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# Microbial community dispersal from wheat grains to sourdoughs: A contribution of participatory research

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## Abstract

Understanding microbial dispersal is critical to understand the dynamics and evolution of microbial communities. However, microbial dispersal is difficult to study because of uncertainty about their vectors of migration. This applies to both microbial communities in natural and human-associated environments. Here, we studied microbial dispersal along the sourdoughs bread-making chain using a participatory research approach. Sourdough is a naturally fermented mixture of flour and water. It hosts a community of bacteria and yeasts whose origins are only partially known. We analysed the potential of wheat grains and flour to serve as an inoculum for sourdough microbial communities using 16S rDNA and ITS1 metabarcoding. First, in an experiment involving farmers, a miller and bakers, we followed the microbiota from grains to newly initiated and propagated sourdoughs. Second, we compared the microbiota of 46 sourdough samples collected everywhere in France, and of the flour used for their back-slopping. The core microbiota detected on the seeds, in the flour and in the sourdough was composed mainly of microbes known to be associated with plants and not living in sourdoughs. No sourdough yeast species were detected on grains and flours. Sourdough lactic acid bacteria were rarely found in flour. When they were, they did not have the same amplicon sequence variant (ASV) as found in the corresponding sourdough. However, the low sequencing depth for bacteria in flour did not allow us to draw definitive conclusion. Thus, our results showed that sourdough yeasts did not come from flour, and suggest that neither do sourdough LAB.

KEYWORDS
bread, dispersal, fermentation, lactic acid bacteria, microbial ecology, yeast

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## 1 | INTRODUCTION

Understanding the functioning and evolution of communities is central to ecological studies. Many of the concepts and debates that have animated this field have arisen from the study of plant communities (Mikkelson, 2005). Microbial communities have also been a subject of increasing interest, and it is now clearly established that they play a central role in the functioning and evolution of many ecosystems. Numerous concepts have been proposed in community ecology but it is only recently that theoretical models have unified them to take account of local evolutionary dynamics and the links between communities. Vellend (2010) defined four factors that shape communities: diversification, selection, dispersal and drift. More recently, Thompson et al. (2020) proposed a meta-community model with three factors: density-independent responses to abiotic conditions, density-dependent biotic interactions and dispersal. These general frameworks offer valuable tools to understand the dynamics of microbial communities but suffer from a lack of empirical data on the selection processes and dispersal of microbial communities.

Microbial dispersal has been mainly studied in natural environments or for human pathogen. The dispersal of beneficial microorganisms in agri-food chains has received less attention. In agri-food chains, humans are expected to contribute to the dispersal of microorganisms, either as a direct vector of transmission or indirectly through the food products they carry. A high dispersal rate would lead to a reduction of diversity throughout the food chain and between food chains. Dispersion through food products could have even greater consequences for long food supply chain, where products are transported over long distance. It is therefore important to better understand the dispersion of microorganisms in food chains to better predict their ability to maintain microbial biodiversity and therefore, their sustainability.

Microbial communities have been used to make fermented foods since the Neolithic era (Tamang & Kailasapathy, 2010) in which they usually display relatively little complexity with regards to wild environments, making them good model systems for ecological studies. They are organized as metacommunities in which the microbial community of each leaven evolves as a function of human practices and may be linked to others through exchanges of the fermented product themselves or of the raw materials used to make them. Among the numerous fermented foods, sourdough microbial communities used for bread-making represent a good meta-community model system. First, sourdough microbial communities are relatively simple, usually containing one to two dominant bacterial and yeast species (Arora et al., 2021; Carbonetto et al., 2018). Second, sourdoughs are made of few ingredients, basically flour and water, which are regularly added to feed the microorganisms, thus limiting the number of sources of microbial species. Third, the microbial communities in sourdough have been widely studied and reviewed (Arora et al., 2021; Calvert et al., 2021; De Vuyst et al., 2016; Gänzle & Ripari, 2016; Gänzle & Zheng, 2019; Gobbetti et al., 2016; Landis et al., 2021; Lau et al., 2021; Van Kerrebroeck et al., 2017). Well-known species such as *Fructilactobacillus sanfranciscensis*, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis* bacteria and *Saccharomyces cerevisiae*, *Kazachstania humilis*, *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* yeasts are frequently encountered. Finally, population genomic analysis of the yeast species *S. cerevisiae* has shown that sourdough yeast populations differ from commercial yeasts and may have undergone specific selection processes when compared to industrial processes (Bigey et al., 2020). The diversity of microbial communities has been studied in home-made and bakery sourdoughs from all over the world (Arora et al., 2021; Landis et al., 2021) and did not display any clear geographical pattern. Several studies revealed that the bakery house microbiota is the main driver of sourdough microbial diversity but the origin of microbes that composed the house microbiota remained to be elucidated. The same species of lactic acid bacteria (LAB) or yeast could be found on the baker's tools (Minervini et al., 2015) or hands (Reese et al., 2020) and in their sourdough. But, no sourdough microorganisms were detected in the bakery air (Minervini et al., 2015) or in the water (Reese et al., 2020; Scheirlinck et al., 2009) used to make the sourdough. Some studies have shown that flour can be a vector for *Lactobacillaceae*. However, this was only shown for three different flours (Minervini, Dinardo, et al., 2018) or for laboratory-made sourdoughs (De Angelis et al., 2019), whose dynamics are not the same as bakery sourdoughs (Minervini et al., 2012). It is still unclear whether flour is a source or/and a vector of sourdough yeast and bacteria.

In this paper, we tested whether wheat grains and flour can be a source of microorganism in newly initiated sourdough and in mature sourdoughs. The study was carried out in French sourdoughs which was found to include most of the yeast species detected world-wide (Carbonetto et al., 2018) and the main lactic acid bacteria species (Lhomme et al., 2015; Michel et al., 2016). The distribution of microbial species in this country suggests that flour could be a vector of dispersion. The majority of sourdoughs contained *F. sanfrancisensis* suggesting that this species is easily dispersed among the sourdoughs, potentially through the flour (Lhomme et al., 2015; Michel et al., 2016). The diversity of yeast species appeared to be structured by the bread-making process. Sourdoughs made with farmer baker's practices mostly exhibit *Kazachstania bulderi*, while sourdoughs made with artisans baker's practices mostly exhibit *Kazachstania humilis*, suggesting a potential role of the type of flour used (Urien et al., 2019). We used a participatory research approach including farmers, bakers and scientists. First, we set up an experiment where 6 wheat populations were used to make 6 flours, which were given to four bakers that make sourdoughs and we follow-up microorganism dispersion along the sourdough making chain from grain to mature sourdough. Second, we studied the microbial species diversity of 44 flours and related mature sourdough samples collected in French bakeries. We did not find any evidence that flour is a vector for sourdough yeasts. Flour rarely carry LAB species and at very low density. We discuss these findings in relation with ecological processes driving microbial community evolution in sourdough.

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## 2 | MATERIAL AND METHODS

### 2.1 | Participatory experimental design for studying microbial community succession from grain to sourdough

#### 2.1.1 | Seeds sampling

Three wheat landraces, (i) 'Saint Priest et le Vernois Rouge', (ii) 'Redon Roux Pâle' and (iii) 'Bladette de Provence' and three modern wheat varieties, (i) Renan, (ii) Pireneo and (iii) Chevalier were grown each on three terroirs. The seeds were sown in autumn 2014 and harvested in July 2015. The grains were sorted with an optical sorter, an air column and a cleaner-separator. They were stored in dehydrated room in 20–25°C till the end of October, then in 10°C without dehydration to fight against weevils. For each terroir, grains from the three landraces were mixed and so were grains from the three modern varieties leading to six lots of grains. Two independent samples of 250 g of these six lots (12 samples) as well as of grains of each varieties from two terroirs (24 samples) were collected, resulting in 26 samples that were stored at −20°C for metabarcoding analyses.

#### 2.1.2 | Milling and flours sampling

The six lots of grains were sent to a French miller (Hélène Chaudy, Ferme d'Orvillier) who is also a farmer-baker (Baker B3). Crushing and sifting were performed with an Astrie stone-mill. To avoid flour contaminations from one lot to another, grains belonging to the miller were ground after each experimental lot milling. For each of the six grain lot, samples of flours were collected at the beginning and at the end of the milling in duplicate leading to 24 flour samples. Flour made with grains belonging to the miller was also sampled at the beginning and at the end of the milling process as control. The 26 flour samples were stored at −20°C for metabarcoding analyses.

#### 2.1.3 | Sourdough sampling

The flours of the six seeds lots were sent to four bakers (MP, PV, HS, LM), who initiated and maintained six sourdoughs for three weeks. Sourdoughs were also initiated at the lab in the same conditions (LL). Each baker received a pallet with 10 kg of each flour, buckets for each sourdough and sample pots for the sampling. They also got a scoresheet to follow the temperature of the flour, of the bakery, of the water; the weight of flour and water at each back-slopping. Each baker made the sourdoughs with his own protocol, although the same one had to be used for the six received flours. They all had to maintain their sourdough for three weeks. Baker MP, PV, HS, LM made 5, 7, 9 and 10 back-slopping respectively. Bakers were asked to have their hands washed between each sourdough back-slopping. The bakers took samples of sourdough, approximately 40 grams, the day of the initiation, after one week, two weeks and three weeks of back-sloppings and stored the samples at −20°C for metabarcoding analysis. The same protocol of sourdough preparation and sampling was done in a laboratory environment as control although 13 back-slopping were carried out in 3 weeks. Overall, 128 samples of sourdoughs were stored for metabarcoding analyses including (i) 30 experimental sourdoughs J0 (ii) 30 sourdoughs collected at sampling time S1 (week 1), (iii) 30 sourdoughs collected at sampling time S2 (week 2) and (iv) 30 sourdoughs collected at sampling time S3 (week 3) (v) 8 baker's own sourdoughs, 4 collected at the beginning of the experiment and 4 collected at the end of the experiment.

### 2.2 | Collection of flour and sourdough from French bakers

#### 2.2.1 | Survey of bread-making practices

A total of 22 bakers and 22 French farmer bakers completed a questionnaire on their bread-making practices, as described by Michel et al. (2019). Questions concerned sourdough management (addition of bran, back-slopping technique, time elapsing since sourdough initiation, sourdough hydration, number of back-slopping procedures per week and between bread-making sessions, temperature at back-slopping), the flour (self-produced or not, type of cereal variety, type of mill) and the bread-making process (use of selected baker's yeast in bread or in other products, mechanical or manual kneading, proportions of sourdough, flour, water and salt in bread dough, fermentation time, quantity of bread produced each week, number of bread-making sessions per week). We also asked the producers if they had shared raw materials (grains, flour or sourdough) or if they had physical contacts with each other. This survey, together with the survey from Michel et al. (2019) were used to choose the experimental design presented above (Section 2.1).

#### 2.2.2 | Sample collection

A total of 46 mature sourdoughs were collected, together with the flour used to make each one. The collection included only sourdoughs made by bakers but no home-made sourdoughs, because the process of domestication and dispersal could be different with these two types of sourdoughs. Forty-four sourdoughs came from different bakeries located everywhere in France, and two bakeries (B64 and B68) sent two sourdoughs, so that 46 sourdough and 44 flour samples were studied. We chose to collect samples at country scale because the millers who produce the fresh flour for bakers, distribute it mainly at this geographical scale. Samples were collected between September 2018 and July 2019 and were received at the laboratory within one to three days. All samples were stored at −20°C in plastic bags and plastic tubes, respectively, before DNA extraction.

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#### 2.2.3 | Sourdough and flour microbial enumeration

All 46 sourdoughs and 21 of the 44 flour samples were plated at reception. 10 g sourdough or 3 g flour were diluted ten times in tryptone-salt buffer (1 g/L tryptone, 8 g/L NaCl). After serial dilutions, lactic acid bacteria (LAB) were enumerated on MRS-5 (Meroth et al., 2003) with 100 μg of cycloheximide and on PCA (6 g/L Tryptone, 2.5 g/L yeast extract, 1 g/L glucose, 15 g/L agar) media while yeasts were enumerated on YEPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 100 mg/L chloramphenicol). Culture-dependent methods were only used for enumeration purposes.

### 2.3 | Metabarcoding analysis

Microbial species diversity of grains, sourdoughs and flours was analysed by amplicon-based DNA metabarcoding. For the collected sourdoughs, two separate Illumina MiSeq runs were used for sourdough and flour to prevent any contamination between the sample types.

#### 2.3.1 | DNA extraction from seeds, flour and sourdough

DNA from seeds microbiota was obtained after maceration of ten grams of seeds in 40 ml of buffer PBS Tween 20 (0.05% v/v) for 40 h at 4°C with constant stirring (5 *g*). The macerates were filtered with Stomacher® bags. After centrifugation at 3075 *g*, during 10 min at 4°C, 2 ml of the supernatant were mixed with the pellet and DNA was extracted using the Powersoil DNA isolation kit (MoBio) as explained in the manufacturer's protocol.

Flour DNA was obtained by mixing five grams of each flour with 35 ml of PBS buffer using a stomacher during 5 min. The filtrate was centrifuged at 123 *g*, for 5 min at 4°C. The supernatant, was then centrifuged at 769 *g* for 15 min at 4°C. The pellet was resuspended in 500 μl of PBS buffer and the DNA extracted following the Powersoil DNA isolation kit procedure (MoBio 12888-100).

Finally, sourdough DNA was extracted directly from 200 mg of material following the Qiagen PowerSoil DNA isolation kit procedure (12888-100). Extraction was then performed in accordance with the manufacturer's instructions.

#### 2.3.2 | Markers amplification and MiSeq sequencing

The 16S V3-V4 region was amplified for bacteria and the ITS1 region for fungi. For fungi, the ITS1 region was targeted with the PCR primers ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (White et al., 1990), while for bacteria, the 16 V3-V4 region was targeted with the PCR primers 343F: (5′-TACGGRAGGCAGCAG-3′) and 784R: (5′-TACCAGGGTATCTAATCCT-3′) (Liu et al., 2007). The primers also included the Illumina tail (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′), and a frame-shift of four, six or eight random nucleotides for forward primers and four, five or six random nucleotides for reverse primers, in order to prevent saturation during sequencing. The primers therefore had the following structure: 5′-Illumina tail—frame-shift—genome targeting region-3′. All the primers used are listed in Table S1. For each forward or reverse primer, an equimolar mix of the three primers containing the different frame-shifts was added to the PCR mix. To prepare the multiplexed Illumina libraries, we employed a strategy based on a two-step PCR approach: a first PCR using the locus-specific primers including the Illumina adapter overhang (with 30 cycles), and a second PCR for the incorporation of Illumina dual-indexed adapters (with 12 cycles). Bead purifications were carried out after each PCR. Quantification, normalization and pooling were performed before sequencing on Illumina MiSeq (Ravi et al., 2018).

#### 2.3.3 | Bioinformatics analyses

The resulting sequences were analysed using R (Team, 2019) workflow combining dada2 v.1.16 (Callahan et al., 2016) and FROGS 3.2.2 (Escudié et al., 2018) software. Reads were filtered, merged and assigned to ASVs with dada2 and the ASVs were assigned to species using the FROGS affiliation tool. Adapters were first removed using cutadapt v. 1.12 with Python 2.7.13. Reads were then filtered using the dada2 filterAndTrim function, with a truncation length of 250 bp for ITS1 forward and reverse reads and 275 and 200 bp for 16S forward and reverse reads, respectively. This truncation reduced the error rate while still allowing the merging of most reads. The error model was then calculated using the learnErrors function. Reads were dereplicated using derepFastq and the dada2 core sample inference algorithm was executed. Forward and reverse reads were then merged with a minimum overlap of 20 bp. The resulting sequences were saved in a sequence table using makeSequenceTable. Chimera were removed using the removeBimeraDenovo function. The amplicon sequence variants (ASV) in the sequence table were then assigned to species using FROGS affiliation v3.2.2 with silva 138 (Quast et al., 2013) for 16S and Unite 8.0 (Nilsson et al., 2019) for ITS1. Unite was completed with ITS1 reference sequences from yeast species usually found in sourdough. Multi-affiliations were dealt with by assigning the lowest common taxonomy level to multi-affiliated ASVs. Samples were rarefied to the minimum number of reads for each barcode, or 1000 reads using the rarefy_even_depth function of the R (v. 4.1.0) phyloseq package (v. 1.24.2) (McMurdie & Holmes, 2013). Samples with a depth of less than 1000 were discarded. If not otherwise specified, the analyses were conducted on the rarefied data.

#### 2.3.4 | Analysis of bread-making practices

Groups of bread-making practices were obtained with an MCA computed with the R package FactoMineR (v. 2.4), and individuals were

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clustered using the HCPC function with two clusters. They were plotted using the factoextra package (v. 1.0.7).

## 2.4 | Statistical analysis

A Wilcoxon–Mann–Witney test was performed to compare the diversity index between the flour and sourdough samples. The correlation between flour and sourdough diversity was computed using a Spearman rank-order correlation test. Both tests were computed using the R package stats v 3.6.2, with the wilcox.test and cor.test functions, respectively. A Mantel test was performed to test the link between geographical distances for sourdoughs and Bray–Curtis distance matrices, using the R ape package (v. 5.5) mantel.test function.

# 3 | RESULTS

## 3.1 | Microbial community succession along the sourdough making chain

To investigate the potential role of wheat grains and flour as an inoculum of yeast and bacteria in newly initiated sourdoughs, we investigate the composition of the microbiota along the sourdough making chain following the sequence of wheat seeds, flours, sourdoughs at initiation and after one, two and three weeks of back-slopping. Overall, a total of 190 samples, including 36 wheat grains, 24 flours and 120 sourdough samples were analysed by metabarcoding on the 16S V3V4 region for bacteria and ITS1 gene for fungi. After trimming and removal of sequences corresponding to mitochondria and chloroplasts, we obtained a median of 38,013, 2178 and 37,165 reads for grains, flours and sourdoughs samples, respectively, for 16S sequencing, and 20,818, 19,597 and 19,125 for ITS1 sequencing (Figure S1).

The level of alpha diversity on the seeds and in the flours were close with 21 and 16 bacterial genus and 33 and 37 fungal genus detected on grains and in flour, respectively. Species richness ranged from 1 to 40 per grain sample and 4 to 18 per flour sample for bacteria and from 6 to 38 and 8 to 29 in grain and flour samples, respectively, for fungi (Figure 1). The level of alpha diversity then decreased over the weeks as sourdoughs were back-slopped to reach an average of 13 bacteria and 13 yeast species in three weeks old sourdoughs (Figure 1). Three weeks old sourdoughs were the only samples to have dominant genus (Freq >0.5). Their microbial communities were indeed typical of a mature sourdough, with one to three dominant species of bacteria and yeasts except for laboratory sourdoughs and sourdoughs made by baker 1 that had only carried five back-slopping during the three weeks. Surprisingly, a lower bacteria alpha diversity was observed in flour compared to newly initiated sourdough. This observation could be explained by the low number of reads in flour samples after having

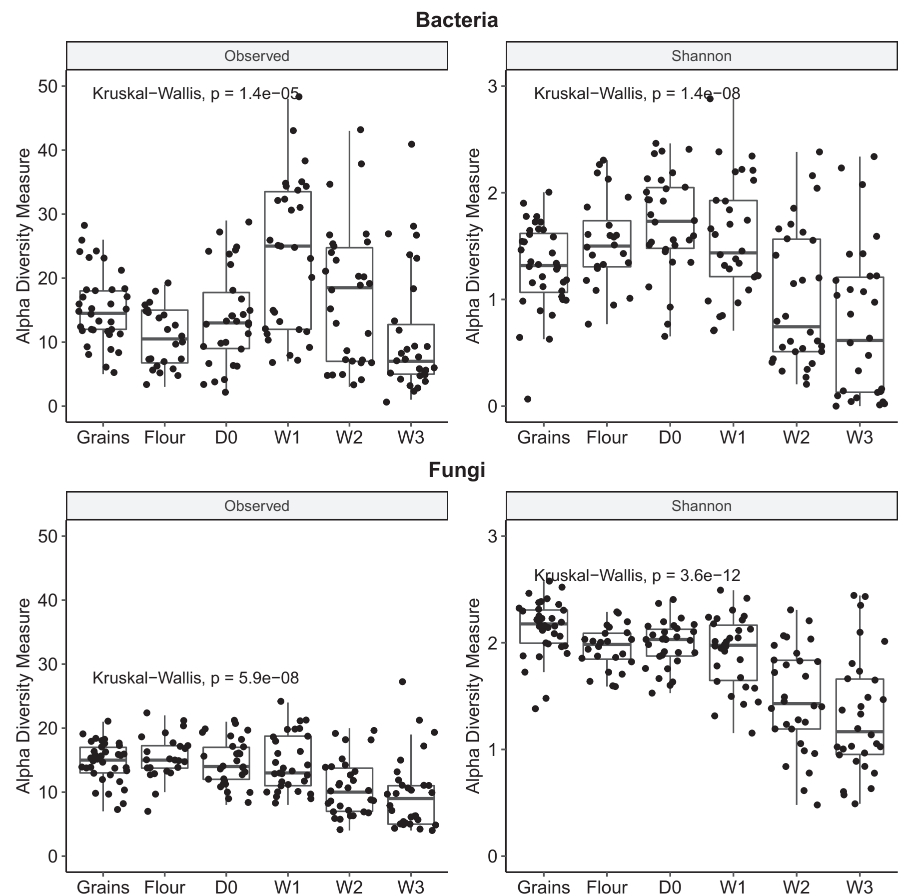


FIGURE 1 Alpha diversity in sourdough and flour samples, estimated from 16S V3-V4 and ITS1 Illumina MiSeq reads assigned to species. Species richness (on the left) and evenness (on the right) are plotted for grains, flours and sourdough at their initiation (D0) and after one, two and three weeks of back-slopping (W1, W2 and W3). Data relative to bacteria are shown at the top and relative to fungi at the bottom.

removed the sequences corresponding to chloroplasts and mitochondria (Figure S1).

The core microbiota, which can be defined as the microbial community consistently present in grains, flours and sourdoughs, was composed of 10 genera of bacteria with a high prevalence of *Pantoea* and *Pseudomonas*. Other genera included *Erwinia*, *Massilia*, *Paenibacillus* and *Sphingomonas*. These genera were strongly represented on the grains, but their frequency decreased along the sourdough making chain. The composition of the bacterial genera on the grains and in the flours was similar, with the exception of one batch of flour containing mainly the genus *Erwinia*. In contrast, several bacterial genera were detected only in the sourdoughs, including *Weisseila*, as well as several genera of the family *Lactobacillaceae*. The proportion of the latter increased until it composed almost all the reads after three weeks of back-slopping. Different species, including *Fructilactobacillus sanfranciscensis Companilactobacillus paralimentarius*, *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* increased in frequency depending on the baker.

Concerning fungi, the core microbiota was composed of 26 genus, with a prevalence of *Alternaria*, *Epicoccum*, *Mycosphaerella*, *Cladosporium*, *Filobasidium*, *Cystofilobasidium* and *Vishniacozyma*. The genera *Fusarium* and *Penicillium*, known to produce mycotoxin, were also present all along the chain but with a lower frequency. The frequency of fungal phylum (Ascomycota and Basidiomycota) changed from the grains to three weeks old sourdoughs, with Basidiomycota mostly found in seeds (freq >0.6) and Ascomycota mostly found in sourdoughs S3 (freq >0.8). The fermenting yeast genera *Saccharomyces*, *Kazachstania*, *Torulaspora* and *Wickerhamomyces* appeared during back-sloppings and were dominant after three weeks in the sourdoughs. Within a bakery, the same species became dominant regardless of the flour used for back-slopping. As for bacteria, different yeast species invaded the community depending on the bakery suggesting an origin from the house microbiota. They included *Saccharomyces cerevisiae*, *Kazachstania bulderi*, *Kazachstania servazzii* and *Wickerhanomyces anomalus*.

Overall, the *Lactobacillaceae* and *Saccharomycetaceae* that are generally present in sourdough and known to be responsible for most of its beneficial functions were detected after sourdough initiation but were not found in the grains and flours, except in two flour samples. One flour sample contained 0.01 percent of *F. sanfranciscensis* and another 0.06 percent of *Kazachstania exigua*. However, the ASVs found in the flour were never found in the sourdough made with this flour.

## 3.2 | Mature sourdoughs microbiota had greater microbial density but less species diversity than the flour microbiota

We also compared 46 mature sourdough samples obtained from 44 bakeries located throughout France with the 44 flour samples used to refresh them, in order to confirm on a larger scale that flours do not contain fermentative species found in sourdough.

On average, microbial density was higher in sourdoughs than in flours, for both bacteria and fungi. Sourdoughs contained on average $1.9\times10^{7}$ (SD = $1.3\times10^{7}$) CFU/g (colony forming units/g) of yeast while flours contained a mean of $2.3\times10^{3}$ (SD = $1.6\times10^{3}$) CFU/g. As for bacteria, the sourdoughs contained $1.3\times10^{9}$ (SD = $1.3\times10^{9}$) CFU/g while flours contained $7.7\times10^{3}$ (SD = $2.0\times10^{4}$) CFU/g or $6.9\times10^{4}$ (SD = $1.0\times10^{5}$) CFU/g, depending on whether the estimation of bacterial density was performed on MRS or PCA. Sourdoughs were only plated on MRS medium, as we expected to find only *Lactobacillaceae*, while flour generally harbours a more diverse bacterial community so we also plated these samples on PCA, which is a less specific medium. The observation of fungal morphology on YEPD petri dishes revealed that most flour samples contained filamentous fungi, some with a typical *Penicillium* morphology, while sourdough samples were characterized by the presence of yeasts.

The composition of the flour and sourdough microbiota were then analysed by metabarcoding on the 16SV3V4 and ITS1 regions. After trimming and removal of sequences corresponding to mitochondria and chloroplasts, we obtained a median of 2031 and 62,421 reads for flours and sourdoughs, respectively, for 16S sequencing, and 27,371 and 44,567 reads for ITS1 sequencing. Although sourdoughs had a higher microbial density than flour, their microbial communities were less diverse than those in flour. Alpha diversity indexes calculated on the number of bacterial and fungal species were significantly lower in sourdough than in flour in terms of both richness (Wilcoxon–Mann–Witney test, bacteria $W = 1725.5$, $p<.001$, fungi $W = 1555.5$, $p<.0001$) and evenness (Wilcoxon–Mann–Witney test, bacteria $W = 1929$, $p<.001$, fungi $W = 1467$, $p<.0001$; Figure 3). This difference was greater for bacteria than for fungi, with averages of four and 11 species for bacteria in sourdough and flour, respectively, and 10 and 13 species for fungi in sourdough and flour, respectively.

Sourdough species diversity was not correlated with flour species diversity for either bacteria (Spearman = 13,617, $p = .86$) or fungi (Spearman = 13,019, $p = .91$).

The microbiota compositions of sourdough and flour were characterized by different families. The bacteria in the sourdoughs were almost entirely composed of *Lactobacillaceae*, while flour contained mainly *Erwiniaceae* and *Pseudomonadaceae*. In sourdough, all samples but three contained *Fructilactobacillus sanfranciscensis* as the dominant bacterial species; the others contained *Companilactobacillus paralimentarius*. Less frequently, the presence of *Levilactobacillus brevis*, *Latilactobacillus* sp. and *Lactilactobacillus* sp. was found. In flour, *Erwiniaceae*, *Pantoea agglomerans*, an unidentified *Pantoae* sp., and *Pseudomonadaceae* were generally detected. Among *Pseudomonas* sp., some were *P. graminis*, *P. rhizospherae* or *P. donghuensis*. As for fungi, *Saccharomycetaceae* was determined in most sourdough samples but was almost absent from flour samples (Figure 4); *S. cerevisiae* was found in 14 sourdough samples, *K. humilis* in seven samples and *K. bulderi* in six. These species were never found in flours. *Pleosporaceae* species (*Alternaria alternata* and *Alternaria infectoria*), *Mycosphaerellaceae* (*Mycosphaerella tassiana*)

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and an unidentified fungus from the *Dothideomycetes* family were detected at a high frequency in almost all flour samples.

### 3.3 | Very little overlap between the microbiotas of mature sourdough and flour

Any overlaps between the mature sourdough and flour communities were analysed using the Weighted Bray–Curtis distance calculated on the basis of species diversity. The Weighted Bray–Curtis distance was used to build two PCoAs, one for the bacterial community and the other for the fungal community. PCoA axis 1 and 2 explained 79.1% and 8.5% of variance for bacteria, and 28.5% and 13.6% of variance for fungi (Figure 5). For bacteria, axis 1 separated the flour and sourdough bacterial communities. For fungi, axis 1 separated many but not all of sourdough fungal communities from flour communities. Over the 46 sourdough fungal communities, 14 co-localized with flour fungal communities. Flour and sourdough dissimilarity matrices were not correlated (Mantel test, $z = 836$, $p = .667$ for bacteria and $z = 854$, $p = .13$ for fungi). Close microbial communities among flours did not lead to close microbial communities among sourdoughs.

We analysed bread-making practices in order to determine whether they might be related to microbial communities in sourdough and flour. Two groups of bread-making practices could be distinguished. Farmer-baker practices (cluster 1) were more frequently associated with the use of non-commercial yeast, ancient wheat landraces, small production runs and lengthy fermentation while artisanal practices (cluster 2) were generally characterized by larger scale production, short fermentation, and the use of commercial yeast and modern wheat varieties. Sourdough from farmer bakers frequently contained *K. bulderi* as the dominant yeast species. However, analysis of the association between sourdough and flour microbial community dissimilarity and the geographical distances between bread-making practices did not reveal any correlation (Mantel test, for flour, $z = 308$, $p = .59$ and $z = 235$, $p = .79$ for bacteria and fungi, respectively; for sourdough, $z = 153$, $p = .60$ and $z = 411$, $p = .32$ for bacteria and fungi, respectively).

The differences between the microbial communities in sourdough and flour were explained by the high abundance in sourdough samples of fermentative microorganisms, which were almost never found in the flour samples (Figure 6).

Overall, fermentative bacteria in the *Lactobacillales* order and yeast in the *Saccharomycetales* order were not detected in most flour samples. Out of 46 samples, ten flour samples contained fermentative bacterial species (*F. sanfranciscensis*, *Lactococcus garviae*, *Carnobacterium divergens*, *Weisella* or *Streptococcus* species) and 13 harboured fermentative yeasts (*Candida saitoana*, an unidentified *Candida* species, *Wickerhamomyces anomalus*, *Mechnikovia* sp. or *Eremothecium coryli*). However, the fermentative species found in flour samples were generally not found in the related sourdoughs. In six cases, *F. sanfranciscensis* was found in both flour and sourdough. Nevertheless, in these cases, the ASVs were not the same except in the case of baker 53 (Figure 7). *Lactococcus garviae* was found in the flour and sourdough used by baker 45 but only one read was present in the sourdough and this ASV differed from that found in the flour. An unidentified *Metschnikowia* species was found in four pairs of sourdough and flour, and *Candida saitoana* and an unidentified *Candida* species in one pair of sourdough and flour samples, although the same ASV was not found in them. Many non-fermentative fungal species were shared between flour and sourdough samples. They were mainly filamentous fungi, and notably species from the genus *Alternaria* or *Mycosphaerella*. For these species, the flour and sourdough samples shared on average 0.98 ASV (SD = 1.48).

## 4 | DISCUSSION

### 4.1 | Interests and limits of the participatory approach

This study could not have been conducted without developing a participatory approach. Farmers, Bakers and scientists all engaged during a four years project to gain new insight into the microbial diversity of the bakery food chain with the aim of promoting the sustainability of this food chain. Farmers and bakers contributed by designing the experiments, growing wheat, milling grains and making experimental sourdoughs. The choice of wheat populations (two types of grain mixture representing landraces and modern varieties), milling technique (stone-mill), sourdough elaboration practices (hydration, number of week of back-slopping, …) were discussed by professionals and researchers. Bakers and farmer bakers also sent samples of their sourdough and flour for analysis at the laboratory. In addition, they participate to the interpretation of the data and dissemination of the results. Overall, 54 farmers, farmer bakers and bakers participated to the study. This approach allowed us to analyse the dispersion of microbes along the sourdough making chain in real agri-food environments. Laboratory-made sourdoughs typically have a different microbial composition than those made in bakeries as show by our study (Figure 2) and others such as in Minervini et al. (2012). Therefore, the analysis of sourdough ecology requires the study of sourdough in situ, which requires the active participation of bakers. This collaboration also allows for knowledge sharing, with bakers learning microbial ecology and scientists learning baking. Indeed, this project has led to the development of a glossary of sourdough baking (Roussel et al. (2020)). Participatory research is time consuming as it requires the development of a common vocabulary but also trust between partners. Conducting experiment in a rigorous manner outside the laboratory is also a challenge. It is therefore common to have missing data. Here, in addition to numerous meetings in different locations to design the experiment, the bakers conducted an experiment over several weeks in addition to their professional activity. Their involvement, as well as that of the researchers, made it possible to obtain all the expected data. It should be noted, however, that it is not possible to carry out all possible experiments in participatory research projects. In particular, in

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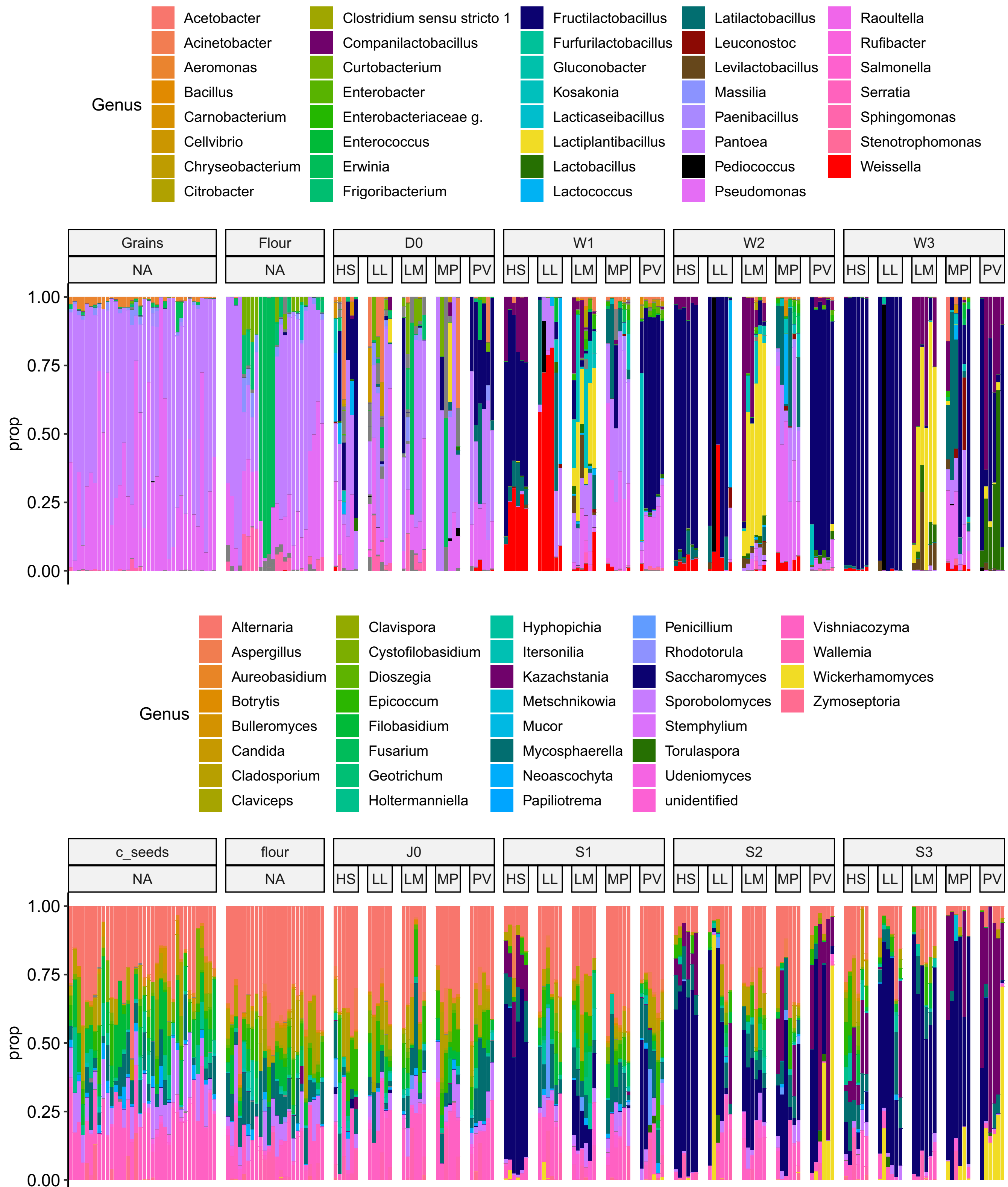


FIGURE 2 Microbiota along the sourdough making food chain. Seeds, flours and newly initiated sourdoughs samples are plotted on the x axis, and their proportion in the sample (based on the unrarefied data) on the y axis. Colours represent genus. The horizontal white bars delimit different ASV. The four bakers (HS, LM, MP, PV) and the lab produced six sourdoughs out of the six batches of flour produced. The sourdoughs were sequenced at their initiation (J0), and once a week (S1, S2, S3). Each flour was sequenced in duplicate at the beginning and at the end of the experiment, resulting in sequencing of 24 samples of flour. [Colour figure can be viewed at wileyonlinelibrary.com]

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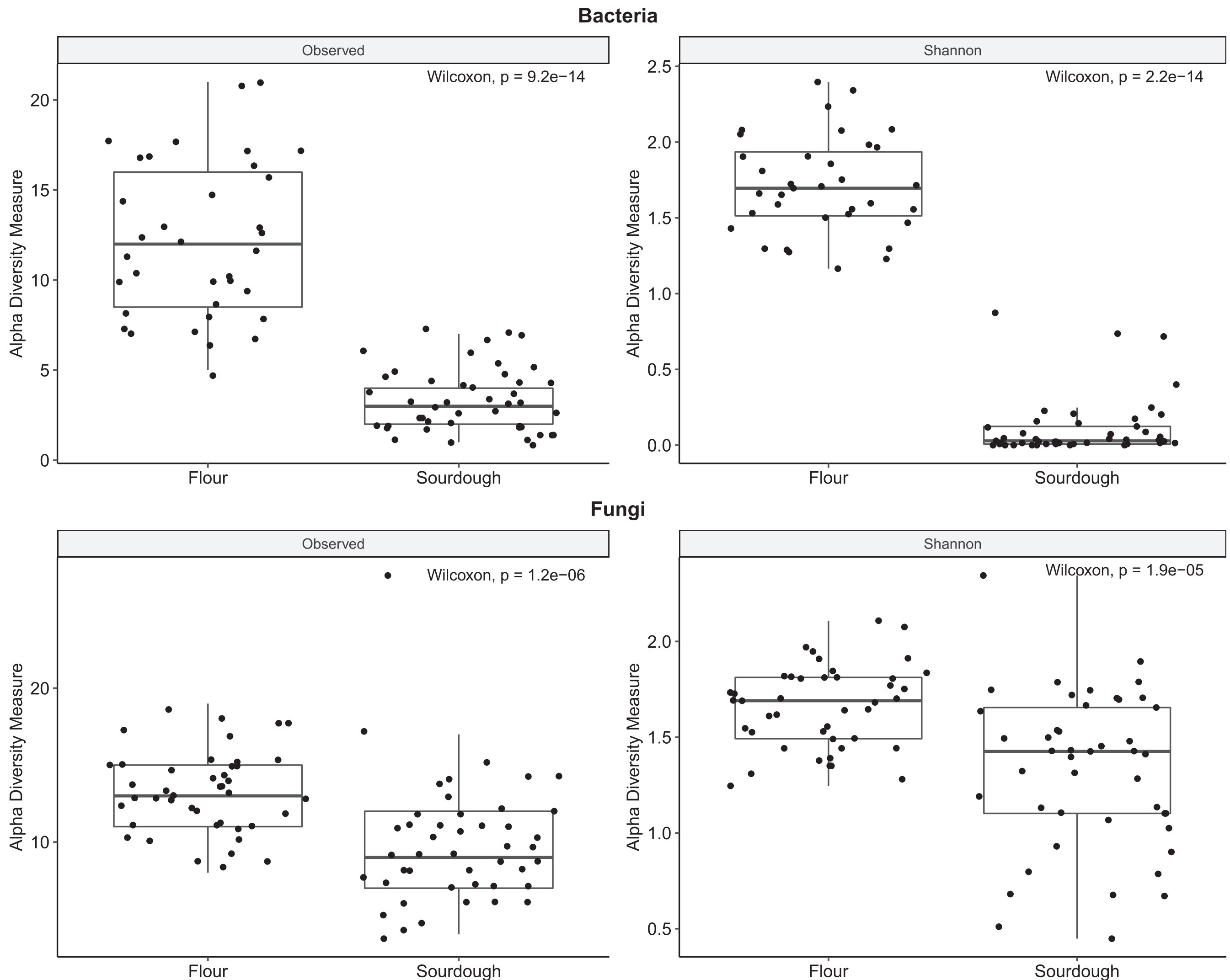


FIGURE 3 Alpha diversity in sourdough and flour samples, estimated from 16S V3-V4 and ITS1 Illumina MiSeq reads assigned to species. Species richness (on the left) and evenness (on the right) are plotted.

our case, the bakers did not want to carry out sourdough invasion trials with strains or sourdoughs from elsewhere, fearing that this would alter the microbiota of their sourdough and their bakery.

## 4.2 | General features of the sourdoughs

The composition of the experimental and collected sourdough microbiota was consistent with previous studies on sourdough. The mean LAB to yeasts ratio was 65.4, which is within the same range as that reported by other studies (Arici et al., 2017; Fraberger et al., 2020; Lhomme et al., 2015; Zhang et al., 2011). As previously detected in French sourdoughs, *F. sanfranscisensis* was the most frequently encountered bacterial species. *S. cerevisiae*, *K. humilis* and *K. bulderi* were the most frequently encountered sourdough dominant yeast species (Lhomme et al., 2015; Michel et al., 2016; Urien et al., 2019). Surprisingly, *Saccharomycetales* accounted for fewer than 5% of the reads in ten collected sourdough samples, yet a typical yeast density and morphology was observed in almost all of these samples. This may have reflected biases in the metabarcoding analysis (van der Loos & Nijland, 2020). DNA could have been poorly extracted or amplified, thus leading to a low number of reads. The reads might also not have passed the quality filtering or merging steps in the bioinformatics analysis, particularly if the ITS region was too long. This is a limitation of the dada2 software, where reads that are too long to be merged are lost. However, this does not concern the ITS database, as in this case the ASV would have been found but not assigned to a species.

## 4.3 | Flour-associated species were mainly plant-associated microorganisms

The microbiotas of the experimental and collected flours as well as that of the seeds mainly comprised plant-associated microorganisms. Several filamentous fungi known to be cereal pathogens, and notably *Alternaria* and *Mycosphaerella* species were detected.

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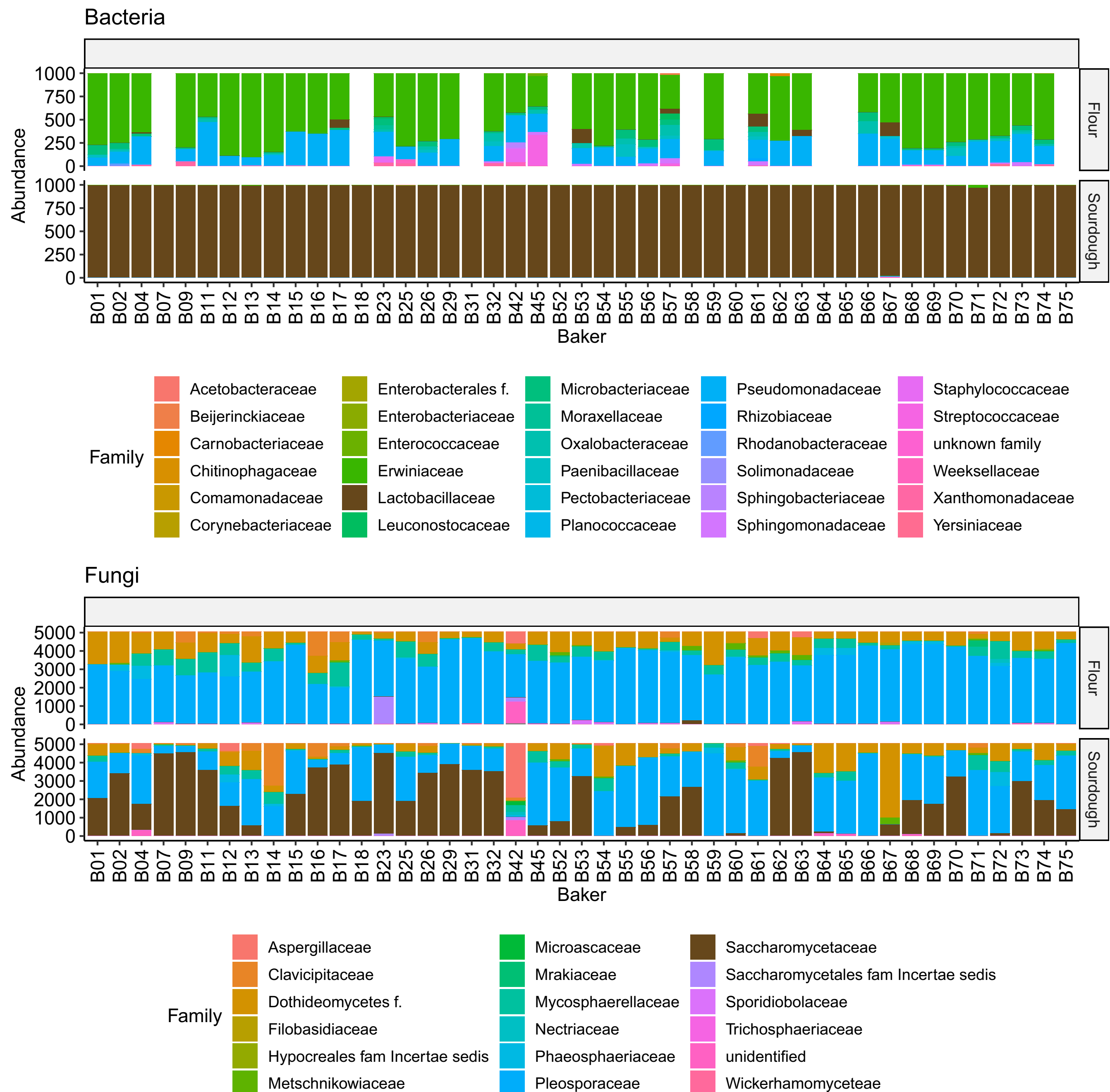


FIGURE 4 Microbial composition of the collected flours and sourdoughs. Colours represent families, their abundance based on the rarefied data is plotted. Horizontal white bars delimit the different ASVs. Data are missing when samples contained less than 1000 reads due to rarefaction, resulting in no data for eight flours. [Colour figure can be viewed at wileyonlinelibrary.com]

Similarly, several bacterial genera such as *Pseudomonas* and *Pantoea* were found. Many species in these genera are plant pathogens or plant-associated species (Dutkiewicz et al., 2016; Preston, 2004).

Most of the species that we detected in seeds and flour during this study had been mentioned in previous studies on wheat seed microbiotas (Kuzniar et al., 2020; Minervini, Lattanzi, et al., 2018; Rozhkova et al., 2021). They had also been mentioned in studies describing flour microbiota (Landis et al. (2021)), and the results were in accordance with those of De Angelis et al. (2019) who compared the microbiotas of soft and *durum* wheat flour using culture independent methods. Minervini, Dinardo, et al. (2018) analysed the microbiotas of three different flours, and found the species *F. sanfranciscensis* in every sample (4% of all the strains isolated from the flour). This was higher than what we found, and could have been related to bias affecting the culture independent analyses, where rare species can go undetected, especially when the sequencing depth is low. In the flour samples, most of the reads were discarded as they corresponded to mitochondria or plasts, thus leading to a lower detection of rare bacteria than in sourdough. We did not have this problem for fungi, and the sequencing depth was the same for

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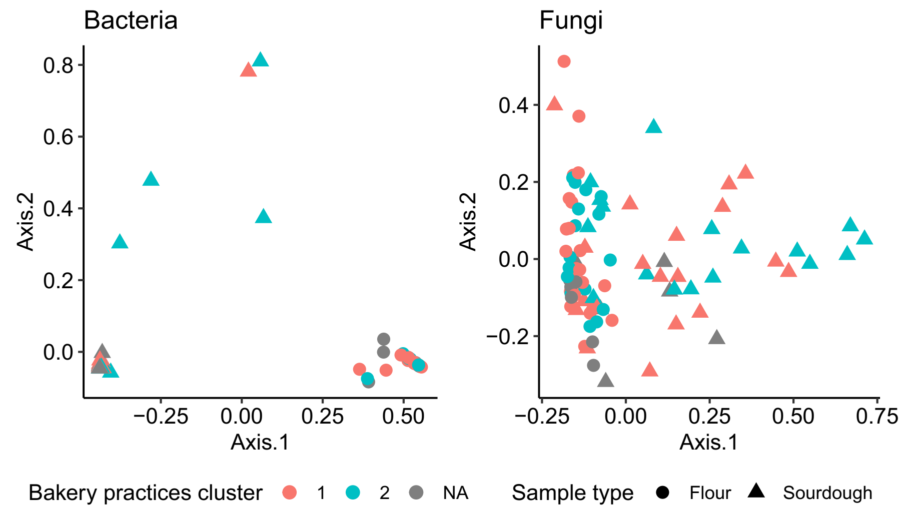


**FIGURE 5** PCoA based on Bray–Curtis dissimilarity for bacteria (left) and fungi (right). Bray–Curtis dissimilarity was computed on the basis of the abundance of the different species. Each point represents a sample. Colours indicate the bakery practices cluster, with farmer-baker practices in red and artisan-baker practices in blue. Sample types are represented by different shapes, flours being shown as circles and sourdoughs as triangles. [Colour figure can be viewed at wileyonlinelibrary.com]

flour and sourdough, so we can be confident that flours do not contain sourdough fermentative yeasts.

The filamentous fungi plant-associated pathogens detected in flour were also detected in sourdoughs, as they have commonly been detected in other studies (Landis et al. (2021); Reese et al. (2020)). However, on average they accounted for 54% of the reads (SD = 30%) in sourdough and 92% (SD = 9.3%) in flour, suggesting that filamentous fungi die in the acidic environment of sourdough and/or are poor competitors with yeasts in this environment. To our knowledge, they have never been detected alive in sourdough, even though they are able to grow on the media classically used to enumerate yeasts (Me & Melvydas, 2007). The presence of their DNA in sourdough suggested that this was partly protected in this environment, possibly thanks to their cell wall structure. However, the high proportion of these fungi in sourdough may also be related to bias affecting DNA extraction and amplification.

Unlike filamentous fungi and several bacteria of the core microbiota, the common plant bacteria *Pseudomonas* was not detected in sourdoughs, suggesting they did not survive in the sourdough ecosystem and that their DNA was degraded. This is highly probable as *Pseudomonas* species generally do not survive at a low pH.

## 4.4 | Microbial succession during the establishment of new sourdough

During the first week of sourdough initiation, LAB of the genera *Pediococcus* and *Weisella* were detected. They were then replaced with species of the family Lactobacillaceae, such as *Fructilactobacillus sanfranciscensis* or *Companilactobacillus paralimentarius*, in accordance with other studies on sourdough initiation (Bessmeltseva et al., 2014). None of the Ascomycota yeast species found in newly initiated sourdough was detected in flour and grains used, suggesting that sourdough yeasts do not come from the flour but rather from the house microbiota. In the experiments, the bakers used spoon to mix flour and water. Therefore, the air and utensils are more likely the vector of microorganisms than their hands.

## 4.5 | Microorganism present in flour did not develop in mature sourdough

The analysis of 46 flours collected all over France confirmed that flour does not contain fermenting yeasts found in mature sourdoughs.

In addition, our results showed that mature sourdoughs did not contain the same LAB as those provided by the flour. *F. sanfranciscensis*, which is the most frequently encountered LAB species in sourdough, was almost never found in flour. The most abundant *F. sanfranciscensis* ASV in sourdoughs, which is shared across all the French sourdoughs studied, was never detected in flour samples. We cannot exclude the fact that this ASV was missed in the flour because the detection threshold of our metabarcoding analysis was not sufficient. However, conversely, rare *F. sanfranciscensis* ASVs were detected in five flour samples, but were not found in the sourdough backslopped with these flours except in one case. It therefore seems that the few *F. sanfranciscensis* strains present in the flour do not establish in the sourdough. This result is contradicted by a previous study (Minervini, Dinardo, et al., 2018) which showed that flour and sourdough share the same strains of *F. sanfranciscensis* in three bakeries.

## 4.6 | Yeast distribution among sourdoughs

Previous work showed that *K. humilis* and *K. bulderi* were commonly found in sourdoughs from French bakers. Their distribution was correlated with the type of bread-making practices. Sourdough made by farmer bakers tended to carry *K. bulderi* while sourdough made according to artisanal practices often contained *K. humilis* (Michel et al., 2019). One of the main difference between these types of bakers is that farmer bakers exchange seeds, share mills or supply each other with flour, while artisanal bakers usually buy their flour from millers who produce and store flour at a larger scale. So one hypothesis explaining this yeast distribution might be that different sources of flour supply may lead to different pathways for microorganism dispersal and explain the structuring of yeast species diversity as a function of bread-making practices. In this study, none of

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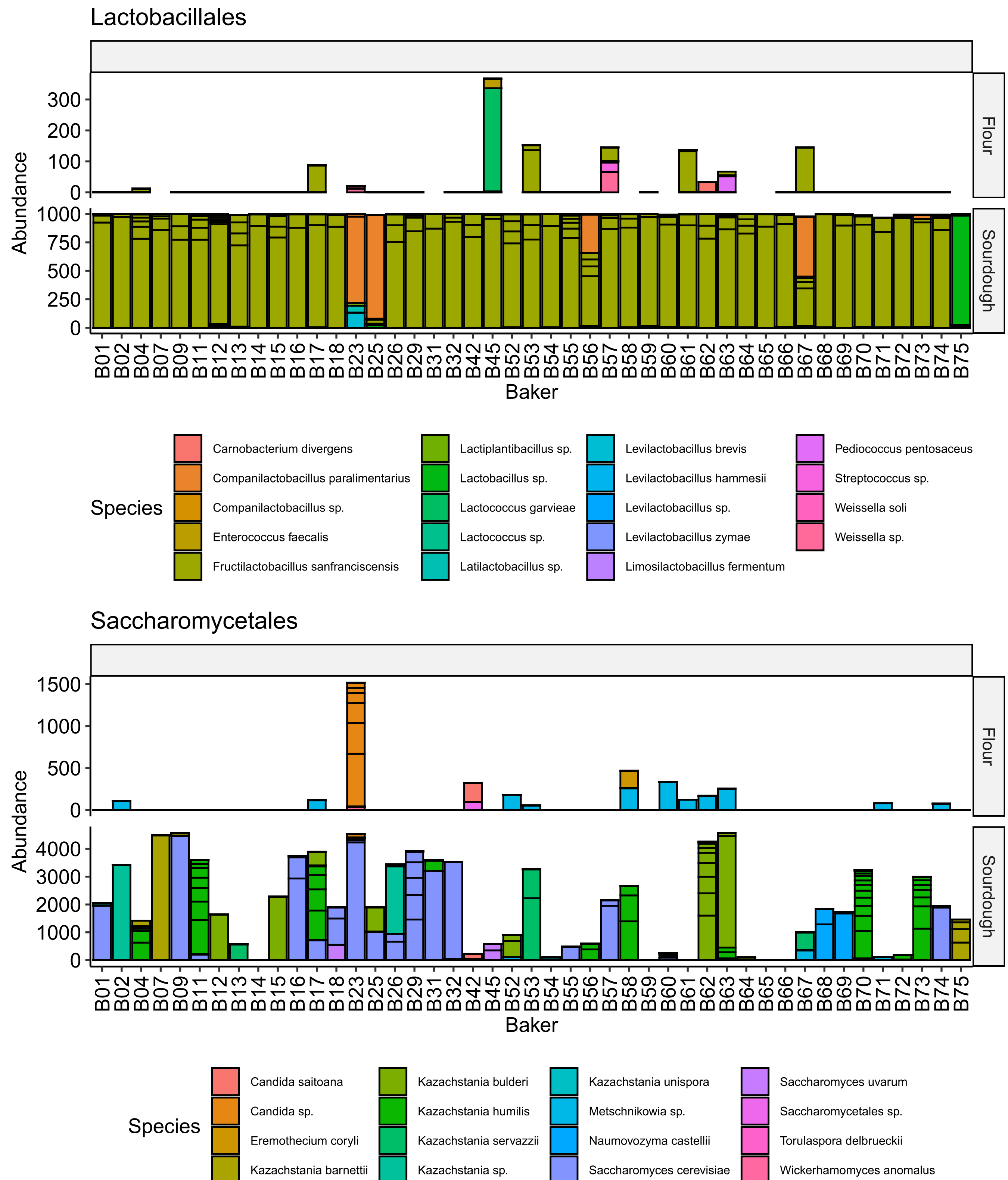


FIGURE 6 Microbial composition of the collected flours and sourdoughs. Samples are plotted on the x axis, and their abundance based on the rarefied data on the y axis. Colours represent species. Horizontal white bars delimit the different ASVs. Only *Lactobacillales* and *Saccharomycetales* are shown. The axes have different scales for abundance in flour and sourdough. [Colour figure can be viewed at wileyonlinelibrary.com]

the yeast species usually found in sourdough was detected in flour indicating that it is unlikely that sourdough yeasts come from the flour. The preferential occurrence of *K. bulderi* in sourdoughs made by farmer bakers or *K. humilis* in artisanal sourdoughs cannot therefore be easily explained by differences in flour and wheat grains supply chains. This finding is in agreement with previous studies which

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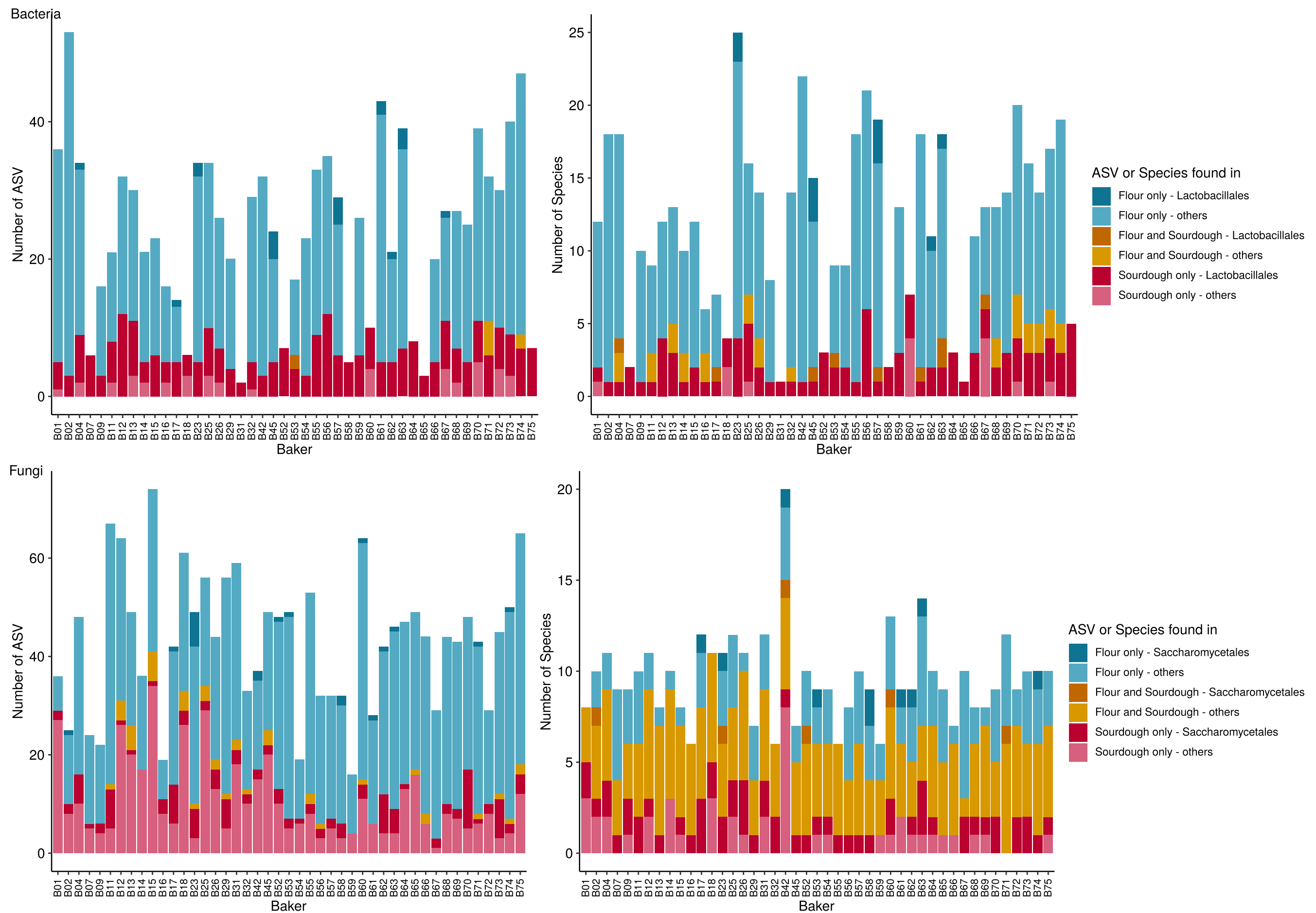


FIGURE 7 Number of shared species (on the right) and ASV (on the left) between sourdoughs and the flour used to make them. Results for bacteria are shown at the top and for fungi at the bottom. [Colour figure can be viewed at wileyonlinelibrary.com]

showed that the species composition of sourdough yeasts depended more on the bakery house than on the cereal flour species used (Comasio et al., 2020; Minervini et al., 2015). The yeast distribution has to be explained by another factor, such as, for example different bread-making practice that may lead to selection of different species. Sourdough yeast may also disperses as a result of exchanges of sourdough between bakers and/or through meetings in the bakery where bakers could exchange utensils, handshakes and contribute to carry the sourdough microorganism. Additional experiments should be carried out to test for these hypothesis.

In conclusion, the evaluation of the bacterial and fungal composition of wheat grain, flour and sourdough showed that microbial species present in the flour are mostly not found in the sourdough. They do not develop in mature sourdough and therefore are not being part of the microbiota active during bread-making process. As a corollary, dispersal from the house of microbiota and selection by the sourdough ecological niche appear as the main drivers of the mature sourdough microbial composition.

## AUTHOR CONTRIBUTIONS

Lucas von Gastrow, Elisa Michel, Judith Legrand, P. Roussel, Véronique Chable, S. Rousselot, Isabelle Goldringer, Xavier Dousset, Estelle Serpolay-Bessoni, Bruno Taupier-Letage, Camille Vindras-Fouillet, Bernard Onno, Florence Valence and Delphine Sicard designed research, Lucas von Gastrow, Elisa Michel, Rémy Amelot, Bernard Onno, Delphine Sicard, Diego Segond, Isabelle Goldringer and Stéphane Guezenec performed research, Lucas von Gastrow, Elisa Michel, Olivier Rué, Judith Legrand, Bernard Onno and Delphine Sicard analysed data and Lucas von Gastrow, Elisa Michel, Florence Valence and Delphine Sicard wrote the paper.

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Moinet who supervised the project (Gluten: mythe ou réalité?) which allowed the collection and analysis of a part of the flour and mature sourdough samples. This work was supported by a grant from the French National Research Agency (ANR-13-ALID-0005) and a grant from the Fondation de France (Gluten: mythe ou réalité?).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

Raw data and scripts are available at: https://data.inrae.fr/dataset.xhtml?persistentId=doi:10.15454/DF0BRL. The sequencing data generated in this study were deposited in the European Nucleotide Archive (ENA) under accession numbers PRJEB52423 for the 46 collected sourdoughs [dataset] von Gastrow (2021) and PRJEB52442 for the experimental sourdough initiation [dataset] Michel (2016).

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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