Title: Artisanal and farmers bread making practices differently shape fungal species diversity in French sourdoughs

Running title: Bread making practices as a driver of yeast species diversity

Authors

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Abstract

Preserving microbial diversity in food systems is one of the many challenges to be met to achieve food security and quality. However, there is still a lack of knowledge about the factors that may influence food microbial diversity, notably in fermented foods. Although industrialization led to the selection and spread of specific fermenting microbial strains, there are still ongoing artisanal processes that may allow the conservation of a wider diversity of microbial species. We examined whether the diversity of artisanal practices could lead to an increased level in fungal species diversity for bread making. We used an interdisciplinary participatory research approach including bakers, psycho-sociologists and microbiologists to analyse French bread making practices and describe fungal communities in naturally fermented sourdough. Bread making practices were clustered in a farmer practices' group and an artisanal practices' group. Surprisingly, the well-known bakery yeast, *Saccharomyces cerevisiae,* was dominant (i.e. with a relative abundance over 50%) in only 24% of sourdoughs and other yeast species of the closely related *Kazachstania* genus were frequent. Bread making practices were found to drive the distribution of these species. The differences in fungal communities were associated with variation in sourdough acidity, maltose concentration and hydration. Overall, our results showed that preserving bread making practices diversity allows the preservation of a higher taxonomic and functional diversity in microbial communities.

Introduction

Humans started to ferment food before the emergence of the Neolithic using naturally fermenting microbial communities. In the 19th century, the industrialization and the increase of knowledge in microbiology resulted in changes in fermented food practices with the use of targeted species and selected strains. This selection led to a reduction in species and genetic diversity for fermented food processing and limited *in situ* conservation of microbial communities in industrialized systems. Domestication of the yeast *Saccharomyces cerevisiae* for the production of beer, wine, cheese, leavened bread or that of the fungus *Penicillium roquefortii* for blue cheese production are well studied cases [1–5]. Recently, in response to global changes, there is a renewed interest in artisanal practices

that make use of naturally fermenting microbial communities. However, the effect of artisanal practices on the distribution of microbial species diversity remains poorly documented. Thus, understanding whether and how artisanal practices may promote microbial diversity is a first step toward the development of food systems with improved sustainability.

Among fermented foods, bread is still a symbol deeply engrained in the history, religious rites and medicine of several cultures. The origin of bread likely originated 14 000 years ago, suggesting bread was made long before plant domestication [6]. Since the Neolithic, bread history is intimately combined with the evolution of cereals, bread making associated tools and the advent of Mediterranean civilizations [7]. Leavened bread was traditionally made with flour, water and a fermenting agent, which was either a fermenting beverage or a fermenting dough, termed sourdough [1, 8]. This sourdough was generally initiated from a mixture of flour and water, naturally colonized by lactic acid bacteria (LAB) and yeasts [1, 9]. Sourdough was then either maintained from one bread making process to the other or initiated again and again, depending on the craftsman [10, 11]. In the 19th century, yeast starters made of *S. cerevisiae,* often called « baker's yeast », were proposed as an alternative to sourdough and these are nowadays commonly used by local bakeries and industries. However, there is currently a renewed interest in the use of naturally fermented sourdoughs for bread leavening.

In France, sourdough breads are made both by bakers and farmer-bakers who also grow and mill their own wheat. The number of farmers-bakers has increased in the 2000s with two motives: to grow wheat varieties meeting their needs and to assert their independence from industry [12]. Although farmer-bakers are less numerous than bakers, they participate in the renewed interest in local wheat varieties and artisanal know-how, which may contribute to the conservation of both socio-cultural diversity and microbial diversity.

Here, we used a participatory research approach involving psycho-sociologists, microbiologists, bio-mathematicians, bakers and farmers-bakers to study whether and how bakers and farmers-bakers contribute to the preservation of socio-cultural and fungal species diversity in sourdough microbial

community. This research emerged from a co-construction by bakers, farmer-bakers and academic researchers.

Materials and methods

A questionnaire survey, face-to-face interviews and focus-groups to collect bread making practices

All materials were collected among bakers and farmer-bakers through a questionnaire survey, interviews and focus groups. The collected variables were related to *i)* the ingredients origin : wheat varieties types (ancient populations also called landraces / modern varieties), whether they produced flour from their own wheat, whether they had their own mill or use an external mill, water origin, *ii)* the sourdough recipe: its age, its hydration state, the origin of the chief sourdough (sample of dough or sourdough), the number of back-sloppings before bread making and per week, the temperature of water used for back-sloppings, *iii)* their bread making practices: the number of bread makings per week, the percentage of sourdough, flour and salt in bread dough, the kneading methods, the total duration of fermentation and the addition of baker's yeast in dough.

Sourdoughs samples, enumeration and strain isolation

Sourdoughs were collected in 39 French bakeries before kneading and referred to as final sourdoughs (Table S1). On the day of collection, they were sent to the lab where yeast and bacteria were enumerated and isolated as in [13, 14], and sourdoughs stored at -20°C in sterile vials for non- culture based analysis. Ethics and rights associated with sourdough collection and strains isolation have been respected.

Sourdough acidity and metabolic analyses

For each sourdough, three independent 1-g replicates were analysed. pH and Total Titrable Acidity were measured as described in (22). Organic acids, alcohol and sugars concentrations (expressed as g/kg of sourdough) were analysed by liquid chromatography using an HPLC HP 1100 LC system (Agilent technologies, Santa clara, CA, USA) equipped with a refractive index detector (RID Agilent G1382A)

106 and a UV detector (Agilent G1314A). Two different columns were used, a Rezex ROA-organic acids
107 column and a Rezex RPM-monosaccharide column (SDVB – Pb+2 8%, 300x7.8mm, Phenomenex,
108 Torrance, CA, USA). The details of the procedure are described in supplementary information (Method
109 S1).

110 **Yeast species identification**

111 The Internal transcribed spacer 1 (ITS1) ribosomal DNA of each 1216 yeast isolates was amplified by
112 PCR from chromosomal DNA, either by using primers ITS1F and ITS2 [15, 16], or primers NSA3 and
113 58A2R [16, 17]. For isolates unidentified with the ITS1 region alone, DNA was extracted according to
114 MasterPure Yeast DNA purification kit (Epicentre, Epibio). PCR reactions targeting partial genes, the
115 D1D2 region of the large subunit of rRNA (LSU), a part of the RNA polymerase II large subunit
116 encoding gene (*RPB1)*, a part of the RNA polymerase II encoding gene (*RPB2)*, a part of the actin
117 encoding gene *Act1* and Transcriptional Elongation Factor TEF were performed. To discriminate three
118 specific isolates, PCR on genes *GHD1*, *FSY1*, *URA3*, *DRC1*, *MET2* were performed [18–21] (Table S4).
119 All PCR products were sent to be sequenced with Sanger sequencing (Eurofins, Germany). Species were
120 identified using NCBI [22], YeastIP [23] and a personal database, which was constructed after *ITS1*,
121 *RPB2*, LSU sequencing of all 33-yeast species reportedly found in sourdoughs in the literature [1].

122 **Sourdough DNA extraction, MiSeq sequencing, bioinformatics**

123 The ITS1 region was targeted with the PCR primers ITS1-F (5′- CTTGGTCATTTAGAGGAAGTAA -
124 3′) and ITS2 (5’-GCTGCGTTCTTCATCGATGC-3’).

125 The sequencing run was performed with MiSeq Reagent Kit v3. 2015 [13, 24]. Sequences were analysed
126 through FROGS “Find Rapidly OTU with Galaxy Solution” [28] and home-made pipelines. Overlapped
127 reads were merged with Flash [29] with a minimum overlap of 10 nucleotides, a maximum overlap of
128 300 nucleotides and a maximum mismatch density of 0.1. Adapters were removed with Cutadapt [30]
129 and data were cleaned with Sickle [25] Reads were clustered with Swarm [31] and chimeras deleted
130 with VSEARCH [32]. Sequences were then filtered on minimum abundance of 0.05% of all sequences.

131 From the OTU's abundance table and for each OTU, the taxonomic affiliation using UNITE Version
132 7.1, Release 2016-11-20 [33], YeastIP [23] and our own databases [13] [24] was obtained by blasting
133 OTUs representative sequences against each database.

134

135 **Data analyses**

136 All statistics and plots (ggplot2 [34], leaflet package [35], with minor esthetical adjustment with
137 Inkscape) have been done with R.

138 To analyse bread making practices, a multiple correspondence analysis (MCA) and hierarchical
139 clustering on principal components based on the first two axes of the MCA were performed using the
140 FactoMineR R package [36].

141 To analyse fungal community, weighted Unifrac distances between sourdough communities were
142 computed from a rooted phylogenetic tree based on the OTUs sequences using the R-packages Phyloseq
143 and GUniFrac [26, 27, 38]. Phylogenetic sequences were aligned with Clustalo [37] and phylogenetic
144 tree were built with the parsimony algorithm, with 100 replicates bootstraps, pairwise ktuple-distances
145 with Seaview [37]. Different roots were tested (*Sporidiobolales sp*., *Bullera globospora*, *Trichosporon*
146 *asahii*, *Udeniomyces pyricola*). Tree architecture did not change with the root. It did not fit the expected
147 phylogeny and, notably, some *Ascomycota* were misclassified among the *Basidiomycota*. However, the
148 dominant sourdough species belonging to the *Saccharomycetaceae* family were clustered according to
149 expected clades or subclades, except that *Kazachstania servazzi* and *Kazachstania unispora* were
150 grouped in a clade closer to *Saccharomyces* species than to other *Kazachstania* species. Using the
151 Unifrac distances matrix, we performed a Principal Coordinate Analysis (PCoA) and clustered
152 sourdough communities using the first two axes of the PCoA. To check the sensitivity of our analysis
153 to this misclassification, we performed the same analyses without the sourdoughs that had one
154 misclassified species representing more than 10% of their reads, *i.e*. sourdoughs B20, B41, B42, and
155 B44 and found the same clustering [27].

For each sourdough, the species richness, Chao1, Shannon and Simpson indexes were computed, and the Shannon and Simpson indices values were converted to the effective number of species per sourdough. This number was estimated from the Shannon diversity index as $exp^{Shannon\ index}$ and from the Simpson diversity index as $\frac{1}{1-Simpson\ index}$ [39, 40]. For probability estimates, the exact 95% confidence intervals were computed using a binomial distribution.

To study the link between microbial diversity and bakery practices, a univariate Permutational Multivariate Analysis (PERMANOVA) on the Unifrac distance matrix was performed for each bakery practice variable. We performed univariate analysis on the 30 bakers who had less than 8 missing values among the 29 bread making practices variables and adjusted the p-value using FDR correction to account for multiple testing. In addition, independence exact Fisher tests between the variable providing fungal community PCoA groups and each of the bread making practices variables were performed. Multiple testing was accounted for using the False Discovery Rate method [41].

The link between the baker practices group, the fungal community group or the yeast dominant species and the variation of each quantitative variable (microbial density, pH, TTA, metabolite concentration) was tested with the following mixed effect model: $Y_{ijk} = \mu + \alpha_i + B_j + \varepsilon_{ijk}$ with $\varepsilon_{ijk} \sim N(0,\sigma^2)$, where α_i is the effect of the fungal community group i modelled as a fixed effect and B_j is the effect of sourdough j modelled as a random effect and k represents the measurement replicates. For sourdough hydration rate, the variable was arcsin transformed but sourdough effect was not included in the model because no repetition was obtained from any sourdough. The model parameters were estimated using the lmertest R package [42]. To test the fixed effects, we used likelihood ratio tests. Multiple comparisons of means were performed using Tukey tests with the multcomp package [43]. p-values were all adjusted for multiple testing with the FDR method. The geographical structuration was tested with a Mantel test on the Unifrac distances matrix and the geographical distances matrix computed with the package geosphere [44] and ade4 [45].

Data and scripts are shared on Zenodo. http://doi.org/10.5281/zenodo.2600170

Results and discussion

Bread making processes and the diversity of bread making practices

We worked with thirty-nine French bakers producing natural sourdough bread and distributed all over France (Table S1). Their bread making practices were analysed through personal interviews (n=12), focus groups (n=3), observation during break-making workshops (n=2), and an online/phone survey (n=36). The general process of sourdough bread making is presented in Figure 1. We analysed 29 variables around this general bread making process, describing variations of the practices at all steps of the bread making process, from wheat grains to baked bread (Figure S1). Three bakers who did not answer the survey on bread making practices, and four others who did not provide enough information about their practices were excluded from the analysis. According to a hierarchical clustering on principal components (HCPC), the 32 other bakers clustered into two groups corresponding to two main types of bread making practices (Figure 2). The first group, hereafter termed "farmer" practices group, includes 6 bakers and 11 farmers-bakers using the following practices: low bread production (<500 kg per week, 81% of the bakers of the "farmer" group), use of ancient wheat populations (56%), manual kneading (63%), working at ambient temperature (88%), long fermentation periods (more than 4 hours for 88%), and no use of commercial baker's yeast (88%). In addition, they tend to make their chief sourdough from dough after kneading (75%). The second group, hereafter called "artisanal" practices group consists of 12 bakers and 4 farmer-bakers having more intensive practices, characterized by a large bread production (>500 kg per week, 81%), mechanical kneading (100%), use of modern wheat varieties (63%), working at ambient temperature (56%), using commercial yeast starters in addition to sourdough for bread making or using commercial yeast starters for pastries and buns making (81%). In this second group, bakers tend to make their chief sourdough from a final sourdough.

204 **Fungal community composition**

205 Sourdough is a mix of flour and water naturally fermented by bacteria and yeasts. Sourdough yeast
206 density ranged from 8.1 10^4 to 5.8 10^8 CFU per gram of sourdough, with a mean value of 2.9 10^7 CFU
207 per gram, as commonly found in sourdoughs from all over the world [1, 17, 46, 47]. We isolated 20 to
208 40 yeast strains from each sourdough and identified species using ITS sequence as well as other barcodes
209 when needed (see M&M section). A total of 1216 strains were characterized from 38 sourdoughs. In
210 addition, we developed an ITS1 meta-barcoding MiSeq sequencing method on sourdough (see sup
211 M&M). After filtering 5,360,620 raw ITS1 sequences for quality, abundance (0.005%) and chimera,
212 3,542,801 sequences were further analyzed. Overall, the sequences clustered in 113 OTUs. The number
213 of reads per sourdough ranged from 8421 to 194 557. Therefore, we carried out our analysis on the
214 rarefied matrix. Among all OTUs, 64 were identified as non-yeast, including 10 assigned to the order
215 *Triticodae* (especially to the species *T. aestivum*), 50 assigned to plant pathogen fungi, such as
216 *Alternaria*, *Aspergillus* or *Fusarium*, *Gibberella*, while 4 OTUs remained unidentified. Among the 40
217 yeast or yeast-like OTUs, 96% of total reads were assigned to the phylum *Ascomycota*, 87.5% to the
218 order *Saccharomycetales* and 85.7% to the family *Saccharomycetaceae*. Only 4% of the total reads were
219 assigned to the phylum *Basidiomycota*. Overall, three OTUs assigned to the species *Kazachstania*
220 *humilis*, *Kazachstania bulderi* and *Saccharomyces cerevisiae* represented 20.3%, 15.5% and 24.1%
221 respectively of the total number of reads and 28.1%, 23.7% and 18.2% respectively of the number of
222 reads identified as yeast species (Figure 3).

223 Both non culture-based and culture-based methods allowed the identification of the same dominant
224 species (defined as a species with an over 50% frequency) for all sourdoughs but five (B09, B20, B22,
225 B25, B41) (Figure 3). In two cases, the discrepancy was explained by the detection of *Cladosporium* sp.
226 at high frequency with metabarcoding while this species could not be isolated in the laboratory (Figure
227 3). In two other cases, it was explained by a high number of *S. cerevisiae* isolated in the laboratory
228 compared to what was observed using metabarcoding sequencing. Finally, in the last case, the
229 identification of *Pichia kudriavzevii* required additional sequencing as it shares an identical ITS with
230 *Candida xylopsoci*. Because metabarcoding allows a deeper characterization of the fungal species

231 diversity with few discrepancy cases, the distribution of fungal species diversity will be further described
232 using metabarcoding data only. Previous analysis of the same sourdoughs revealed that *L.*
233 *sanfranciscensis* was the dominant bacterial species in all analysed sourdoughs but two, where the
234 dominant species was either *L. curvatus* or *L. heilongjiangensis* [13, 14]. Therefore, we decided to study
235 the distribution pattern of microbial species in the fungal community only.

236 **Distribution of fungal species diversity over sourdoughs**

237 All sourdoughs but two had a dominant yeast species with a relative abundance over 50% and
238 many species with a lower relative abundance (Figure 3). Within sourdoughs, fungal species richness
239 ranged from 10 to 33, with a 23.5 median (Table S2). The effective number of species per sourdough
240 calculated from the Shannon diversity index ranged from 1 to 6.8 (Table S2), with 70% of sourdoughs
241 having an index below 2 (Table S2). Between-sourdoughs species diversity was analysed using weighted
242 Unifrac distances, computed from a phylogenetic tree built from the distances between OTUs using
243 *Sporidiobolales species* as root (Figure S2). Unifrac distances computed with four differently rooted
244 trees were highly positively correlated (Figure S2). Unifrac distances between sourdoughs ranged from
245 0.0005 and 0.71, with a median of 0.49 and a mean of 0.52. There was no significant correlation between
246 the Unifrac distances and geographical distances between sourdoughs (Mantel test, P=0.35) (Figure 4).

247 We then analysed specifically the distribution of yeast species diversity as yeast species,
248 together with lactic acid bacteria, are the main functional player in a sourdough ecosystem and for bread
249 quality. Over the 40 yeast species detected in the 38 sourdoughs, 12 had a relative abundance over 50%
250 in at least one sourdough, four had a relative abundance between 20% and 50% and 24 had a relative
251 abundance below 10%. All dominant species (relative abundance over 50%) were fermentative yeast
252 species, except in one sourdough that had a C*ladosporium* species. We found all the sourdough yeast
253 genera (*Saccharomyces*, *Candida*, *Kazachstania*, *Pichia*, *Torulaspora* and *Hyphopichia)* commonly
254 reported in the literature except the *Wickerhamomyces* genus that we did not detect in our samples [47–
255 49].

256 **The baker's yeast, *Saccharomyces cerevisiae* is not the most widespread yeast species in**
257 **sourdoughs**

258 The well-known baker's yeast, *Saccharomyces cerevisiae*, was found in 53% of all sourdoughs (95%
259 confidence intervals=36% - 69%) but was dominant (relative abundance over 50%) in only 24% (95%
260 confidence intervals=11% - 40%) (Figure 3). In two cases, *S. cerevisiae* co-occurred with another yeast
261 species at similar relative abundance. In the first case, *S. cerevisiae* was present at a relative abundance
262 of 40% with *Candida sake* at a 41% relative abundance. In the second case, it was found at a relative
263 abundance of 47% with *Pichia kudriavzevii* at a relative abundance of 52%. In all the other cases, *S.*
264 *cerevisiae* had a relative abundance below 21% and was found with other dominant yeast species, such
265 as *Kazachstania australis*, *Kazachstania humilis*, *Saccharomyces uvarum* or *Torulaspora delbrueckii*.
266 This suggests that *S. cerevisiae* did not displace other species and can indeed be out-competed by other
267 species in sourdoughs. Therefore, despite the recurrent use of *S. cerevisiae* as starter, its massive use in
268 some bakeries and homes, and its occurrence in a wide range of habitats such as soil, trees, and humans
269 [2, 3], this species does not appear to have overwhelmingly colonized French traditional sourdoughs
270 (Figure 3).

271 **The *Kazachstania* genus is highly represented in sourdoughs**

272 *Kazachstania* was the most represented yeast genus over all sourdoughs, when considering both the
273 number of reads over all sourdoughs and the number of detected species. Indeed, this genus represented
274 57% of the total number of reads while *Saccharomyces* represented 26% of the total number of reads.
275 In addition, eight species of the *Kazachstania* genus were found in sourdough, a much higher figure
276 than for the *Saccharomyces* genus, represented by only two species (*S. uvarum* and *S. cerevisiae*) (Figure
277 3). *Kazachstania* species dominated in 54% (95% confidence intervals=36%-69%) of sourdoughs while
278 *Saccharomyces* species dominated in 27% only (95% confidence intervals=13%-43%). *K. humilis*,
279 followed by *K. bulderi* were the most commonly dominant *Kazachstania* species, and found in
280 respectively 21% (95% confidence intervals=10%-37%) and 15% of sourdoughs (95% confidence
281 intervals=6%-31%) (Figure 3). *K. humilis* is common in sourdoughs and has been found in many

282 countries, viz. China, Ethiopia, Finland, Germany, Morocco, USA, Italy, Belgium and France [49–52].
283 *K. bulderi* has been reported for the first time in anaerobic maize silage in the Netherlands and in
284 fermented liquid feed for piglets [53, 54] and more recently in French wheat sourdoughs [14, 17]. A yet
285 undescribed *Kazachstania* species was also identified in five sourdoughs (4.5%-29%) and found
286 dominant in three (1.7%-22%). Strains of this species were closely related to a strain previously isolated
287 from boza, a Bulgarian fermented drink, as estimated with ITS and LSU (D1D2) barcodes (Source:
288 NCBI, GenBank: KC118125.1 and KX369579.1). In addition, *Kazachstania saulgeensis,* a recently
289 described species [55, 56], was dominant in one sourdough (0.07%-14%). Finally, several *Kazachstania*
290 species were detected for the first time as dominant in sourdoughs, whereas they had been previously
291 found in other environments, like soil (*K. australis*), sauerkraut (*K. barnettii*), fermented milk (*K.*
292 *unispora*), or feces (*K. unispora*) [55, 57, 58]. None of the previous studies on sourdough have
293 evidenced as many *Kazachstania* species [1].

294 **The diversity of sourdough fungal communities was associated with differences in bread making**
295 **practices**

296 We tested whether sourdough fungal community composition could be explained by bread making
297 practices. To do so, we performed univariate PERMANOVA analysis on the 30 bakers with less than 8
298 missing values for the 29 bread making practices variables (Table S3). The univariate analysis revealed
299 that the weighted Unifrac distance between sourdoughs varied significantly ($P<0.05$) with the use of
300 commercial yeast in bakery. It also varied significantly with sourdough age, chief sourdough origin
301 (dough, sourdough or both), the quantity of bread produced per week, the milling method (cylinder,
302 millstone, Astrie, Tyrol), the type of wheat variety (ancient, modern or a mix thereof) and the
303 fermentation duration. However, after FDR correction for taking into account multiple testing, none of
304 these variables significantly explained Unifrac distances.

305 In order to understand further the relationship between sourdough fungal community composition
306 and bread making practices, we clustered sourdoughs according to their fungal community composition,
307 on the basis of the PCoA of their weighted Unifrac distances. Then, we tested the link between the

308 fungal community group and the bread making practice group (farmers/artisanal practices group) as well
309 as the link between the fungal community group and each of the different bread making practices (Figure
310 5). Sourdoughs were clustered into three fungal community groups. Group 1 clustered all sourdoughs
311 (but two) having *Kazachstania* species as dominant species (K. *humilis K. barnettii, K. bulderi, K.*
312 *saulgeensis, K. sp)*. Group 2 contained sourdoughs with *Saccharomyces sp*., *K. servazzi* or *K. unispora*
313 as dominant species. Group 3 sourdoughs harbored *S. cerevisiae* together with other species such as
314 *Pichia kudriavzevii*, *Candida sake*, or a Dipodascaceae sp. Group 1 sourdoughs were mostly made by
315 bakers having farmer's bread making practices while group 2 and 3 sourdoughs were mostly made by
316 bakers using artisanal practices (exact Fisher test, P=0.035). The fungal community groups were
317 significantly related to two specific bread making practice variables: the quantity (in kg) of bread made
318 per week (Exact Fisher test, P=0.001) and the use of commercial yeast (Exact Fisher test, P=0.05). All
319 sourdoughs in group 2 but one were found in bakeries making between 500 kg and 1000 kg of bread per
320 week, while groups 1 and 3 sourdoughs originated from bakeries producing very different amounts of
321 bread (ranging from amounts below 250 kg to over 1000 kg). In addition, group 1 sourdoughs were
322 more frequently found in bakeries that do not use commercial yeast while group 2 and 3 were more
323 frequently found in bakeries using the commercial yeast *S. cerevisiae* (Exact Fisher test, P=0.01).
324 Interestingly, group 1 sourdoughs harbored *S. cerevisiae* either at a relative abundancy below 1% or not
325 at all, while all groups 2 and 3 sourdoughs had *S. cerevisiae* at a relative abundancy over 20%, except
326 in three cases where it was either absent or at a relative abundancy below 6%.

327 To test more specifically the link between bread making practices and the distribution of
328 *Kazachstania* species, we analyzed more in depth group 1 sourdoughs. Within this group, 8 sourdoughs
329 had *K. humilis* as dominant species, 6 had *K. bulderi*, 3 had the undescribed *Kazachstania* species and
330 the remainder had still other *Kazachstania* species. All sourdoughs made with artisanal practices carried
331 *K. humilis* as dominant species or, in one case, the yet undescribed *Kazachstania* sp. By contrast,
332 sourdoughs made with farmers' practices had as dominant species *Kazachstania bulderi*, *K. australis*,
333 *K. barnettii*, *K. saulgeensis* or the yet undescribed *Kazachstania* species (exact Fisher test, P=0.004).

334 Other bread making practices than the ones studied here could also explain the distribution of yeast
335 species diversity. Interviews with the 5 bakers working with sourdough hosting *Kazachstania bulderi*
336 and the underscribed *Kazachstania* species suggested the role of dispersion of these species in French
337 sourdoughs. Indeed, these bakers have been connected over the years either through seed exchanges,
338 sourdoughs mixing or gifts, bread making training in common or working in one another's bakery.
339 Although sourdough bacteria have been shown to originate from the bakery house microbiota and flour
340 [59, 60], the origins of yeast species found in sourdough are yet unknown.

341

342 **Fungal community composition was partly related to sourdough acidity, maltose**
343 **concentration and hydration**

344 The composition of fungal community may affect sourdough metabolic content (sugars, acids, alcohols)
345 via fungal strains metabolite consumption and production. Inversely, the presence and concentration of
346 different compounds (sugars, acids, alcohols) may also be one of the driver of fungal communities'
347 composition as those parameters may affect differently the different fungal strains fitness. For example,
348 lactic acid bacteria (LAB) are the main producers of acidity in sourdough, but yeasts also produce acetic
349 acid and may also indirectly affect acidity through positive or negative interaction with bacteria.

350 To investigate the relation between sourdough fungal communities and metabolic compounds, we
351 quantified sourdough hydration, yeast density, bacteria density, sourdough pH, Total Titrable Acidity
352 (TTA), sourdough concentration in seven sugars (maltose, glucose, fructose, raffinose, arabinose,
353 mannose, xylose), four alcohols (glycerol, ethanol, mannitol, meso-erythtritol), six acids (lactate,
354 acetate, glutarate, pyruvate, malate, succinate) and calculated the fermentative quotient (lactate over
355 acetate ratio). For each variable, there was a wide range of variation (Table S5). The principal component
356 analysis based on all variables showed no evidence of sourdoughs grouping (Fig S4). As expected in
357 fermentation, yeast density was positively correlated to ethanol ($r=0.74$, $P<0.001$), glycerol ($r=0.67$,
358 $P<0.001$), and acetate ($r=0.6$, $P<0.001$) concentration. However, it was not significantly correlated to

359 sugar concentrations. This might be explained by sugar competition and cross feeding that may exist
360 between yeasts and bacteria.

361 We then tested whether bread making practices group (farmers' practices and artisanal practices)
362 was associated with the variation of each quantitative variable separately. There was no significant effect
363 of the bread making practice group except for sourdough hydration that was significantly higher in
364 sourdoughs made using farmers' practices ($F_{1,94}$=11,69, P<0.001). Sourdoughs made with farmer's
365 practices had in average 55% water while sourdoughs made with artisanal practices had in average 49%
366 of water.

367 In addition, we tested whether differences in fungal community were associated with any variation
368 in a quantitative variable (Table S5). Group 3 microbial community sourdoughs (definied by PCoA
369 clustering on Unifrac distance, see below), which contain *S. cerevisiae* in co-dominance with a second
370 yeast species (*Candida sake*, *Pichia kudriavzevii* or a *Dipodascus* species), had a significantly higher
371 mean pH (mean pH_{group3}=4.2 against pH_{group1}=3.8, Tukey Contrasts, P<0.001), lower TTA (mean TTA
372 $_{group3}$=7.7 against TTA_{group1}=17.1, Tukey Contrasts, P=0.002), and a higher maltose concentration (mean
373 $Maltose_{group3}$=52.8 mg/gr of sourdough against $Maltose_{group1}$=24.1 mg/gr of sourdough, Tukey Contrasts
374 P=0.002) than group1, having a *Kazachstania* dominant species. Compared to group 2 having in most
375 cases *S. cerevisiae* as dominant species, it also had higher pH (pH_{group2}=3.9, Tukey Contrasts, P=0.003),
376 and higher maltose concentration ($Maltose_{group2}$=23.7, Tukey contrast, P=0.003). These data may reflect
377 a lower fermentative activity for group 3 fungal community having two co-dominant species, and/or a
378 negative interaction effect of group3 fungal community on the activity of lactic acid bacteria (LAB),
379 which are the main producers of sourdough acids. Previous studies on the bacteria content of the same
380 sourdoughs showed that *L. sanfranciscensis* was most generally the dominant species, although *L.*
381 *heilongjiangensis, L. curvatus* or *L. brevis* were also found as dominant species [13, 14]. We found no
382 significant correlation between LAB and yeast densities (r= -0.15, p= 0.45, Fig S4) but the link between
383 fungal and bacteria community might be species and strains dependent. Additional studies on the
384 interactions between fungal and bacterial communities need to be performed to better understand how
385 they may drive sourdough acidity and sugar content.

386 Finally, we analysed whether the difference in dominant yeast species was associated with some
387 variation of any of the studied quantitative variables. We only considered the 26 sourdoughs having
388 either *S. cerevisiae* (9 sourdoughs), *K. humilis* (8 sourdoughs), *K. bulderi* (6 sourdoughs) or *K. sp* (3
389 sourdoughs) as dominant species, since the other yeast species were found dominant only once. The
390 differences in dominant species was not significantly associated to variation in sourdough sugar, acids
391 or alcohol concentration. However, *K. bulderi* was found in significantly more hydrated sourdoughs
392 (63% water content in average) compared to the three other dominant species, *K. humilis*, *K. sp*., and *S.*
393 *cerevisiae,* found in sourdoughs having respectively 49%, 47%, 53 % water content in average (Tukey
394 Contrasts, $P<0.001$; $P<0.001$; $P<0.001$). *K. bulderi* was found to be dominant only in sourdoughs made
395 using farmers' practices, a bread making practice group that was also found to be associated with more
396 hydrated sourdoughs. Additional experiments should be carried out to test whether this species has
397 indeed a better fitness in more hydrated sourdoughs or whether its presence in more hydrated sourdoughs
398 is related to covariation with other farmer practices.

399 Conclusion and perspectives

400 In conclusion, a great diversity of bread making practices and fungal community composition
401 was found in our sample of French sourdoughs. Surprisingly, the well-known baker's yeast
402 *Saccharomyces cerevisiae* was found dominant only in one fourth of the sampled sourdoughs. By
403 contrast, several species of the neighbouring genus *Kazachstania* (including one yet undescribed
404 species) were detected at high frequency, revealing a major role for this mostly unknown genus in the
405 study of fungal domestication and in bread making. The fungal community diversity was partly
406 explained by the diversity in bread making practices. It was also partly associated with sourdough acidity
407 and maltose concentration, suggesting the role of fungi/bacteria interaction in sourdough functional
408 diversity. To our knowledge, this is the first evidence of the influence of artisanal practices on taxonomic
409 and functional diversity in microbial communities. Therefore, our results highlight the necessity of
410 maintaining socio-cultural diversity to maintain microbial diversity in food systems. These findings

411 could not have been evidenced without the collaboration of bakers and scientists, showing the
412 importance of participatory research projects to gain new insight into biodiversity preservation.

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568

569 **Figure Legends**

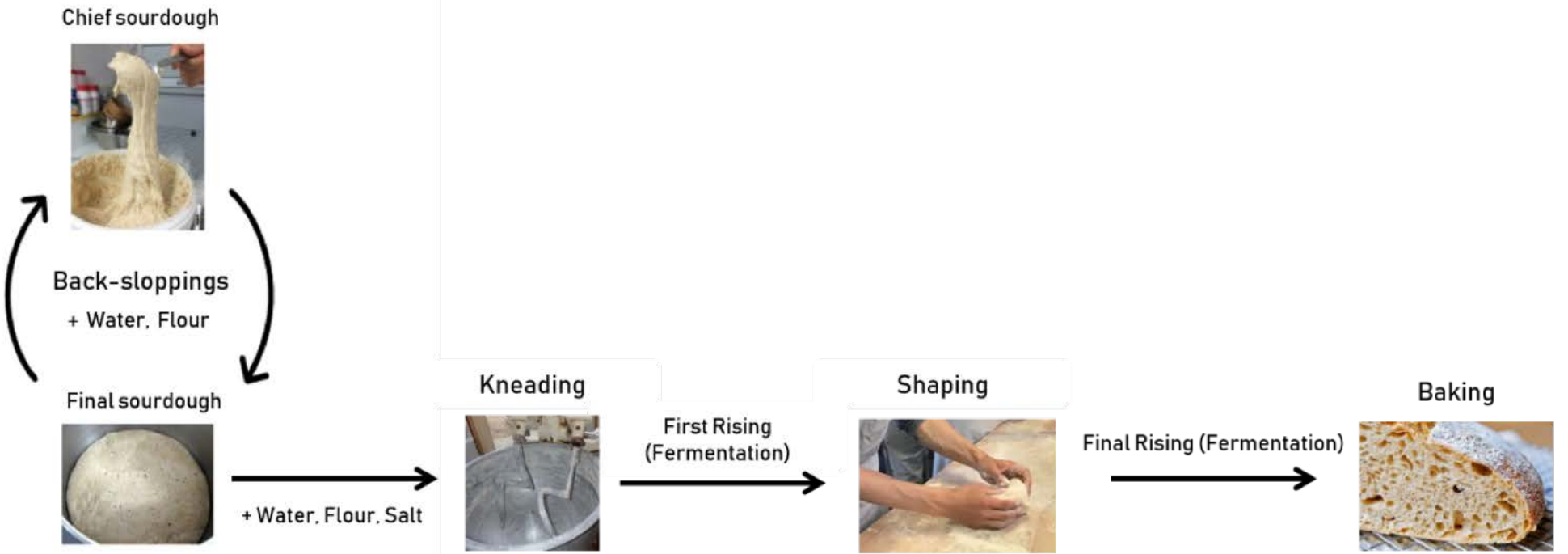
570 **Figure 1.** The sourdough bread making process. Sourdough is a mix of flour and water naturally
571 fermented by bacteria and yeasts. It is initiated by mixing flour, water and occasionally other ingredients.
572 It is then fed by regularly adding flour and water, a process termed back-slopping. Once considered
573 mature by bakers based on their acidity, flavour and bubbling activity, the sourdough is called "chief",
574 or "mother" sourdough, and can then be used for bread making. The bread making process starts from
575 this "chief sourdough", or from a piece of dough or sourdough sampled from the preceding bread making
576 process, or initiated from a mix of flour and water naturally colonized by yeasts and LAB following
577 several back-sloppings. The chief sourdough is refreshed to constitute the final sourdough, which is used
578 for bread making. This final sourdough is mixed with flour, water, and other ingredients (salt, seeds,
579 yeasts starters, etc.) during kneading to constitute the dough. After kneading, primary fermentation
580 occurs during the first rising. The dough is then divided and shaped. The pieces of dough are then left
581 to rise during a second fermentation and finally oven-baked.

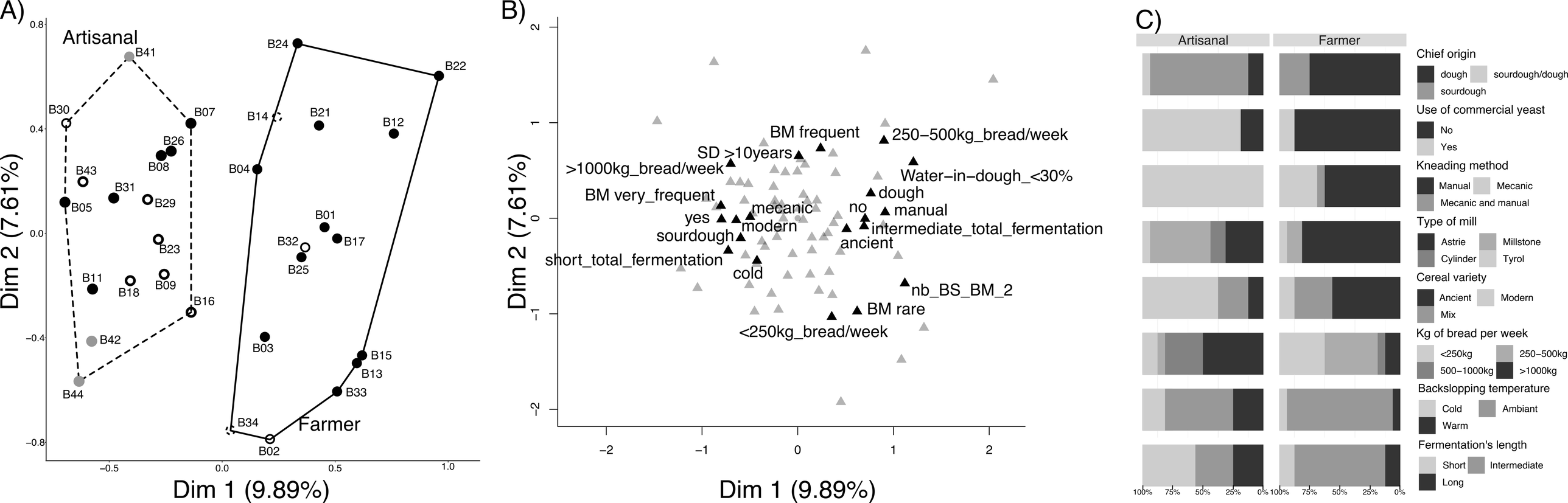
582

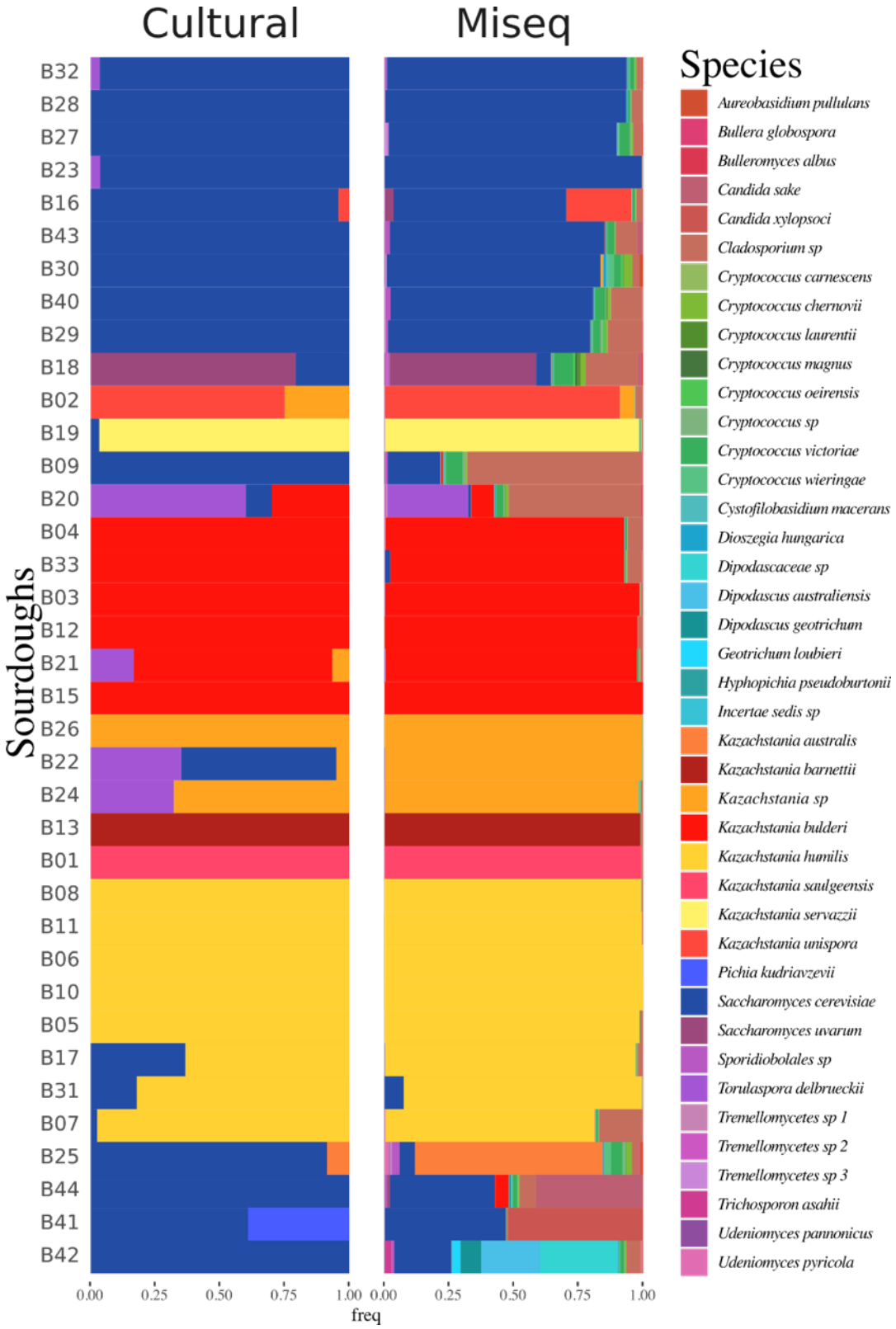
583 **Figure 2.** Multiple Correspondence Analysis (MCA) based on 28 categorical variables describing
584 bread making practices.

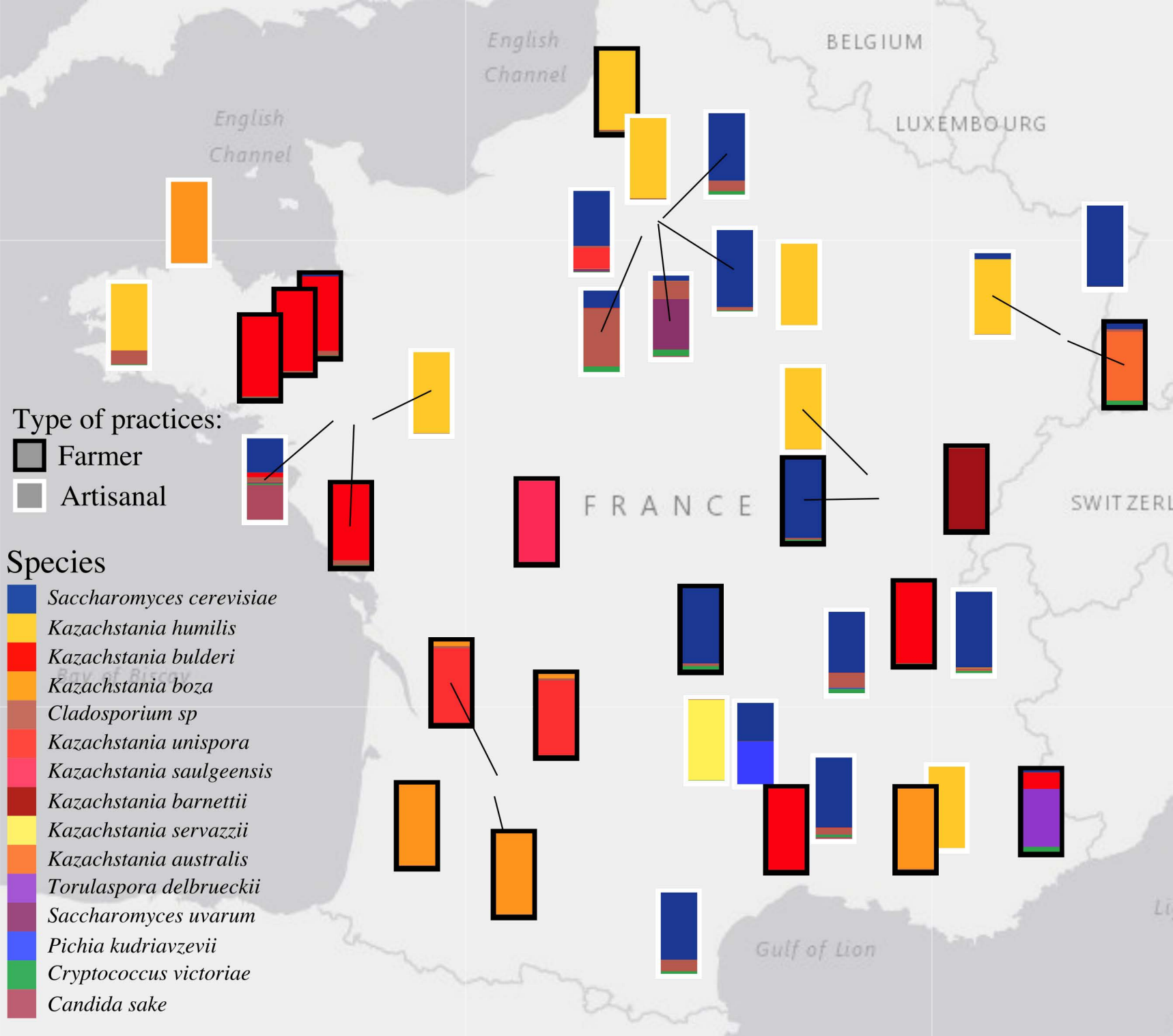
585 A) Representation of bakers. Each point represents a bakery. The left area brings together baker with
586 "artisanal" practices and the right area bakers with "farmer" practices. Centroid of each group is
587 designed by a triangle. The dot's colors indicate the PCoA cluster of the sourdough fungal community.
588 Black dots for group 1, white for group 2, grey for group 3. The fungal community of the sourdough of
589 baker 14 (in red) was not studied. B) Representation of the 20 first categories that mostly contributed
590 the MCA axis. The category is written next to the triangle. C) Percentage of different categories in each
591 bread making practices group. Only variables that mostly explained differences between bread making
592 practice groups are shown: use of commercial yeast, kneading method, chief origin, kg of bread
593 production per week, number of bread making per week, percent of water in dough, number of back-
594 sloppings before making bread, water origin, sourdough age and flour percentage in dough

595 **Figure 3.** Yeast species diversity was analyzed for 38 out of the 39 sourdoughs with both cultural and
596 metabarcoding methods. Sourdough B14 was excluded from microbial analyses, because of difficulties
597 in isolating strains from the sample, suggesting that this sourdough's microflora was no longer active.
598 Left: species were identified by traditional microbial isolation and identification using ITS sequencing.
599 Right: species were identified using ITS1 metabarcoding.
600
601 **Figure 4.** Distribution of yeast species diversity across French sourdough.
602 Each bar represents the yeast species diversity of one sourdough and is placed on the map where the
603 baker is located.
604
605 **Figure 5.** Representation of fungal community weighted Unifrac distances between sourdoughs.
606 The first line shows the clustering of sourdough according to their Unifrac distance on a tree (left) or on
607 a PCoA (right). Sourdough fungal community can be clustered in three groups according to their
608 weighted Unifrac distances. The lines below show the distribution of bread making practices or the
609 distribution of the dominant or most frequent species among fungal community. Each line corresponds
610 to a variable. On the left: a tree constructed from the Unifrac distance matrix colored by groups of
611 sourdough defined by the clustering analysis. In the center: sourdoughs represented on the first 2 axes
612 of the PCoA colored according to the group of sourdough. On the right: the distribution of modalities of
613 the variable for each group of sourdough (number of each group on the x-axis).
614

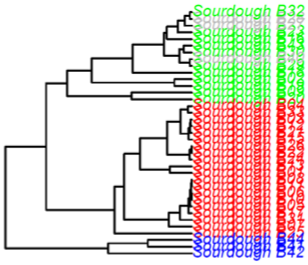

Chief sourdough
Back-sloppings
+ Water, Flour
Final sourdough
+ Water, Flour, Salt
Kneading
First Rising
(Fermentation)
Shaping
Final Rising (Fermentation)
Baking


A)
Artisanal
Farmer
B41
B30
B07
B26
B08
B43
B31
B29
B05
B23
B11
B18
B09
B16
B42
B44
B24
B22
B14
B21
B12
B04
B01
B32
B17
B25
B03
B15
B13
B33
B34
B02
Dim 1 (9.89%)
Dim 2 (7.61%)
B)
BM frequent
250−500kg_bread/week
SD >10years
>1000kg_bread/week
Water−in−dough_<30%
BM very_frequent
dough
mecanic
no
manual
yes
modern
intermediate_total_fermentation
sourdough
ancient
short_total_fermentation
cold
nb_BS_BM_2
BM rare
<250kg_bread/week
Dim 1 (9.89%)
Dim 2 (7.61%)
C)
Artisanal
Farmer
Chief origin
dough
sourdough/dough
sourdough
Use of commercial yeast
No
Yes
Kneading method
Manual
Mecanic
Mecanic and manual
Type of mill
Astrie
Millstone
Cylinder
Tyrol
Cereal variety
Ancient
Modern
Mix
Kg of bread per week
<250kg
250−500kg
500−1000kg
>1000kg
Backslopping temperature
Cold
Ambiant
Warm
Fermentation's length
Short
Intermediate
Long
100%
75%
50%
25%
0%

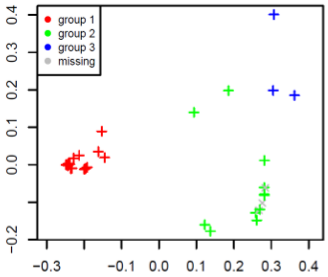

Cultural
Miseq
Sourdoughs
B32
B28
B27
B23
B16
B43
B30
B40
B29
B18
B02
B19
B09
B20
B04
B33
B03
B12
B21
B15
B26
B22
B24
B13
B01
B08
B11
B06
B10
B05
B17
B31
B07
B25
B44
B41
B42
0.00
0.25
0.50
0.75
1.00
freq
Species
Aureobasidium pullulans
Bullera globospora
Bulleromyces albus
Candida sake
Candida xylopsoci
Cladosporium sp
Cryptococcus carnescens
Cryptococcus chernovii
Cryptococcus laurentii
Cryptococcus magnus
Cryptococcus oeirensis
Cryptococcus sp
Cryptococcus victoriae
Cryptococcus wieringae
Cystofilobasidium macerans
Dioszegia hungarica
Dipodascaceae sp
Dipodascus australiensis
Dipodascus geotrichum
Geotrichum loubieri
Hyphopichia pseudoburtonii
Incertae sedis sp
Kazachstania australis
Kazachstania barnettii
Kazachstania sp
Kazachstania bulderi
Kazachstania humilis
Kazachstania saulgeensis
Kazachstania servazzii
Kazachstania unispora
Pichia kudriavzevii
Saccharomyces cerevisiae
Saccharomyces uvarum
Sporidiobolales sp
Torulaspora delbrueckii
Tremellomycetes sp 1
Tremellomycetes sp 2
Tremellomycetes sp 3
Trichosporon asahii
Udeniomyces pannonicus
Udeniomyces pyricola


English Channel
English Channel
BELGIUM
LUXEMBOURG
FRANCE
SWITZERL
Gulf of Lion
Type of practices:
Farmer
Artisanal
Species
Saccharomyces cerevisiae
Kazachstania humilis
Kazachstania bulderi
Kazachstania boza
Cladosporium sp
Kazachstania unispora
Kazachstania saulgeensis
Kazachstania barnettii
Kazachstania servazzii
Kazachstania australis
Torulaspora delbrueckii
Saccharomyces uvarum
Pichia kudriavzevii
Cryptococcus victoriae
Candida sake

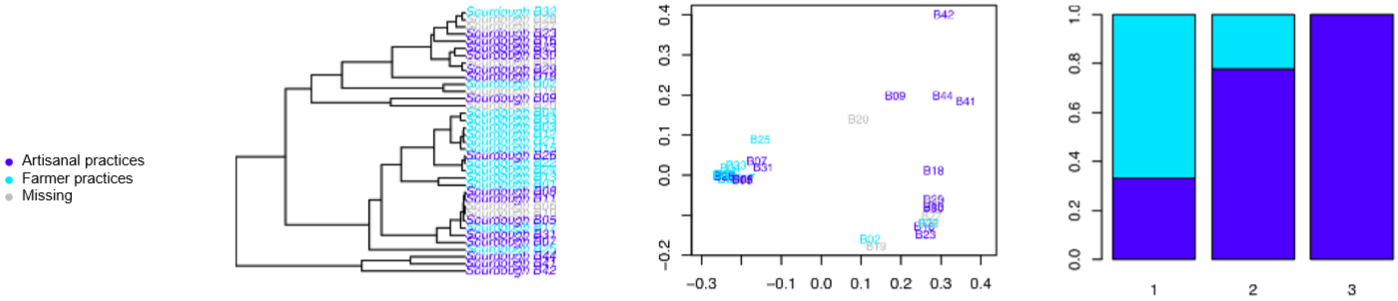
Unifrac tree, clustered in 3 groups



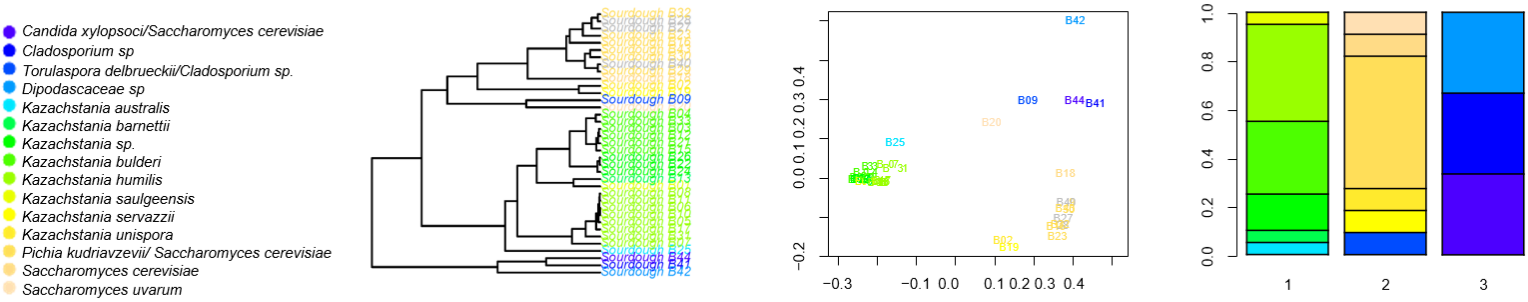
PCoA, clustering in 3 groups



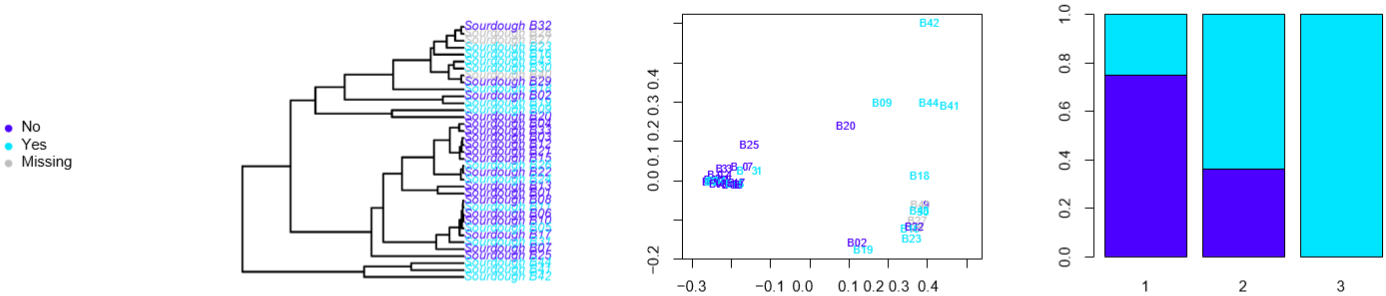
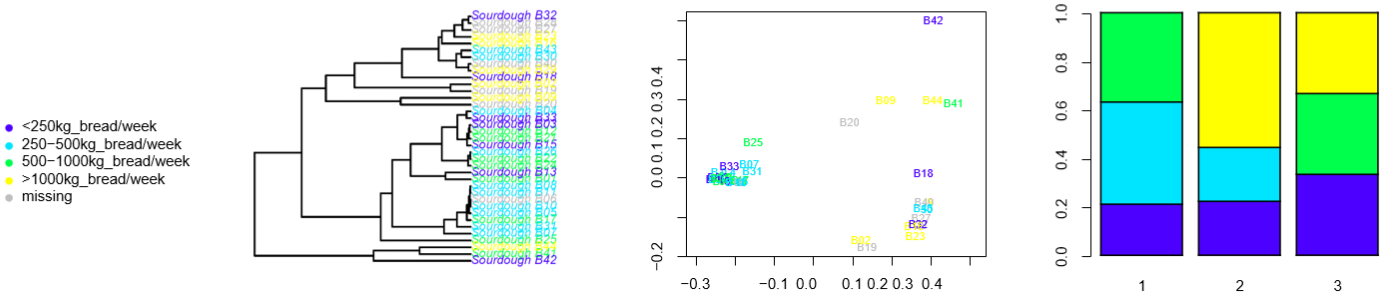
Practices clustering



Dominant species



Use of commercial yeast



Quantity of bread per week