1 # Development of a method for the direct fermentation of semolina by
2 # selected sourdough lactic acid bacteria

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Abstract

Three obligately heterofermentative lactic acid bacteria (LAB) strains (*Lactobacillus sanfranciscensis* PON100336, *Leuconostoc citreum* PON10079 and *Weissella cibaria* PON10030) were used in this study as a multi-species starter culture for sourdough production. The starter inoculum was prepared and propagated in sterile semolina extract (SSE) broth. Acidification kinetics, microbiological counts detected on specific media for sourdough LAB, polymorphic profile comparison and species-specific PCRs evidenced a stability of the liquid inoculum over time determining its suitability for direct addition to semolina. In order to validate this innovative method for the production of durum wheat (*Triticum durum* Desf) sourdoughs, 15 semolinas (from ten old and five modern genotypes cultivated in Sicily, southern Italy) were used to prepare the SSEs and to produce sourdoughs and finally breads. Chemical and microbiological analyses of the sourdoughs and the evaluation of the quality parameters (weight loss, height, crumb and crust colour, image analysis and volatile organic compound generation) of the resulting breads indicated that the direct addition of the liquid inocula propagated in SSE is a valuable method to stabilise the production of sourdoughs. The differences registered during the technological characterisation of the breads were underlined by the sensory tests and the multivariate analysis and are mainly imputable to the type of semolina.

Keywords: Fermentation; Lactic acid bacteria; Quality parameters; Starter culture; Sourdough; *Triticum durum* genotypes; Volatile organic compounds

1. Introduction

Bread production can be assumed as a simple process. The ingredients, mainly flour, water, salt and a leavening agent are mixed together. However, the dough is left to ferment for a while when the leavening is carried out by biological agents, in order to develop the desired characteristics. Baker's yeast is the primary biological agent in dough formation, but typical breads are often produced with

38 the sourdough technology (De Vuyst et al., 2009). Sourdough is an extremely complex ecosystem
39 where several lactic acid bacteria (LAB) and yeasts cohabit (Corsetti and Settanni, 2007).
40 In general, the raw materials, the microbiota developing during the fermentation process and the
41 technological parameters applied during bread making affect consistently the characteristics of the
42 final products (Corsetti et al., 2000). In particular, the microbial composition of sourdough plays a
43 major role during fermentation (De Vuyst and Neysens, 2005). However, a series of intrinsic and
44 extrinsic factors may in turn influence the composition of the sourdough microbiota (De Vuyst et
45 al., 2014).
46 The vast majority of bread is traditionally produced from wheat (Goesaert et al., 2005). Although
47 bread is generally produced with the flour from common wheat (*Triticum aestivum* L*)*, for this
48 reason also called "bread wheat", the use of semolina from durum wheat (*Triticum durum* Desf) in
49 bread production is quite common in southern Italy (Corsetti et al., 2001; Quaglia, 1988). Several
50 typical breads produced in Sicily are made with semolina applying the sourdough technology
51 (Ventimiglia et al., 2015).
52 The dominating LAB populations of a given type of sourdough are quite stable at species level
53 (Meroth et al., 2003). Regarding the Italian sourdoughs, basically included in Type I sourdough
54 produced with traditional techniques and characterized by continuous, daily refreshments and
55 fermentation at ambient temperature (De Vuyst and Neysen, 2005), a few species are often found to
56 dominate the lactic acid microbiota. The species most frequently found at the highest levels in
57 Italian sourdoughs is undoubtedly *Lactobacillus sanfranciscensis* (Picozzi et al., 2010; Siragusa et
58 al., 2009), but other obligately heterofermentative LAB, such as *Leuconostoc* and *Weissella* species,
59 are often found to dominate this ecosystem (Coppola et al., 1996). However, during refreshments,
60 the addition of flours stored in different conditions and obtained from wheat crops grown in
61 different environmental conditions, cropping systems and genotypes might determine a variation of
62 the microbial composition of sourdoughs over time (De Vuyst et al., 2014). Alfonzo et al. (2013)
63 reported that flours and semolinas used to produce sourdough breads in a restricted area in Sicily

64 (southern Italy) were characterised by different strains of the same species. Thus, a certain
65 succession of the dominant strains during sourdough propagation for long times cannot be excluded.
66 The variability of the dominant strains is reflected in a limited reproducibility of the final
67 characteristics of a given bread typology. When a starter culture is added to a sourdough ecosystem,
68 it is important to ensure its stability over time, in order to warrant a certain reproducibility of the
69 characteristics of the resulting bread. For this reason, a new method for the preparation of the starter
70 culture for sourdough production has been developed in this study. The innovative method is based
71 on the daily addition of a direct liquid inoculum to semolina. The methodology was validated with
72 several semolinas from old and modern genotypes of durum wheat cultivated in Sicily.
73

74 **2. Materials and methods**

75 *2.1. Starter strains*

76 In this study, three obligately heterofermentative LAB strains (*Lactobacillus sanfranciscensis*
77 PON100336, *Leuconostoc citreum* PON10079 and *Weissella cibaria* PON10030) were used as a
78 multi-species starter culture for sourdough production. The strains, belonging to the culture
79 collection of the Department of Agricultural and Forest Sciences – University of Palermo (Italy),
80 were previously isolated from wheat semolinas produced from durum wheats cultivated in Sicily
81 (southern Italy) (Alfonzo et al., 2013) and selected for their potential during the production of
82 experimental sourdough breads (Settanni et al., 2013). Recently, the performances of *Ln. citreum*
83 and *W. cibaria* have been evaluated under industrial conditions without obligate heterofermentative
84 *Lactobacillus* species, evidencing their specific abilities to carry out the sourdough fermentation
85 (Corona et al., in press). The strains *Ln. citreum* PON10079 and *W. cibaria* PON10030 were
86 propagated overnight at 30°C in modified-de Man-Rogosa-Sharpe (mMRS) broth, prepared from
87 MRS (Oxoid, Milan, Italy) added with maltose and fresh yeast extract at the final concentration of
88 1% and 10%, respectively, and adjusted to pH 5.6 with 5M lactic acid, while *Lb. sanfranciscensis*

PON100336 was propagated overnight at 30°C in Sour Dough Bacteria (SDB) broth prepared as described by Kline and Sugihara (1971).

2.2. Preparation and propagation of the liquid inoculum

Each broth culture for the preparation of the multi-species strain starter was grown overnight in the optimal conditions, centrifuged at 5000 × g for 5 min and washed twice in Ringer's solution (Sigma-Aldrich, Milan, Italy) before re-suspending to the value 1.00 of optical density at 600 nm using the 6400 Spectrophotometer (Jenway Ltd., Felsted, Dunmow, UK). The cell suspensions of each LAB strain included in this study contained approximately 10^9 CFU/mL, as previously evaluated by plate count on agar mMRS and SDB by Settanni et al. (2013).

The multi-species starter was prepared in form of liquid culture. To this purpose, the sterile semolina extract (SSE) broth was prepared with a modification of the method reported by Alfonzo et al. (2013). Briefly, 200 g of commercial semolina (Mulini Gaspare Salvia, Partinico, Italy) were suspended in 1 L distilled H_2O, sterilised by autoclaving at 121°C for 20 min and, after precipitation and removal of the solid components of semolina, adjusted to pH 5.6 with filtered lactic acid (0.5 M). The washed cells from the three strains were transferred together in 10 mL SSE at the final concentration of 10^6 CFU/mL to prepare the first inoculum. After development at 30°C for 24 h, the mixed broth culture was used to prepare a second inoculum (1 % v/v) in SSE which was left to ferment for 24 h. Following this procedure, the propagation of the broth culture occurred for other 13 d for a total of 15 consecutive inocula. At each step of preparation and propagation, the liquid inoculum was subjected to the measurement of pH, determined electrometrically with the Lab pH Meter Model pH 50 (XS Instruments, Carpi, Italy), and to the plate counts carried out, after decimal dilutions in Ringer's solution, on mMRS and SDB agar (1.5%, w/v) incubated at 30°C for 48 h.

The isolates from the highest dilutions of the fermented liquid broths were randomly picked up (3 – 4 per morphology) and subjected to DNA extraction performed with the InstaGene Matrix kit (Bio-

115 Rad, Hercules, CA, USA), following the manufacturer's instructions. The DNAs were analysed by
116 the randomly amplified polymorphic DNA (RAPD)-PCR technique with the primer M13 as
117 described by Settanni et al. (2012). The PCR products were separated on 1.5% (w/v) agarose gels
118 (Gibco BRL, Cergy Pontoise, France) stained with the SYBR® safe DNA gel stain (Molecular
119 probes) and acquired under UV trans-illumination. The comparison of the RAPD patterns resulting
120 from the DNA of the isolates with those of the starter strains was performed with the GelCompar II
121 software version 6.5 (Applied-Maths, Saint-Marten-Latem, Belgium).
122 In order to unequivocally confirm the species of the dominating strains the isolates were processed
123 by the species-specific PCRs for *Lb. sanfranciscensis* (Settanni et al., 2005), *Ln. mesenteroides*
124 (Lee et al., 2000) and *W. cibaria* (Fusco et al., 2011).
125
126 *2.3. Sourdough production*
127 In order to evaluate the efficacy of the direct liquid inoculum addition to different semolinas to
128 produce sourdough breads (Fig. 1), several genotypes were individually tested. The study included
129 the semolina from five modern and ten old genotypes (Table 1) cultivated in adjacent experimental
130 fields (6 m × 50 m) at the farm "Pietranera" (37°30' N, 13°31' E; 178 m elevation), located in Santo
131 Stefano di Quisquina (AG, Italy). The commercial semolina used to evaluate the stability of the
132 liquid inoculum (paragraph 2.2) was used as control. The semolina were used to prepare the SSEs,
133 which were inoculated with the three starter strains at 10^6 CFU/mL as reported above. At the fifth
134 step of propagation in SSE, the 16 liquid inocula were used for the direct addition to semolina. The
135 inocula were also analysed by plate count and the colonies subjected to RAPD profile comparison
136 and species-specific PCRs as reported above.
137 The doughs (300 g) were prepared with a dough yield (weight of the dough/weight of the semolina
138 × 100) of 160 in 1 L sterile glass beckers under a flow laminar hood. Each semolina (187.5 g) was
139 inoculated with the microbial suspension (112.5 mL) from the corresponding fermented SSE diluted
140 in sterile tap H_2O to an approximately 10^6 CFU/g of starter culture inoculum and manually mixed

141 by means of a sterile spoon. Soon after mixing, each dough was divided in two aliquots; 200 g were
142 left in becker and covered with parafilm and the remaining 100 g were transferred to a metal pan
143 (see paragraph 2.6 for the characteristics) covered with aluminium foil. The doughs were incubated
144 at 30°C for 8 h. The trials were carried out in duplicate and repeated after two weeks.
145
146 *2.4. Analysis of sourdoughs*
147 The acidification of the sourdoughs (10 g) kept in beckers was followed by pH measurement,
148 performed on 10 g of sample, collected aseptically, by direct immersion of the pH-meter probe, and
149 total titratable acidity (TTA), determined by titratation with 0.1 N NaOH. TTA was evaluated on
150 the same 10 g of doughs used for pH determination which were homogenised in distilled H_2O (90
151 mL) by stomacher (BL Smart, Astori Tecnica, Poncarale, Italy) for 2 min at the highest speed. The
152 samples were analysed at T_0 and at 2 h-interval until 8 h and the results of TTA were expressed in
153 terms of mL of NaOH/10 g of sourdough.
154 The concentrations of lactic and acetic acid and, consequently the fermentation quotient (FQ, lactic
155 acid/acetic acid molar ratio), of each sourdough were determined at T_0 and at the end of
156 fermentation. The chemical determinations were carried out by high performances liquid
157 chromatography as described by Alfonzo et al. (2013) on sourdough samples (10 g) homogenised
158 with distilled H_2O (90 mL) with a Sorvall Omni-Mixer (Dupont Instruments, Newtown, CT) and
159 treated as reported by Ventimiglia et al. (2015). Data were acquired and processed with the
160 PerkinElmer software specific to the HPLC instrument (TotalChrom Workstation 2008 rev. 6.3.2).
161 The 16 sourdoughs were also microbiologically investigated by plate count just after mixing of
162 semolina with the bacterial inoculum (T_0) and after 8 h of fermentation. Ten grams of each sample
163 were suspended in 90 mL of Ringer's solution, homogenised by stomacher as reported above and
164 serially diluted. Total mesophilic microorganisms (TMM) were inoculated on plate count agar
165 (PCA) (Oxoid), incubated aerobically at 30°C for 72 h. LAB were plated on SBD agar and mMRS
166 agar, incubated aerobically and anaerobically, respectively, at 30°C for 48 h. Yeasts were plated on

167 yeast potato dextrose (YPD) agar, incubated aerobically at 25°C for 72 h. Semolina samples (10 g)
168 were also microbiologically analysed as reported for the sourdoughs.
169 The dominating LAB were also identified at strain and species level as reported above.
170
171 *2.5. Bread making and analysis*
172 After preparation, 100 g of each dough were transferred to rectangular stainless steel baking pans of
173 the dimensions (143 × 79 mm, top inside; 129 × 64 mm, bottom outside; depth inside 57 mm)
174 indicated by the Method 10-10B of the American Association of Cereal Chemists (AACC, 2000)
175 and fermented for 8 h at 30°C. The resulting sourdoughs were baked in the industrial oven Air-o-
176 steam (Electrolux, Pordenone, Italy) applying the following 3-step baking program: 1 min at 190°C;
177 8 min at 180°C with 70% relative humidity (RH); 10 min at 185°C with 20% RH.
178 The breads were cooled at ambient temperature before analysis. The breads were first weighted to
179 determine the weight loss and then cut transversely in two halves long about 6 cm. The height of the
180 breads was measured on the central slices (Schober et al., 2005). The Chroma Meter CR-400
181 (Minolta, Osaka, Japan) was used for colour determination by the Hunter's scale parameters (L*, a*
182 and b*) measured on crust (four points) and crumb of the central slices (three points). The hardness
183 of crumb was determined by means of the Instron-5564 (Instrom Corp., Canton, MA) following the
184 method reported by Corsetti et al. (2000).
185 The two central slices of each loaf were subjected to the image analysis to calculate void fraction
186 (the fraction of the total area corresponding to the bread pores), cell density (number of cells/cm^2)
187 and mean cell area (in mm^2). The images were scanned (Epson Perfection 4180 Photo, Seiko Epson
188 Corp., Japan) with 350 dpi of resolution, saved in TIFF format, analysed with the ImageJ software
189 (National Institutes Health, Bethesda, Md, USA), cropped to a square of 207 × 207 pixels
190 (representing 15×15 mm of the slice) and converted to grey-level image (8 bit). The Otsu's
191 threshold algorithm was applied to obtain binary images.

192 The solid phase micro-extraction (SPME) isolation technique was applied to determine the volatile
193 organic compounds (VOCs) emitted by the breads. Each sample of bread (5 g) was heated to 60°C
194 in a vial to generate a headspace that was collected for 40 min through the DBVCarboxen-PDMS
195 fibers (Supelco, Bellefonte, PA). The Finnegan TraceMS for GC/MS (Agilent 6890 Series GC
196 system, Agilent 5973 NetWorkMass Selective Detector, Milan, Italy) equipped with a DB-WAX
197 capillary column (Agilent Technologies, 30m, 0.250mm i.d., film thickness 0.25 μm, part n° 122-
198 7032) was used to analyse the SPME fibre as reported by Alfonzo et al. (2013). The methodology
199 described by Settanni et al. (2013) was applied for the identification of the compounds. All solvents
200 and reagents were purchased from WWR International (Milan, Italy).
201 All determinations on breads were performed in triplicate.
202
203 *2.6. Sensory analysis*
204 The final breads were subjected to the sensory analysis performed by a descriptive panel consisting
205 of 11 tasters (six women and five men; age, 26-60 years old) familiar with the sensory analysis of
206 foods, but not specifically trained in the evaluation of sourdough breads. The panel was trained for
207 descriptive analysis according to the guidelines in the ISO 8586 to evaluate 23 descriptors chosen
208 among those reported by Comendador et al. (2012) and evaluated by other authors (Martins et al.,
209 2015; Rodrigues et al., 2014), including crust colour, crust thickness, crumb colour, porosity,
210 alveolation, alveolation uniformity, odor intensity, bread odor, yeast odor, sourdough odor,
211 unpleasant odor, aroma intensity, bread aroma, yeast aroma, sourdough aroma, unpleasant aroma,
212 salty, acid, bitter, taste persistency, adhesiveness in mouth, crispness and the overall assessment.
213 The analysis was carried out according to the guidelines in the ISO 6658. The taster expressed the
214 intensity of each attribute with a mark on a 6-point hedonic scale (5 = extremely high; 0 =
215 extremely low).
216
217 *2.7. Statistical and explorative multivariate analyses*

218 Chemical, physical and microbiological data were analysed with the ANOVA linear model
219 according to a repeated measure design (GLM procedure of SAS 9.1.2 software, 2004) which
220 included the effects of the semolina as repeated measure. Comparison among LS means were
221 performed by Tukey's test; differences were considered significant at $P<0.05$.
222 In order to represent graphically the values of VOCs, a heat map clustered analysis, based on double
223 hierarchical dendrogram with heat map plot, was performed. The relative values of VOCs were
224 depicted by colour intensity from yellow (lowest concentration) to red (highest concentration). Heat
225 map analysis of the volatile levels was performed using the auto-scaled data. Graphic construction
226 were achieved by using XLStat software version 2014.5.03. (Addinsoft, New York, USA) for excel.
227 To better investigate the relationship among data obtained from the breads produced with the
228 different semolina, an explorative multivariate analysis was carried out. A hierarchical cluster
229 analysis (HCA) (joining, tree clustering) was carried out for grouping the trials according to their
230 similarity, measured by Euclidean distances, whereas cluster aggregation was based on the single
231 linkage method (Todeschini, 1998). The different productions were grouped by principal
232 component analysis (PCA). The input matrix used for HCA and PCA consisted of the values of the
233 measurements (23 variables) performed on sourdoughs and breads, except the data on sensory
234 evaluation. The number of principal factors was selected according to the Kaiser criterion (Jolliffe,
235 1986) and only factors with eigen-values higher than 1.00 were retained. All data were preliminary
236 evaluated by using the Barlett's sphericity test (Dillon and Goldstein, 1984; Mazzei et al., 2010) in
237 order to check the statistically significant difference among samples within each data set. XLStat
238 software version 2014.5.03 (Addinsoft, New York, USA) for excel was used for data processing
239 and graphic construction.
240

241 **RESULTS**

242 *3.1. Stability of the liquid inoculum*

243 The results of the microbiological monitoring of the liquid inoculum from the preparation (day 1)
244 until the 14th day of propagation (days 2 – 15) showed that SSE allowed a development of LAB at
245 levels above 10^8 CFU/mL (Fig. 2). The cell densities registered on mMRS and SDB were not
246 statistically different, indicating that *Lb. sanfranciscensis* PON100336 was able to grow in SSE at
247 levels similar to those reached by the other starter strains.
248 The pH kinetics during the preparation of the multi-species starter culture in SSE was followed
249 frequently until 24 h and it was also compared to the pH drop showed by the three individual strains
250 inoculated in SSE (Fig. 3). *Lb. sanfranciscensis* PON100336 was acidified SSE slower than *Ln.*
251 *citreum* PON10079 and *W. cibaria* PON10030 during the first 6 h of observation. However, all
252 strains acidified the SSEs until pH 3.8 at 24 h. The pH drop of the multi-species culture was slightly
253 more rapid than the single strains *Ln. citreum* PON10079 and *W. cibaria* PON10030 and was
254 registered at 3.7 at 24 h. The pH of the mixed culture in SSE monitored during the successive
255 consecutive 14 days of propagation (Fig. 4) was quite constant ranging between 3.5 – 3.7.
256 The direct comparison of the polymorphic profiles of the isolates collected from the agar plates
257 (Fig. 5) and the species-specific PCRs confirmed the presence of all three added strains in the range
258 10^7 – 10^8 CFU/mL after the daily SSE fermentation. These results showed that the inoculum
259 prepared and propagated daily in SSE was stable.
260
261 *3.2. Chemical and microbiological characterisation of sourdoughs*
262 Once the stability of the inoculum in SSE over time was ascertained with the commercial semolina,
263 the liquid inocula were prepared in SSEs obtained from each semolina included in this study. The
264 liquid inocula were then propagated for five days and added directly to the semolina to produce the
265 doughs. The pH kinetics of these doughs during the 8 h of fermentation showed a similar trend (Fig.
266 6A). All initial pHs were in the range 5.9 – 6.1. The most consistent pH drop was registered at 4 h.
267 At this time, the doughs of the control trial showed a pH value of 4.8, while all the other doughs
268 showed higher pH values; in particular, the pHs of the doughs made with the five modern genotype

semolinas were 5.1 – 5.2, whereas those of the majority of the doughs made with the semolina from the old genotypes were above 5.3. However, the final pH value reached by all doughs at 8 h ranged between 4.2 – 4.4.

TTA (Fig. 6B) was correlated linearly with pH, since a decrease of pH corresponded to an increase of the mL of NaOH/10 g of dough. In fact, a significant increase for all trials was registered at 4 h, but the highest increase was not shown by CT, as expected on the basis of the pH value. After 8 h of fermentation, the highest TTA values were registered for the doughs processed with the semolina of two old genotypes (T9 and T10) and two modern genotypes (T11 and T12). The analyses showed that the doughs produced in this study were characterised by pH and TTA evolution typical of traditional sourdoughs.

All doughs contained an initial level of lactic acid of 0.01 – 0.02 mg/g, while acetic acid was not revealed. At the end of fermentation, the levels of both acids increased in all sourdoughs (Table 2). In particular, the concentration of lactic acid was between 3.60 and 5.96 mg/g, while that of acetic acid was around 1.00 mg/g in all sourdoughs. The resulting FQs were in the range 2.20 – 3.64.

The microbiological characterisation of the sourdoughs is reported in Table 3. TMM were generally detected at lower levels than LAB. The direct comparison with the microbial loads of the 16 semolina (PCA = <2 – 4.6 Log CFU/g; mMRS = <1 – 3.85 Log CFU/g; SDB = <2 – 3.45 Log CFU/g; YPD = <2 – 4.4 Log CFU/g) indicated that the TMM loads registered at T_0 were due to the LAB added through the liquid inoculum. Just after mixing of semolina with the microbial suspension, all doughs were characterised by LAB inocula at levels ranging between 5.66 and 7.21 Log CFU/g on mMRS and 5.68 and 7.78 Log CFU/g on SDB. After 8 h of fermentation, LAB increased at high numbers until levels around 10^9 CFU/g for the majority of the sourdoughs. In general, higher levels of LAB were registered on SDB rather than mMRS, reaching cell densities of 10.13 and 10.16 Log CFU/g in the sourdoughs T12 and T13, respectively. The lowest LAB levels (7.78 Log CFU/g) were displayed by the sourdough T5 on mMRS. Yeasts were below the detection limit in ten doughs at T_0, but their levels increased consistently at 8 h in nine sourdoughs, including

295 all those made with the modern genotype semolina. However, the levels of the yeasts were on
296 average five order of magnitude lower than those of LAB.
297
298 *3.3. Monitoring of the starter strains*
299 The comparison of the RAPD profiles of the colonies developed from the highest dilutions of the
300 cell suspensions to those of the pure cultures (Fig. 7) indicated that, after 8 h of fermentation, *Lb.*
301 *sanfranciscensis* PON100336 dominated the LAB populations of all sourdoughs obtained with the
302 semolina from the modern genotypes and the sourdoughs T2, T5, T7 and T9 made with the old
303 genotypes while a codominance of the three starter strains was found for the other sourdoughs.
304 *Leuconostoc citreum* PON10079 could not be detected on the Petri dishes inoculated with the
305 highest dilutions of the sourdough T5. *Weissella cibaria* PON10030 was often identified from SDB.
306
307 *3.4. Characteristics of the experimental breads*
308 The final breads were evaluated for several quality characteristics. The weight of the breads were
309 almost comparable among the different trials (Table 4). However, the highest weight loss was
310 registered for the breads of the trials T1 and T10. The height of the central slices was between 2.20
311 and 3.59 cm (Table 4). The highest variability in height was registered among the breads produced
312 from the semolina of the old genotypes, while all breads made with the modern genotype semolina
313 showed a similar height. The three colour parameters of the crumb and the parameter L* of the crust
314 were comparable for all breads. For the crust, a few differences were registered for the parameter
315 b*, while the parameter a* clearly distinguished the breads produced with the semolina from the old
316 genotypes from those made with the semolina from the modern genotypes. Regarding firmness
317 (Table 4), the lower the value the softer the breads and CT breads were those with the lowest
318 values. The breads from the old genotypes T4, T5 and T8 showed a softness comparable to those of
319 the breads obtained with the majority of the modern genotype semolina (T13 – T15). Furthermore,

320 the breads with the highest values of firmness were those from the trials characterised by the highest
321 weight loss (T1 and T10).
322 The image analysis showed that all trials were quite different from one another for almost all
323 parameters (void fraction, cell density and mean cell area) evaluated (Table 5).
324 Eighteen VOCs were emitted from the breads produced in this study (Fig. 8). The dendrogram
325 resulting from the cluster analysis and the heat map showed the relationships among the breads
326 based on the amount of each VOC, determining the formation of three main clusters. All breads
327 produced with the semolina from the modern genotypes were included into a single cluster, while
328 the breads made with the semolina from the old genotypes were divided into two distant clusters.
329 Interestingly, the breads made with the commercial semolina clustered with the breads of the trials
330 T2, T4 – T6 and T10. Aldehydes (280.3 – 595.3 μg/kg) constituted the major class of VOCs for all
331 breads, esters (7.3 – 318.6 μg/kg) were particularly represented in the breads of the trials T5 and T8,
332 ketones (31.3 – 123.7 μg/kg) were at relevant concentrations in the breads of the trials T5, T7 – T9,
333 while acids (26.2 – 184.2 μg/kg) in those of trials T9, T13, T15 and CT. Phenol was detected at
334 very low levels in almost all breads, except those made with the old genotypes T1, T7 – T9. Within
335 the major classes, the main compounds found for all breads were nonanal for the aldehydes, acetic
336 acid for the acids and geranylacetone for ketones. 2-Penthylfuran was the only compound detected
337 for the esters. The other classes identified were terpenes, with a single compound (D-limonene),
338 hydrocarbons and alcohols.
339
340 *3.5. Sensory evaluation*
341 The results of the sensory tests on the breads are reported in Table 6. The highest scores for crust
342 colour were registered for the trials T6 and T12, while the trial T9 showed reached the highest
343 evaluation for the colour of crumb. The thickness of the crust resulted generally low. The porosity,
344 the alveolation and the alveolation uniformity were variable among the breads. The highest value of
345 crust elasticity was registered for the trial T11, while the lowest for the trial T5. The odour intensity,

346 the bread odour, the sourdough odour, the aroma intensity, the bread aroma and sourdough aroma
347 were quite high for the majority of the breads. On the contrary, yeast and unpleasant odour, as well
348 as yeast and unpleasant aroma were scored at very low level, often zero. All breads resulted slightly
349 salted, sour and bitter. The taste persistency was almost at the same level for the trials CT, T6 and
350 T11. Only the bread of trial T1 resulted similar to that of the control trial for the crispness. The
351 bread of the control trial was characterised by the lowest gummy crumb. Finally, the highest score
352 for the overall assessment was displayed by the breads of the trial T6, followed by the breads of the
353 control trial. Except for yeast odour and aroma and unpleasant odour and aroma, which were not
354 significantly different among judges and breads, all other sensory attributes were scored different.
355 The less notable differences were evidenced for crust thickness, salty, acid and bitter taste among
356 judges.
357

358 *3.6. Multivariate analysis*

359 HCA classified the trials in accordance to their mutual dissimilarity and relationship (Fig. 9). This
360 analysis basically generated two main mega-clusters. It is worth of note that all sourdough and
361 bread productions performed with the semolina from the modern genotypes were gathered together
362 with the control trial and this mega-cluster also included one trial (T10) carried out with the old
363 genotype Senatore Cappelli. Interestingly, seven of the 10 trials performed with the semolina from
364 the old genotypes were included in the other mega-clusters.
365 In order to condense the information into a reduced number of Factors, data were then subjected to
366 PCA. The Barlett's sphericity test was applied to all data matrix inputs (microbiological, chemical,
367 physical and quality parameters of sourdoughs and breads) and differences statistically ($p < 0.0001$)
368 significant were found among trials. The results of the PCA (Fig. 10) showed that only three eigen-
369 values were higher than 1, which accounted for 28.41 e 16.54 % of variability. This indicated that
370 the initial 23 variables might be expressed as linear combination of only two Factors explaining
371 44.95% of total variance. The components of the PCA were correlated to variables as shown in the

372 Fig. 10a. The discrimination of the trials can be visualized in the plot of scores (Fig. 10b). In detail,
373 the last figure shows the projection of the cases onto the planes as function of the Factors 1 and 2.
374 The trials were significantly separated along Factor 1 on the basis of flavour profile, pH, firmness
375 and void fraction. On the other hand, the variables associated to the Factor 2 significantly
376 contributed to discriminate the trials obtained with the semolina from the modern genotypes (T11-
377 T15 and TC) from those obtained from the old genotypes (T1-T10).
378

379 **DISCUSSION**

380 This work was carried out to answer a specific request of bread makers concerning the stabilization
381 of the sourdough inoculum to avoid differences among bread productions. To this purpose, in the
382 first part of the present work, the preparation of a liquid inoculum was performed and its stability
383 over time was evaluated. The medium used for the development of the starter culture was the SSE
384 which has the characteristics of being simple and fast to prepare and cost-effective. The pH was
385 adjusted to 5.6 to favour the growth of sourdough LAB. This because the pH of wheat flour, as well
386 as semolina, is close to the maximum pH value for the growth of *Lb. sanfranciscensis* (Gänzle et
387 al., 1998) which is considered the key sourdough LAB (Lhomme et al., 2015; Messens and De
388 Vuyst, 2002), particularly for Italian productions (Gobbetti and Corsetti, 1997). The starter culture
389 applied in this study was composed by a multi-species combination including three obligately
390 heterofermentative strains. Besides *Lb. sanfranciscensis*, *Ln. citreum* and *W. cibaria* were also
391 chosen. These species, found to be dominant among the LAB community associated to the semolina
392 used in Sicily (Alfonzo et al., 2013), are commonly present in the sourdough ecosystems produced
393 in different countries (Choi et al., 2012; Coppola et al., 1996; De Vuyst et al., 2002; Iacumin et al.,
394 2009; Robert et al., 2006, 2009; Valmorri et al., 2006) and might also contribute to the structure of
395 the final dough by exopolysaccharides (Choi et al., 2012; Di Cagno et al., 2006; Galle et al., 2010;
396 Maina et al., 2008). Specifically, the strains *Lb. sanfranciscensis* PON100336, *Ln. citreum*
397 PON10079 and *W. cibaria* PON10030 were included in the starter culture because they showed

398 interesting results when tested individually during experimental sourdough productions (Settanni et
399 al., 2013) and stability at industrial level production (Corona et al., in press).
400 Due to the importance of the obligately heterofermentative species for sourdough fermentation,
401 facultative heterofermentative species and least of all obligately homofermentative LAB were not
402 used in this study. First of all, they can increase the FQ due to the production of high amount of
403 lactic acid and do not contribute to the production of CO_2 and, secondly, they are provided by the
404 Sicilian durum wheat semolina (Alfonzo et al. 2013). For example, the species *Lb. plantarum* has
405 been recently reported to codominate with the heterofermentative species during the production of
406 sourdough in different areas of western Sicily (Ventimiglia et al., 2015). Thus, it was not added in
407 this study to promote the stabilization of a specific consortium of the solely obligately
408 heterofermentative species.
409 The simple and time-saving method for the direct inoculation of the selected LAB to the semolina
410 developed in this work was based on the daily propagation of the mixed strain culture in SSE. At
411 the beginning of the process, *Lb. sanfranciscensis* was found to be the slowest acidifier. This result
412 was not surprising and it could be due to the stress imposed by the preparation of the inoculum. In
413 fact, the cells were washed and the pH was not too acidic. Corsetti et al. (2007) registered a similar
414 behaviour for *Lb. sanfranciscensis* during *in situ* tests with semolina. However, the pH and LAB
415 counts, registered on average levels of 3.6 and 10^8 CFU/mL, respectively, and the genetic
416 monitoring of the starter strains showed that the liquid inoculum propagated daily in SSE made with
417 the commercial semolina was stable. Thus, in order to validate the methodology independently on
418 the semolina used, the liquid inocula were prepared and propagated in SSE produced from different
419 semolinas. To this purpose, 15 semolinas produced from old and modern durum wheat genotypes
420 were tested with this innovative technique for the sourdough starter preparation and addition.
421 Specifically, the three strains were inoculated in SSEs produced from the 15 genotypes to prepare
422 the liquid inocula which were then propagated (by inoculation of the previous day liquid inoculum

423 from each SSE) for five consecutive days before direct addition to unsterile semolina and left to
424 ferment for 8 h.
425 The breeding programs on wheat grains determined the gradual replacement of old varieties or
426 landraces, and the establishment of new, more responsive to new agronomic principles of intensive
427 farming and the modern food technology, which required genotypes with high rates of gluten to be
428 devoted to pasta (Palumbo et al., 2000). The recent revaluation of old durum wheat genotypes, used
429 to diversify agricultural production and to expand the portfolio of products available to consumers,
430 made necessary to deepen the study on their semolina and dough for bread making. Semolinas used
431 in this study were obtained from wheat genotypes chosen according to a temporal gradient, from the
432 early 1900s (Timilia) to 2004 (Saragolla).
433 The values of pH and TTA registered after fermentation were comparable to those commonly found
434 in mature durum wheat sourdoughs (Minervini et al., 2012a; Pepe et al., 2013; Rizzello et al., 2015;
435 Ventimiglia et al., 2015). Thus, regarding the acidification of the doughs, the liquid starter culture
436 behaved like a mature sourdough inoculum. Furthermore, the chemical analyses showed levels of
437 lactic and acetic acid comparable to those reported by Ventimiglia et al. (2015) for the sourdoughs
438 produced in Sicily. The resulting FQ of all doughs reached at the end of the fermentation period was
439 in the range considered to affect positively the aroma profile and the structure of sourdough breads
440 (Spicher, 1983). Some of the sourdoughs produced in this study reached even a FQ included in the
441 optimal range of 2.0 – 2.7 indicated by Hammes and Gänzle (1998), evidencing clearly that the
442 innovative method stabilized the ratio between lactic and acetic acid for the sourdoughs produced
443 with different semolinas.
444 The microbiological analysis of the semolinas showed that the raw materials hosted different levels
445 of the microorganisms investigated, mainly LAB and yeasts. However, after the fermentation with
446 the starter inocula, the microbiological counts increased consistently in almost all sourdoughs.
447 Independently on the microbiological characteristics of the semolina, the addition of the multi-
448 species culture in form of liquid inoculum prepared in SSE determined the development of high

449 numbers of LAB until the levels commonly found in mature sourdoughs produced in Sicily
450 (Minervini et al., 2012a; Ventimiglia et al., 2015), confirming that the method developed in this
451 study is effective for the development of sourdoughs. However, the recognition of the added strains
452 was necessary to state that the cultures added with the liquid inocula dominated the fermentation
453 process over the indigenous LAB hosted in semolina. RAPD profile comparison indicated a quite
454 stable codominance of the three starter strains in the majority of sourdoughs. The strain monitoring
455 also showed a certain aptitude of *W. cibaria* to grow well on SDB.
456 The analyses of the breads evidenced a certain capacity of the starter LAB culture used in this study
457 to act as a leavening agent. This phenomenon is basically due to the CO_2 produced through 6-
458 phosphogluconate/phosphoketolase pathway (heterolactic fermentation) (Axelsson, 1998). Some
459 differences were registered among the breads for the hardness, weight loss, colour of the crust, void
460 fraction, cell density and mean cell area. These results are mainly imputable to the different
461 semolina characteristics. On the other hand, it is well known that plant breeding activities conducted
462 on the species in the last decades has led to a considerable grain yield increase but has consistently
463 modified the semolina quality and particularly the protein content and gluten properties influencing
464 strength, extensibility, and toughness (De Vita et al 2007; Ruisi et al., 2015).
465 The number of VOCs emitted from the breads produced in this study was in agreement with the
466 numbers of VOCs generally reported for sourdough breads (Hansen and Schieberle, 2005; Salim-
467 ur-Rehman et al., 2006). These findings indicated that the liquid inoculum prepared in SSE
468 generated a complexity of compounds in bread comparable to that due to the mature sourdoughs.
469 Since the VOCs have different odour activity (Reiners and Grosch, 1998), their different
470 composition might be defining for the sensory evaluation of the breads. Czerny and Schieberle
471 (2002) stated that LAB influence the flavour compounds already present in flour. However, since in
472 our study we demonstrated that, during fermentation, the starter LAB added with the liquid inocula
473 dominated over the LAB populations present in semolina and that yeasts are at very low levels, we
474 can consider that the differences in VOCs among the breads are mainly a consequence of the type

475 of semolina. Among the key odour compounds of bread (Birch et al., 2013), hexanal, 2-nonenal and
476 2-penthylfuran, which derive from enzymatic oxidation and/or autoxidation of the lipid fraction of
477 the wheat (Frankel, 1982; Hann and Morrison, 1975), and benzaldehyde were detected. In
478 particular, hexanal and 2-penthylfuran were found at higher concentrations in the breads from the
479 old genotypes and those from CT. All breads were mainly characterized by aldehydes (hexanal,
480 nonanal, 2-octenal, furfuraldehyde, decanal, 2-nonenal, isophthalaldehyde and benzaldehyde).
481 The microbiological, chemical, physical and quality parameters of sourdoughs and breads were
482 subjected to the multivariate analysis to evaluate the differences/variabilities among the trials. The
483 correlation analysis among the data considered showed that there were many significant
484 relationships among them. HCA generated two main clusters, separating almost sharply the
485 productions made with the semolina from the modern genotypes from those from the old genotypes.
486 PCA confirmed this trend and clearly showed that the trials with the modern genotypes and CT
487 were closed to one another, almost all in the same quadrant, while the trials with the old genotypes
488 were wide spread among the four quadrants. The position of the majority of the trials performed
489 with the semolina from the old genotypes is basically explained by the VOCs, as visible from the
490 loading plot. This indicated that each bread produced from the old genotypes was characterized by
491 unique aromas. These results could also explain why analysing exclusively the results of the VOCs
492 of the breads, CT clustered with the breads obtained with the semolina from the old genotypes, even
493 though a modern genotype represented the highest percentage of the semolina mixture used in this
494 trial.
495 The panel of judges was able to evidence the differences among the sensory attributes of the breads.
496 A higher complexity of odours (odour intensity and sourdough odour) and sourdough aroma were
497 registered for the breads produced with the semolima from the old genotypes (especially T6) and
498 from CT, in agreement with the concentrations of VOCs.
499
500 **CONCLUSIONS**

501 The direct application of the liquid inocula developed with the multi-species strain starter in SSE to
502 several semolina allowed the production of sourdoughs with characteristics commonly reported for
503 mature sourdoughs. The specific conclusions of this work are as follows: the liquid inocula were
504 stabilized for all semolina preparing an *ad hoc* SSE for each durum wheat genotype; the dominance
505 of the added strains was verified; the adaptation of the selected LAB in SSE stimulated their rapid
506 development in dough; the added multi-species strain starter determined high numbers of LAB in
507 the sourdoughs, independently on the microbiological characteristics of semolina. Regarding the
508 characterisation of the products obtained with the different semolinas, the trials with the modern
509 genotypes showed almost comparable results, whereas the trials with the old genotypes were
510 characterized by marked differences. However, the real effectiveness of the innovative method
511 depends on the daily propagation of the liquid inoculum by the bread maker. Furthermore, it has to
512 be considered that the production of sourdoughs at laboratory- or bakery-level might influence the
513 composition of the fermenting microbiota (Minervini et al., 2012b). Thus, works are being prepared
514 to apply the system reported in this study at large scale level in industrial bakeries and transfer this
515 new technological approach for sourdough production to the bread makers.
516
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690

691 **Table 1.** Durum wheat semolina used in this study.

Semolina	Genotype name	Genotype group (year of release)
1	Timilia	old
2	Russello	old
3	Biancuccia	old
4	Realforte rosso	old
5	Tripolino	old
6	Scorsonera	old
7	Perciasacchi	old
8	Aziziah	old
9	Bidì	old
10	Senatore Cappelli	old (1915)
11	Iride	modern (1996)
12	Creso	modern (1974)
13	Vertola	modern (2003)
14	Saragolla	modern (2004)
15	Simeto	modern (1988)
C	Commercial	70% modern genotype; 30 old landraces

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694 **Table 2.** Organic acids of the experimental sourdoughs.

Trials	Lactic acid (mg/g)	Acetic acid (mg/g)	FQ
T1	4.61 ± 0.12^{EF}	1.24 ± 0.15^{A}	2.48
T2	5.39 ± 0.05^{BC}	1.28 ± 0.05^{A}	2.81
T3	4.98 ± 0.08^{DE}	1.31 ± 0.23^{A}	2.53
T4	4.56 ± 0.09^{F}	1.19 ± 0.13^{A}	2.55
T5	3.60 ± 0.21^{G}	1.09 ± 0.06^{A}	2.20
T6	5.07 ± 0.18^{CD}	1.26 ± 0.15^{A}	2.68
T7	5.14 ± 0.14^{CD}	1.14 ± 0.25^{A}	3.01
T8	4.92 ± 0.07^{DEF}	1.14 ± 0.20^{A}	2.88
T9	5.59 ± 0.11^{B}	1.21 ± 0.09^{A}	3.08
T10	5.41 ± 0.04^{BC}	0.99 ± 0.11^{A}	3.64
T11	5.96 ± 0.12^{A}	1.21 ± 0.04^{A}	3.28
T12	5.29 ± 0.14^{BCD}	1.19 ± 0.08^{A}	2.96
T13	6.01 ± 0.07^{A}	1.36 ± 0.16^{A}	2.95
T14	4.99 ± 0.19^{DE}	1.29 ± 0.19^{A}	2.58
T15	5.38 ± 0.07^{BC}	1.02 ± 0.21^{A}	3.52
CT	5.10 ± 0.17^{CD}	1.15 ± 0.10^{A}	2.96
Statistical significance	***	N.S.	n.d.

695 Abbreviations: CT, control trial; FQ, fermentation quotient; n.d., not determined.; N.S., not significant

696 Results indicate mean values ± SD of four measurements (carried out in duplicate for two independent fermentations)

697 Data within a column followed by the same letter are not significantly different according to Tukey's test

698 P value: ***, $P < 0.001$

Table 3. Microbiological loads (Log CFU/g) of the experimental sourdoughs.

Trials	Media							
	PCA		mMRS		SDB		YPD	
	0 h	8 h	0 h	8 h	0 h	8 h	0 h	8 h
T1	5.57 ± 0.27^{BCDE}	8.33 ± 0.65^{A}	6.51 ± 0.27^{AB}	8.85 ± 0.00^{ABC}	6.69 ± 0.14^{BCD}	8.90 ± 0.36^{ABCD}	3.35 ± 0.09^{B}	3.78 ± 0.31^{ABCD}
T2	5.48 ± 0.15^{CDE}	7.56 ± 0.39^{A}	6.59 ± 0.35^{AB}	8.86 ± 0.19^{ABC}	6.97 ± 0.23^{B}	9.50 ± 0.54^{ABC}	4.15 ± 0.11^{A}	4.46 ± 0.23^{A}
T3	5.65 ± 0.18^{BCDE}	8.34 ± 0.45^{A}	6.47 ± 0.22^{AB}	8.98 ± 0.25^{ABC}	6.82 ± 0.26^{BC}	9.04 ± 0.32^{ABCD}	2.40 ± 0.07^{D}	4.22 ± 0.34^{AB}
T4	5.11 ± 0.29^{E}	8.67 ± 0.49^{A}	6.55 ± 0.31^{AB}	9.06 ± 0.10^{ABC}	6.36 ± 0.06^{BCDE}	9.11 ± 0.49^{ABCD}	2.30 ± 0.17^{D}	3.48 ± 0.20^{CD}
T5	6.38 ± 0.21^{BC}	8.39 ± 0.56^{A}	6.39 ± 0.36^{AB}	7.78 ± 0.18^{D}	6.60 ± 0.17^{BCD}	8.59 ± 0.28^{CD}	$< 2.00^{E}$	$< 2.00^{E}$
T6	5.20 ± 0.28^{DE}	7.96 ± 0.35^{A}	6.99 ± 0.42^{A}	8.63 ± 0.34^{BCD}	6.99 ± 0.28^{AB}	8.22 ± 0.17^{D}	$< 2.00^{E}$	$< 2.00^{E}$
T7	5.97 ± 0.44^{BCDE}	8.27 ± 0.41^{A}	7.21 ± 0.61^{A}	8.56 ± 0.42^{BCD}	6.89 ± 0.38^{BC}	9.44 ± 0.67^{ABC}	$< 2.00^{E}$	$< 2.00^{E}$
T8	5.91 ± 0.37^{BCDE}	8.53 ± 0.37^{A}	7.16 ± 0.28^{A}	8.72 ± 0.39^{ABCD}	7.02 ± 0.15^{AB}	8.89 ± 0.37^{BCD}	$< 2.00^{E}$	$< 2.00^{E}$
T9	5.41 ± 0.11^{CDE}	8.00 ± 0.37^{A}	5.82 ± 0.16^{B}	8.83 ± 0.45^{ABC}	5.96 ± 0.06^{DE}	9.25 ± 0.23^{ABCD}	$< 2.00^{E}$	3.89 ± 0.14^{ABC}
T10	5.77 ± 0.24^{BCDE}	8.34 ± 0.41^{A}	5.66 ± 0.33^{B}	9.55 ± 0.54^{AB}	6.67 ± 0.22^{BCD}	9.83 ± 0.31^{AB}	$< 2.00^{E}$	4.33 ± 0.23^{AB}
T11	6.64 ± 0.32^{AB}	8.13 ± 0.14^{A}	6.23 ± 0.17^{AB}	8.99 ± 0.31^{ABC}	5.68 ± 0.13^{E}	9.84 ± 0.41^{AB}	$< 2.00^{E}$	3.18 ± 0.23^{D}
T12	5.27 ± 0.14^{DE}	8.75 ± 0.27^{A}	5.81 ± 0.25^{B}	9.74 ± 0.37^{A}	7.78 ± 0.35^{A}	10.13 ± 0.12^{A}	$< 2.00^{E}$	4.13 ± 0.24^{ABC}
T13	6.20 ± 0.31^{BCD}	8.12 ± 0.46^{A}	6.49 ± 0.46^{AB}	8.72 ± 0.42^{ABCD}	6.51 ± 0.15^{BCD}	10.16 ± 0.30^{A}	$< 2.00^{E}$	4.11 ± 0.12^{ABC}
T14	5.78 ± 0.19^{BCDE}	8.14 ± 0.16^{A}	6.40 ± 0.24^{AB}	8.47 ± 0.30^{CD}	6.69 ± 0.27^{BCD}	9.55 ± 0.42^{ABC}	$< 2.00^{E}$	3.18 ± 0.16^{D}
T15	7.51 ± 0.87^{A}	7.97 ± 0.24^{A}	6.54 ± 0.43^{AB}	8.77 ± 0.24^{ABCD}	6.74 ± 0.54^{BCD}	9.39 ± 0.21^{ABCD}	$< 2.00^{E}$	4.21 ± 0.21^{AB}
CT	5.18 ± 0.47^{DE}	7.71 ± 0.30^{A}	6.48 ± 0.27^{AB}	8.55 ± 0.31^{BCD}	6.12 ± 0.29^{CDE}	9.41 ± 0.50^{ABC}	3.04 ± 0.22^{C}	3.70 ± 0.38^{BCD}
Statistical significance	***	N.S.	***	***	***	***	***	***

Abbreviations: CT, control trial; mMRS, modified-de Man-Rogosa-Sharpe agar for mesophilic rod LAB; SDB, sourdough bacteria agar for typical sourdough LAB; YPD, yeast peptone dextrose

Results indicate mean values± SD of four plate counts (carried out in duplicate for two independent productions)

Data within a column followed by the same letter are not significantly different according to Tukey's test

P value: ***, $P < 0.001$; N.S., not significant

Table 4. Characteristics of the experimental breads.

Trials	Weight loss (g)	Height (cm)	Crumb colour			Crust colour			Firmness
			L*	a*	b*	L*	a*	b*	
T1	15.92 ± 1.45^{AB}	2.61 ± 0.11^{ABCD}	62.08 ± 4.21^{A}	-0.74 ± 0.30^{A}	19.52 ± 1.57^{AB}	60.42 ± 3.88^{A}	5.56 ± 0.27^{ABC}	30.00 ± 3.27^{C}	20.53 ± 0.45^{B}
T2	13.99 ± 1.07^{AB}	2.57 ± 0.10^{BCD}	61.97 ± 4.30^{A}	-0.84 ± 0.29^{A}	20.15 ± 2.15^{AB}	60.90 ± 3.80^{A}	3.89 ± 0.18^{DEF}	29.92 ± 2.57^{C}	18.05 ± 0.61^{CD}
T3	14.21 ± 0.97^{AB}	2.76 ± 0.08^{ABCD}	61.13 ± 3.67^{A}	-0.96 ± 0.32^{A}	20.71 ± 2.40^{AB}	59.96 ± 4.64^{A}	4.96 ± 0.15^{BCD}	31.35 ± 3.41^{C}	14.54 ± 0.44^{F}
T4	14.85 ± 2.27^{A}	3.59 ± 0.90^{A}	61.36 ± 2.99^{A}	-1.13 ± 0.28^{A}	21.22 ± 2.37^{AB}	58.65 ± 4.54^{A}	5.97 ± 0.35^{AB}	32.50 ± 3.01^{C}	8.48 ± 0.24^{I}
T5	14.46 ± 0.84^{AB}	2.35 ± 0.27^{D}	61.56 ± 3.00^{A}	-0.97 ± 0.39^{A}	21.08 ± 1.94^{AB}	58.70 ± 4.84^{A}	6.45 ± 0.44^{A}	32.82 ± 2.80^{C}	6.36 ± 0.22^{J}
T6	13.15 ± 1.25^{AB}	2.85 ± 0.05^{ABCD}	62.21 ± 2.81^{A}	-0.75 ± 0.45^{A}	21.00 ± 1.32^{AB}	60.72 ± 3.95^{A}	6.08 ± 0.27^{A}	33.38 ± 2.12^{C}	14.08 ± 0.47^{F}
T7	15.15 ± 4.27^{B}	2.20 ± 0.09^{D}	62.44 ± 3.00^{A}	-0.48 ± 0.16^{A}	20.99 ± 0.56^{AB}	61.42 ± 2.99^{A}	5.68 ± 0.26^{AB}	32.85 ± 2.58^{C}	16.27 ± 0.41^{E}
T8	13.61 ± 1.57^{AB}	2.45 ± 0.11^{CD}	62.96 ± 3.19^{A}	-0.83 ± 0.60^{A}	19.08 ± 3.41^{AB}	62.29 ± 2.49^{A}	5.91 ± 0.25^{AB}	32.66 ± 4.72^{C}	9.42 ± 0.38^{HI}
T9	14.62 ± 1.07^{AB}	3.30 ± 0.10^{ABC}	62.79 ± 3.34^{A}	-1.19 ± 0.61^{A}	16.96 ± 3.70^{AB}	62.82 ± 2.27^{A}	5.78 ± 0.33^{AB}	32.19 ± 5.00^{C}	17.26 ± 0.21^{DE}
T10	16.34 ± 1.38^{AB}	3.39 ± 0.15^{AB}	61.74 ± 4.92^{A}	-1.52 ± 0.33^{A}	14.55 ± 3.10^{B}	65.08 ± 3.28^{A}	4.55 ± 0.41^{CDE}	45.55 ± 4.80^{AB}	22.16 ± 0.51^{A}
T11	14.69 ± 1.00^{AB}	2.93 ± 0.22^{ABCD}	60.53 ± 4.50^{A}	-1.74 ± 0.51^{A}	17.92 ± 6.19^{AB}	65.25 ± 3.12^{A}	3.27 ± 0.58^{FGH}	45.81 ± 4.80^{AB}	18.71 ± 0.40^{C}
T12	14.85 ± 0.77^{AB}	3.34 ± 0.17^{AB}	60.94 ± 4.27^{A}	-2.01 ± 0.48^{A}	21.84 ± 6.57^{AB}	66.17 ± 3.06^{A}	2.38 ± 0.52^{H}	46.51 ± 4.80^{A}	12.41 ± 0.11^{G}
T13	14.72 ± 0.24^{A}	3.53 ± 0.32^{A}	61.52 ± 2.43^{A}	-2.25 ± 0.29^{A}	25.74 ± 2.17^{A}	65.95 ± 2.51^{A}	2.23 ± 0.47^{H}	33.76 ± 3.27^{C}	6.00 ± 0.17^{J}
T14	14.50 ± 1.73^{AB}	2.80 ± 0.21^{ABCD}	62.89 ± 4.27^{A}	-1.74 ± 1.24^{A}	26.02 ± 1.81^{A}	64.76 ± 3.69^{A}	2.63 ± 0.33^{GH}	34.03 ± 3.00^{C}	9.82 ± 0.21^{H}
T15	14.42 ± 1.58^{AB}	3.04 ± 0.18^{ABCD}	63.30 ± 5.30^{A}	-1.52 ± 1.17^{A}	25.82 ± 1.58^{A}	63.22 ± 4.47^{A}	3.14 ± 0.20^{FGH}	34.09 ± 2.49^{C}	9.82 ± 0.20^{H}
CT	15.31 ± 0.52^{AB}	3.29 ± 0.27^{ABC}	63.56 ± 5.15^{A}	-1.41 ± 1.11^{A}	25.93 ± 1.30^{A}	61.90 ± 3.85^{A}	3.70 ± 0.21^{EFG}	35.15 ± 2.09^{BC}	4.60 ± 0.25^{K}
Statistical significance	**	***	N.S.	N.S.	**	N.S.	***	***	***

Abbreviations: CT, control trial

Results indicate mean values± SD of four determinations (carried out in duplicate for two independent productions)

Data within a column followed by the same letter are not significantly different according to Tukey's test

P value: **, $P < 0.01$; ***, $P < 0.001$; N.S., not significant

721 **Table 5.** Image analysis of the experimental breads.

Trials	Void fraction (%)	Cell density (n/cm^2)	Mean cell area (mm^2)
T1	41.14 ± 0.93^{F}	37.67 ± 0.95^{EFG}	4.95 ± 0.54^{DEF}
T2	49.19 ± 1.30^{BCD}	12.00 ± 0.88^{J}	0.25 ± 0.08^{I}
T3	40.91 ± 1.00^{F}	39.67 ± 0.69^{DE}	6.29 ± 0.82^{BCDE}
T4	30.97 ± 0.85^{HI}	55.67 ± 0.56^{B}	6.50 ± 0.38^{BCD}
T5	42.88 ± 1.04^{EF}	28.67 ± 0.78^{I}	3.49 ± 0.64^{F}
T6	28.04 ± 0.73^{I}	35.00 ± 1.11^{GH}	4.83 ± 0.30^{EF}
T7	55.62 ± 1.20^{A}	12.00 ± 0.88^{J}	1.63 ± 0.62^{HI}
T8	52.47 ± 1.10^{AB}	14.33 ± 0.89^{J}	0.75 ± 0.04^{HI}
T9	47.61 ± 1.02^{CD}	39.00 ± 1.27^{DEF}	1.80 ± 0.13^{GH}
T10	50.93 ± 0.44^{BC}	36.67 ± 1.00^{EFG}	0.21 ± 0.07^{I}
T11	42.27 ± 1.58^{F}	42.00 ± 0.99^{D}	4.01 ± 0.71^{F}
T12	43.92 ± 1.09^{EF}	32.67 ± 1.32^{H}	5.85 ± 0.17^{CDE}
T13	33.85 ± 1.23^{GH}	50.67 ± 1.05^{C}	7.59 ± 0.32^{B}
T14	34.37 ± 1.55^{G}	60.33 ± 1.02^{A}	10.63 ± 0.55^{A}
T15	45.89 ± 0.67^{DE}	36.33 ± 1.04^{FG}	3.28 ± 0.56^{FG}
CT	40.99 ± 1.21^{F}	53.67 ± 0.89^{BC}	6.95 ± 0.72^{BC}
Statistical significance	***	***	***

722 Abbreviations: CT, control trial

723 Results indicate mean values± SD of four determinations (carried out in duplicate for two independent productions)

724 Data within a column followed by the same letter are not significantly different according to Tukey's test

725 P value: ***, $P < 0.001$

Table 6. Sensory characteristics of the experimental breads.

Attributes	Experimental trials																SEM	Statistical significance	
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	CT		Judges	Bread
Crust colour	2.04^{C}	2.04^{C}	2.61^{B}	1.38^{E}	1.53D^{E}	3.24^{A}	1.93^{C}	1.22^{E}	1.82C^{D}	2.07^{C}	1.45D^{E}	3.12^{A}	0.62^{F}	1.27^{E}	2.10^{C}	2.61^{B}	0.18	**	***
Crumb colour	1.25^{IJ}	1.68^{FGH}	1.81^{EFG}	0.78^{K}	2.25^{BCD}	2.14^{CDE}	1.99^{DEF}	2.44^{BC}	3.24^{A}	1.76^{FG}	2.51^{B}	1.66^{FGH}	0.92^{JK}	2.26^{BCD}	1.42^{HI}	1.59^{GHI}	0.16	**	***
Crust thickness	0.45^{CDE}	0.36^{DE}	0.58^{ABCDE}	0.45^{CDE}	0.32^{E}	0.63^{ABC}	0.47^{CDE}	0.59^{ABCD}	0.48^{BCDE}	0.50^{BCDE}	0.36^{DE}	0.69^{ABC}	0.49^{BCDE}	0.36^{DE}	0.74^{AB}	0.80^{A}	0.04	*	***
Porosity	1.17^{HI}	1.41^{GH}	2.01^{DE}	1.57^{FG}	0.75^{J}	2.94^{A}	1.83^{EF}	0.97^{IJ}	2.52^{BC}	2.78^{AB}	2.78^{AB}	2.84^{AB}	2.89^{A}	1.02^{IJ}	2.36^{CD}	2.23^{CD}	0.19	**	***
Alveolation	1.21^{DE}	1.05^{EFG}	0.80^{FGH}	0.76^{FGHI}	0.89^{EFGH}	1.82^{BC}	0.44^{I}	0.74^{GHI}	2.68^{A}	1.88^{B}	1.77^{BC}	1.24^{DE}	2.52^{A}	0.59^{HI}	1.46^{CD}	1.09^{EF}	0.17	**	***
Alveolation uniformity	1.62^{EF}	1.77^{DE}	2.16^{BC}	1.64^{EF}	1.63^{EF}	2.66^{A}	2.03^{CD}	2.15^{BC}	1.36^{FG}	1.75^{DE}	1.99^{CD}	2.44^{AB}	1.87^{CDE}	1.17^{G}	1.81^{DE}	2.72^{A}	0.11	**	***
Crust elasticity	1.82^{DEF}	1.59^{EFG}	1.79^{EF}	1.43^{G}	0.90^{H}	2.66^{BC}	1.88^{DE}	0.83^{H}	1.74^{EF}	2.81^{B}	3.22^{A}	2.50^{C}	2.61^{BC}	1.00^{H}	2.15^{D}	1.55^{FG}	0.18	**	***
Odor intensity	1.46^{K}	2.63^{GH}	2.24^{IJ}	2.82^{EFG}	2.94^{EF}	4.48^{A}	2.41^{HI}	2.20^{IJ}	2.69^{FGH}	2.75^{EFG}	3.27^{CD}	3.03^{DE}	3.47^{C}	2.10^{J}	3.55^{C}	4.05^{B}	0.19	***	***
Bread odor	1.65^{G}	3.02^{BC}	2.44^{EF}	2.93^{CD}	2.39^{EF}	3.68^{A}	2.64^{DE}	2.20^{F}	2.19^{F}	2.69^{CDE}	2.88^{CD}	3.29^{B}	3.89^{A}	1.83^{G}	2.90^{CD}	3.98^{A}	0.17	**	***
Yeast odor	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.05^{A}	0.10^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.05^{A}	0.00^{A}	0.01^{A}	0.00^{A}	0.00^{A}	0.05^{A}	0.01	N.S.	N.S.
Sourdough odor	1.49^{K}	2.78^{EFG}	2.98^{DE}	2.55^{FGH}	1.81^{JK}	4.49^{A}	2.70^{EFGH}	2.33^{HI}	2.96^{DEF}	3.20^{CD}	2.01^{IJ}	3.22^{CD}	3.47^{C}	2.38^{GHI}	3.26^{CD}	3.89^{B}	0.19	***	***
Unpleasant odor	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00	N.S.	N.S.
Aroma intensity	1.68^{GHI}	1.44^{I}	2.44^{BC}	1.80^{FGH}	2.01^{DEFG}	2.08^{DEF}	1.65^{HI}	1.33^{I}	2.01^{DEFG}	2.27^{BCD}	1.87^{EFGH}	2.09^{DEF}	2.15^{CDE}	1.67^{HI}	2.49^{B}	3.08^{A}	0.11	**	***
Bread aroma	2.31^{HI}	3.36^{DE}	4.19^{A}	2.85^{FG}	3.09^{EF}	3.72^{BCD}	3.58^{CD}	1.90^{I}	2.70^{GH}	2.85^{FG}	3.14^{EF}	3.85^{ABC}	3.89^{ABC}	3.14^{EF}	4.02^{AB}	3.77^{BC}	0.16	**	***
Yeast aroma	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.10^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.01	N.S.	N.S.
Sourdough aroma	1.62^{DE}	1.34^{E}	1.87^{CD}	2.10^{ABC}	1.94^{BCD}	2.37^{A}	1.42^{E}	1.65^{DE}	1.47^{E}	1.63^{DE}	1.84^{CD}	2.15^{ABC}	2.20^{ABC}	2.12^{ABC}	1.86^{CD}	2.25^{AB}	0.08	*	***
Unpleasant aroma	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00	N.S.	N.S.
Salty	0.39^{BC}	0.76^{A}	0.31^{BC}	0.29^{BC}	0.29^{BC}	0.27^{C}	0.31^{BC}	0.17^{C}	0.23^{C}	0.33^{BC}	0.35^{BC}	0.35^{BC}	0.47^{ABC}	0.39^{BC}	0.47^{ABC}	0.64^{AB}	0.04	*	***
Acid	1.22^{BC}	1.14^{C}	1.14^{C}	1.10^{C}	1.14^{C}	1.13^{C}	1.10^{C}	1.11^{C}	1.23^{ABC}	1.27^{ABC}	1.21^{BC}	1.25^{ABC}	1.54^{AB}	1.17^{C}	1.41^{ABC}	1.56^{A}	0.04	*	***
Bitter	0.23^{AB}	0.14^{AB}	0.09^{B}	0.10^{B}	0.17^{AB}	0.15^{AB}	0.15^{AB}	0.09^{B}	0.12^{AB}	0.15^{AB}	0.35^{AB}	0.41^{A}	0.19^{AB}	0.14^{AB}	0.31^{AB}	0.25^{AB}	0.02	*	**
Taste persistency	0.60^{FG}	1.07^{CD}	1.09^{BCD}	1.00^{DE}	0.94^{DE}	1.44^{A}	0.87^{DEF}	0.50^{G}	1.16^{ABCD}	0.89^{DEF}	1.33^{ABC}	0.93^{DEF}	1.00^{DE}	0.75^{EFG}	1.09^{BCD}	1.38^{AB}	0.06	**	***
Crispness	1.35^{A}	0.56^{C}	0.59^{BC}	0.63^{BC}	0.63^{BC}	0.74^{BC}	0.71^{BC}	0.56^{C}	0.67^{BC}	0.73^{BC}	0.59^{BC}	0.54^{C}	0.48^{C}	0.46^{C}	0.92^{B}	1.30^{A}	0.07	**	***
Gummy crumb	0.68^{DEF}	0.79^{CDE}	0.89^{BCD}	0.53^{DEF}	1.23^{AB}	0.61^{DEF}	1.25^{AB}	1.34^{A}	1.12^{ABC}	0.64^{DEF}	0.72^{DEF}	0.58^{DEF}	0.46^{EF}	1.18^{AB}	0.62^{DEF}	0.38^{F}	0.08	**	***
Overall assessment	1.68^{GH}	2.00^{FG}	2.40^{DE}	2.23^{EF}	1.72^{GH}	3.49^{A}	2.23^{EF}	1.92^{FG}	1.01^{I}	2.21^{EF}	2.78^{CD}	2.88^{BC}	2.53^{CDE}	1.38^{H}	2.49^{DE}	3.20^{AB}	0.16	**	***

Abbreviations: SEM, standard error of means; N.S., not significant, *, P≤0.05; **, P≤0.01; ***, P≤0.001.

Result indicate mean value.

Data within a line followed by the same letter are not significantly different according to Tukey's test.

732 **Legends to Figures**

733 **Fig. 1.** Schematic representation of the innovative method for sourdough production.

734 **Fig. 2.** Microbiological counts of the liquid inoculum prepared in SSE. Empty columns, mMRS;
735 black columns, SDB.

736 **Fig. 3.** Kinetics of acidification of the starter strains during the first 24 h of monitoring in SSE
737 incubated at 30°C. Symbols: square, *Lb. sanfranciscensis* PON100336; rhombus, *W. cibaria*
738 PON10030; triangle, *Ln. citreum* PON10079; circle, triple inoculums.

739 **Fig. 4.** pH values of the multi-species starter culture propagated in SSE at 30°C for 24 h.

740 **Fig. 5.** Monitoring of the LAB added as starter culture in form of liquid inoculums by RAPD-PCR
741 profile comparison. The numbers indicate the highest levels of detection of the strains on agar plates
742 which correspond to the minimum densities present in the liquid broths. Lanes: M, molecular
743 marker (GeneRuler 100 bp plus DNA ladder); *W. cibaria* PON10030; *Ln. citreum* PON10079; *Lb.*
744 *sanfranciscensis* PON100336.

745 **Fig. 6.** Monitoring of pH (A) and TTA (B) of the doughs obtained with different semolina during
746 fermentation.

747 **Fig. 7.** Monitoring of the LAB after fermentation by RAPD-PCR profile comparison. The numbers
748 indicate the highest levels of detection of the strains on agar plates which correspond to the
749 minimum densities present in the fermented doughs. Lanes: M, molecular marker (GeneRuler 100
750 bp plus DNA ladder); *W. cibaria* PON10030; *Ln. citreum* PON10079; *Lb. sanfranciscensis*
751 PON100336.

752 **Fig. 8.** Distribution of the volatile organic compounds among breads from the different trials. The
753 double hierarchical dendrogram is based on the values of VOCs. The heat map plot depicts the
754 relative percentage of each compound within each bread.

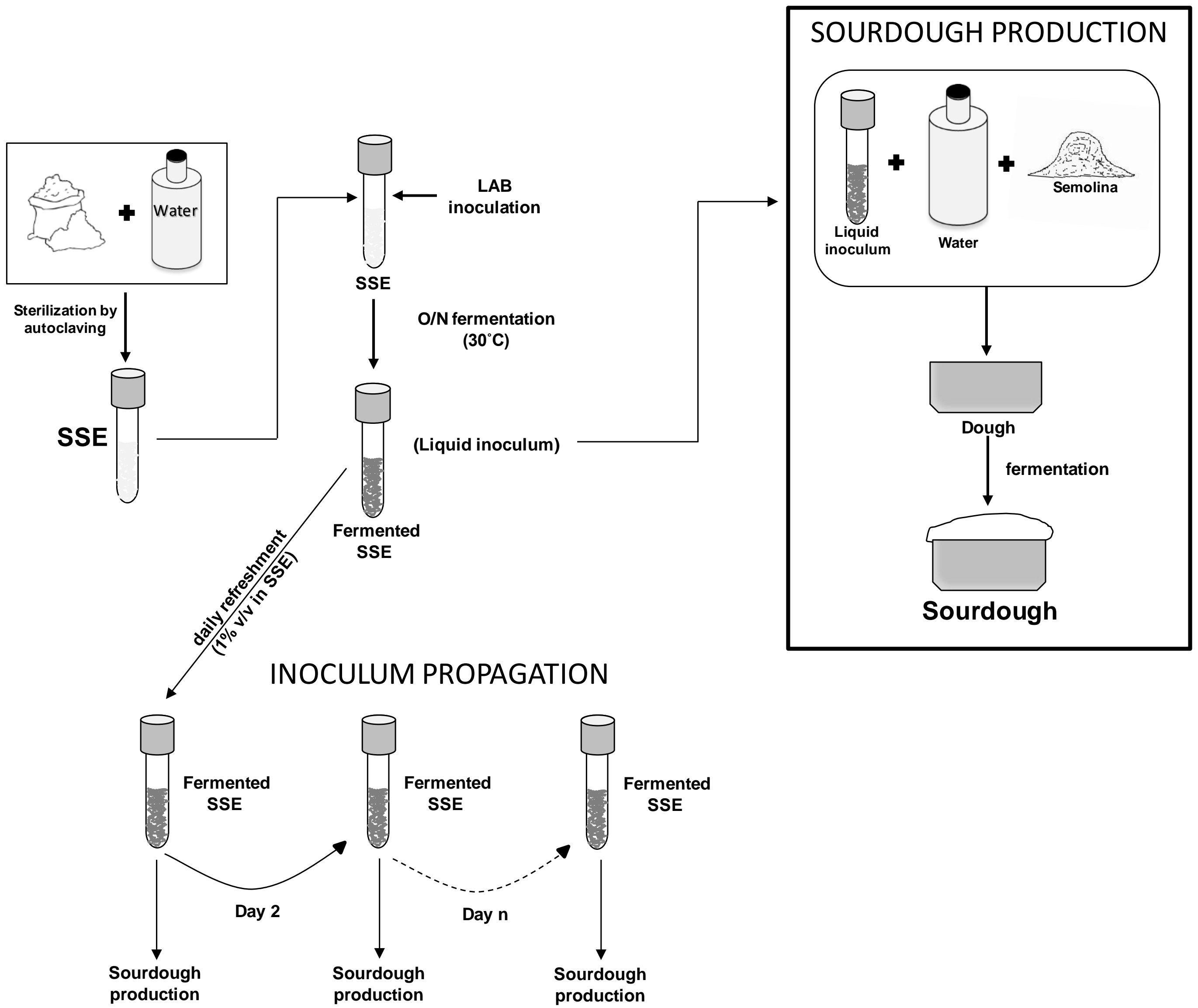
755 **Fig. 9.** Dendrogram resulting from hierarchical cluster analysis on 23 variables determined on
756 sourdoughs and breads.

757 **Fig. 10.** Loading plot (A) and score plot (B) resulting from principal component analysis on 23

758 variables determined on sourdoughs and breads.

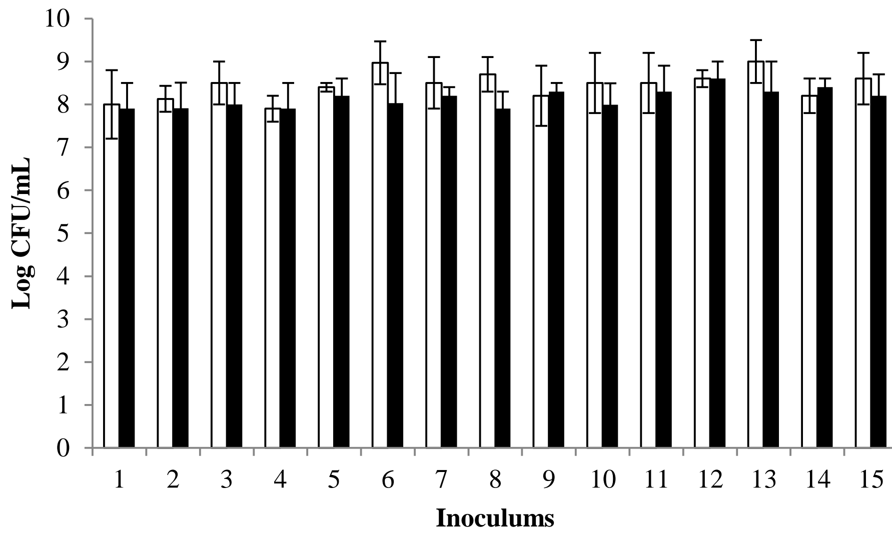
759

760 **Fig. 1.**



761
762

763 **Fig. 2.**



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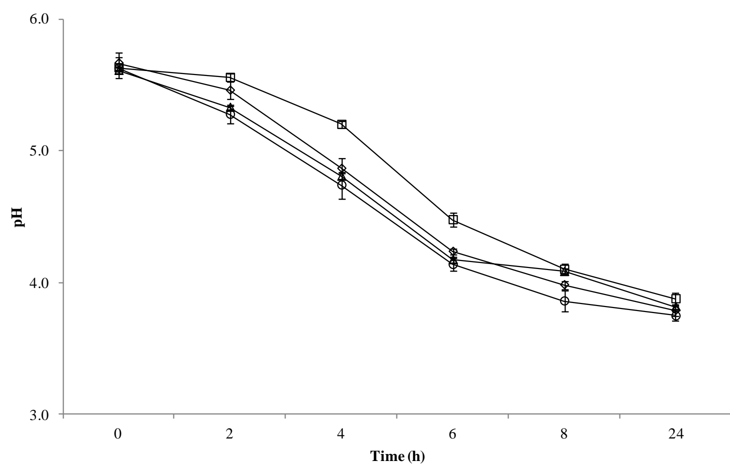
765 **Fig. 3.**



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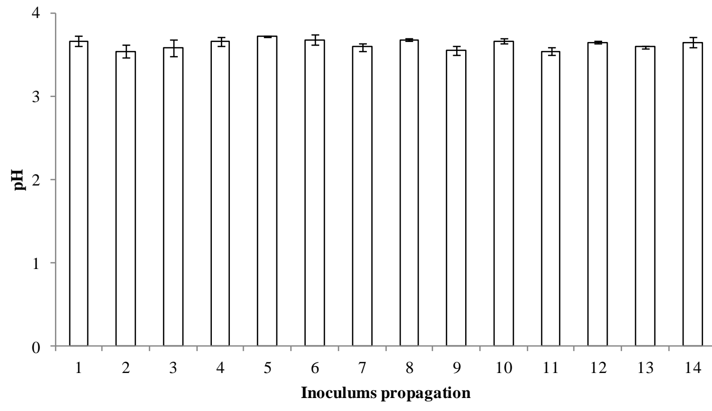
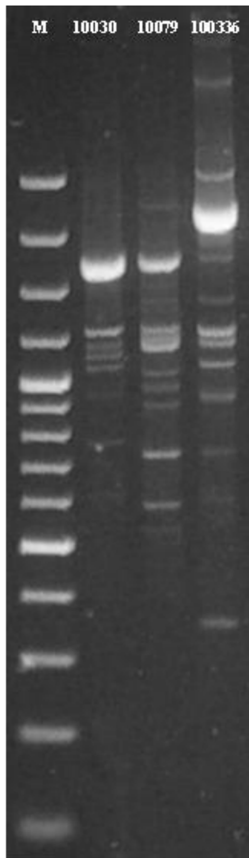
767 **Fig. 4.**



768

769 **Fig. 5.**

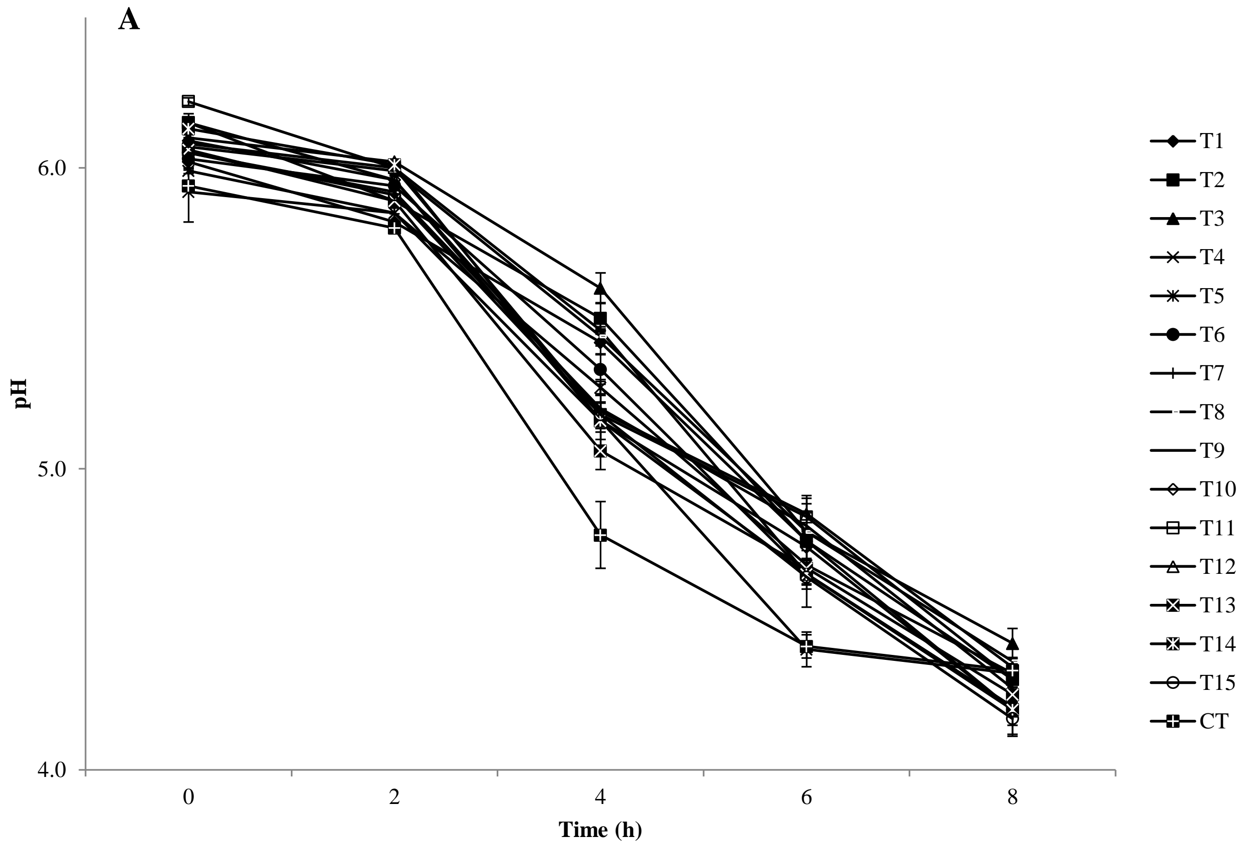
Highest level of detection (Log CF/mL)			Step	Day
7	7	7	preparation	1
8	7	7	propagation	2
8	8	7	propagation	3
7	7	7	propagation	4
8	8	8	propagation	5
8	8	7	propagation	6
8	8	8	propagation	7
8	8	7	propagation	8
8	7	8	propagation	9
8	8	7	propagation	10
8	8	8	propagation	11
8	8	8	propagation	12
8	8	8	propagation	13
8	7	8	propagation	14
8	8	8	propagation	15

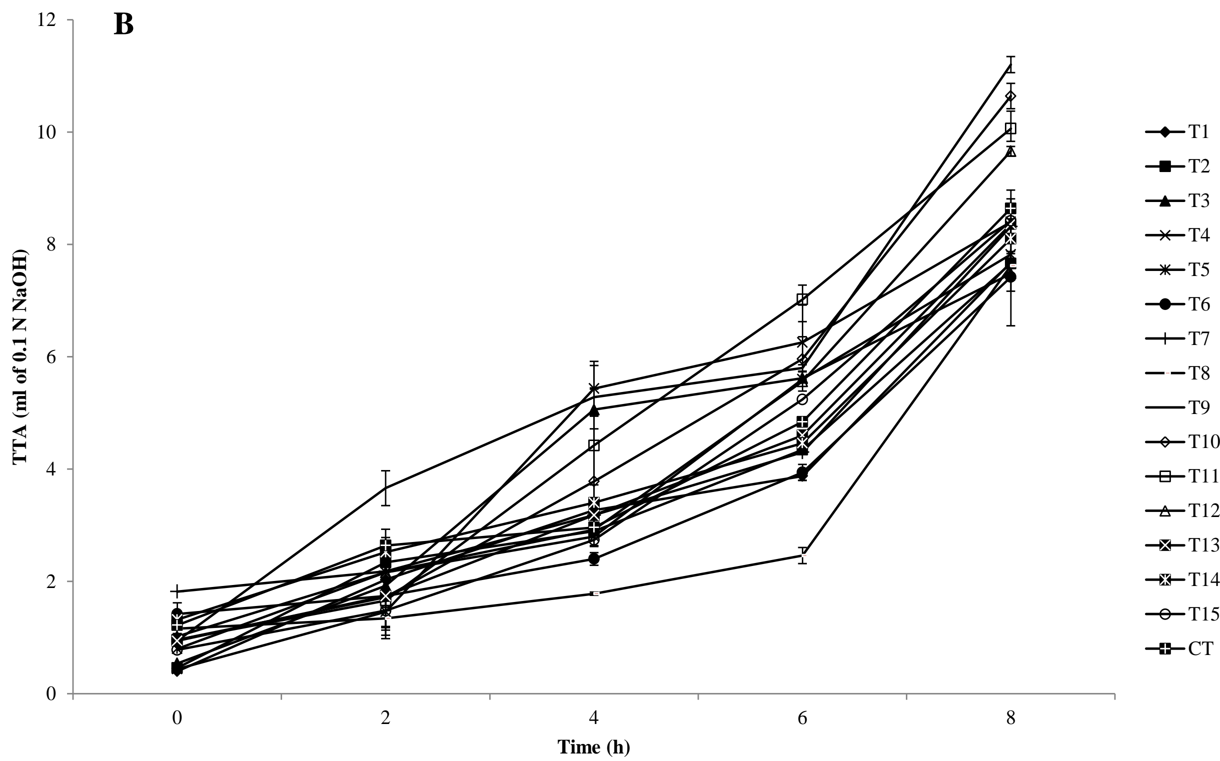


770

771 **Fig. 6.**

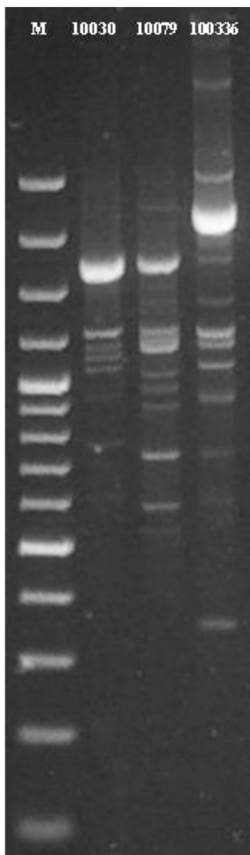
772





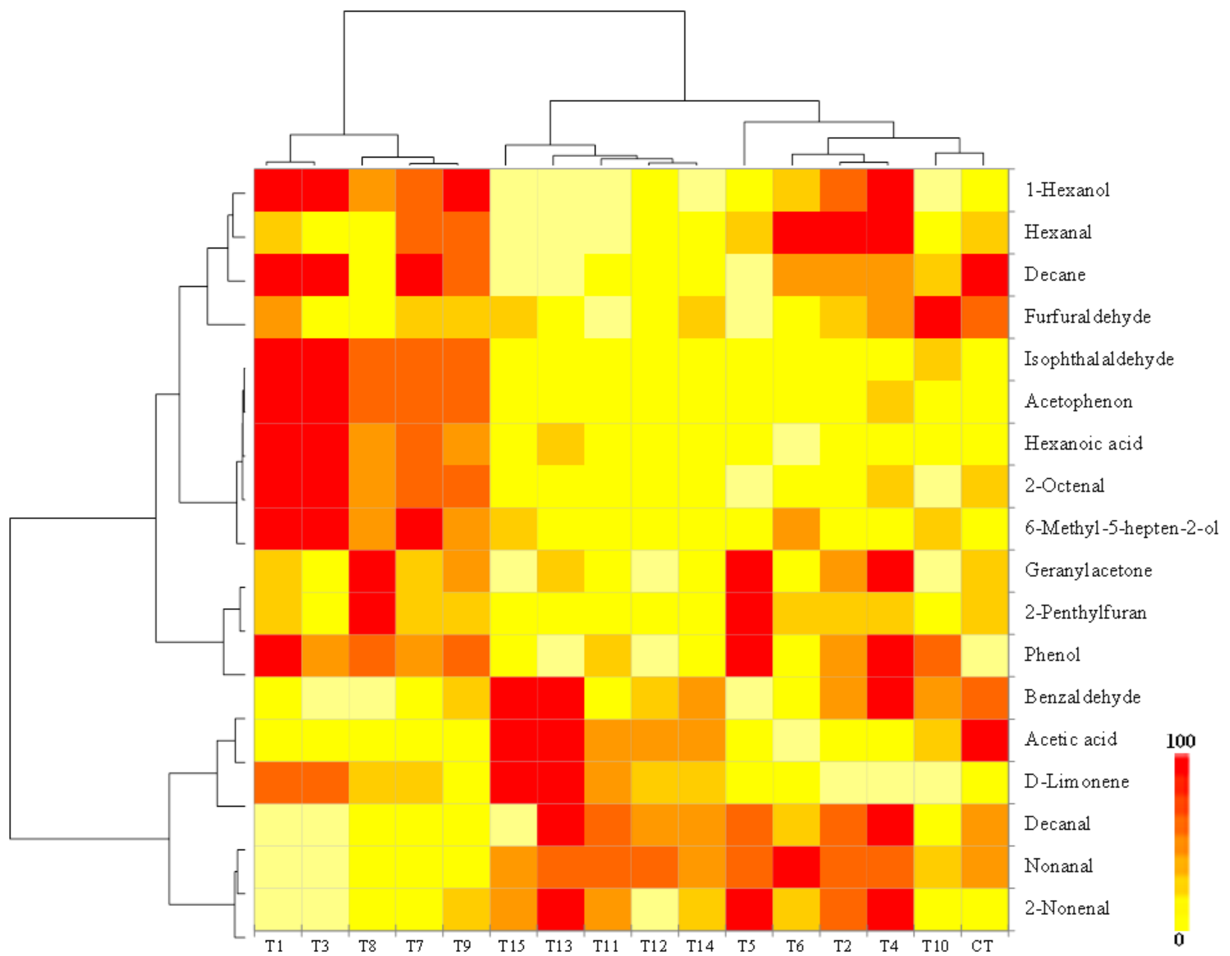
773 **Fig. 7.**

Highest level of detection (Log CF/g)			Trial
8	8	8	T1
8	8	9	T2
9	8	8	T3
9	8	8	T4
8	7	9	T5
8	8	8	T6
8	8	9	T7
8	8	8	T8
8	8	9	T9
9	8	8	T10
8	8	9	T11
9	8	10	T12
9	8	10	T13
8	8	9	T14
8	8	9	T15
8	8	9	CT



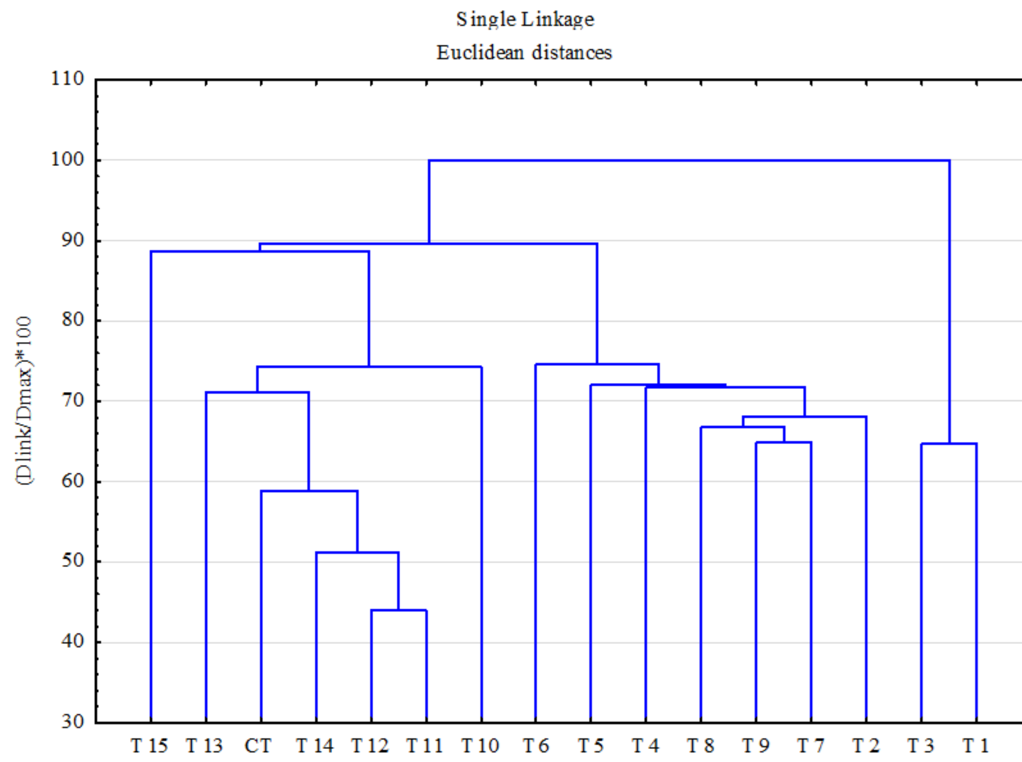
774

775 **Fig. 8.**



776
777

778 **Fig. 9.**



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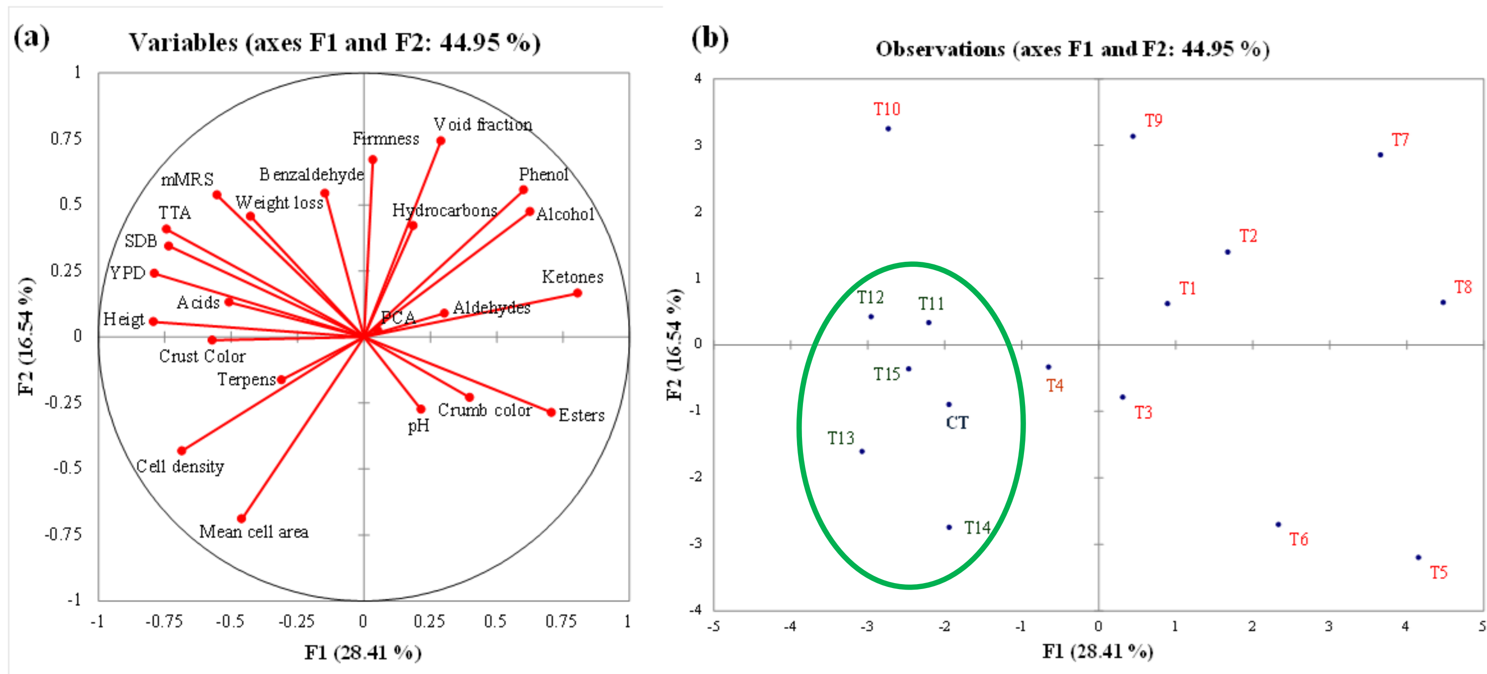
780

781 **Fig. 10.**



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