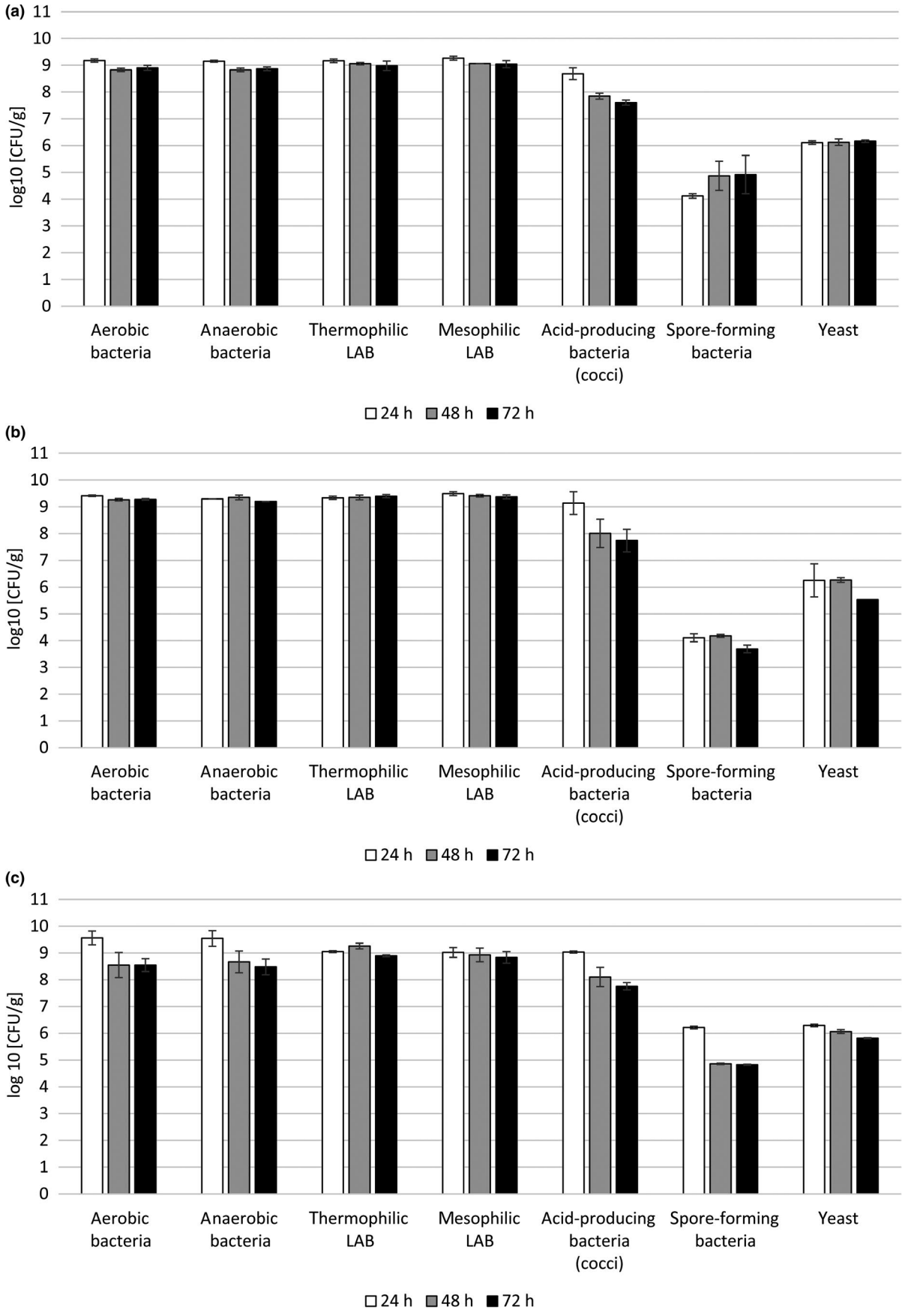

FIGURE 1 Counts of distinct microbial groups present in spontaneous sourdoughs from wholegrain flours made from common wheat (a), spelt wheat (b), and rye (c) during 3 days of backslopping

TABLE 1 Identification of bacteria isolated from wheat, spelt, and rye sourdoughs after 24, 48, and 72 hr of fermentation

Identification based on sequencing of 16S rRNA genes	No. of isolates obtained from different sourdough samples									Total no. of isolates
	Wheat			Spelt			Rye			
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	
Lactobacillus plantarum[a]	8	5	9	6	4	5	4	5	4	50
Lactobacillus brevis	2	3	6	4	7	3	1	3	3	32
Enterococcus sp.	7	8	2	0	0	1	3	6	4	31
Pediococcus pentosaceus	4	5	4	0	0	0	3	3	6	25
Weissella sp. (*confusa*, *cibaria*)	9	0	0	5	0	0	7	0	0	21
Lactococcus lactis	2	0	0	5	2	0	4	2	0	15
Lactobacillus sp. (*paralimentarius*, *kimchii*)	4	3	2	0	2	1	0	0	2	14
Staphylococcus epidermidis	1	0	0	1	3	0	0	1	1	7
Carnobacterium maltaromaticum	0	0	0	0	1	4	1	0	0	6
Escherichia coli	1	0	0	3	1	1	0	0	0	6
Leuconostoc sp. (*lactis*, *holzapfelii*)	0	0	0	0	2	0	2	1	0	5
Lactobacillus sp. (*crustorum*, *farciminis*, *mindensis*)	0	0	1	0	0	0	1	1	1	4
Leuconostoc sp. (*lactis*, *holzapfelii*, *citreum*)	2	0	0	0	1	0	0	0	0	3
Streptococcus thermophilus	0	0	0	0	1	2	0	0	0	3
Lactobacillus coryniformis	0	0	0	1	0	0	0	1	0	2
Lactobacillus sp. (*heilongjiangensis*, *farciminis*, *futsaii*, *nantensis*)	0	0	0	0	0	0	0	1	1	2
Staphylococcus hominis	0	2	0	0	0	0	0	0	0	2
Staphylococcus warneri	1	1	0	0	0	0	0	0	0	2
Lactobacillus sp. (*farciminis*, *nantensis*)	0	0	0	0	0	0	0	1	0	1
Pediococcus acidilactici	0	0	0	0	1	0	0	0	0	1
Lactobacillus sp. (*casei*, *paracasei*)	0	0	0	0	0	1	0	0	0	1
Lactobacillus sp. (*curvatus*, *graminis*, *sakei*)	1	0	0	0	0	0	0	0	0	1
Shigella sonnei	1	0	0	0	0	0	0	0	0	1
Staphylococcus capitis	0	0	0	1	0	0	0	0	0	1
Staphylococcus haemolyticus	0	1	0	0	0	0	0	0	0	1
Bacillus licheniformis	0	1	0	0	0	0	0	0	0	1
Chryseobacterium sp.	0	0	0	0	0	0	1	0	0	1
Total	43	29	24	26	25	18	27	25	22	239

[a]Isolates belonging to *Lb. plantarum* phylogenetic group were classified to *Lb. plantarum* species using species-specific *recA* gene-derived PCR primers.

coverage estimator (Table 2) and the rarefaction curves that most closely approximated horizontal lines (Figure A1 in Appendix), the most deeply sequenced samples were those obtained from rye sourdoughs. However, the Good's coverage values for all samples were in the range of 97%–99%, indicating that we managed to capture the majority of the bacterial biodiversity in each sample. OTU diversity in the samples was quantified using the inverse Simpson parameter and Shannon's diversity index. Both diversity indices suggest that the lowest heterogeneity of organisms was present in rye sourdoughs and that higher, similar biodiversity was present in the common wheat and spelt wheat samples.

A detailed breakdown of the taxonomic assignments of the OTUs is presented in figures prepared using Krona charts (Ondov, Bergman, & Phillippy, 2011) (Figure A2 in Appendix). The analyzed sequences were classified into three major phyla (with abundance > 0.7%), *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*. The abundance of the dominant *Lactobacillales* order was high in all rye sourdough samples during backslopping (81% of *Bacteria* at 24 hr, 91% at 48 hr, and 80% at 72 hr) and increased during fermentation in two types of wheat sourdoughs (common wheat: 66% of *Bacteria* at 24 hr, 76% at 48 hr, and 80% at 72 hr and spelt wheat: 65% of *Bacteria* at 24 hr, 74% at 48 hr, and 83% at 72 hr). An overview of the taxonomic assignments across all samples within the *Lactobacillales* order is presented in Figure 2. On the third day, the sourdough environment was dominated by bacteria from the *Lactobacillus* genus. The next most numerous types among *Lactobacillales* were unclassified *Lactobacillales*, unclassified

TABLE 2 Basic statistics of nine sequenced samples obtained using Mothulity software

Sourdough sample	Sample code	No. sequences	No. OTUs	Good's coverage	Inverse simpson	Shannon
Wheat 24 hr	SP1IB	21,558	666	0.98	7.55	2.70
Wheat 48 hr	SP1IIB	43,930	2,016	0.97	5.13	3.05
Wheat 72 hr	SP1IIIB	34,532	867	0.98	7.61	2.66
Spelt 24 hr	SO1IB	31,844	1,574	0.97	6.22	3.02
Spelt 48 hr	SO1IIB	40,859	1,439	0.98	5.95	2.76
Spelt 72 hr	SO1IIIB	36,300	1,031	0.98	3.33	2.09
Rye 24 hr	SZ2IB	38,292	323	0.99	1.56	0.96
Rye 48 hr	SZ2IIB	48,661	565	0.99	1.69	1.15
Rye 72 hr	SZ2IIIB	43,130	568	0.99	1.61	1.12

Note: OTUs were defined using a threshold of dissimilarity of 0.03.

Lactobacillaceae, *Weissella* and, depending on the sourdough sample, *Pediococcus*, *Lactococcus*, or *Leuconostoc* (at various ratios depending on the type of flour). Comparison of the rye sourdough samples obtained at three time points (Figure 2) showed that after 24 hr of fermentation, the abundance of *Weissella* was higher than *Lactobacillus* (reaching 36% of *Lactobacillales* for *Weissella* compared with 30% for *Lactobacillus*); however, as fermentation progressed, the *Weissella* abundance decreased to 11% at 48 hr and to 5% at 72 hr in favor of the *Lactobacillus* genus (52% at 48 hr, and 67% at 72 hr). The abundance of the *Weissella* genus in other sourdoughs (common wheat and spelt) also decreased with time during backslopping.

3.4 | Comparison of the bacterial diversity of sourdoughs within the 72-hr fermentation period and between flour types

The structures of the communities present in all samples were compared using the Yue and Clayton measure (Figure 3) (Yue & Clayton, 2005). A comparison of spontaneous sourdoughs made from different wholegrain flours showed that sourdoughs made with spelt and wheat flour were more similar to each other than to the rye sourdough. Evaluation of the population changes that occurred over 3 days of backslopping indicated that the samples obtained on the second (48 hr) and third (72 hr) days from sourdoughs made with single flour type were similar. Among them, the most similar samples were those from rye sourdoughs. The sourdough samples obtained after 24 hr of fermentation were more similar to each other than to sourdoughs made from the same flours but collected at different time points.

4 | DISCUSSION

In the present work, the biodiversity of spontaneous sourdoughs made from wheat, spelt, and rye wholemeal flours was analyzed by culturing and by a metagenetic approach using high-throughput sequencing (HTS). Wheat (*Triticum aestivum*), spelt (*Triticum spelta*), and rye (*Secale cereale*) liquid sourdoughs were fermented under industrial conditions using continuous stirring at 30°C for 3 days with daily backslopping.

From the microbiological point of view, sourdough ecosystems are characterized by higher cell counts of LAB compared to yeasts (De Vuyst et al., 2014). Characterization of the microbial populations of the sourdough samples analyzed in this work demonstrated that the number of mesophilic LAB (9.0 log CFU/ml) was three orders of magnitude higher than the number of yeasts for all flour types. Similar results, in which the number of LAB reached stable values greater than 9.0 log CFU/g and the ratio between LAB and yeasts stabilized at ca. 100:1, were previously reported by Ercolini and coworkers for rye and wheat sourdoughs (Ercolini et al., 2013). In another study, total LAB counts varied from 7.2 log to 9.6 log CFU/g, while yeast counts were 6 log to 7.6 log CFU/g and the ratio between LAB and yeast for majority of French organic sourdoughs was less than 100:10 reaching the highest ratio of 1,000:1.5 (Michel et al., 2016). The numbers of acid-producing cocci in sourdoughs made from all three flour types decreased after 24 hr of fermentation and reached similar values in sourdoughs at 48 and 72 hr. Previous research has established that stabilization of LAB consortia in spontaneously started sourdoughs occurs within 5–10 days and is manifested by a decrease in and stabilization of acidity and by LAB counts that are characteristic of mature sourdough (De Vuyst & Neysens, 2005; Ercolini et al., 2013; Van der Meulen et al., 2007; Weckx et al., 2010). However, it was also found that stabilization of sourdough ecosystems occurred more rapidly at 30°C than at lower temperatures and that LAB were already prevalent after the first fermentation cycle (after 24 hr) at levels reaching 7.2×10^8 CFU/g (Bessmeltseva et al., 2014). After the third backslopping cycle (day 3), the viable count of bacteria in all sourdoughs exceeded 10^9 CFU/g. Previous studies also demonstrated that only one fermentation cycle at 30°C is required to enrich LAB in sourdough (Ercolini et al., 2013; Vrancken, Rimaux, Weckx, Leroy, & De Vuyst, 2011), whereas up to three renewal cycles are needed at a lower temperature (23°C) (Vrancken et al., 2011). The sourdoughs produced in our studies were monitored for 3 days by determining the active acidity (pH) and the total titratable acidity (expressed as mL 0.1 N NaOH/10 g) (TTA) (Litwinek et al., 2017). During fermentation, the pH decreased to 3.7

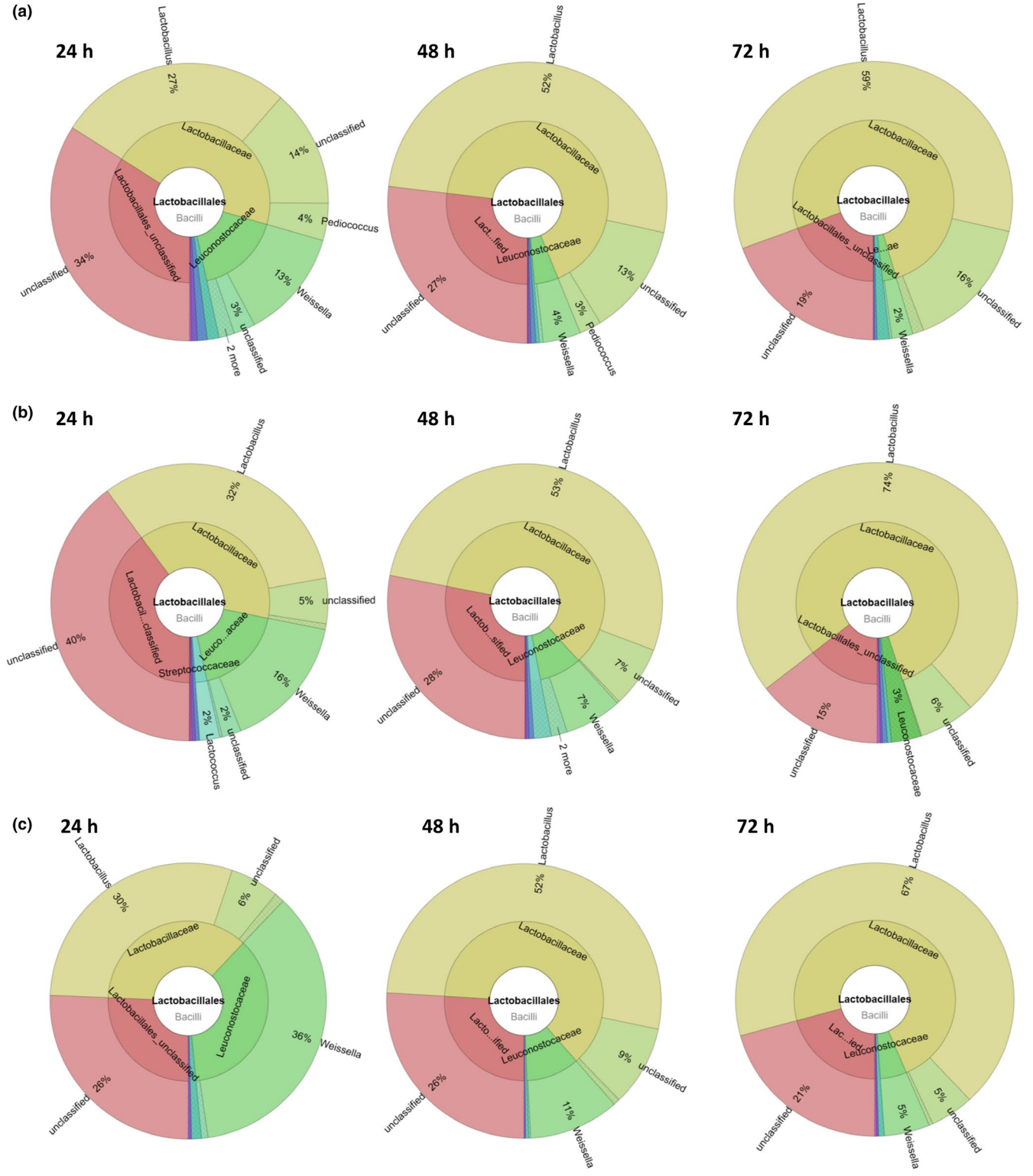

FIGURE 2 Taxonomic structure of the *Lactobacillales* community in spontaneous sourdoughs made from wheat (a), spelt (b), and rye (c) wholemeal flour during 3 days of backslopping. The figure was prepared using Krona Charts (Ondov et al., 2011)

in all samples obtained from rye and spelt sourdoughs and to 3.8 in samples obtained from the wheat sourdough. The TTA reached an average value of 25 in sourdoughs made from rye and spelt flour and an average value of 19.5 in the sourdough prepared from common wheat. Besmeltseva and coworkers showed that during fermentations conducted at 30°C, the maximum TTA occurred on day 10 and reached ca. 22.5 (Bessmeltseva et al., 2014).

The use of a culture-dependent method for the evaluation of biodiversity of spontaneous sourdoughs revealed that the predominant bacteria present in samples obtained after 72 hr belonged to the *Lb.*

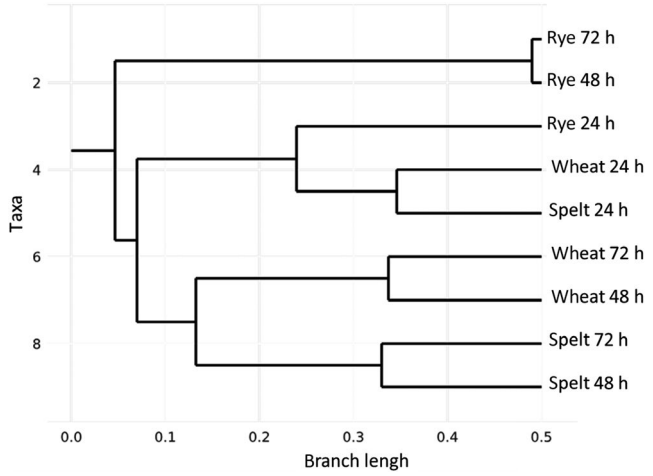

FIGURE 3 Dendrogram showing the similarity among bacterial communities in spontaneous sourdoughs made from wheat, spelt, and rye wholemeal flour based on the Yue and Clayton measurement (Yue & Clayton, 2005) (relative scale)

plantarum and *Lb. brevis* genera. *Lb. plantarum* and *Lb. brevis* are prevalent LAB in sourdoughs and have been identified in 44% and 22% of sourdoughs, respectively (Van Kerrebroeck et al., 2017). This observation is also consistent with the data review made by Gänzle and Zheng (2019) showing that spontaneous sourdoughs harbor environmental or nomadic microorganisms, particularly *Lb. fermentum*, *Lb. plantarum*, and *Lb. brevis* and do not contain *Lb. sanfranciscensis*. The second most frequently isolated groups of LAB (except in the spelt sourdough) were bacteria belonging to *Enterococcus* sp. and *Pediococcus* sp. Their presence was detected in 15% and about 10% of sourdoughs, respectively (Van Kerrebroeck et al., 2017). According to literature data, spontaneous sourdoughs often harbor enterococci, lactococci, and pediococci (Gänzle & Zheng, 2019). Interestingly, LAB belonging to the genera *Weissella*, *Leuconostoc*, and *Lactococcus* were frequently isolated in our studies irrespective of flour type, mostly from samples obtained after 24 hr of fermentation, and were no longer detected in sourdoughs after 72 hr. This might be the reason for the detection of *Weissella*, *Leuconostoc*, and *Lactococcus*, respectively, in only 18%, 15%, and 10% of sourdoughs considered as mature (Van Kerrebroeck et al., 2017). A decrease in the prevalence of *Weissella* spp. in wheat and rye sourdoughs at the late fermentation stage was observed previously (Ercolini et al., 2013). The decrease in the prevalence of at least some of these groups of LAB (presumably *Leuconostoc* and/or *Lactococcus*) in sourdoughs after 24 hr of fermentation is likely correlated with the decrease in acid-producing cocci that was revealed by microbial group counts. The activity of acid-producing bacteria during the first day of fermentation might have resulted in a rapid increase in the acidity of the analyzed sourdoughs. Our results obtained using the classical microbiological approach based on culturing are consistent with general observations that sourdough microbial consortia often contain one to several LAB species (De Vuyst et al., 2014; Minervini, De Angelis, Di Cagno, & Gobbetti, 2014).

Culture-independent analyses, including HTS, have been extensively used to complement classical culture-based methods for determining the biodiversity in various food products, such as milk, fermented dairy products, and plant-, meat-, and fish-derived fermented foods (Alegría et al., 2012; Ercolini, 2013; Kowalczyk et al., 2012; Mayo et al., 2014). In bread technology, the evaluation of microbial diversity, population structure, and population dynamics is generally performed during the sourdough fermentation process. In this study, culture-independent methods were applied for elucidation of microbial diversity and community dynamics in spontaneous sourdoughs made from wheat, spelt, and rye wholemeal or wholegrain flour. Using such approach, bacteria belonging to three phyla, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*, were identified in majority of sourdough samples. The relative number of *Proteobacteria* decreased during the fermentation process (except for the rye sourdough after 72 hr of fermentation). This is consistent with previous findings showing that several bacterial phyla in addition to *Firmicutes* (e.g., *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, and *Proteobacteria*) may be present in the dough before fermentation is initiated. The majority of these phyla represent non-active populations or are outcompeted by *Firmicutes* during the first fermentation cycle (Ercolini et al., 2013; Rizzello et al., 2015). LAB population dynamics are typically characterized by a three-phase evolution (De Vuyst et al., 2014; Van der Meulen et al., 2007; Weckx et al., 2010). The first phase is distinguished by the dominance of LAB species belonging to the genera *Enterococcus*, *Lactococcus*, and *Leuconostoc*. In our study, this phase was probably completed prior to the end of the first day of fermentation for the wholegrain sourdoughs as the overall abundance of these bacteria did not exceed 2% of *Bacteria*. The second phase, which is characterized by an increased prevalence of sourdough-specific LAB, such as species belonging to the genera *Lactobacillus*, *Pediococcus*, and *Weissella*, consequently began within the first 24 hr; this is supported by the fact that the highest abundance of these genera was detected in samples obtained after 24 hr of fermentation and was slightly lower in samples obtained after 48 hr. Finally, according to the literature, the third phase of fermentation is distinguished by the dominance of well-adapted sourdough strains belonging to heterofermentative species (e.g., *Lb. sanfranciscensis* and *Lactobacillus fermentum*) and to *Lb. plantarum*. In our case, the third phase was reached after 72 hr of fermentation and was characterized by dominance of the *Lactobacillus* genus and the highest number of isolates belonging to the *Lb. plantarum* species.

Alpha diversity, defined as the species richness in a single batch of sourdough taking into account the number of species and the proportion in which each species is represented in the community, was quantified using the inverse Simpson parameter and Shannon's diversity index. The first of these measures is sensitive to abundant species, whereas the second is equally sensitive to rare and abundant species (Morris et al., 2014). The inverse Simpson calculator is preferred to other measures of alpha diversity since it indicates the richness of a community with uniform evenness that has the same level of diversity and thus has some biological interpretation (https://www.mothur.org/wiki/Invsimpson). Despite attempts to identify an ideal diversity measure, a single indicator cannot be recommended, and it is suggested that at least two measures must be reported (Morris et al., 2014). In our study, both diversity indices indicated

that the lowest heterogeneity of organisms occurred in the rye sourdoughs (Shannon's diversity index in rye at 72 hr was 1.12) and that higher, similar biodiversity occurred in the common wheat and spelt wheat samples (Shannon's diversity index in wheat and spelt at 72 hr was 2.66 and 2.09, respectively). The most commonly used and the simplest metric representing alpha diversity is richness. This indicator reports the number of species, but not their abundance, and is sensitive to the presence of rare species. According to the literature, alpha diversity in sourdough represented by species richness is rather limited; usually, fewer than 6 different species or strains account for more than 99% of the microbial cells present (Gänzle & Ripari, 2016). At the same time, the gamma diversity of sourdoughs, a measure of the overall number of species present in different sourdoughs, is much higher and typically includes more than 80 bacterial species. Finally, beta diversity, which is defined as species diversity between ecosystems, was compared using the Yue and Clayton measure (Yue & Clayton, 2005). Beta diversity analysis revealed the differences between wheat (common and spelt) and rye sourdoughs. Furthermore, all sourdough samples obtained after 24 hr of fermentation were clustered and separated from sourdough samples obtained from the same flour at other time points. This observation reflects the relative similarity of the initial microbiota present in sourdoughs irrespective of flour type. The similarity of the samples obtained from a single flour type on the second (48 hr) and third (72 hr) days was greatest for the rye sourdough. This suggests that the fermentation phase was close to maturity at these time points and demonstrates the influence of the fermentation process on the development of specific sourdough-typical LAB and highly adapted sourdough-typical LAB.

In conclusion, this work was carried out to determine the microbial biodiversity of sourdoughs that are made from wheat, spelt, and rye wholemeal flour and can be used as a source of strains for specific starter cultures for functional bread production. The research was conducted on an industrial scale and with the use of wholegrain flours, which are anticipated to influence the microbial diversity in sourdoughs.

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CONFLICT OF INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

Jakub Boreczek took the lead in investigation, validation, and visualization; equally contributed to writing—original draft; and made supporting role in writing—review and editing; Dorota Litwinek equally contributed to conceptualization and made supporting role in supervision and writing—review and editing; Joanna Żylińska-Urban made supporting role in investigation; Dariusz Izak equally contributed to data curation and took the lead in formal analysis; Krzysztof Buksa and Jacek Bardowski made supporting role in validation; Jan Gawor and Robert Gromadka equally contributed to data curation; Magdalena Kowalczyk equally contributed to conceptualization; took the lead in funding acquisition, project administration, supervision, and writing—review and editing; and equally contributed to writing—original draft.

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

All data are provided in full in the results section of this paper apart from the Illumina sequencing raw data, which were deposited in the NCBI Sequence Read Archive (SRA) database under the BioProject accession number PRJNA541497, https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA541497.

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APPENDIX

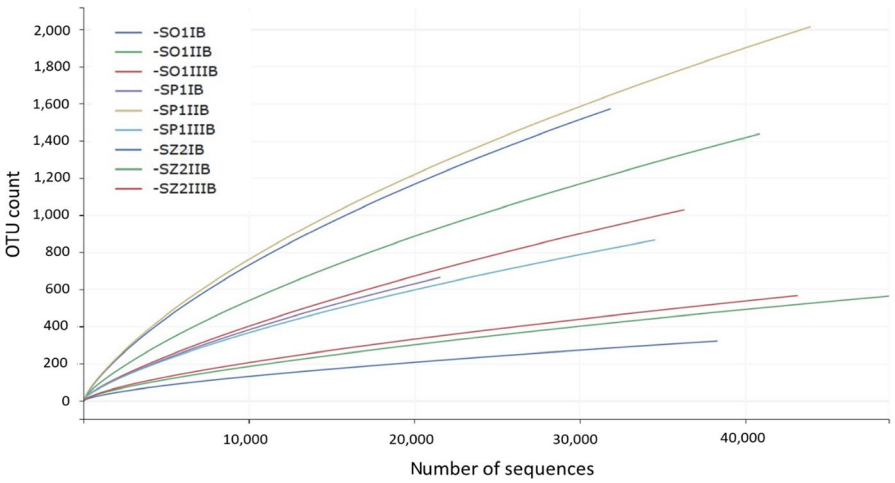

FIGURE A1 Rarefaction curves of the observed OTUs at a genetic distance of 0.03

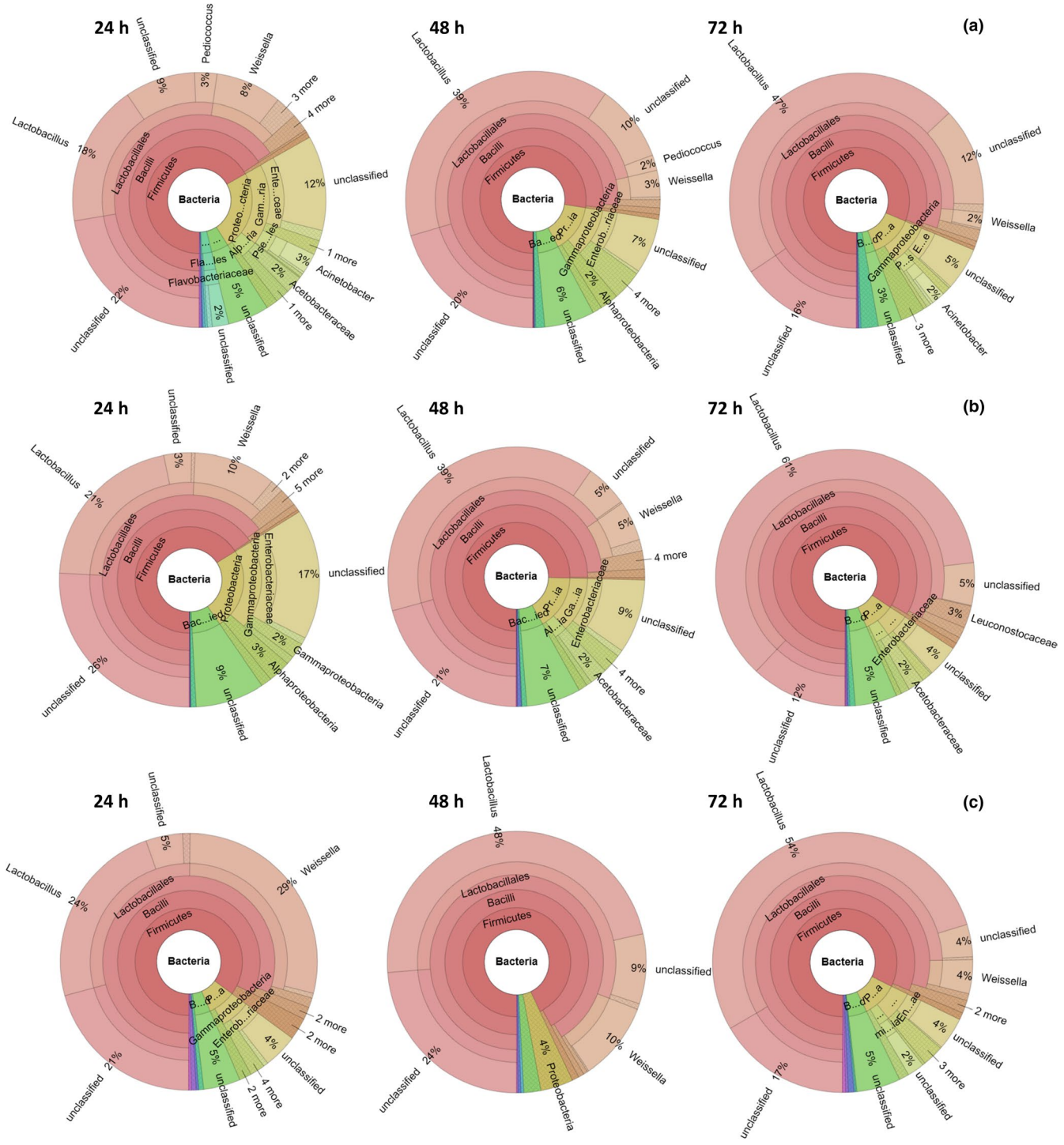

FIGURE A2 Taxonomic structure of the bacterial community in spontaneous sourdoughs made from common wheat (a), spelt (b), and rye (c) during 3 days of backslopping. The figure was prepared using Krona Charts (Ondov et al., 2011)