Microbial Diversity of Type I Sourdoughs Prepared and Back-Slopped with Wholemeal and Refined Soft (Triticum aestivum) Wheat Flours

Microbial diversity of type I sourdoughs prepared and back-sloped with wholemeal and refined soft (*Triticum aestivum*) wheat flours

Short version of the title: biodiversity of type I sourdoughs

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Abstract: Type I sourdough fermentations were carried out for 20 days with daily back-slopping under laboratory and artisan bakery conditions using one wholemeal and two refined soft wheat (*Triticum aestivum*) flours, preliminarily subjected to chemical and rheological testing. The bacterial and yeast diversity and dynamics were investigated though plate counting and a combined culture-dependent and -independent PCR-DGGE approach; pH, total titrable acidity and concentration of key organic acids (phytic, lactic and acetic) were investigated through conventional tests. The three flours differed for both chemical and technological traits. Regarding the establishment and stabilization of microbial consortia during the continuous sourdough propagation, a microbial succession was seen, with atypical sourdough species detected at day 0 (such as f.i. *Lactococcus lactis* and *Leuconostoc holzapfelii/citreum* group for bacteria, or *Candida silvae* and *Wickerhamomyces anomalus* for yeasts) progressively replaced by taxa more adapted to the sourdough ecosystem (*Lactobacillus brevis*, *Lb. alimentarius/paralimentarius*, *Saccharomyces cerevisiae*). In the mature sourdoughs, a quite different species composition was seen. As sourdoughs propagated with the same flour at laboratory and artisan bakery level were compared, an influence of both the substrate and the propagation environment on the sourdough microbial diversity was assumed.

Practical Applications: Foods manufactured with wholemeal flours boast numerous health benefits, mainly due to their high content in bran fibre; however, these flours are poorly exploited in bread-making due to their scarce leavening ability and their richness in anti-nutrients (phytates). The use of wholemeal sourdoughs might represent a valid alternative to bakers' yeast for the future development of technologically and nutritionally superior raw materials for all cereal foods, such as bread, breakfast and snack products manufacture.

Keywords: High rate extraction rate flour, high-fibre sourdough, high-fibre bread-making, sourdough microbiota, PCR-DGGE.

63 **Introduction**

64 Soft wheat (*Triticum aestivum*) is the most widely used cereal for the production of bakery products and, in particular,
65 bread. The latter can be manufactured with both high and low extraction rate flours; a 100% extraction rate flour
66 corresponds to wholemeal flour containing all the caryopsis components, whereas low extraction rate flours are refined,
67 whiter flours, commonly classified as type 00 or 0, which are progressively deprived of increasing amounts of bran and
68 germ, and thus B vitamins, fat, iron and other minerals.

69 Refined flours have a higher aptitude to leavening and are preferred to wholemeal flours for the manufacture of some
70 baked leavened products, such as special occasion cakes (i.e. Panettone, Pandoro or Colomba) (Garofalo and others
71 2008; Garofalo and others 2011). On the contrary, wholemeal flours have a lower gluten content, which explains the
72 production of doughs with a lower stability, resistance and extensibility (Azizi and others 2006), but are richer in
73 phytates (Buddrick and others 2014), which sequester and, hence, prevent the intestinal absorption of some essential
74 minerals, such as calcium, zinc, iron and magnesium (Kumara and others 2010). However, wholemeal flours are richer
75 in bran fiber, which confers not only improved sensory and nutritional properties to bread but also health benefits, such
76 as the reduction of blood cholesterol and glycemic index, and the promotion of weight loss by increased satiety
77 (Stevenson and others 2012). Previous studies clearly demonstrated that the use of high extraction rate flours also leads
78 to a reduction in the bread staling rate (Venkateswara and others 1985; Azizi and others 2006).

79 In ancient times unrefined flours were typically processed with sourdough (Poutanen and others 2009). Sourdough is a
80 biological ecosystem, composed of lactic acid bacteria (LAB) and yeasts, which may be deliberately added to doughs
81 made of flour and water or spontaneously selected during sourdough continuous propagation (De Vuyst and others
82 2014). The use of sourdough has undoubtedly a great potential for the manufacture of leavened bakery products with
83 increased sensory properties, nutritional value, and prolonged shelf-life (Gobbetti and others 2014). Doughs started with
84 sourdoughs are characterized by more balanced biochemical (f.i. fermentation quotient, profile of volatile compounds,
85 etc.) and rheological (f.i. structural stability) traits than doughs started with the sole baker's yeast, where alcoholic
86 fermentation end-products largely predominate (Cossignani and others 1996). In the case of doughs prepared with
87 wholemeal flours, the use of sourdough appears particularly advantageous for the activation of endogenous phytases
88 during prolonged fermentation (Buddrick and others 2014), which directly implies the improvement of bread mineral
89 bioavailability (Lopez et al., 2003). Leavening and bread-making trials conducted with flours characterized by low
90 technological aptitudes, such as barley (Zannini and others 2009; Mariotti and others 2014) or even gluten-free flours
91 (Arendt and others 2007) demonstrated the usefulness of sourdough technology for the improvement of bread qualities.

92 In the past decades, this technology was successfully explored for the improvement of volume and shelf-life of high-
93 fibre breads (Salmenkallio-Marttila and others 2001; Katina and others 2005) and a new type of sourdough, consisting

of wheat bran pre-fermented with selected yeasts and lactic acid bacteria, was supplemented to bread doughs, with encouraging results.

The composition of the sourdough microbiota plays a main role for taste and quality of baked goods. For this reason, scientific research focuses largely on the characterization of microbiota of sourdoughs fermented under different conditions, on selection of suitable starter cultures for the baking industry, and on metabolic properties of sourdough microorganisms. Despite intense research conducted in the last decades, it is still unclear to which extent the sourdough microbiota is affected and selected by the kind of substrate, the process parameters (f.i. temperature and number of refreshments, dough yield, etc.), and the establishment of diverse microbial interactions.

This study deals with the question how the composition of the sourdough microbiota is influenced by the substrate and the sourdough propagation conditions. More specifically, the focus was laid on both the flour extraction rate and the back-sloping environment. Based on the above premises, this study was aimed at investigating the biochemical (pH, total titratable acidity, concentration of phytic acid (phytate), lactic acid and acetic acid, and fermentation quotient) and microbiological traits of spontaneously fermented sourdoughs prepared and propagated daily at artisan and laboratory scale with wholemeal soft wheat (*Triticum aestivum*) flour, with a view to their further exploitation in high-fibre bread-making. Conventional sourdoughs produced with refined flour from soft wheat grains processed by roll milling or stone grinding were also analyzed, in parallel. Preliminary chemical, farinograph, alveograph, and micro-visco-amylograph tests were also conducted on the three flours whereas the evolving microbial diversity of the high-fibre and conventional type I sourdoughs were investigated through a combined PCR-DGGE approach, relying on the analysis of the bacterial and yeast DNA extracted directly from the (sour)dough samples and the bulks of colonies harvested from selected dilution agar plates used for viable counting.

Materials and Methods

Composition and traits of the three flours

Three soft wheat (*T. aestivum*) flours designed for making bread were used in this study. Wheat was cultivated during the growing season 2013-2014, at the farmers' cooperative "Il Biroccio" (Filottrano, Ancona, Italy). A wholemeal (hereafter referred to as "F_A") and two type 0 (hereafter referred to as "F_B" and "F_C") flours were obtained through steel roller milling (F_A and F_B) or stone grinding (F_C). The grains were thoroughly cleaned by winnowing, sieving and sorting. Flours were kept in sealed containers at -20 °C before use.

Chemical analyses

For each flour, the following chemical analyses were performed according to international standard methods: moisture (UNI EN ISO 712:2010); ash (UNI-ISO 2171:2010); falling number (UNI EN ISO 3093:2007); and protein (UNI EN ISO 20483:2007). Commercial kits were used for the determination of phytic acid (phytate/total phosphorous) (K-PHYT 12/12, Megazyme, Wicklow, Ireland).

Rheological analyses

The three flours were subjected to farinograph (UNI 10790:1999), alveograph (UNI EN ISO 29971:2008), and micro-visco-amylograph tests (UNI 10872:2000), according to international standard methods, using the following equipment: Chopin alveograph (alveo-consistograph with alveolink NG, Chopin, Villeneuve-la-Garenne, France), Brabender farinograph (Farinograph-E, Brabender, Duisburg, Germany), Brabender micro-visco-amylograph (Micro visco-amylo-graph, Brabender, Duisburg, Germany).

Propagation of type I sourdoughs

Type I sourdough preparation was started as follows: 100 mL of tap water and 150 g of the flours F_A, F_B or F_C were mixed to produce 250 g of dough [dough yield (dough weight × 100/flour weight), 166], according to a traditional protocol (Zannini and others 2009) without the use of starter cultures or baker's yeast. The doughs were kneaded manually for 5–10 min until the correct consistency was obtained and fermented for 24 h in glass containers covered with a lid. The resulting sourdoughs were propagated daily over a period of 20 days according to the back-slopping procedure, where 100 g of the sourdough from the day before was used as the inoculum [40% (w/w)] of a new mixture of flour (100 g) and tap water (50 mL) (dough yield, 156). Sourdough propagation was performed at both artisan bakery and laboratory level, at room temperature; in the first case, it was performed at an artisan bakery in the Marche region (central Italy), where the sourdough technology had never been used before, and the manufacture of bread is based on the use of compressed bakers' yeast. Sourdoughs propagated at laboratory and artisan bakery level with the three flours F_A, F_B and F_C are hereafter referred to as "S_A-l", "S_B-l", and "S_C-l" and "S_A-b", "S_B-b", "S_C-b", respectively.

Sampling was performed on the first unfermented doughs (t_0) and the sourdoughs immediately before each daily refreshment step; for each type of sourdough, a total of 21 samples were collected, cooled to 4 °C and analyzed in duplicate within 2 h of collection. At each refreshment step all samples were subjected to the measurement of pH and total titratable acidity (TTA), whereas the sole samples collected after 0 (t_0), 5 (t_5), 10 (t_{10}), and 20 (t_{20}) days of daily back-slopping were subjected to both plate counting of presumptive LAB and yeasts and PCR-DGGE analyses.

Organic acids content of laboratory and artisan bakery sourdoughs was determined at the end of the 20-day propagation.

156 Viable counts of presumptive lactic acid bacteria (LAB) and yeasts

157 LAB and yeast cell counts were determined by mixing 10 g of each dough and sourdough sample with 90 mL of a
158 sterile aqueous peptone solution (0.1% peptone, w/v), followed by homogenization with a Stomacher apparatus (400
159 Circulator, International PBI, Milan, Italy) at 260 rpm for 3 min. Presumptive lactobacilli were counted on modified de
160 Man Rogosa Sharpe (mMRS) agar, containing 1% maltose (w/v) plus 5% fresh yeast extract (v/v) and Sourdough
161 Bacteria (SDB) medium modified according to Vogel et al. (1994), (hereafter referred to as mSDB), the latter being
162 elective for *Lactobacillus sanfranciscensis* (Foschino and others 2004). Both media were supplemented with
163 cycloheximide (0.1 g L^{-1}) to inhibit the growth of yeasts. Plates were incubated in sealed jars with the Anaerogen
164 system (Oxoid, Basingstoke, UK) at 30 °C for 48 h. Yeasts were counted on Wallestein Laboratory Nutrient (WLN)
165 medium added with chloramphenicol (0.1 g L^{-1}) to inhibit the growth of bacteria and incubated at 25 °C for 72 h. The
166 results of the viable counts were expressed as means of the Log of colony forming units (cfu) per gram of sample ±
167 standard deviation. After counting, plates spiked with the highest (-6, -7 or -8) and lowest dilution (-2) were used for
168 bulk formation, following the procedure previously described by Garofalo and others (2015).
169
170 Determination of pH, total titratable acidity (TTA), organic acids (phytic, lactic and acetic acid), and fermentation
171 quotient (FQ)

172 The pH measurements on doughs and sourdoughs were carried out with a pH meter equipped with a solid electrode
173 (HI2031, Hanna Instruments, Padua, Italy) which was inserted directly into the sample. Total titratable acidity (TTA)
174 was measured on 10 g of dough or sourdough homogenized with 90 mL of distilled water using a Stomacher apparatus
175 (International PBI). TTA was expressed as the amount (mL) of 0.1 N NaOH needed to achieve a final pH of 8.5. Lactic
176 and acetic acids were determined in the water-soluble extracts of the 20-day back-slopped sourdoughs, according to the
177 following procedure. Ten grams of each sourdough were homogenized with 90 mL of Tris-HCl 50 mM buffer (pH 8.8).
178 After incubation at 25 °C for 30 min under stirring the suspension was centrifuged at 4.000 rpm for 10 min at 4 °C, and
179 the supernatant was analyzed using the commercial kits K-ACETIC 02/11 and K-DLATE 12/12 (Megazyme). The
180 fermentation quotient (FQ) was determined as the molar ratio between D,L-lactic and acetic acids. Phytic acid
181 (phytate/total phosphorous) was determined using the commercial kit K-PHYT 12/12 (Megazyme).
182
183 Bacterial and fungal DNA extraction from (sour)doughs and cell bulks

184 Microbial DNA extraction from the dough and sourdough samples was performed using the PowerFood™ Microbial
185 DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA) following the kit manufacturer's instructions. DNA
186 extraction from the LAB and yeast bulk cell suspensions was performed according to the procedures described by

187 Hynes and others (1992) and Makimura and others (1999), respectively. The procedure indicated by Hynes and others
188 (1992) was slightly modified as described by Osimani and others (2015). All the DNA suspensions were subjected to
189 optical readings at 260, 280 and 234 nm with a UV-Vis Shimadzu UV-1800 Spectrophotometer (Shimadzu
190 Corporation, Kyoto, Japan) for the assessment of the DNA quantity and purity.
191
192 Amplification reactions
193 Two microliters of each microbial DNA suspension (adjusted to 25 ng/μL) were amplified in 25 μL reaction volume
194 using bacterial universal primer sets (338f_{GC}- 518r), targeting the variable regions V3 of the 16S rRNA gene, as
195 previously described (Osimani and others 2015). Two microliters of each microbial DNA suspension (adjusted to 25
196 ng/μL) were amplified in 25 μL reaction volume using universal primers for the eumycetes (NL1$_{GC}$-LS2), as previously
197 described (Osimani and others 2015). All the PCR reactions were performed in the thermal cycler My Cycler (Bio-Rad
198 Laboratories, Segrate, Italy); aliquots (5 μL) of the PCR products were routinely checked on 2% agarose (w/v) gels,
199 prior to PCR-DGGE analysis.
200
201 Polyacrylamide gel preparation and DGGE runs
202 PCR products were analyzed using the DGGE Bio-Rad D-code™ apparatus (Bio-Rad Laboratories). DGGE runs were
203 performed in 8% polyacrylamide (acrylamide/bis-acrylamide mix 37.5:1, w/v) gels with a 30-60% urea-formamide
204 (w/v) gradient. Gels were subjected to a constant voltage of 130 V for 4 h at 60 °C. After electrophoresis, gels were
205 stained in 1X TAE buffer containing SYBR Green I (1× final concentration; Molecular Probes, Eugene, OR, USA) and
206 photographed under UV transillumination, using the Complete Photo XT101 system (Explera, Jesi, Italy).
207
208 Band sequencing and identification
209 For DGGE band sequencing, bands of interest were excised by gel cutting tips, resuspended in 50 μL of sterile water,
210 and incubated overnight at 4 °C to allow diffusion of DNA from the gel cuts. Five microliters of the eluted DNA were
211 re-amplified using the conditions described above, with the forward primer without the GC clamp and sent to Beckman
212 Coulter Genomics (Hope End, Takeley, United Kingdom) for purification and sequencing. The sequences obtained in
213 FASTA format were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/)
214 using the basic blast search tools. An identity of the partial 16S and 26S rRNA gene sequences to type strain sequences
215 ≥ 97% was used as the criterion for species identification. Due to the slowly diverging nature of the 16 rRNA gene
216 (Felis and Dellaglio 2007), a few sequences could not be univocally assigned to a single species, but to a microbial
217 group, including strictly correlated species, namely the *Lactobacillus graminis*/*curvatus* group, the *Lactobacillus*

alimentarius/*paralimentarius* group, the *Lactobacillus pentosus*/*plantarum*/*paraplantarum* group, the *Weissella confusa*/*cibaria* group, and the *Leuconostoc holzapfelii*/*citreum* group. Similarly, a discrimination between phylogenetically closely related species, namely *Kazachstania unispora, Kazachstania servazzii, Kazachstania aerobia* was not achieved through the analysis of the D1 region of yeast DNA, as previously reported (Garofalo and others 2015).

Statistical analysis

Chemical and rheological properties of three flours were analyzed by one-way ANOVA. All the data referring to dough and sourdough microbial communities (viable counts of LAB and yeasts) and biochemical traits (pH, TTA, concentration of phytic acid, lactic acid and acetic acid, FQ) were subjected to two-way ANOVA focusing on both the influence of the flour and the sourdough propagation environment, and on their cross interaction. Due to the presence of a cross interaction for several parameters a Tukey's HSD test at $P < 0.05$ was performed for mean separation of all possible combinations of the two factors. Principal component analysis (PCA) using a correlation matrix was carried out to visualize the effects of the flour and the sourdough propagation environment on the above parameters. Finally, a two way cluster permutation test was performed using all the data referring to microbial communities and biochemical traits, and used to overlay confidence ellipses on the PCA score plots. For cluster analysis the Ward's method was applied. All the statistical analyses were performed using JMP 9 (SAS Institute Inc., Cary, NC).

Results and Discussion

Chemical and rheological traits of the three soft wheat flours

The results of chemical and rheological analyses carried out on the three flours are shown in Table S1 in the supplementary material. With the exception of moisture, for all the chemical traits investigated, F_A differed from F_B and F_C. Analogously, differences were seen in some rheological properties of wholemeal (F_A) and type 0 flours (F_B and F_C), namely consistency, water absorption, dough development time, mixing tolerance index after 10 min, maximum viscosity, falling number, phytic acid.

It has previously been reported that the reduced moisture of high extraction rate flours is mainly dependent on their content in wheat bran, the latter being characterized by a lower moisture with respect to aleuronic particles (Azizi and others 2006). However, in this study, no significant reduction in such a quality parameter was seen in F_A compared with F_B and F_C. According to what was reported by Azizi and others (2006), even the significantly lower falling number of F_A, which indicates a higher alfa-amylase activity, and the higher ash and protein content of this flour might be directly

249 imputable to its higher bran content. It has previously been reported that the alfa-amylase activity of wheat flour
250 depends on its extraction rate and quality, with wholemeal flour, and especially the bran fraction, having the highest
251 enzyme activity (Martinez-Anaya 2003). As expected, F_A was also characterized by a higher phytic acid content than
252 the refined flours F_B and F_C. In fact, it is known that the flour refining process reduces the levels of phytate (Buddrick
253 and others 2014), which are concentrated in the aleuronic layers of grains (Poutanen and others 2009).
254 As far as the rheological traits of the three flours are concerned, a high water absorption, which significantly differed
255 from that of F_B and F_C, was seen in F_A. The occurrence of a linear correlation of this trait with the flour extraction rate
256 has previously been elucidated by Azizi and others (2006), who reported an increase in this parameter in high extraction
257 rate flours, which are generally characterized by coarser particle sizes. Even the weaker resistance of F_A to mechanical
258 shocks (revealed by a significantly lower mixing tolerance index after 10 min) and the markedly higher dough
259 development time might be attributed to the higher content in wheat bran, which translates into a reduction in the gluten
260 network and quality. In terms of acidification, the results collected demonstrated that just two refreshment steps were
261 needed to significantly reduce the pH of all the sourdoughs to mean values < 4.5, whereas a higher variability was seen
262 in both the maximum increase in TTA during the 20-day propagation and the number of refreshments needed to reach
263 TTA values > 8.0. It has previously been proposed that sourdoughs achieve their maturity, in terms of constant
264 acidification and leavening capacity, within 5 to 7 days of continuous propagation (Ercolini and others 2013).
265
266 Microbial viable counts and acidification kinetics during sourdough propagation
267 After 5 days of propagation at laboratory level, the mean viable counts of presumptive LAB of S_A-l, S_B-l, and S_C-l
268 reached values ≥ 9.2 Log cfu/g, irrespective of the growth medium used (mSDB or mMRS), whereas at the same
269 sampling time, the artisan bakery sourdoughs were characterized by mean cell numbers of presumptive LAB ≥ 9.0 Log
270 cfu/g (S_A-b on mMRS; S_A-b, S_B-b, and S_C-b, on mSDB) and > 8.0 Log cfu/g (S_B-b and S_C-b on mMRS), respectively
271 (Figure 1). The viable cell counts estimated by plating on mSDB stayed almost constant during subsequent propagation
272 in all the sourdoughs analyzed, whereas those estimated by plating on mMRS showed variable fluctuations, consisting
273 in a slight decrease in laboratory sourdoughs (S_A-l, S_B-l, and S_C-l) and, conversely, a slight increase in artisan bakery
274 sourdoughs (S_A-b, S_B-b, and S_C-b).
275 No yeasts could be enumerated in all the initial doughs (t_0) prepared at laboratory level, considering a detection limit of
276 2 Log cfu/g, whereas doughs prepared at artisan bakery level contained a significantly ($P > 0.001$) higher (at least 1.0
277 log unit) yeast cell density with respect to presumptive LAB (Figure 1). Nevertheless, after 20 days of daily
278 propagation, all the sourdoughs were characterized by a ratio between LAB and yeasts stabilized at ca. 100:1 (Figure 1).

279 In all the sourdoughs analyzed, a pH drop below 4.5 was seen between the 1st and the 2nd day of propagation, whereas
280 differences were seen in the refreshment step, where pH values $\leq$ 4.0 were reached (remaining almost constant until the
281 end of the 20-day propagation period), namely: days 10, 3 and 7 in S_A-l, S_B-l and S_C-l, respectively, and days 5, 4 and 4
282 in S_A-b, S_B-b and S_C-b, respectively (Figure 1).
283 Differences were also seen between the sourdoughs propagated at laboratory and artisan bakery level in the significant
284 maximum increase in TTA reached during the 20-day propagation, namely: $\geq$ 20 mL in S_A-l and S_A-b, and $\geq$ 11 mL in
285 S_B-l, S_B-b, S_C-l and S_C-b, respectively. Analogously, there were also variations in the propagation day when the first
286 significant increase in sourdough TTA was recorded, namely: days 2, 1 and 2 in S_A-l, S_B-l and S_C-l, respectively, and
287 day 2 in S_A-b, S_B-b and S_C-b (Figure 1).
288 A mean fermentation quotient (FQ) > 5 was seen in all the 20-day back-slopped sourdoughs analyzed, with the
289 exception of S_B-l, which was characterized by a mean molar ratio of lactic and acetic acid of 1.92. In the two laboratory
290 sourdoughs S_B-l and S_C-l, this molar ratio reached levels as high as 13.74 and 12.55, respectively.
291
292 Biochemical characteristics of the mature 20-day back-slopped sourdoughs
293 The values of pH, TTA, concentration of phytic acid, lactic acid and acetic acid, and FQ of the mature high-fibre and
294 conventional type I sourdoughs are shown in Table 1. The mean pH and TTA values recorded at the end of the 20-day
295 propagation were, with a few exceptions, in the typical ranges of 3.6-3.8 and 8-13, respectively (Brummer and Lorenz
296 1991). The significantly higher TTA observed in the sourdoughs propagated with wholemeal flour (S_A-l and S_A-b) is
297 feasibly correlated to the higher ash content of F_A compared with F_B and F_C, which has been reported as having an
298 influence on sourdough buffering ability (Mariotti and others 2014). As regards the content in lactic acid and acetic
299 acid, mean values notably higher than those reported for wheat sourdoughs, ranging from 600 to 800 mg/100 g
300 sourdough and from 80 to 160 mg/100 g sourdough, respectively (Barber and others 1992; Hansen and Hansen 1994),
301 were seen. Fermentation quotients exceeding 1 (and in most cases higher than 5) were also seen in all the 20-day back-
302 slopped sourdoughs, thus suggesting a predominance of the species with an obligately homofermentative or
303 facultatively heterofermentative metabolism.
304 Although the phytic acid content of flour and mature sourdough is not directly comparable, at the end of the 20-day
305 continuous propagation, a trend toward the reduction of this parameter was seen. More in detail, a complete
306 hydrolytation of this compound was observed in S_B and S_C, whereas a noticeable decrease was seen in S_A sourdoughs
307 propagated with wholemeal flour (F_A), containing the highest amount of this anti-nutrient. Sourdough fermentation has
308 been found to decrease the amount of phytates, either through a mere pH drop to values < 5.5, sufficient to activate the
309 endogenous phytates, or through the phytase activity of LAB and yeasts (Poutanen and others 2009; Mariotti and others

2014). In particular, in whole-wheat flours, the solubilization of minerals due to fermentation has been shown to be strictly dependent on the bran particle size, being effective in finely milled bran, but not in coarse bran (Lioger and others 2007).

Statistical correlations between propagation environment, flour, microbial communities and biochemical traits

The two way ANOVA highlighted a cross interaction of the sourdough propagation environment and the type of flour on TTA ($P = 0.0008$), acetic acid content ($P < .0001$), FQ ($P = 0.0014$), yeast ($P = 0.0075$) and LAB viable counts (the latter assessed on mMRS) ($P < .0001$). Both the variability factors considered had a significant effect on all the chemical/microbiological traits analyzed; the only exceptions were represented by phytic acid content, which was not influenced by the sourdough propagation environment, and viable counts of LAB (assessed on mSBD), not influenced by the type of flour (Table 1).

PCA indicated eigenvalues of 4.24, 3.44 and 0.79 for the first 3 components, respectively, thus suggesting the adoption of two principle components. These two components accounted for 47.1% and 38.3% of total variance of the data, respectively. The first principle component was dominated by pH, TTA, and content of phytic acid and lactic acid. The second component had the highest scores for acetic acid, FQ, LAB and yeast viable counts. The PCA analysis indicated that the type of flour strongly influenced PC1 whereas the sourdough propagation environment had less influence. Although the correlation with PC2 of the type of flour in the sourdoughs produced at laboratory level was minimal, it was magnified in the sourdoughs propagated at the artisan bakery (Figure 2). According to the permutation test, sourdoughs were distributed into two major clusters (A and B). Irrespective to the propagation environment, cluster B included the sourdoughs refreshed with the flour F_A, characterized by the highest values of pH, lactic acid content, TTA, and phytic acid content. Cluster A included sourdoughs S_B and S_C, whose further clustering (A_I and A_{II}) was influenced by the propagation environment. Acetic acid content and LAB viable counts assessed on mMRS were higher in the sourdoughs propagated at laboratory level whereas FQ and LAB viable counts assessed on mSBD were lower (Figures 2 and 4).

PCR-DGGE analyses

The results of PCR-DGGE analyses are shown in Figure 3 and in Tables S2 to S5, in the supplementary material.

Overall, multiple genera (*Lactobacillus*, *Leuconostoc*, *Weissella*, *Lactococcus,* and *Staphylococcus*) were identified in both the high-fibre and refined-flour sourdoughs with the double PCR-DGGE approach used; these genera correspond to those commonly retrieved in *T. aestivum* flour (Ercolini and others 2013). In almost all the mature sourdoughs analyzed (with the exception of S_C-b) the closest relatives to *Lb. brevis* and the *Lb. alimentarius*/*paralimentarius* group

were stably detected in both the mMRS and mSDB bulks, irrespective of the flour and, hence, the flour extraction rate or the propagation environment. *Lb. brevis* is an obligate heterofermentative species, which is frequently isolated from sourdoughs (De Vuyst and others 2009). This species has been used with some success for the pre-fermentation of wheat bran destined for the manufacture of wheat bread supplemented with bran (Salmenkallio-Marttila and others 2001), thus clearly demonstrating the adaptability and even competitiveness of such a microorganism in high-fibre cereal environments. *Lb. paralimentarius* is another common sourdough inhabitant which has first been isolated from this peculiar ecosystem (Cai and others 1999).

The detection of both these species at the highest dilutions suggests their occurrence at cell densities $\geq$ 6 Log cfu/g. Although newly-developed sequencing techniques, also referred to as next generation sequencing (NGS) methods, have recently been applied to the study of complex food ecosystems, a recent investigation exploiting and comparing NGS and PCR-DGGE clearly demonstrated how this latter technique still represents a valid tool for the profiling of the sourdough core microbiota (Ercolini and others 2013).

In the present study, an apparently flour-dependent occurrence of *Weissella* spp. was seen in S_A-l and S_A-b, and in S_B-l and S_B-b, whereas for other LAB, a greater influence of the propagation environment might be assumed, as was the case for the sourdoughs refreshed in the laboratory, which were characterized by the establishment of the closest relatives to the *Lb. graminis*/*curvatus* group (detected in S_A-l, S_B-l and S_C-l) and *Lb. plantarum/pentosus*/*paraplantarum* group (detected in S_B-l and S_C-l).

Together with *Lb. fermentum*, which occurred in the sole laboratory sourdough S_C-l, the *Lb. plantarum/pentosus*/*paraplantarum* group is recognized as a highly adapted sourdough-typical LAB (Van der Meulen and others 2007; Weckx and others 2010), stably co-dominating with *Lb. fermentum* in laboratory wheat sourdough fermentations carried out under semi-aseptic conditions (Vogelmann and others 2009).

Finally, for the occurrence of other bacteria, a contribution of both parameters might be hypothesized, as in the case of the *Leuc. holzapfelii/citreum* group and *Leuc. mesenteroides/pesudomesenteroides*, which were exclusively detected in S_A-l, and of *Pediococcus pentosaceus* and the two minority taxa *Leuc. holzapfelii*/*citreum* group and *Staphylococcus pasteuri* detected in the sole S_C-b. Insight into the impact of both these parameters on the sourdough microbiota is readily available in the literature; several investigations have proved the influence of the substrate (Vogelmann and others 2009; Zannini and others 2009; Ercolini and others 2013), whereas a number of other studies comparing the LAB and yeast diversity of sourdoughs back-slopped at laboratory and artisan bakery level pointed out the effect of the bakery environment (equipment, air, staff) on the establishment of an in-house dominant microbiota (Scheirlinck and others 2007; Van der Meulen and others 2007; Vrancken and others 2010; Weckx and others 2010; Minervini and others 2012).

372 Regarding the yeast dynamics, to our knowledge, this is the first study investigating the spontaneously developing yeast
373 microbiota in Italian sourdoughs in controlled laboratory fermentations without the addition of any starter culture.
374 A simpler evolution with respect to LAB was observed, and an overall low number of yeast species was identified, in
375 agreement with what was previously reported in an investigation aimed at assessing the yeast species composition of
376 artisan bakery and spontaneous laboratory sourdoughs (Vrancken and others 2010). In terms of yeast cell densities, in
377 both the laboratory and artisan bakery sourdoughs analyzed in this study, a progressive increase was seen in viable
378 counts to Log cfu/g ranging from mean values of 7.47 to 7.97; however, at day 0, an initial yeast population markedly
379 lower (up to 4 orders of magnitude) with respect to artisan bakery sourdoughs was found in the sourdoughs prepared in
380 the laboratory, feasibly due to a very poor yeast contamination of this latter propagation environment. In a further
381 research study, carried out on seven sourdoughs propagated at artisan bakery and laboratory level, after 20 and 80 days
382 of continuous back-slopping, most of the laboratory sourdoughs showed a yeast cell density markedly lower than that of
383 artisan bakery sourdoughs, not exceeding 3 Log cfu/g (Minervini and others 2012).
384 In the present study, a clear dominance of *S. cerevisiae* was seen along the whole 20-day propagation, irrespective of
385 the back-slopping environment or the type of flour. This finding is in agreement with what was very recently found by
386 Ercolini and others (2013) and other authors investigating the yeast ecology of this peculiar ecosystem (Stolz 2003;
387 Garofalo and others 2011), thus confirming the high adaptation of this yeast to wheat sourdoughs.
388 However, for the other yeasts identified, interesting remarks can be made by comparing the sourdoughs prepared with
389 the same flour and propagated at laboratory or artisan bakery level; in more detail, except for S_B-b, the co-dominance of
390 members of the *K. unispora*/*servazzii/aerobia* group with *S. cerevisiae* was established in the sole artisan bakery
391 sourdoughs S_A-b and S_C-b and the laboratory sourdoughs S_A-l. To date, *K. unispora* has rarely been documented as
392 existing in a sourdough ecosystem, namely once in Belgian artisan wheat sourdough (Huys and others 2013) and twice
393 in rye sourdoughs (Bessmeltseva and others 2014). In S_A-b *P. kudriavzevii*, formerly known as *Issatchenkia orientalis*,
394 the anamorph *Candida krusei*, was also found to stably occur with the previous two species from days 5 to 20 of daily
395 propagation. Interestingly, highly phytase-active stains ascribed to *P. kudriavzevii* have previously been isolated from
396 two Lithuanian rye sourdoughs and a Danish rye sourdough made with wholemeal flour (Nuobariene and others 2012),
397 thus corroborating the positive association of this yeast with whole grain foods containing considerable amounts of
398 phytic acid.
399
400 **Conclusions**
401 To the authors knowledge this is the first report on the exploitation of the back-slopping technique for the production of
402 type I sourdoughs with wholemeal flour. In general, the results collected demonstrated the suitability of this flour for

403 this specific purpose, encouraging further research for its future exploitation in high-fibre bread-making. In particular,
404 wholemeal sourdoughs offer interesting challenges for the quality improvement of high-fibre baked leavened goods, as
405 the positive modification of texture, flavour, nutritional value and shelf-life and the overcome of the deleterious effect
406 of wheat bran on bread volume.
407 When the sourdoughs propagated at artisan bakery and laboratory level are comparatively evaluated irrespective of the
408 type of flour, the overall findings support, on the one hand, what was previously reported about the role of the bakery
409 environment (equipment, air, or staff) on the contamination of sourdough batches, while on the other hand they
410 underline the equally important influence of the substrate on the adaptation capacity of microorganisms (in terms of
411 growth rate and competitiveness).
412
413 **Author Contributions**
414 Conceived and designed the experiments: L. Aquilanti, M. Taccari. performed the experiments: M. Taccari, V.
415 Milanović. Analyzed the data: L. Aquilanti, F. Clementi, S. Polverigiani, A. Osimani. Contributed
416 reagents/materials/analysis tools: S. Polverigiani, C. Garofalo, F. Clementi. Wrote the paper: M. Taccari, L. Aquilanti.
417
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425
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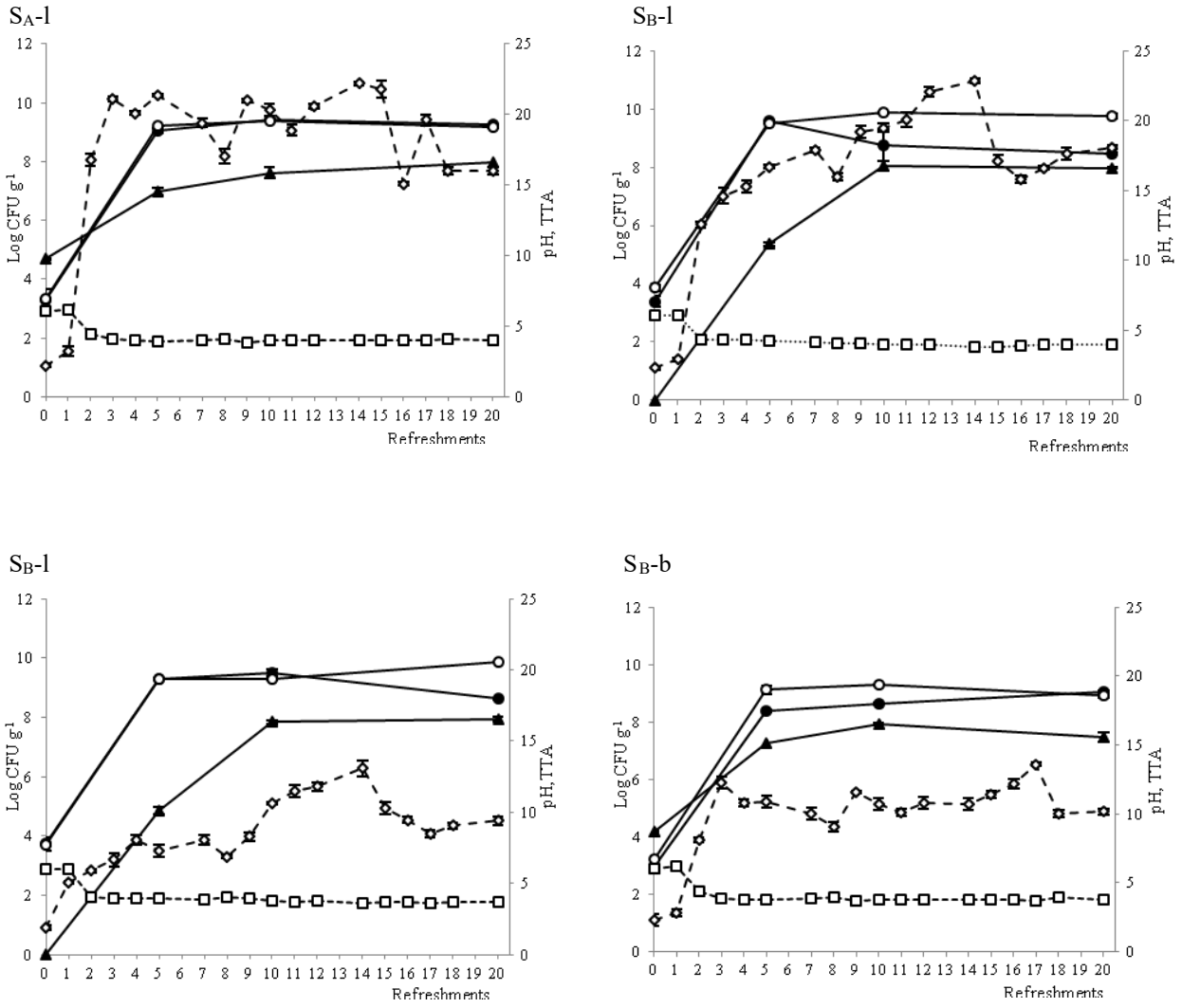


Figure 1-Viable counts of presumptive lactic acid bacteria and yeasts, assessed on selective media mMRS (—●—), mSDB (—○—) and WLN(—▲—) and kinetics of acidification (pH ···□··· and TTA - -◇- -) of wholemeal (S_A) and type 0 (S_B and S_C) sourdoughs daily propagated under laboratory (labeled "l") and artisan bakery (labeled "b") conditions for 20 days; numbers on the χ axis indicate days of propagation. Day 0 corresponds to the first water and flour dough sampled and analyzed prior to fermentation, whereas days 1 to 20 correspond to sourdough sampled immediately before each daily refreshment. Means of two independent experiments ± standard deviations are shown.

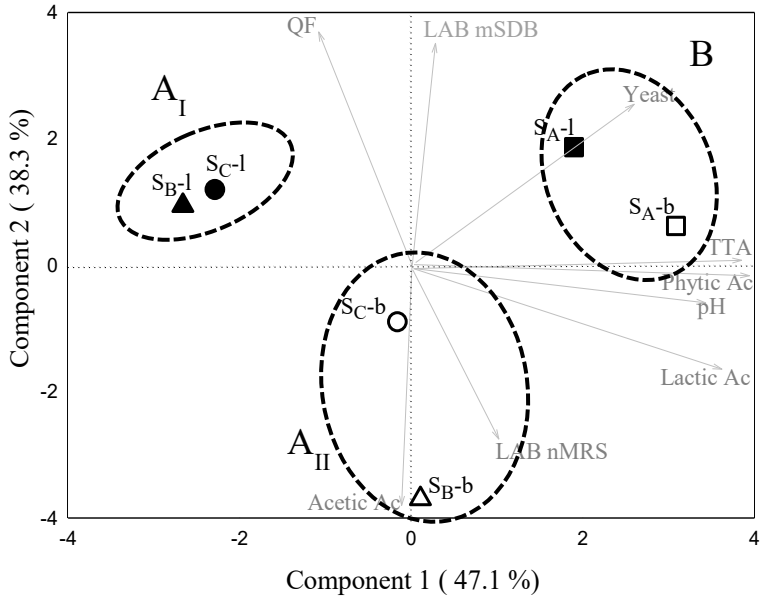
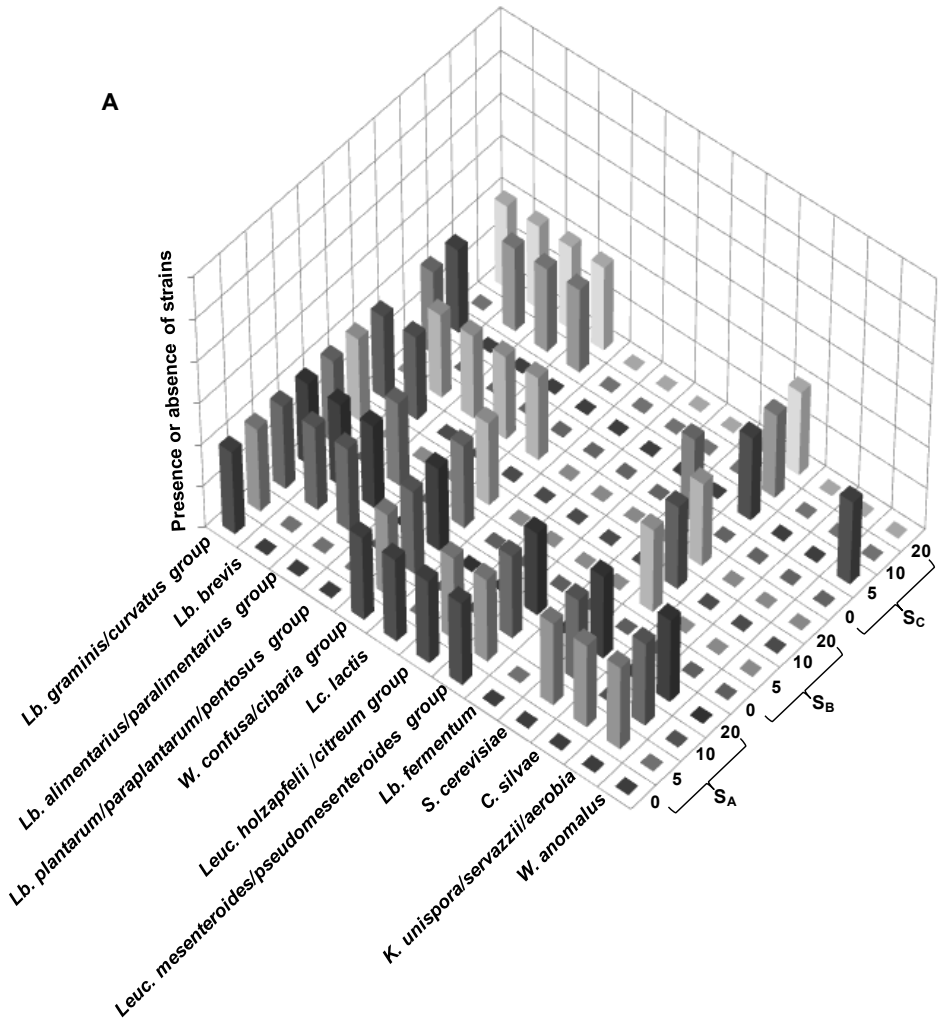


Figure 2-Score and loading plots of first and second principal components based on microbial community and biochemical characteristics data means from sourdoughs produced with different flours (A, B and C) and in two different propagation environments (laboratory, "l" and artisan bakery, "b"). Clusters A_I, A_{II} and B are indicated by dotted ellipses and correspond to those derived from the cluster analysis shown in Fig. 4.


A
Presence or absence of strains
Lb. graminis/curvatus group
Lb. brevis
Lb. alimentarius/paralimentarius group
Lb. plantarum/paraplantarum/pentosus group
W. confusa/cibaria group
Lc. lactis
Leuc. holzapfelii /citreum group
Leuc. mesenteroides/pseudomesenteroides group
Lb. fermentum
S. cerevisiae
C. silvae
K. unispora/servazzii/aerobia
W. anomalus
0
5
10
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S_A
0
5
10
20
S_B
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5
10
20
S_C

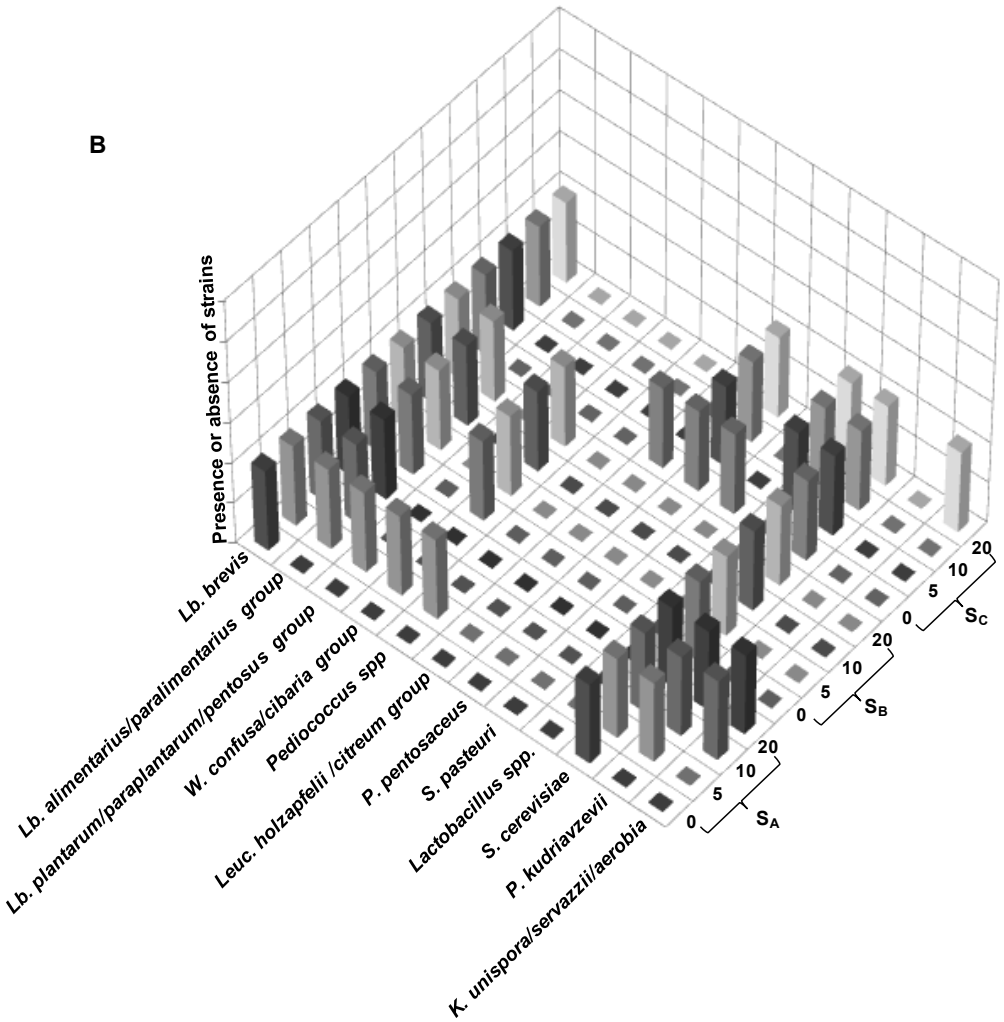

Figure 3-Lactic acid bacteria (LAB) and yeast species identified through the combined PCR-DGGE approach in the sourdoughs propagated at artisan (S_A-b, S_B-b, S_C-b) and laboratory (S_A-l, S_B-l, S_C-l) level for 1, 5, 10 and 20 days using a wholemeal (F_A) and two type 0 (F_B and F_C) flours. The *z*-axis indicates only the presence or absence of a given

species, resulting from either the analysis of the DNA extracted directly from the dough and sourdough samples or the bulk of colonies harvested from selected dilution plates. (A) Laboratory, (B) Artisan bakery.

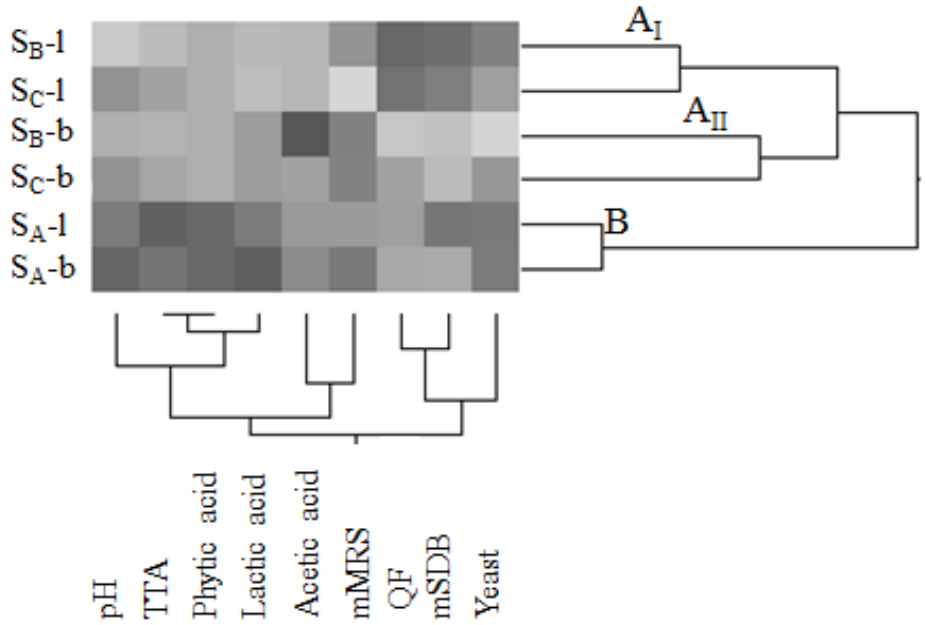

Figure 4-Dendrogram obtained from two way cluster analysis based on the microbial community and biochemical characteristics of sourdoughs propagated with the three flours (F_A, F_B or F_C) at the laboratory ("l") or artisan bakery ("b").

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Table 1-Biochemical properties and microbial viable counts of wholemeal (S_A) and type 0 (S_B and S_C) mature sourdoughs after 20 days propagation under laboratory (labeled "l") and artisan bakery (labeled "b") conditions.

Mature sourdough	pH	TTA (mL 0.1 N NaOH)	Phytic acid (g/100g)	Lactic acid (g/100 g)	Acetic acid (g/100 g)	FQ	Yeast (Log cfu/g)	LAB (on mMRS) (Log cfu/g)	LAB (on mSDB) (Log cfu/g)
ANOVA									
Flour	0.0001*	<.0001*	<.0001*	0.0003*	<.0001*	0.0177*	0.0079*	<.0001*	0.1431
Site	0.0274*	0.0117*	1	0.0041*	<.0001*	<.0001*	0.0193*	<.0001*	<.0001*
Flour x Site	0.1924	0.0008*	1	0.9458	<.0001*	0.0014*	0.0075*	<.0001*	0.0572
Mean separation test									
TREATMENT									
S_A-b	4.02 ± 0.02^{a}	16.00 ± 0.20^{b}	0.12 ± 0.00^{a}	17.20 ± 0.10^{a}	1.96 ± 0.00^{b}	5.85 ± 0.09^{ab}	7.97 ± 0.02^{a}	9.26 ± 0.01^{a}	9.19 ± 0.03^{b}
S_B-b	3.79 ± 0.01^{cd}	10.15 ± 0.15^{d}	n.d.	11.60 ± 0.51^{bc}	4.01 ± 0.08^{a}	1.92 ± 0.04^{c}	7.47 ± 0.11^{b}	9.05 ± 0.01^{b}	8.93 ± 0.05^{b}
S_C-b	3.87 ± 0.00^{bc}	11.40 ± 0.10^{c}	n.d.	11.63 ± 0.22^{bc}	1.18 ± 0.10^{c}	6.61 ± 0.67^{b}	7.81 ± 0.02^{a}	9.00 ± 0.00^{b}	9.01 ± 0.11^{b}
S_A-l	3.95 ± 0.05^{ab}	18.10 ± 0.10^{a}	0.13 ± 0.00^{a}	14.66 ± 0.88^{ab}	1.43 ± 0.05^{c}	6.83 ± 0.62^{b}	7.97 ± 0.02^{a}	8.45 ± 0.02^{d}	9.75 ± 0.05^{a}
S_B-l	3.72 ± 0.00^{d}	9.40 ± 0.20^{d}	n.d.	9.35 ± 0.83^{c}	0.45 ± 0.00^{d}	13.74 ± 1.28^{a}	7.93 ± 0.06^{a}	8.62 ± 0.02^{c}	9.85 ± 0.00^{a}
S_C-l	3.87 ± 0.00^{bc}	11.70 ± 0.30^{c}	n.d.	8.94 ± 0.99^{c}	0.47 ± 0.01^{d}	12.55 ± 1.10^{a}	7.78 ± 0.00^{ab}	7.00 ± 0.00^{e}	9.65 ± 0.03^{a}

Values represent means ± st. err. (n.= 2). Means followed by a different letter were significantly different according to the Tukey's test ($P < 0.05$).

FQ: Fermentation Quotient; n.d. not detectable.