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

Antonio Alfonzo, Valeria Urso, Onofrio Corona, Nicola Francesca, Gaetano Amato, Luca Settanni ⁎, Giuseppe Di Miceli

Dipartimento Scienze Agrarie e Forestali, Università degli Studi di Palermo, Viale delle Scienze 4, 90128 Palermo, Italy

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A B S T R A C T

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, polymeric 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, crust and crumb color, 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.

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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 the sourdough technology (De Vuyst et al., 2009). Sourdough is an extremely complex ecosystem where several lactic acid bacteria (LAB) and yeasts cohabit (Corsetti and Settanni, 2007).

In general, the raw materials, the microbiota developing during the fermentation process and the technological parameters applied during bread making affect consistently the characteristics of the final products (Corsetti et al., 2000). In particular, the microbial composition of sourdough plays a major role during fermentation (De Vuyst and Neyesens, 2005). However, a series of intrinsic and extrinsic factors may in turn influence the composition of the sourdough microbiota (De Vuyst et al., 2014).

The vast majority of bread is traditionally produced from wheat (Goesaert et al., 2005). Although bread is generally produced with the flour from common wheat (Triticum aestivum L.), for this reason also called “bread wheat”, the use of semolina from durum wheat (Triticum durum Desf) in bread production is quite common in southern Italy (Corsetti et al., 2001; Quaglia, 1988). Several typical breads produced in Sicily are made with semolina applying the sourdough technology (Ventimiglia et al., 2015).

The dominating LAB populations of a given type of sourdough are quite stable at species level (Meroth et al., 2003). Regarding the Italian sourdoughs, basically included in Type I sourdough produced with traditional techniques and characterised by continuous, daily refreshments and fermentation at ambient temperature (De Vuyst and Neyesens, 2005), a few species are often found to dominate the lactic acid microbota. The species most frequently found at the highest levels in Italian sourdoughs is undoubtedly Lactobacillus sanfranciscensis (Picozzi et al., 2010; Siragusa et al., 2009), but other obligately heterofermentative LAB, such as Leuconostoc and Weissella species, are often found to dominate this ecosystem (Coppola et al., 1996). However, during refreshments, the addition of flours stored in different conditions and obtained from wheat crops grown in different environmental conditions, cropping systems and genotypes might determine a variation of...
the microbial composition of sourdoughs over time (De Vuyst et al., 2014). Alfonzo et al. (2013) reported that flours and semolinas used to produce sourdough breads in a restricted area in Sicily (southern Italy) were characterised by different strains of the same species. Thus, a certain succession of the dominant strains during sourdough propagation for long times cannot be excluded.

The variability of the dominant strains is reflected in a limited reproducibility of the final characteristics of a given bread typology. When a starter culture is added to a sourdough ecosystem, it is important to ensure its stability over time, in order to warrant a certain reproducibility of the characteristics of the resulting bread. For this reason, a new method for the preparation of the starter culture for sourdough production has been developed in this study. The innovative method is based on the daily addition of a direct liquid inoculum to semolina. The methodology was validated with several semolinas from old and modern genotypes of durum wheat cultivated in Sicily.

2. Materials and methods

2.1. Starter strains

In this study, three obligately heterofermentative LAB strains (*Lb. sanfranciscensis* PON100336, *Leuconostoc citreum* PON10079 and *Weissella cibaria* PON10030) were used as a multi-species starter culture for sourdough production. The strains, belonging to the culture collection of the Department of Agricultural and Forest Sciences – University of Palermo (Italy), were previously isolated from wheat semolinas produced from durum wheats cultivated in Sicily (southern Italy) (Alfonzo et al., 2013) and selected for their potential during the production of experimental sourdough breads (Settanni et al., 2013). Recently, the performances of *Ln. citreum* and *W. cibaria* have been evaluated under industrial conditions without obligate heterofermentative *Lactobacillus* species, evidencing their specific abilities to carry out the sourdough fermentation (Corona et al., 2016). The strains *Ln. citreum* PON10079 and *W. cibaria* PON10030 were propagated overnight at 30 °C in modified-de Man-Rogosa-Sharpe (mMRS) broth, prepared from MRS (Oxoid, Milan, Italy) added with maltose and fresh yeast extract at the final concentration of 1% and 10%, respectively, and adjusted to pH 5.6 with 5 M 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 10^8 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 first step of preparation and propagation, the liquid inoculum was sub-cultured 16 times from the starter inoculum. After development at 30 °C for 24 h, the propagation of the broth culture occurred for other 13 d for a total of 15 consecutive inocula. At the fifth step of propagation in SSE, the 16 liquid inocula were used to prepare the 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-Rad, Hercules, CA, USA), following the manufacturer’s instructions. The DNAs were analysed by the randomly amplified polymorphic DNA (RAPD)-PCR technique with the primer M13 as described by Settanni et al. (2012). The PCR products were separated on 1.5% (w/v) agarose gels (Gibco BRL, Cergy Pontoise, France) stained with the SYBR® safe DNA gel stain (Molecular probes) and acquired under UV trans-illumination. The comparison of the RAPD patterns resulting from the DNA of the isolates with those of the starter strains was performed with the GelCompar II software version 6.5 (Applied-Maths, Saint-Martens-Latem, Belgium).

In order to unequivocally confirm the species of the dominating strains the isolates were processed by the species-specific PCRs for Lb. sanfranciscensis (Settanni et al., 2005), Leuconostoc mesenteroides (Lee et al., 2000) and W. cibaria (Fusco et al., 2011).

### 2.3. Sourdough production

In order to evaluate the efficacy of the direct liquid inoculum addition to different semolinas to produce sourdough breads (Fig. 1), several genotypes were individually tested. The study included the semolina from five modern and ten old genotypes (Table 1) cultivated in adjacent experimental fields (6 m × 50 m) at the farm “Pietranera” (37°30′ N, 13°31′ E; 178 m elevation), located in Santo Stefano di Quisquina (AG, Italy). The commercial semolina used to evaluate the stability of the liquid inoculum (paragraph 2.2) was used as control. The semolina were used to prepare the SSEs, which were inoculated with the three starter strains at 10^6 CFU/mL as reported above. At the fifth step of propagation in SSE, the 16 liquid inocula were used for the direct addition to semolina. The inocula were also analysed by plate count and the colonies subjected to RAPD profile comparison and species-specific PCRs as reported above.

The doughs (300 g) were prepared with a dough yield (weight of the dough / weight of the semolina × 100) of 160 in 1 L sterile glass beckers under a flow laminar hood. Each semolina (187.5 g) was inoculated with the microbial suspension (112.5 mL) from the corresponding fermented SSE diluted in sterile tap H_2O to an approximately 10^6 CFU/g of starter culture inoculum and manually mixed by means of a sterile spoon. Soon after mixing, each dough was divided in two aliquots; 200 g were left in becker and covered with parafilm and the remaining 100 g were transferred to a metal pan (see paragraph 2.6 for the characteristics) covered with aluminium foil. The doughs were incubated at 30 °C for 8 h. The trials were carried out in duplicate and repeated after two weeks.

![Fig. 2. Microbiological counts of the liquid inoculum prepared in SSE. Empty columns, mMRS; black columns, SDB.](image-url)
2.4. Analysis of sourdoughs

The acidification of the sourdoughs kept in beckers was followed by pH measurement, performed on 10 g of sample, collected aseptically, by direct immersion of the pH-meter probe, and total titratable acidity (TTA), determined by titration with 0.1 N NaOH. TTA was evaluated on the same 10 g of doughs used for pH determination which were homogenised in distilled H2O (90 mL) by stomacher (BL Smart, Astori Tecnica, Poncarale, Italy) for 2 min at the highest speed. The samples were analysed at T0 and at 2 h-interval until 8 h and the results of TTA were expressed in terms of mL of NaOH/10 g of sourdough.

The concentrations of lactic and acetic acid and, consequently, the fermentation quotient (FQ, lactic acid/acetic acid molar ratio) of each sourdough were determined at T0 and at the end of fermentation. The chemical determinations were carried out by high performances liquid chromatography as described by Alfonzo et al. (2013) on sourdough samples (10 g) homogenised with distilled H2O (90 mL) with a Sorvall Omni-Mixer (Dupont Instruments, Newtown, CT) and treated as reported by Ventimiglia et al. (2015). Data were acquired and processed with the PerkinElmer software specific to the HPLC instrument (TotalChrom Workstation 2008 rev. 6.3.2).

The 16 sourdoughs were also microbiologically investigated by plate count just after mixing of semolina with the bacterial inoculum (T0) and after 8 h of fermentation. Ten grams of each sample were suspended in 90 mL of Ringer’s solution, homogenised by stomacher as reported above and serially diluted. Total mesophilic microorganisms (TMM) were inoculated on plate count agar (PCA) (Oxoid), incubated aerobically at 30 °C for 72 h. LAB were plated on SBD agar and mMRS agar, incubated aerobically and anaerobically, respectively, at 30 °C for 48 h. Yeasts were plated on yeast potato dextrose (YPD) agar, incubated aerobically at 25 °C for 72 h. Semolina samples (10 g) were also microbiologically analysed as reported for the sourdoughs.

The dominating LAB were also identified at strain and species level as reported above.

2.5. Bread making and analysis

After preparation, 100 g of each dough were transferred to rectangular stainless steel baking pans of the dimensions (143 × 79 mm, top inside; 129 × 64 mm, bottom outside; depth inside 57 mm) indicated by the Method 10-10B of the American Association of Cereal Chemists (AACC, 2000) and fermented for 8 h at 30 °C. The resulting sourdoughs were baked in the industrial oven Air-o-steam (Electrolux, Pordenone, Italy) applying the following 3-step baking program: 1 min at 190 °C; 8 min at 180 °C with 70% relative humidity (RH); 10 min at 185 °C with 20% RH.

The breads were cooled at ambient temperature before analysis. The breads were first weighed to determine the weight loss and then cut transversely in two halves long about 6 cm. The height of the breads was measured on the central slices (Schober et al., 2005). The Chroma Meter CR-400 (Minolta, Osaka, Japan) was used for colour determination by the Hunter’s scale parameters (L*, a* and b*) measured on crust (four points) and crumb of the central slices (three points). The hardness of crumb was determined by means of the Instron-5564 (Instron Corp., Canton, MA) following the method reported by Corsetti et al. (2000).

The two central slices of each loaf were subjected to the image analysis to calculate void fraction (the fraction of the total area corresponding to the bread pores), cell density (number of cells/cm²) and mean cell area (in mm²). The images were scanned (Epson Perfection 4180 Photo, Seiko Epson Corp., Japan) with 350 dpi of resolution, saved in TIFF format, analysed with the ImageJ software (National Institutes Health, Bethesda, Md, USA), cropped to a square of 207 × 207 pixels (representing 15 × 15 mm of the slice) and converted to grey-level image (8 bit). The Otsu’s threshold algorithm was applied to obtain binary images.

The solid phase micro-extraction (SPME) isolation technique was applied to determine the volatile organic compounds (VOCs) emitted from the breads. Each sample of bread (5 g) was heated at 60 °C in a vial to generate a headspace that was collected for 40 min through the DBVCarboxen-PDMS fibers (Supelco, Bellefonte, PA). The Finnegan TraceMS for GC/MS (Agilent 6890 Series GC system, Agilent 5973 NetWorkMass Selective Detector, Milan, Italy) equipped with a DB-WAX capillary column (Agilent Technologies, 30 m, 0.250 mm i.d.,
film thickness 0.25 μm, part n° 122-7032) was used to analyse the SPME fibre as reported by Alfonzo et al. (2013). The methodology described by Settanni et al. (2013) was applied for the identification of the compounds. All solvents and reagents were purchased from WWR International (Milan, Italy).

All determinations on breads were performed in triplicate.

2.6. Sensory analysis

The final breads were subjected to the sensory analysis performed by a descriptive panel consisting of 11 tasters (six women and five men; age, 26–60 years old) familiar with the sensory analysis of foods, but not specifically trained in the evaluation of sourdough breads. The panel was trained for descriptive analysis according to the guidelines in the ISO 8586 to evaluate 23 descriptors chosen among those reported by Comendador et al. (2012) and evaluated by other authors (Martins et al., 2015; Rodrigues et al., 2014), including crust colour, crust thickness, crumb colour, porosity, alveolation, alveolation uniformity, odor intensity, bread odor, yeast odor, sourdough odor, unpleasant odor, aroma intensity, bread aroma, yeast aroma, sourdough aroma, unpleasant aroma, salty, acid, bitter, taste persistency, adhesiveness in mouth, crispness and the overall assessment. The analysis was carried out according to the guidelines in the ISO 6658. The tasters expressed the intensity of each attribute with a mark on a 6-point hedonic scale (5 = extremely high; 0 = extremely low).

2.7. Statistical and explorative multivariate analyses

Chemical, physical and microbiological data were analysed with the ANOVA linear model according to a repeated measure design (GLM procedure of SAS 9.1.2 software, 2004) which included the effects of the semolina as repeated measure. Comparison among LS means were performed by Tukey’s test; differences were considered significant at P < 0.05.

In order to represent graphically the values of VOCs, a heat map clustered analysis, based on double hierarchical dendrogram with heat map plot, was performed. The relative values of VOCs were depicted by colour intensity from yellow (lowest concentration) to red (highest concentration). Heat map analysis of the volatile levels was performed using the auto-scaled data. Graphic construction were achieved by using XLStat software version 2014.5.03. (Addinsoft, New York, USA) for excel.

To better investigate the relationship among data obtained from the breads produced with the different semolinas, an explorative multivariate analysis was carried out. A hierarchical cluster analysis (HCA) (joining, tree clustering) was carried out for grouping the trials according to their similarities, measured by Euclidean distances, whereas cluster aggregation was based on the single linkage method (Todeschini, 1998). The different productions were grouped by principal component analysis (PCAn). The input matrix used for HCA and PCAn consisted of the values of the measurements (23 variables) performed on sourdoughs and breads, except the data on sensory evaluation. The number of principal factors was selected according to the Kaiser criterion (Jolliffe, 1986) and only factors with eigen-values higher than 1.00 were retained. All data were preliminary evaluated by using the Barlett’s sphericity test (Dillon and Goldstein, 1984; Mazzei et al., 2010) in order to check the statistically significant differences among samples within each data set. XLStat software version 2014.5.03 (Addinsoft, New York, USA) for excel.
New York, USA) for Excel was used for data processing and graphic construction.

3. Results

3.1. Stability of the liquid inoculum

The results of the microbiological monitoring of the liquid inoculum from the preparation (day 1) until the 14th day of propagation (days 2–15) showed that SSE allowed a development of LAB at levels above $10^8$ CFU/mL (Fig. 2). The cell densities registered on mMRS and SDB were not statistically different, indicating that *Lb. sanfranciscensis* PON100336 was able to grow in SSE at levels similar to those reached by the other starter strains.

The pH kinetics during the preparation of the multi-species starter culture in SSE was followed frequently until 24 h and it was also compared to the pH drop showed by the three individual strains inoculated in SSE (Fig. 3). *Lb. sanfranciscensis* PON100336 was acidified SSE slower than *Ln. citreum* PON10079 and *W. cibaria* PON10030 during the first 6 h of observation. However, all strains acidified the SSEs until pH 3.8 at 24 h. The pH drop of the multi-species culture was slightly more rapid than the single strains *Ln. citreum* PON10079 and *W. cibaria* PON10030 and was registered at 3.7 at 24 h. The pH of the mixed culture in SSE monitored during the successive consecutive 14 days of propagation (Fig. 4) was quite constant ranging a value between 3.5 and 3.7.

The direct comparison of the polymorphic profiles of the isolates collected from the agar plates (Fig. 5) and the species-specific PCRs confirmed the presence of all three added strains in the range $10^7$–$10^8$ CFU/mL after the daily SSE fermentation. These results showed that the inoculum prepared and propagated daily in SSE was stable.

3.2. Chemical and microbiological characterisation of sourdoughs

Once the stability of the inoculum in SSE over time was ascertained with the commercial semolina, the liquid inocula were prepared in SSEs obtained from each semolina included in this study. The liquid inocula were then propagated for five days and added directly to the semolinas to produce the doughs. The pH kinetics of these doughs during the 8 h of fermentation showed a similar trend (Fig. 6A). All initial pHs were in the range 5.9–6.1. The most consistent pH drop was registered at 4 h. At this time, the doughs of the control trial showed a pH value of 4.8, while all the other doughs 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 and 4.4.

![Fig. 6. Monitoring of pH (A) and TTA (B) of the doughs obtained with different semolinas during fermentation.](image-url)
Table 2

Organic acids of the experimental sourdoughs.

<table>
<thead>
<tr>
<th>Trials</th>
<th>Lactic acid (mg/g)</th>
<th>Acetic acid (mg/g)</th>
<th>FQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>4.61 ± 0.12&lt;sup&gt;EF&lt;/sup&gt;</td>
<td>1.24 ± 0.15&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.48</td>
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<tr>
<td>T2</td>
<td>5.39 ± 0.05&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>1.28 ± 0.05&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.81</td>
</tr>
<tr>
<td>T3</td>
<td>4.98 ± 0.08&lt;sup&gt;DE&lt;/sup&gt;</td>
<td>1.31 ± 0.23&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.53</td>
</tr>
<tr>
<td>T4</td>
<td>4.56 ± 0.09&lt;sup&gt;F&lt;/sup&gt;</td>
<td>1.19 ± 0.13&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.55</td>
</tr>
<tr>
<td>T5</td>
<td>3.80 ± 0.21&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>1.09 ± 0.06&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.20</td>
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<tr>
<td>T6</td>
<td>5.07 ± 0.18&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>1.26 ± 0.15&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.68</td>
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<tr>
<td>T7</td>
<td>5.14 ± 0.14&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>1.14 ± 0.25&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.01</td>
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<td>T8</td>
<td>4.92 ± 0.07&lt;sup&gt;DE&lt;/sup&gt;</td>
<td>1.14 ± 0.20&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.88</td>
</tr>
<tr>
<td>T9</td>
<td>5.59 ± 0.11&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.21 ± 0.09&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.08</td>
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<tr>
<td>T10</td>
<td>5.41 ± 0.04&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>0.99 ± 0.11&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.64</td>
</tr>
<tr>
<td>T11</td>
<td>5.96 ± 0.12&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.21 ± 0.04&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.28</td>
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<tr>
<td>T12</td>
<td>5.29 ± 0.14&lt;sup&gt;BCD&lt;/sup&gt;</td>
<td>1.19 ± 0.06&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.96</td>
</tr>
<tr>
<td>T13</td>
<td>6.01 ± 0.07&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.36 ± 0.16&lt;sup&gt;A&lt;/sup&gt;</td>
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<td>T14</td>
<td>4.99 ± 0.19&lt;sup&gt;DE&lt;/sup&gt;</td>
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<td>T15</td>
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<td>1.02 ± 0.21&lt;sup&gt;A&lt;/sup&gt;</td>
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<tr>
<td>CT</td>
<td>5.10 ± 0.17&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>1.15 ± 0.10&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.96</td>
</tr>
</tbody>
</table>

Statistical significance: *** N.S. n.d.

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

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

3.3. Monitoring of the starter strains

The comparison of the RAPD profiles of the colonies developed from the highest dilutions of the cell suspensions to those of the pure cultures (Fig. 7) indicated that, after 8 h of fermentation, *Lb. sanfranciscensis* PON100336 dominated the LAB populations of all sourdoughs obtained with the semolina from the modern genotypes and the sourdoughs T2, T5, T7 and T9 made with the old genotypes while a codominance of the three starter strains was found for the other sourdoughs. *Lb. citreum* PON10097 could not be detected on the Petri dishes inoculated with the highest dilutions of the sourdough T5. *W. cibaria* PON10090 was often identified from SDB.

3.4. Characteristics of the experimental breads

The final breads were evaluated for several quality characteristics. The weight of the breads were almost comparable among the different trials (Table 4). However, the highest weight loss was registered for the breads of the trials T1 and T10. The height of the central slices was
between 2.20 and 3.59 cm (Table 4). The highest variability in height was registered among the breads produced from the semolina of the old genotypes, while all breads made with the modern genotype semolinas showed a similar height. The three colour parameters of the crumb and the parameter L* of the crust were comparable for all breads. For the crust, a few differences were registered for the parameter b*, while the parameter a* clearly distinguished the breads produced with the semolina from the old genotypes from those made with the semolina from the modern genotypes. Regarding firmness (Table 4), the lower the value the softer the breads and CT breads were those with the lowest values. The breads from the old genotypes T4, T5 and T8 showed a softness comparable to those of the breads obtained with the majority of the modern genotype semolinas (T13–T15). Furthermore, the breads with the highest values of firmness were those from the trials characterised by the highest weight loss (T1 and T10).

The image analysis showed that all trials were quite different from one another for almost all parameters (void fraction, cell density and mean cell area) evaluated (Table 5).

Eighteen VOCs were emitted from the breads produced in this study (Fig. 8). The dendrogram resulting from the cluster analysis and the heat map showed the relationships among the breads based on the amount of each VOC, determining the formation of three main clusters. All breads produced with the semolina from the modern genotypes were included into a single cluster, while the breads made with the semolina from the old genotypes were divided into two distant clusters. Interestingly, the breads made with the commercial semolina clustered with the breads of the trials T2, T4–T6 and T10. Aldehydes (280.3–595.3 μg/kg) constituted the major class of VOCs for all breads, esters (7.3–318.6 μg/kg) were particularly represented in the breads of the trials T5 and T8, ketones (31.3–123.7 μg/kg) were at relevant concentrations in the breads of the trials T5, T7–T9, while acids (26.2–184.2 μg/kg) in those of trials T9, T13, T15 and CT. Phenol was detected at very low levels in almost all breads, except those made with the old genotypes T1, T7–T9. Within the major classes, the main compounds found for all breads were nonanal for the aldehydes, acetic acid for the acids and geranylacetone for ketones. 2-Penthylfuran was the only compound detected for the esters. The other classes identified were terpenes, with a single compound (α-limonene), hydrocarbons and alcohols.

3.5. Sensory evaluation

The results of the sensory tests on the breads are reported in Table 6. The highest scores for crust colour were registered for the trials T6 and T12, while the trial T9 showed the highest evaluation for the colour of crumb. The thickness of the crust resulted generally low. The porosity, the alveolation and the alveolation uniformity were variable among the breads. The highest value of crust elasticity was registered for the trial T11, while the lowest for the trial T5. The odor intensity, the bread odor, the sourdough odor, the aroma intensity, the bread aroma and sourdough aroma were quite high for the majority of the breads. On the contrary, yeast and unpleasant odor, as well as yeast and unpleasant aroma were scored at very low level, often zero. All breads resulted slightly salted, sour and bitter. The taste persistency was almost at the same level for the trials CT, T6 and T11. Only the breads of trial T1 resulted similar to those of the control trial for the crispness. The bread of the control trial were characterised by the lowest gummy crumb. Finally, the highest score for the overall assessment was displayed by the breads of the trial T6, followed by the breads of the
control trial. Except for yeast odor and aroma and unpleasant odor and aroma, which were not significantly different among judges and breads, all other sensory attributes were scored different. The less notable differences were evidenced for crust thickness, salty, acid and bitter taste among judges.

3.6. Multivariate analysis

HCA classified the trials in accordance to their mutual dissimilarity and relationship (Fig. 9). This analysis basically generated two main mega-clusters. It is worth of note that all sourdough and bread productions performed with the semolina from the modern genotypes were gathered together with the control trial and this mega-cluster also included one trial (T10) carried out with the old genotype Senatore Cappelli. Interestingly, seven of the 10 trials performed with the semolina from the old genotypes were included in the other mega-clusters.

In order to condense the information into a reduced number of Factors, data were then subjected to PCA. The Bartlett’s sphericity test was applied to all data matrix inputs (microbiological, chemical, physical and quality parameters of sourdoughs and breads) and differences statistically (P < 0.0001) significant were found among trials. The results of the PCA (Fig. 10) showed that only three eigen-values were higher than 1, which accounted for 28.41 to 16.54% of variability. This indicated that the initial 23 variables might be expressed as linear combination of only two Factors explaining 44.95% of total variance. The components of the PCA were correlated to variables as shown in the Fig. 10a. The discrimination of the trials can be visualized in the plot of scores (Fig. 10b). In detail, the last figure shows the projection of the cases onto the planes as function of the Factors 1 and 2. The trials were significantly separated along Factor 1 on the basis of flavour profile, pH, firmness and void fraction. On the other hand, the variables associated to the Factor 2 significantly contributed to discriminate the trials obtained with the semolina from the modern genotypes (T11–T15 and TC) from those obtained with the old genotypes (T1–T10).

4. Discussion

This work was carried out to answer a specific request of bread makers concerning the stabilization of the sourdough inoculum to avoid differences among bread productions. To this purpose, in the first part of the present work, the preparation of a liquid inoculum was performed and its stability over time was evaluated. The medium used for the development of the starter culture was the SSE which has the characteristics of being simple and fast to prepare and cost-effective. The pH was adjusted to 5.6 to favour the growth of sourdough LAB. This because the pH of wheat flour, as well as semolina, is close to the maximum pH value for the growth of Lb. sanfranciscensis (Gänzle et al., 1998) which is considered the key sourdough LAB (Lhomme et al., 2015; Messens and De Vuyst, 2002), particularly for Italian productions (Gobetti and Corsetti, 1997). The starter culture applied in this study was composed by a multi-species combination including three obligately heterofermentative strains. Besides Lb. sanfranciscensis, L. ciferri and W. cibaria were also chosen. These species, found to be dominant among the LAB community associated to the semolina used in Sicily.
are commonly present in the sourdough ecosystems produced in different countries (Choi et al., 2012; Di Cagno et al., 2006; Galle et al., 2010; Maina et al., 2008). Specifically, the strains Lb. sanfranciscensis PON100336, Ln. citreum PON10079 and W. cibaria PON10030 were included in the starter culture because they showed interesting results when tested individually during experimental sourdough productions (Settanni et al., 2013) and stability at industrial level production (Corona et al., 2016).

The simple and time-saving method for the direct inoculation of the selected LAB to the semolina developed in this work was based on the daily propagation of the mixed strain culture in SSE. At the beginning of the process, Lb. sanfranciscensis was found to be the slowest acidifier. This result was not surprising and it could be due to the stress imposed by the preparation of the inoculum. In fact, the cells were washed and the pH was not too acidic. Corsetti et al. (2007) registered a similar behaviour for Lb. sanfranciscensis during in situ tests with soft wheat flour. However, the pH and LAB counts, registered on average levels of 3.6 and \(10^6\) CFU/mL, respectively, and the genetic monitoring of the starter strains showed that the liquid inoculum propagated daily in SSE made with the commercial semolina was stable. Thus, in order to validate the methodology independently on the semolina used, the liquid inocula were prepared and propagated in SSE produced from different semolinas. To this purpose, 15 semolinas produced from old and modern durum wheat genotypes were tested with this innovative technique for the sourdough starter preparation and addition. Specifically, the three strains were inoculated in SSEs produced from the 15 genotypes to prepare the liquid inocula which were then propagated (by inoculation of the previous day liquid inoculum from each SSE) for five consecutive days before direct addition to unsterile semolina and left to ferment for 8 h.

Due to the importance of the obligately heterofermentative species for sourdough fermentation, facultative heterofermentative species and least of all obligately homofermentative LAB were not used in this study. First of all, they can increase the FQ due to the production of high amount of lactic acid and do not contribute to the production of CO2 and, secondly, they are provided by the Sicilian durum wheat semolina (Alfonzo et al., 2013). For example, the species Lactobacillus plantarum has been recently reported to codominate with the heterofermentative species during the production of sourdough in different areas of western Sicily (Ventimiglia et al., 2015). Thus, it was not added in this study to promote the stabilization of a specific consortium of the solely obligately heterofermentative species.

The breeding programs on wheat grains determined the gradual replacement of old varieties or landraces, and the establishment of new, more responsive to new agronomic principles of intensive farming and the modern food technology, which required genotypes with high rates of gluten to be devoted to pasta (Palumbo et al., 2000). The recent revaluation of old durum wheat genotypes, used to diversify agricultural production and to expand the portfolio of products available to consumers, made necessary to deepen the study on their semolina and dough for bread making. Semolinas used in this study were obtained from wheat genotypes chosen according to a temporal gradient, from the early 1900s (Timilia) to 2004 (Saragolla).

The values of pH and TTA registered after fermentation were comparable to those commonly found in mature durum wheat sourdoughs (Minervini et al., 2012a; Pepe et al., 2013; Rizzello et al., 2015; Ventimiglia et al., 2015). Thus, regarding the acidification of the doughs, the liquid starter culture behaved like a mature sourdough inoculum. Furthermore, the chemical analyses showed levels of lactic and acetic acid comparable to those reported by Ventimiglia et al. (2015) for the sourdoughs produced in Sicily. The resulting FQ of all doughs reached at the end of the fermentation period was in the range considered to affect positively the aroma profile and the structure of sourdough breads (Spicher, 1983). Some of the sourdoughs produced in this study reached even a FQ included in the optimal range of 2.0–2.7 indicated by Hammes.
### Table 6
Sensory characteristics of the experimental breads.

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Experimental trials</th>
<th>SEM</th>
<th>Statistical significance</th>
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<tbody>
<tr>
<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
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<tr>
<td>Crust colour</td>
<td>2.04</td>
<td>2.04</td>
<td>2.61</td>
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<tr>
<td>Crumb colour</td>
<td>1.25</td>
<td>1.68</td>
<td>1.81</td>
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<td>Crust thickness</td>
<td>0.45</td>
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<td>0.58</td>
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<tr>
<td>Porosity</td>
<td>1.17</td>
<td>1.41</td>
<td>2.01</td>
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<tr>
<td>Alveolation</td>
<td>1.21</td>
<td>1.05</td>
<td>0.80</td>
</tr>
<tr>
<td>Alveolation uniformity</td>
<td>1.62</td>
<td>1.72</td>
<td>2.16</td>
</tr>
<tr>
<td>Crust elasticity</td>
<td>1.82</td>
<td>1.59</td>
<td>1.79</td>
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<tr>
<td>Odor intensity</td>
<td>1.46</td>
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<td>2.24</td>
</tr>
<tr>
<td>Bread odor</td>
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<td>3.02</td>
<td>2.44</td>
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<tr>
<td>Yeast odor</td>
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<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Sourdough odor</td>
<td>1.49</td>
<td>2.78</td>
<td>2.98</td>
</tr>
<tr>
<td>Unpleasant odor</td>
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<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Aroma intensity</td>
<td>1.68</td>
<td>1.44</td>
<td>2.44</td>
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<tr>
<td>Bread aroma</td>
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<td>4.15</td>
</tr>
<tr>
<td>Yeast aroma</td>
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<td>0.00</td>
<td>0.00</td>
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<tr>
<td>Sourdough aroma</td>
<td>1.62</td>
<td>1.34</td>
<td>1.87</td>
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<tr>
<td>Unpleasant aroma</td>
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<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Salty</td>
<td>0.39</td>
<td>0.76</td>
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<td>Acid</td>
<td>1.22</td>
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<td>1.14</td>
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<tr>
<td>Bitter</td>
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<td>Taste persistency</td>
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<td>Crispness</td>
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<td>Gummy crumb</td>
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<tr>
<td>Overall assessment</td>
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<td>2.00</td>
<td>2.40</td>
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</table>

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

Results indicate mean value.

Data within a line followed by the same letter are not significantly different according to Tukey's test.
and Hammes and Gänzle (1998), evidencing clearly that the innovative method stabilized the ratio between lactic and acetic acid for the sourdoughs produced with different semolinas.

The microbiological analysis of the semolinas showed that the raw materials hosted different levels of the microorganisms investigated, mainly LAB and yeasts. However, after the fermentation with the starter inocula, the microbiological counts increased consistently in almost all sourdoughs. Independently on the microbiological characteristics of the semolina, the addition of the multi-species culture in form of liquid inoculum destabilized the ratio between lactic and acetic acid for the sourdoughs produced in Sicily (Minervini et al., 2012a; Ventimiglia et al., 2015), evidencing clearly that the liquid inoculum prepared in SSE generated a complexity of compounds in bread comparable to that due to the mature sourdoughs. Since the VOCs have different odor activity (Reiners and Grosch, 1998), their different composition might be defining for the sensory evaluation of the breads. Czerny and Schieberle (2002) stated that LAB influence the flavour compounds already present in flour. However, since in our study we demonstrated that, during fermentation, the starter LAB added with the liquid inocula dominated over the LAB populations present in semolina and that yeasts are at very low levels, we can consider that the differences in VOCs among the breads are mainly a consequence of the type of semolina. Among the key odor compounds of bread (Birch et al., 2013), hexanal, 2-nonenal and 2-pentylfuran, which derive from enzymatic oxidation and/or autoxidation of the lipid fraction of the wheat (Frankel, 1982; Hann and Morrison, 1975), and benzaldehyde were detected. In particular, hexanal and 2-pentylfuran were found at higher concentrations in the breads from the old genotypes and those from CT. All breads were mainly characterised by aldehydes (hexanal, nonanal, 2-octenal, furfuraldehyde, decanal, 2-nonenal, isophthalaldehyde and benzaldehyde).

The microbiological, chemical, physical and quality parameters of sourdoughs and breads were subjected to the multivariate analysis to evaluate the differences/variances among the trials. The correlation analysis among the data considered showed that there were many significant relationships among them. HCA generated two main clusters, separating almost sharply the productions made with the semolina from the modern genotypes from those obtained with the old genotypes. PCA confirmed this trend and clearly showed that the trials with the modern genotypes and CT were closed to one another, almost all in the same quadrant, while the trials with the old genotypes were widely spread among the four quadrants. The position of the majority of the trials performed with the semolina from the old genotypes is basically explained by the VOCs, as visible from the loading plot. This indicated that each bread produced from the old genotypes was characterised by unique aromas. These results could also explain why analysing exclusively the results of the VOCs of the breads, CT clustered with the breads obtained with the semolina from the old genotypes.

Some differences were registered among the breads for the hardness, weight loss, colour of the crust, void fraction, cell density and mean cell area. These results are mainly imputable to the different semolina characteristics. On the other hand, it is well known that plant breeding activities conducted on the species in the last decades has led to a considerable grain yield increase but has consistently modified the semolina quality and particularly the protein content and gluten properties influencing strength, extensibility, and toughness (De Vita et al., 2007; Ruisi et al., 2015).

The number of VOCs emitted from the breads produced in this study was in agreement with the numbers of VOCs generally reported for sourdough breads (Hansen and Schieberle, 2005; Salim-ur-Rehman et al., 2006). These findings indicated that the liquid inoculum prepared in SSE generated a complexity of compounds in bread comparable to that due to the mature sourdoughs. Since the VOCs have different odor activity (Reiners and Grosch, 1998), their different composition might be defining for the sensory evaluation of the breads. Czerny and Schieberle (2002) stated that LAB influence the flavour compounds already present in flour. However, since in our study we demonstrated that, during fermentation, the starter LAB added with the liquid inocula dominated over the LAB populations present in semolina and that yeasts are at very low levels, we can consider that the differences in VOCs among the breads are mainly a consequence of the type of semolina. Among the key odor compounds of bread (Birch et al., 2013), hexanal, 2-nonenal and 2-pentylfuran, which derive from enzymatic oxidation and/or autoxidation of the lipid fraction of the wheat (Frankel, 1982; Hann and Morrison, 1975), and benzaldehyde were detected. In particular, hexanal and 2-pentylfuran were found at higher concentrations in the breads from the old genotypes and those from CT. All breads were mainly characterised by aldehydes (hexanal, nonanal, 2-octenal, furfuraldehyde, decanal, 2-nonenal, isophthalaldehyde and benzaldehyde).

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even though a modern genotype represented the highest percentage of the semolina mixture used in this trial. The panel of judges was able to evidence the differences among the sensory attributes of the breads. A higher complexity of odors (odor intensity and sourdough odor) and sourdough aroma were registered for the breads produced with the semolina from the old genotypes (especially T6) and from CT, in agreement with the concentrations of VOCs.

5. Conclusions

The direct application of the liquid inocula developed with the multi-species strain starter in SSE to several semolinas allowed the production of sourdoughs with characteristics commonly reported for mature sourdoughs. The specific conclusions of this work are as follows: the liquid inocula were stabilized for all semolinas preparing an ad hoc SSE for each durum wheat genotype; the dominance of the added strains was verified; the adaptation of the selected LAB in SSE stimulated their rapid development in dough; the added multi-species strain starter determined high numbers of LAB in the sourdoughs, independently on the microbiological characteristics of semolina. Regarding the characterisation of the products obtained with the different semolinas, the trials with the modern genotypes showed almost comparable results, whereas the trials with the old genotypes were characterised by marked differences. However, the real effectiveness of the innovative method depends on the daily propagation of the liquid inoculum by the bread maker. Furthermore, it has to be considered that the production of sourdoughs at laboratory- or bakery-level might influence the composition of the fermenting microbiota (Minervini et al., 2012b).

Thus, works are being prepared to apply the system reported in this study at large scale in industrial bakeries and transfer this new technological approach for sourdough production to the bread makers.

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