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Industrial application of selected lactic acid bacteria isolated from local semolinas for typical sourdough bread production

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ABSTRACT

Four obligate heterofermentative lactic acid bacteria (LAB) strains (*Weissella cibaria* PON10030 and PON10032 and *Leuconostoc citreum* PON 10079 and PON10080) were tested as single strain starters, mono-species dual strain starters, and multiple strain starter for the preparation and propagation of sourdoughs for the production of a typical bread at industrial level. The kinetics of pH and TTA during the daily sourdough refreshments indicated a correct acidification process for all trials. The concentration of lactic and acetic acid increased consistently during fermentation. The resulting molar ratios between these two organic acids in the experimental trials were lower than those observed in the control trial. The microbiological investigation showed levels of approximately 10^9 CFU/mL in almost all sourdoughs and the comparison of the genetic polymorphisms of the dominating LAB with those of the pure cultures evidenced the persistence of the added strains over time. The resulting breads were evaluated for several quality parameters. The breads with the greatest height were obtained with the quadruple combination of leuconostocs and weissellas. The highest softness was registered for the breads obtained from fermentations performed by *W. cibaria* PON10032 alone and in combination. The different inocula influenced also the color, the void fraction, the cell density and the mean cell area of the breads. Different levels of acids, alcohols, aldehydes, esters, hydrocarbons, ketones, terpenes, furans and phenol were emitted by the breads. The sensory tests indicated the breads from the sourdoughs fermented with the seven LAB inocula as sweeter and less acidic than control breads and the breads from the trials with the highest complexity of LAB inoculums were those more appreciated by tasters. A multivariate approach found strong differences among the trials. In particular, control breads and the breads obtained with different starter LAB were quite distant and a more strict relation was found among the productions carried out by *W. cibaria* strains. This study proved the suitability of the selected strains of *L. citreum* and *W. cibaria* for industrial-scale level applications in sourdough bread production.

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1. Introduction

In recent years, new consumers are experiencing traditional and typical foods. These foods are often made with local ingredients in a given restricted geographic area through process technologies that belong to the cultural heritage of the indigenous populations (Settanni and Moschetti, 2014) and their preparation methods are expression of the local folklore (Weichselbaum et al., 2009). In Italy, several types of typical breads are produced; they differ for ingredients, recipes and technology of production (Minervini et al., 2010). The majority of these breads are produced applying the sourdough technology (Ottogalli et al., 1996). However, the use of the sourdough is well known in Italy, since a long time, also for the production of bakery products different from bread, such as pizza (Coppola et al., 1996) and several sweet baked goods (Foschino et al., 1999).

Sourdough is a complex matrix originated from a mixture of flour and water fermented mainly by indigenous lactic acid bacteria (LAB) and yeasts present in flour (Vogel et al., 1999; De Vuyst and Vancanneyt, 2007). Yeasts are primarily responsible for the leavening of dough, while LAB determine the process of acidification, even though heterofermentative LAB partly contribute to the mass blowing (Gobbetti et al., 1995). However, the composition of the sourdough microbiota is strongly influenced by a series of intrinsic

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and extrinsic factors (De Vuyst et al., 2014).

In contrast to the use of mostly homofermentative species of LAB in the majority of (fermented) food applications, heterofermentative species play a major role in sourdough fermentation (Salovaara, 1998), especially when sourdoughs are prepared in a traditional manner (Corsetti et al., 2003). Members of the *Lactobacillus* genus are frequently isolated from this ecosystem, including strains with different fermenting metabolisms, since they belong to species that are obligate homofermentative (OHo), obligate heterofermentative (OHe) or facultative heterofermentative (FHe) (Corsetti et al., 2005). For this reason, lactobacilli are reported to be the typical LAB associated to sourdough fermentation (Corsetti and Settanni, 2007). However, species belonging to *Leuconostoc, Weissella* and *Pediococcus*, and at a lesser extent also *Lactococcus, Enterococcus* and *Streptococcus* genera have been isolated from sourdoughs (De Vuyst and Neysens, 2005; Ehrmann and Vogel, 2005; Corsetti et al., 2007; Corsetti and Settanni 2007).

Within the OHe LAB, *Leuconostoc* and *Weissella* species are often found to dominate the sourdough ecosystems (Coppola et al., 1996). In particular, *Leuconostoc citreum* and *Weissella cibaria*, reported to be dominant among the LAB communities associated to the local flours (from *Triticum aestivum*) and semolinas (from *Triticum durum*) produced in Sicily (Alfonzo et al., 2013), are commonly present in sourdoughs of different countries (Coppola et al., 1996; De Vuyst et al., 2002; Robert et al., 2006; Valmorri et al., 2006; Iacumin et al., 2009; Choi et al., 2012). Several strains of these species of sourdough origin are producers of exopolysaccharides (Di Cagno et al., 2006; Maina et al., 2008; Galle et al., 2010; Choi et al., 2012) and might contribute to the structure of the final doughs.

In this study, strains of *L. citreum* and *W. cibaria*, previously isolated from local semolinas produced in Sicily (southern Italy) (Alfonzo et al., 2013) and selected for their pro-technological characteristics during experimental sourdough bread productions (Settanni et al., 2013), were applied at industrial-scale level in several mono- and dual-species combinations with the aim of evaluating the performances of OHe non-*Lactobacillus* species during driven sourdough fermentations for the production of a typical Sicilian bread, namely “Pane di Piana degli Albanesi” (PPA). The specific objectives of this work were to: (i) evaluate the acidification kinetics, the microbial populations and the organic acids generated during sourdough production and propagation; (ii) characterize the final breads produced when the sourdoughs reached their microbial stability for the quality parameters, volatile profiles and sensory attributes.

2. Materials and methods

2.1. Lactic acid bacteria, growth conditions and composition of starter cultures

Four LAB strains [*Weissella cibaria* PON10030 (W_1) and PON10032 (W_2); *Leuconostoc citreum* PON 10079 (L_1) and PON10080 (L_2)], belonging to the culture collection of the Department of Agricultural and Forestry Sciences – University of Palermo (Italy), were used in this study. The strains, were propagated in MRS broth (Oxoid, Milan, Italy) overnight at 30 °C. These LAB were tested as single strain starters (four trials: L_1; L_2; W_1; W_2), mono-species dual strain starters [two trials: $L_1 + L_2$ (L_{12}); $W_1 + W_2$ (W_{12})] and multiple strain starter [one trial: $L_1 + L_2 + W_1 + W_2$ (LW)], forming a total of seven different starter cultures, for the production of the typical sourdough PPA bread.

2.2. Sourdough production and sample collection

Starter cultures were prepared as follows: after growth, LAB were centrifuged at 5000*g* for 5 min, washed twice and resuspended in Ringer’s solution (Oxoid) until reaching an optical density at 600 nm of ca. 1.00, corresponding to a concentration of about 10^9 CFU mL^{-1}. Each sourdough was produced by mixing 10 L of tap water, 200 mL of the LAB inoculums and 10 kg of commercial semolina (Salvia Gaspare, Partinico, Italy) in a fermenter (FLNi Fermentatore, Novasilos S.r.l., Forlì, Italy). Each LAB inoculums was first diluted in water, heated at 28 °C, and then the semolina was added under agitation to obtain a homogenous dough with a starter concentration of approximately 10^6 CFU g^{-1}. The mass was fermented at 28 °C for 16 h (Sourdough 0). Ten kilograms of sourdough were refrigerated and used the day after for the first refreshment obtained adding 5 kg of semolina and 5 L of water and fermented for 16 h. Six refreshments were performed weekly for each LAB inoculums (Sourdoughs 1–6). A control sourdough production (trial CT) was performed as described for the experimental ones (trials L_1, L_2, W_1, W_2, L_{12}, W_{12}, and LW) inoculated with 200 g of the traditional sourdough refreshed by the company since two years used in place of the suspension of the selected LAB. Each production week was repeated after two months, for a total of two sourdough formations and 12 refreshments for each inoculums. Samples of sourdough were collected just after ingredient mixing (initial time, T_i) and at 16 h of fermentation (T_f).

2.3. Analyses of sourdoughs

Drop of pH was determined electrometrically with the pH meter BASIC 20+ (Crison Instrument S.A., Barcelona, Spain). Total titratable acidity (TTA) was determined by titration with 0.1 N NaOH and expressed in terms of mL of NaOH/10 g of sourdough. pH and TTA were measured in triplicate.

The concentration of lactic and acetic acid in sourdough samples were determined by high performance liquid chromatography (HPLC) analysis carried out as described by Alfonzo et al. (2013). To this purpose, 10 g of each sample were homogenised with 90 mL distilled H_2O and 10 mL of the resulting solution were added with 5 mL of 0.1 mmol/L $HClO_4$ solution. After centrifugation at 4.000 × g for 15 min at 15 °C, the supernatants were acidified to pH 3.0 ± 0.1 with 1 mmol/L $HClO_4$. Distilled H_2O was added to reach the final volume of 25 mL and the solutions were left in ice for 30 min before being filtered through 0.45 μm cellulose filters (Millipore). PerkinElmer software specific to the HPLC instrument (TotalChrom Workstation 2008 rev. 6.3.2) was used to acquire and process data. Chemical determinations were carried out in triplicate.

The molar ratio between lactic and acetic acid was also determined because it represents an important parameter, known as fermentation quotient (FQ), affecting the aroma profile of the sourdoughs (Corsetti and Settanni, 2007).

Microbiological determinations were carried out on 10 g of samples. Sourdoughs were first suspended in Ringer’s solution (90 mL) and homogenised in a stomacher (BagMixer® 400; Interscience, Saint Nom, France) for 2 min at maximum speed, and then subjected to the serial decimal dilutions. Cell suspensions were analysed for: total mesophilic count (TMC) on plate count agar (PCA), spread-plated and incubated aerobically at 30 °C for 72 h; LAB on modified-de Man-Rogosa-Sharpe (mMRS) agar, prepared from MRS 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, added with cycloheximide (10 mg mL^{-1}) to avoid fungal growth, pour-plated and incubated anaerobically at 30 °C for 48 h; and total yeasts on Wallerstein laboratory (WL) nutrient agar, added with chloramphenicol (0.05 mg mL^{-1}) to avoid

bacterial growth, spread-plated and incubated aerobically at 28 °C for 72 h. All media and chemical components were purchased from Oxoid. Plate counts were performed in duplicate.

2.4. Monitoring of the added strains

The dominance of the strains added in the trials L_1, L_2, L_{12}, W_1, W_2, W_{12} and LW was verified at the end of fermentation of the sixth refreshment applying the random amplified polymorphic DNA (RAPD)-PCR technique. After plate count, the colonies developed from the highest dilutions of the cell suspensions were randomly collected based on their appearance (at least three identical colonies), purified by successive sub-culturing on mMRS and tested for Gram reaction, performed by the KOH (3%, w/v) method, and catalase activity, determined with H_2O_2 (5%, v/v). The axenic broth cultures, grew overnight in the optimal conditions, were subjected to DNA extraction with the InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instruction. PCRs were carried out in a 25-μL reaction mix using the primer M13 as described by Settanni et al. (2012). The amplicons were separated by electrophoresis on 1.5% (wt/vol) agarose gels (Gibco BRL, Cergy Pontoise, France). GeneRuler 100 bp Plus DNA ladder (M Medical Srl, Milan, Italy) was loaded as molecular size marker. The gels were stained with the SYBR® safe DNA gel stain (Molecular probes, Eugene, OR, USA) and visualised by UV *trans*-illumination. The RAPD patterns were analysed using GelCompar II software version 6.5 (Applied-Maths, Saint-Marten-Latem, Belgium). The recognition of the added strains was performed by comparison of the polymorphic profiles of the isolates from the highest dilutions of the sourdoughs to those of *W. cibaria* PON10030 and PON10032 and *L. citreum* PON 10079 and PON10080. Colonies from the plate counts of sourdoughs from the control trial were also isolated and subjected to the comparison of the polymorphic profiles.

2.5. Bread production

Sourdough breads were produced applying the traditional recipe for PPA bread. Sourdoughs were considered ready to be used as leavening agent for PPA by the bread maker when the value of pH was approximately 4.1. The experimental sourdoughs were applied in PPA bread production at the sixth refreshment (S6). To prepare 10 kg of dough, with a dough yield (weight of the dough/weight of the flour × 100) of 175, 2.5 kg of sourdough developed with each starter culture were mixed with 5.7 kg of semolina and 1.8 L of warm (20–25 °C) tap water. The dough mass was divided into aliquots of 200 g which were placed in rectangular (60 mm × 40 mm × 4 mm) stainless steel baking pans. Fermentation was performed in a heated (30 °C) chamber for 5 h. Dough samples were collected before cooking (T_{bc}) and analysed for pH, TTA, lactic and acetic acid concentrations and microbiological determinations. Bread cooking was performed at 240 °C for 40 min in an industrial convection oven (Modular 80012 DH, Tornati Forno S.r.l, Montelabbate, Italy).

2.6. Bread analysis

After cooking, the resulting breads were cooled at ambient temperature and evaluated for several quality attributes. Breads were cut transversely in two halves and the central slice was measured with a caliper (Schober et al., 2005). Four points of the crust and three points of the crumb of the central slices were analysed by means of a colorimeter (Chroma Meter CR-400C, Minolta, Osaka, Japan) and the Hunter's scale parameters (L^*, a^* and b^*) were measured. The hardness of crumb was determined as reported by Corsetti et al. (2000) by means of the Instron-5564 (Instron Corp., Canton, MA).

Both central slices of each bread were scanned (Epson Perfection 4180 Photo, Seiko Epson Corp., Japan) with 350 dpi of resolution. The images were saved in TIFF format and analysed with the ImageJ software (National Institutes Health, Bethesda, Md, USA). After cropping to a square of 207 × 207 pixels (15 × 15 mm), the images were converted to grey-level (8 bit). The Otsu's threshold algorithm was applied and void fraction (the fraction of the total area corresponding to the bread pores), cell density (number of cells/cm^2) and mean cell area (in mm^2) were calculated.

Volatile organic compounds (VOCs) were determined applying the solid phase micro-extraction (SPME) isolation technique on 5 g of bread. Each sample was heated to 60 °C in a vial and the headspace was collected by a DBVCarboxen-PDMS fibers (Supelco, Bellefonte, PA) for 40 min. The SPME fibre was inserted into 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). The analyses were conducted as reported by Alfonzo et al. (2013). The identification of the compounds was performed following the methodology described by Settanni et al. (2013). All solvents and reagents were purchased from WWR International (Milan, Italy). All chemical and physical determinations on breads were performed in triplicate.

2.7. Sensory analysis

The final breads were subjected to the sensory analysis performed by a descriptive panel consisting of 12 tasters (four women and eight men; age, 22–62 years old) familiar with the sensory analysis of foods, but not specifically trained in the evaluation of sourdough breads. The analysis was carried out according to the guidelines in the ISO 6658. The judges were asked to score 20 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 color, crust thickness, crumb color, porosity, alveolation, alveolation uniformity, odor intensity, bread odor, unpleasant odor, aroma intensity, bread aroma, unpleasant aroma, salty, acid, astringent, bitter, taste persistency, adhesiveness in mouth, crispness and the overall assessment. The tasters expressed the intensity of each attribute on a 9-point hedonic scale (9 = extremely good; 1 = extremely bad).

2.8. Statistical analyses

Chemical, physical, technological, microbiological and sensory 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 starter LAB as repeated measures. 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 color 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 combination of strains, 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 mutual dissimilarity,

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) performed with data obtained from sourdoughs and breads. The number of principal factors was selected according to the Kaiser criterion (Jolliffe, 1986) and only factors with eigenvalues higher than 1.00 were retained. All data were preliminary evaluated by 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 was used for data processing and graphic construction.

3. Results

3.1. Evolution of chemical and microbiological parameters of sourdoughs

The kinetics of pH and TTA during sourdough formation and refreshment are reported in Fig. 1. Just after mixing (T_{i0}) of semolina with water and inocula, the pH values of all trials, including control production, were in the range 6.0–6.1. After 16 h of fermentation (T_{f0}), the pH dropped consistently, reaching the lowest value (4.1) for L_1 and L_{12} sourdoughs (Fig. 1B,D) and the highest (4.5) for W_2 sourdough (Fig. 1F). From the first refreshment onward, the initial pH did not exceed the value 4.6, which was registered only in correspondence of W_2 sourdough. From the third refreshment, all sourdoughs, except W_2, showed an almost stable behaviour in terms of pH at T_i, registered around 4.2–4.3, and pH at T_f, dropped at 4.0–4.1. W_2 sourdough reached the value 4.1 at the fourth refreshment. TTA data were correlated linearly with pH values. The highest increase of acidity was displayed after the first 16 h of fermentation for all trials. From the fifth refreshment, the final TTA exceeded 10 mL of NaOH/10 g for almost all sourdoughs except W_2, for which it was at the highest level (9 mL of NaOH/10 g) at the sixth refreshment. TTA of LW (Fig. 1H) sourdough was higher than 10 mL of NaOH/10 g from the fourth refreshment.

The concentration of organic acids (lactic and acetic acid) were determined soon after sourdough preparation and at each step of propagation also in order to establish the FQ resulting from the action of the different bacterial inocula (Table 1). At the end of each fermentation, lactic acid concentration registered for the control trial ranged between 3.92 and 7.01 mg/g which was generally higher than the levels recorded for all seven trials inoculated with the selected LAB, alone as well as in combination. The level of lactic acid in the inoculated trials did not exceed 4.38 mg/g, registered at the sixth refreshment for sourdough W_{12}. On the contrary, the concentrations of acetic acid displayed by the control trial was, on average, lower than those detected in the other sourdoughs during production and propagation, with the exception of the trials L_1 and, especially W_2 for which the highest level registered for this acid was 0.67 and 0.50 mg/g, respectively. In general, the concentrations of both organic acids increased with the number of bacteria inoculated. In particular, the generation of acetic acid increased more than the production of lactic acid for the trials with the multiple combination of leuconostocs and weissellas together. Regarding the FQ, values below 3.0 characterised the refreshments of almost all inoculated trials, except the trial L_1 for which this parameter was between 3.56 and 3.98. In particular, the lowest FQ, ranging from 2.03 to 2.89, was found for all fermented sourdoughs of the trial LW. The FQ of the control sourdoughs was higher than those registered in presence of the added selected LAB with values between 5.03 and 6.69.

The microbiological investigation during the different steps of sourdough production is reported in Fig. 2. The initial levels of LAB in the sourdoughs started with the control inoculums was at around 10^6 CFU/g, while higher levels were reached in the trials inoculated with the selected LAB. In particular, the highest cell densities for this group (7.7 Log CFU/g) were displayed by the trial LW. The final level of LAB at each fermentation step was abundantly above 8.0 Log CFU/g for almost all trials, except the trial L_2 that was characterised by 8.0 Log CFU/g as the maximum result for plate count. From the second step of propagation, the behaviour of LAB was quite constant for all trials indicating the persistence of the starter cultures. The trend of TMM followed that of LAB, but the levels were a bit lower. Regarding yeast populations, they started from an initial level in the range 2.1–2.8 Log CFU/g and increased their concentrations at each step of sourdough propagation, reaching a final density at the last refreshment of 6.2–6.6 Log CFU/g. In particular, yeasts stabilised their levels at 10^6 CFU/g for all trials from the fifth refreshment.

3.2. Persistence of the starter strains

The RAPD profiles of the isolates collected after plate counts were compared to those of the pure cultures of the added LAB (Fig. 3), in order to evaluate their persistence. The direct comparison of the polymorphic profiles of the isolates from all trials performed with single strains (L_1, L_2, W_1 and W_2) indicated that the LAB populations were dominated by the strain inoculated. The trial L_{12} was dominated by *L. citreum* PON 10079, while the trial W_{12} showed both *W. cibaria* strains at similar levels. The sourdoughs of the trial with the quadruple inoculums showed the dominance of *L. citreum* PON 10079 and *W. cibaria* PON10032 over the other starter strains. However, some isolates of *W. cibaria* PON10030 were detected only at 1 Log lower than the dominant strains in sourdough of the trial LW. The four RAPD profiles of the added LAB were not found associated to any isolate from the control sourdoughs, confirming that the dominant microbiota of this trial did not included the strains used to develop the experimental trials.

3.3. Characteristics of the experimental breads

After baking, the experimental PPA breads were cooled for about 50 min at room temperature and subjected to the evaluation of the quality characteristics. The results of the characterization of the final breads are reported in Table 2. The height of the breads, at the central slices, ranged between 38.25 and 53.00 mm. The lowest height was registered for the breads obtained after fermentation with *L. citreum* PON10080. The inoculum *W. cibaria* PON10032 generated breads comparable to those of the control trial. The breads with the greatest height were obtained with the quadruple combination of leuconostocs and weissellas. Furthermore, the double combination of leuconostocs determined the production of breads with almost the same height of those fermented by *L. citreum* PON10079, while the breads obtained from the double *W. cibaria* inocula were as tall as the breads produced with *W. cibaria* PON10032 alone.

The three color parameters of the crust and the parameter b^* of the crumb were comparable for all breads. A few differences were registered for the parameter L^* and a^* of the crumb. In particular, all experimental PPAs resulted similar to one another and all different from the control breads for the parameter a^*, while all breads obtained with weissellas, in single and double combinations, were clearly distinguished from all other breads with regards to the parameter L^*.

The firmness of the breads varied from 11.05 to 18.62 N. The softest breads were those obtained from sourdoughs fermented by *W. cibaria* PON10032 and those from all trials with multiple combinations of strains. The breads from the trial carried out in

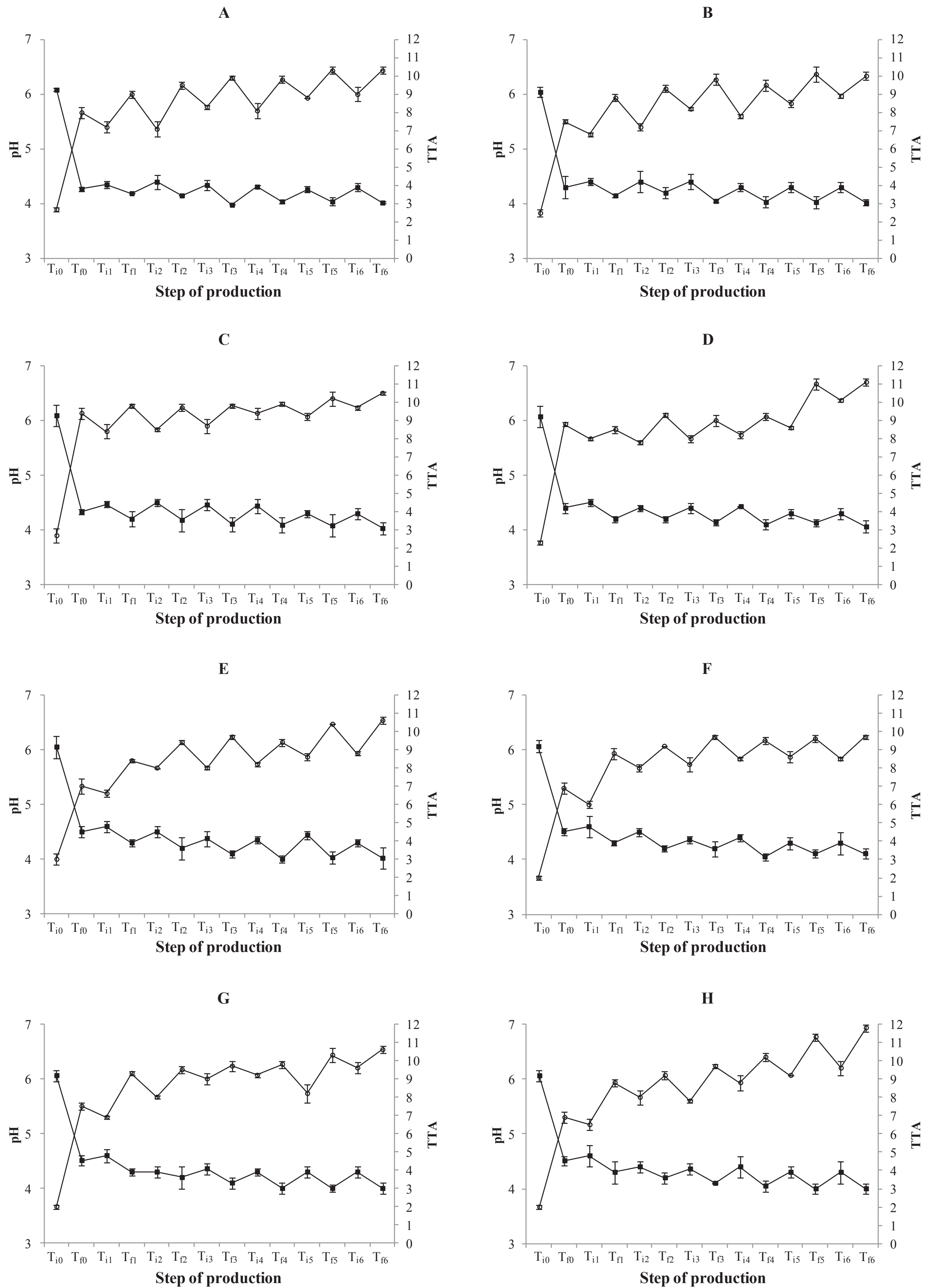

Fig. 1. Kinetics of acidification during sourdough production. **A**, Control; **B**, *Leuconostoc citreum* PON10079; **C**, *L. citreum* PON10080; **D**, *L. citreum* PON10079 + *L. citreum* PON10080; **E**, *Weissella cibaria* PON10030; **F**, *W. cibaria* PON10032; **G**, *W. cibaria* PON10030 + *W. cibaria* PON10032; **H**, *L. citreum* PON10079 + *L. citreum* PON10080 + *W. cibaria* PON10030 + *W. cibaria* PON10032. Symbols: ■, pH; ○, TTA.

Table 1
Organic acids produced during sourdough production and propagation.

Trials	Time													
	T_{i0}	T_{f0}	T_{i1}	T_{f1}	T_{i2}	T_{f2}	T_{i3}	T_{f3}	T_{i4}	T_{f4}	T_{i5}	T_{f5}	T_{i6}	T_{f6}
CT														
Lactic acid	0.53 ± 0.08	4.40 ± 0.04	3.60 ± 0.04	3.92 ± 0.04	3.47 ± 0.07	5.52 ± 0.09	5.10 ± 0.09	6.04 ± 0.06	5.35 ± 0.06	6.96 ± 0.09	6.24 ± 0.07	6.79 ± 0.08	6.27 ± 0.09	7.01 ± 0.07
Acetic acid	0.13 ± 0.01	0.48 ± 0.05	0.35 ± 0.01	0.52 ± 0.02	0.43 ± 0.02	0.55 ± 0.01	0.42 ± 0.01	0.62 ± 0.01	0.69 ± 0.01	0.74 ± 0.02	0.68 ± 0.03	0.72 ± 0.02	0.64 ± 0.02	0.72 ± 0.02
FQ	3.21	6.11	8.86	5.03	5.38	6.69	8.09	6.49	5.17	6.27	6.12	6.29	6.53	6.49
L_1														
Lactic acid	0.23 ± 0.01	2.85 ± 0.02	2.08 ± 0.04	3.56 ± 0.08	2.81 ± 0.04	3.54 ± 0.06	2.85 ± 0.04	3.70 ± 0.08	2.84 ± 0.04	3.68 ± 0.08	2.77 ± 0.04	3.74 ± 0.08	2.69 ± 0.05	3.58 ± 0.04
Acetic acid	0.00 ± 0.00	0.53 ± 0.01	0.46 ± 0.01	0.63 ± 0.02	0.48 ± 0.02	0.66 ± 0.03	0.53 ± 0.02	0.62 ± 0.09	0.53 ± 0.01	0.64 ± 0.03	0.52 ± 0.02	0.66 ± 0.02	0.51 ± 0.02	0.67 ± 0.01
FQ	n.d.	3.58	3.01	3.77	3.90	3.58	3.58	3.98	3.57	3.83	3.55	3.78	3.52	3.56
L_2														
Lactic acid	0.34 ± 0.02	3.15 ± 0.03	2.28 ± 0.02	3.76 ± 0.04	2.92 ± 0.03	3.74 ± 0.04	2.95 ± 0.03	3.70 ± 0.04	2.74 ± 0.03	3.88 ± 0.04	3.07 ± 0.03	3.74 ± 0.04	2.99 ± 0.03	3.78 ± 0.04
Acetic acid	0.05 ± 0.00	0.64 ± 0.01	0.54 ± 0.00	0.67 ± 0.01	0.56 ± 0.01	0.75 ± 0.01	0.61 ± 0.01	0.78 ± 0.01	0.64 ± 0.01	0.84 ± 0.01	0.71 ± 0.01	0.87 ± 0.01	0.73 ± 0.01	0.86 ± 0.01
FQ	4.53	3.28	2.81	3.74	3.48	3.32	3.22	3.16	2.85	3.08	2.88	2.87	2.73	2.93
L_{12}														
Lactic acid	0.27 ± 0.02	3.26 ± 0.03	2.58 ± 0.08	3.53 ± 0.04	2.72 ± 0.03	3.84 ± 0.09	3.07 ± 0.04	3.79 ± 0.08	2.94 ± 0.03	3.78 ± 0.07	3.17 ± 0.03	3.74 ± 0.09	2.81 ± 0.03	3.98 ± 0.04
Acetic acid	0.10 ± 0.00	0.62 ± 0.01	0.51 ± 0.01	0.69 ± 0.01	0.56 ± 0.01	0.77 ± 0.03	0.66 ± 0.01	0.82 ± 0.01	0.71 ± 0.01	0.86 ± 0.02	0.75 ± 0.01	0.90 ± 0.02	0.75 ± 0.02	0.86 ± 0.01
FQ	1.80	3.51	3.37	3.41	3.24	3.32	3.10	3.08	2.76	2.93	2.82	2.77	2.50	3.09
W_1														
Lactic acid	0.34 ± 0.01	2.45 ± 0.03	2.02 ± 0.02	3.14 ± 0.02	2.43 ± 0.03	3.74 ± 0.11	2.78 ± 0.08	3.79 ± 0.09	2.98 ± 0.03	3.88 ± 0.11	2.91 ± 0.03	3.79 ± 0.08	2.92 ± 0.05	3.68 ± 0.05
Acetic acid	0.00 ± 0.00	0.43 ± 0.00	0.36 ± 0.01	0.58 ± 0.01	0.42 ± 0.01	0.68 ± 0.02	0.55 ± 0.06	0.72 ± 0.02	0.58 ± 0.01	0.79 ± 0.03	0.66 ± 0.01	0.86 ± 0.01	0.71 ± 0.01	0.84 ± 0.02
FQ	n.d.	3.80	3.74	3.61	3.86	3.67	3.37	3.51	3.43	3.27	2.94	2.94	2.74	2.92
W_2														
Lactic acid	0.25 ± 0.00	2.83 ± 0.02	2.05 ± 0.02	3.42 ± 0.03	2.58 ± 0.03	3.85 ± 0.08	2.84 ± 0.03	3.86 ± 0.04	3.10 ± 0.03	3.85 ± 0.09	2.92 ± 0.03	3.56 ± 0.06	2.86 ± 0.03	3.44 ± 0.08
Acetic acid	0.00 ± 0.00	0.26 ± 0.01	0.17 ± 0.00	0.33 ± 0.01	0.25 ± 0.00	0.41 ± 0.02	0.32 ± 0.01	0.48 ± 0.05	0.34 ± 0.01	0.42 ± 0.03	0.33 ± 0.01	0.47 ± 0.02	0.37 ± 0.01	0.50 ± 0.00
FQ	n.d.	7.26	8.04	6.91	6.88	6.26	5.92	5.36	6.08	6.11	5.90	5.05	5.15	4.59
W_{12}														
Lactic acid	0.30 ± 0.01	3.07 ± 0.03	2.35 ± 0.02	3.83 ± 0.04	2.96 ± 0.02	4.14 ± 0.09	3.37 ± 0.04	4.19 ± 0.03	3.24 ± 0.03	4.28 ± 0.07	3.29 ± 0.03	4.19 ± 0.07	3.31 ± 0.03	4.38 ± 0.04
Acetic acid	0.10 ± 0.00	0.72 ± 0.00	0.61 ± 0.02	0.79 ± 0.02	0.68 ± 0.01	0.87 ± 0.02	0.76 ± 0.02	0.92 ± 0.01	0.80 ± 0.01	0.96 ± 0.01	0.85 ± 0.02	0.94 ± 0.02	0.82 ± 0.02	0.96 ± 0.01
FQ	2.00	2.84	2.57	3.23	2.90	3.17	2.96	3.04	2.70	2.97	2.58	2.97	2.69	3.04
LW														
Lactic acid	0.35 ± 0.02	3.32 ± 0.07	2.49 ± 0.03	3.85 ± 0.03	3.06 ± 0.03	4.12 ± 0.09	3.47 ± 0.06	4.09 ± 0.00	3.19 ± 0.04	4.02 ± 0.04	3.32 ± 0.03	4.10 ± 0.11	3.30 ± 0.03	4.08 ± 0.06
Acetic acid	0.10 ± 0.00	0.79 ± 0.02	0.69 ± 0.02	0.89 ± 0.01	0.78 ± 0.02	0.95 ± 0.02	0.84 ± 0.02	0.99 ± 0.05	0.85 ± 0.02	1.06 ± 0.01	0.95 ± 0.01	1.14 ± 0.03	1.02 ± 0.01	1.34 ± 0.02
FQ	2.33	2.80	2.41	2.88	2.62	2.89	2.75	2.75	2.50	2.53	2.33	2.40	2.16	2.03
Statistical significance														
Lactic acid	**	***	***	*	**	***	*	***	***	***	***	***	***	***
Acetic acid	N.S.	***	*	*	*	*	*	*	*	**	*	**	**	*

Abbreviations: CT, control trial; L_1, *Leuconostoc citreum* PON10079; L_2, *L. citreum* PON10080; L_{12}, *L. citreum* PON10079 + *L. citreum* PON10080; W_1, *Weissella cibaria* PON10030; W_2, *W. cibaria* PON10032; W_{12}, *W. cibaria* PON10030 + *W. cibaria* PON10032; LW, *L. citreum* PON10079 + *L. citreum* PON10080 + *W. cibaria* PON10030 + *W. cibaria* PON10032; FQ, fermentation quotient; n.d., not determined.
Results indicate mean values ± SD of six measurements (carried out in triplicate for two independent fermentations).
P value: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; N.S., not significant.

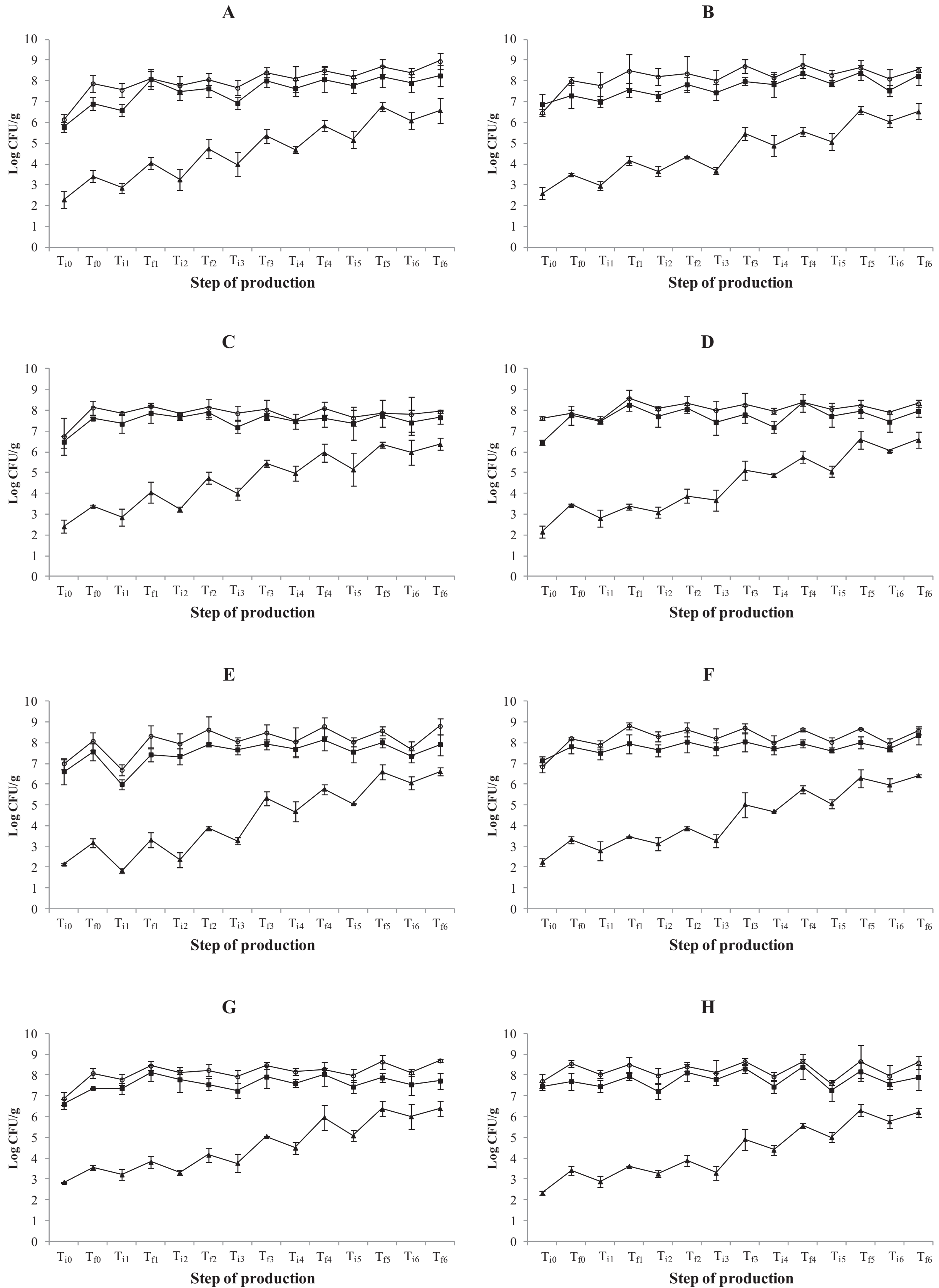

Fig. 2. Levels of microorganisms during sourdough production. **A**, Control; **B**, *Leuconostoc citreum* PON10079; **C**, *L. citreum* PON10080; **D**, *L. citreum* PON10079 + *L. citreum* PON10080; **E**, *Weissella cibaria* PON10030; **F**, *W. cibaria* PON10032; **G**, *W. cibaria* PON10030 + *W. cibaria* PON10032; **H**, *L. citreum* PON10079 + *L. citreum* PON10080 + *W. cibaria* PON10030 + *W. cibaria* PON10032. Symbols: ■, TMM on PCA; ○, LAB on mMRS agar; ▲, yeasts on WL agar.

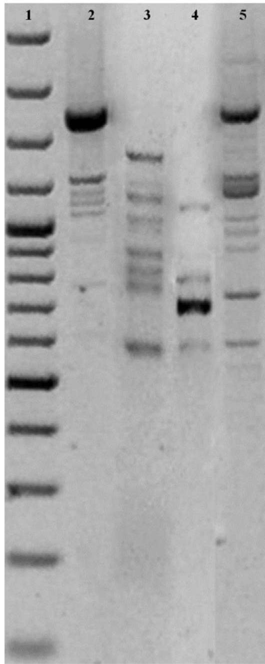

Fig. 3. RAPD-PCR profiles of LAB added as starter strains obtained with primer M13. Lanes: 1, GeneRuler 100 bp plus DNA ladder; 2, *Weissella cibaria* PON10030; 3, *W. cibaria* PON10032; 4, *L. citreum* PON10079; 5, *L. citreum* PON10080.

presence of *L. citreum* PON10080 were harder than control breads.

The image analysis evidenced some differences among the breads. The breads from the trials L_1, L_{12}, W_2, W_{12} and LW were characterised by a similar void fraction. The cell density was different among all breads, but the mean cell area showed a high similarity of the breads obtained with the multiple (dual and quadruple) strain combinations.

The breads produced in this study emitted 39 VOCs belonging to 9 classes of chemicals, including acids, alcohols, aldehydes, esters, hydrocarbons, ketones, terpenes, furans and phenol (Table S1). Fig. 4 shows the dendrogram resulting from the cluster analysis and the heat map; the relationships among the breads are based on the amount of each VOC. The cluster analysis determined the formation of three main clusters. All breads produced with the LAB inoculated singly were included into a single cluster. The breads obtained with the double *W. cibaria* strain combination clustered with those made with all strains together, while the breads from trial L_{12} were closed to those of the control trial. Alcohols (30.60–61.37%), aldehydes (19.11–40.28%), and esters (4.09–23.68%) constituted the major classes of VOCs for all breads. The class of acids represented a consistent percentage of the VOCs of the breads obtained with the leuconostocs together, both in dual as well as in quadruple inocula. The double inoculums of weissellas generated the lesser amount of acids in the final breads (barely 3.20%), but determined the highest percentage of esters (23.68%) among the breads of the different trials. The classes of hydrocarbons and ketones were represented only by two compounds each, while D-limonene was the only compound detected for terpenes. The compound found at the highest concentrations in all breads was phenylethyl alcohol, ranging from 14.64 to 24.23% of total VOCs. The other compounds found at consistent percentages among the majority of breads were ethanol, hexanal, 3-methyl-1-butanol, nonanal, ethyl octanoate, acetic acid, furfuraldehyde, ethyl decanoate, furfuryl alcohol, 6-methyl5-hepten-2-ol, benzaldehyde, 2-nonenal and 5-methylfurfural. The breads from trial W_2 were the only samples that did not emit isophthalaldehyde, while those from trial L_2 did

Table 2
Characteristics of breads.

Strain	Height (mm)	Crust color			Crumb color			Firmness value (N)	Void fraction (%)	Cell density (n cm^{-2})	Mean cell area (mm^2)
		L*	a*	b*	L*	a*	b*				
CT	43.50 ± 2.65^{bc}	52.27 ± 5.78^{a}	10.73 ± 2.33^{a}	26.12 ± 4.87^{ab}	62.83 ± 4.16^{ab}	-2.33 ± 0.25^{b}	14.42 ± 1.17^{a}	16.31 ± 2.55^{ab}	30.00 ± 0.12^{c}	38.33 ± 0.57^{f}	0.26 ± 0.02^{a}
L_1	49.75 ± 2.50^{ab}	59.88 ± 5.80^{a}	8.50 ± 2.02^{a}	28.48 ± 4.81^{ab}	63.09 ± 3.64^{ab}	-1.60 ± 0.23^{a}	14.18 ± 0.96^{a}	13.71 ± 2.43^{ab}	44.79 ± 0.87^{a}	47.67 ± 0.04^{e}	0.28 ± 0.02^{a}
L_2	38.25 ± 2.06^{c}	60.74 ± 4.35^{a}	8.37 ± 3.10^{a}	33.43 ± 3.16^{a}	70.54 ± 1.83^{a}	-1.91 ± 0.20^{ab}	16.68 ± 1.23^{a}	18.62 ± 2.36^{a}	29.37 ± 0.03^{c}	69.33 ± 0.01^{b}	0.32 ± 0.05^{a}
L_{12}	51.25 ± 1.26^{a}	55.53 ± 5.55^{a}	8.93 ± 2.48^{a}	25.07 ± 3.17^{ab}	65.44 ± 2.47^{ab}	-2.12 ± 0.19^{ab}	16.32 ± 1.05^{a}	11.56 ± 0.90^{b}	47.50 ± 0.31^{a}	73.00 ± 0.64^{a}	0.22 ± 0.02^{b}
W_1	44.25 ± 3.10^{bc}	54.92 ± 3.21^{a}	9.03 ± 2.05^{a}	27.71 ± 4.44^{ab}	61.72 ± 2.70^{b}	-1.91 ± 0.14^{ab}	16.48 ± 1.30^{a}	17.62 ± 1.03^{a}	40.08 ± 0.50^{b}	39.00 ± 0.45^{f}	0.27 ± 0.06^{a}
W_2	47.75 ± 0.96^{ab}	57.39 ± 4.07^{a}	9.03 ± 5.62^{a}	23.61 ± 6.50^{ab}	61.65 ± 1.41^{b}	-1.85 ± 0.06^{ab}	16.78 ± 1.02^{a}	11.40 ± 1.54^{b}	47.37 ± 0.85^{a}	50.67 ± 0.79^{d}	0.25 ± 0.05^{a}
W_{12}	51.50 ± 1.91^{a}	57.00 ± 3.56^{a}	7.13 ± 1.25^{a}	21.99 ± 1.51^{ab}	62.08 ± 2.02^{b}	-2.11 ± 0.17^{ab}	17.51 ± 1.84^{a}	11.82 ± 1.46^{b}	46.66 ± 0.65^{a}	31.33 ± 0.61^{g}	0.21 ± 0.03^{b}
LW	53.00 ± 1.63^{a}	56.58 ± 0.93^{a}	6.25 ± 1.30^{a}	21.26 ± 0.82^{b}	65.12 ± 1.21^{ab}	-1.76 ± 0.09^{a}	15.64 ± 0.43^{a}	11.05 ± 0.70^{b}	48.08 ± 1.06^{a}	61.00 ± 0.09^{c}	0.21 ± 0.04^{b}
Statistical significance	$P < 0.001$	N.S.	N.S.	$P < 0.01$	$P < 0.01$	$P < 0.01$	N.S.	***	***	***	**

Abbreviations: CT, control trial; L_1, *Leuconostoc citreum* PON10079; L_2, *L. citreum* PON10080; L_{12}, *L. citreum* PON10079 + *L. citreum* PON10080; W_1, *Weissella cibaria* PON10030; W_2, *W. cibaria* PON10032; W_{12}, *W. cibaria* PON10030 + *W. cibaria* PON10032; LW, *L. citreum* PON10079 + *L. citreum* PON10080 + *W. cibaria* PON10030 + *W. cibaria* PON10032.
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; N.S., not significant.

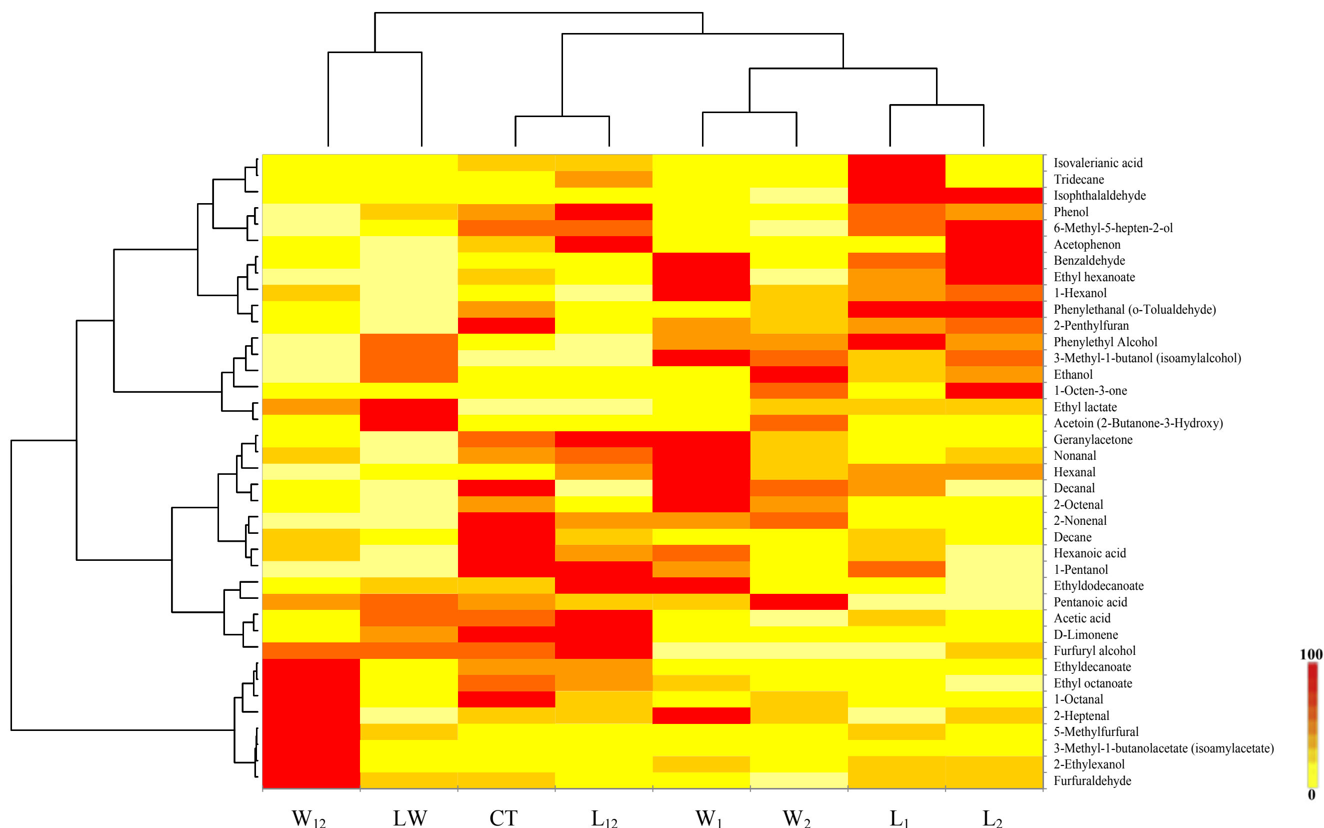

Fig. 4. Distribution of the volatile organic compounds among breads from the different trials. The double hierarchical dendrogram is based on the values of VOCs. The heat map plot depicts the relative percentage of each compound within each sourdough. Abbreviations: CT, control trial; L_1, *Leuconostoc citreum* PON10079; L_2, *L. citreum* PON10080; L_{12}, *L. citreum* PON10079 + *L. citreum* PON10080; W_1, *Weissella cibaria* PON10030; W_2, *W. cibaria* PON10032; W_{12}, *W. cibaria* PON10030 + *W. cibaria* PON10032; LW, *L. citreum* PON10079 + *L. citreum* PON10080 + *W. cibaria* PON10030 + *W. cibaria* PON10032.

not generate hexanoic acid, ethyl octanoate and ethyl dodecanoate. Tridecane was found only for the breads from the trials L_1 and L_{12}. D-limonene, registered at 5.76% in control breads, was found, at lower levels, only in L_{12} and LW breads.

3.4. Sensory attributes of breads

The judges evaluated all breads for their sensory profiles. The results of the sensory tests are reported in Table 3. The breads were quite different from one another for 18 attributes out of the 22 object of evaluation. Except strange aroma sensation, whose differences among judges were not statistically significant, strange odor, astringent and bitter taste were recognised as different both among breads and judges. The porosity of the breads obtained with leuconostocs was found to be highly similar to that of the control breads. All the breads obtained within the trials inoculated with the selected LAB were characterised by high scores of odor intensity and bread odor. Similar results were registered for aroma intensity, bread aroma and taste persistency. The breads from the sourdoughs fermented with the seven LAB inocula were also sweeter than control bread, but the acid sensation was less pronounced. The overall assessment clearly indicated the breads from the trial LW as those more appreciated.

3.5. Multivariate analysis

The data from the eight trials were found to be statistically different by the Barlett's sphericity test. The HCA, that is an exploratory data analysis tool which aims at sorting different objects into groups in a way that the degree of association between two objects is maximal if they belong to the same group and minimal otherwise (Dziedzic et al., 2016), indicated the eight trials as separate objects cutting the hierarchical tree diagram at 77.7% (Fig. 5). Furthermore, with the cut-off line at 95%, control breads and the breads obtained with the quadruple starter LAB inoculums clustered separately from the other breads obtained with single and dual combinations of LAB (included into a single cluster).

The results of PCAn showed seven eigen-values higher than 1, with the first four accounting for 82.60% of total variability (data not showed). However, Factor 1 and Factor 2 together explained 53.42% of total variability. For this reason, the 44 variables were expressed as linear combination of the first two factors.

The score plot (Fig. 6A) clearly shows the far distance among the breads produced with different LAB strains and the control breads. A strict relation was found between W_1 and W_{12} experimental breads. In particular, the highest differences were found for control and LW breads along Factor 1 which has the highest incidence (34.91%) on the total variability. Another relevant difference was found for the breads of trial L_2, although they differed from the other breads mainly along Factor 2 which has a lower incidence (18.51%) than Factor 1. As shown by the loading plot (Fig. 6B), the Factor 1 was mainly affected by bread aroma, odor intensity, strange odor and sweetness that showed the highest loading values, while the variability associated to Factor 2 was mainly explained by porosity, ketones, crust color and overall assessment.

Table 3
Evaluation of the sensory attributes of the experimental breads.

Attributes	Trial								SEM	Statistical significance	
	Control	L_1	L_2	L_{12}	W_1	W_2	W_{12}	LW		Judges	Bread
Crust color	4.25^{b}	3.83^{c}	3.47^{d}	4.06^{b}	4.53^{a}	4.47^{a}	4.05^{b}	3.61^{d}	0.22	**	***
Crumb color	4.03^{bc}	3.34^{d}	3.83^{c}	3.36^{d}	4.39^{a}	4.13^{b}	4.42^{a}	3.42^{d}	0.26	**	***
Crust thickness	3.71^{b}	3.34^{d}	2.92^{f}	3.54^{c}	4.03^{a}	3.94^{a}	3.56^{bc}	3.14^{e}	0.22	**	***
Porosity	4.18^{cd}	4.12^{d}	4.32^{c}	4.28^{cd}	6.07^{a}	6.08^{a}	5.85^{b}	6.07^{a}	0.56	***	***
Alveolation	5.68^{a}	4.84^{c}	5.74^{a}	3.73^{d}	5.26^{b}	5.29^{b}	5.79^{a}	3.75^{d}	0.49	***	***
Regularity alveolation	5.28^{d}	5.89^{b}	5.01^{e}	6.87^{a}	5.65^{c}	5.56^{c}	4.88^{e}	6.86^{a}	0.44	***	***
Odor Intensity	4.54^{e}	5.37^{b}	4.85^{d}	5.61^{a}	5.14^{c}	5.55^{a}	5.09^{c}	5.66^{a}	0.23	**	***
Bread odor	3.95^{e}	4.76^{b}	4.23^{d}	5.07^{a}	4.55^{c}	4.96^{a}	4.47^{c}	5.09^{a}	0.24	**	***
Strange odor	1.33^{a}	1.14^{a}	1.23^{a}	1.11^{a}	1.25^{a}	1.19^{a}	1.28^{a}	1.12^{a}	0.05	N.S.	N.S.
Crumb elasticity	3.34^{d}	4.74^{b}	3.43^{cd}	4.82^{ab}	3.55^{c}	4.74^{b}	3.43^{cd}	4.89^{a}	0.42	***	***
Aroma intensity	4.56^{c}	5.35^{b}	4.81^{d}	5.66^{ab}	5.16^{d}	5.52^{b}	5.03^{d}	5.68^{a}	0.23	**	***
Bread aroma	3.53^{f}	4.39^{c}	3.83^{e}	4.63^{b}	4.14^{d}	4.55^{b}	4.08^{d}	4.69^{a}	0.24	**	***
Strange aroma	1.39^{a}	1.11^{b}	1.23^{ab}	1.14^{ab}	1.26^{ab}	1.14^{ab}	1.23^{ab}	1.12^{b}	0.05	N.S.	**
Sweet	2.53^{d}	3.82^{a}	2.85^{c}	3.76^{a}	3.13^{b}	3.79^{a}	3.13^{b}	3.82^{a}	0.29	**	***
Salty	1.69^{b}	1.83^{b}	1.66^{b}	2.25^{a}	1.77^{b}	2.14^{a}	1.78^{b}	2.12^{a}	0.13	*	**
Acid	1.44^{a}	1.25^{ab}	1.28^{ab}	1.21^{ab}	1.23^{ab}	1.39^{ab}	1.14^{b}	1.23^{ab}	0.06	*	**
Astringent	1.31^{a}	1.36^{a}	1.24^{a}	1.25^{a}	1.31^{a}	1.29^{a}	1.28^{a}	1.38^{a}	0.03	N.S.	N.S.
Bitter	1.34^{a}	1.32^{a}	1.27^{a}	1.23^{a}	1.39^{a}	1.26^{a}	1.28^{a}	1.34^{a}	0.03	N.S.	N.S.
Taste persistency	2.36^{b}	2.82^{a}	2.48^{b}	2.85^{a}	2.89^{a}	2.95^{a}	2.48^{b}	2.94^{a}	0.14	*	*
Crispy crust	2.95^{b}	2.54^{c}	2.17^{d}	2.77^{b}	3.29^{a}	3.16^{a}	2.79^{b}	2.34^{d}	0.22	**	***
Adhesiveness (mouth)	1.85^{a}	1.88^{a}	1.65^{b}	1.61^{bc}	1.12^{d}	1.16^{d}	1.45^{c}	1.16^{d}	0.18	*	***
Overall assessment	5.06^{c}	5.08^{c}	5.06^{c}	5.05^{c}	5.62^{b}	6.05^{a}	5.53^{b}	6.13^{a}	0.26	**	**

Abbreviations: CT, control trial; L_1, *Leuconostoc citreum* PON10079; L_2, *L. citreum* PON10080; L_{12}, *L. citreum* PON10079 + *L. citreum* PON10080; W_1, *Weissella cibaria* PON10030; W_2, *W. cibaria* PON10032; W_{12}, *W. cibaria* PON10030 + *W. cibaria* PON10032; LW, *L. citreum* PON10079 + *L. citreum* PON10080 + *W. cibaria* PON10030 + *W. cibaria* PON10032. Result indicate mean value.
Data within a line 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.

4. Discussion

All baked products made with sourdough technology are characterised by a regional variability. Sicily is a southern Italian region with a long history of production of baked goods, probably due to the availability of wheat flour. In fact, Sicily was considered the granary of the Roman Empire (Katz and Weaver, 2003). This region condensed different cultures for its central position in the Mediterranean Sea. For this reason, Sicily offers a wide range of choice of traditional and typical breads and other products. In the last years,

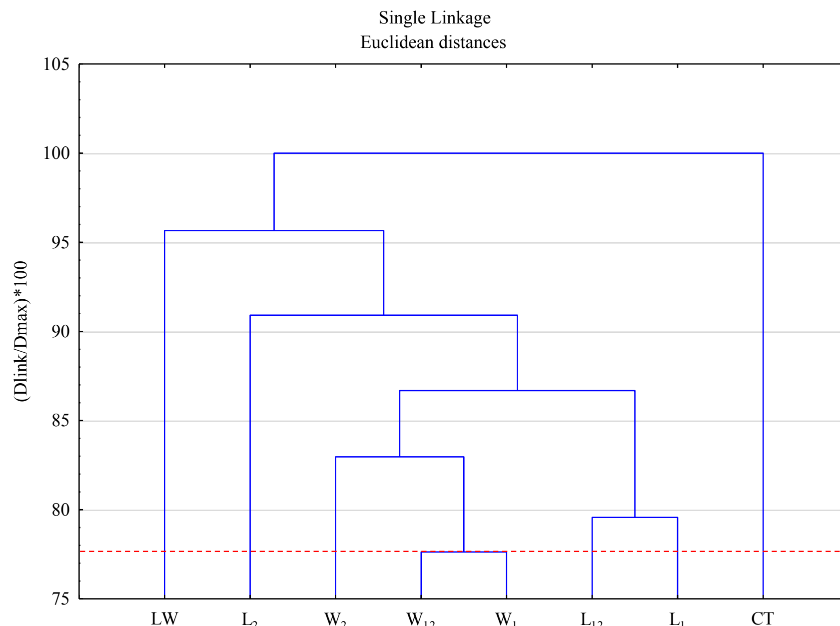

Fig. 5. Dendrogram resulting from hierarchical cluster analysis on 44 variables determined on sourdoughs and breads. Abbreviations: D, distance; link, linkage; max, maximum of linkage Euclidean distance; L_1, *Leuconostoc citreum* PON10079; L_2, *L. citreum* PON10080; L_{12}, *L. citreum* PON10079 + *L. citreum* PON10080; W_1, *Weissella cibaria* PON10030; W_2, *W. cibaria* PON10032; W_{12}, *W. cibaria* PON10030 + *W. cibaria* PON10032; LW, *L. citreum* PON10079 + *L. citreum* PON10080 + *W. cibaria* PON10030 + *W. cibaria* PON10032. The dashed red line indicates the cut-off level to separate the eight trials as distinct objects. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

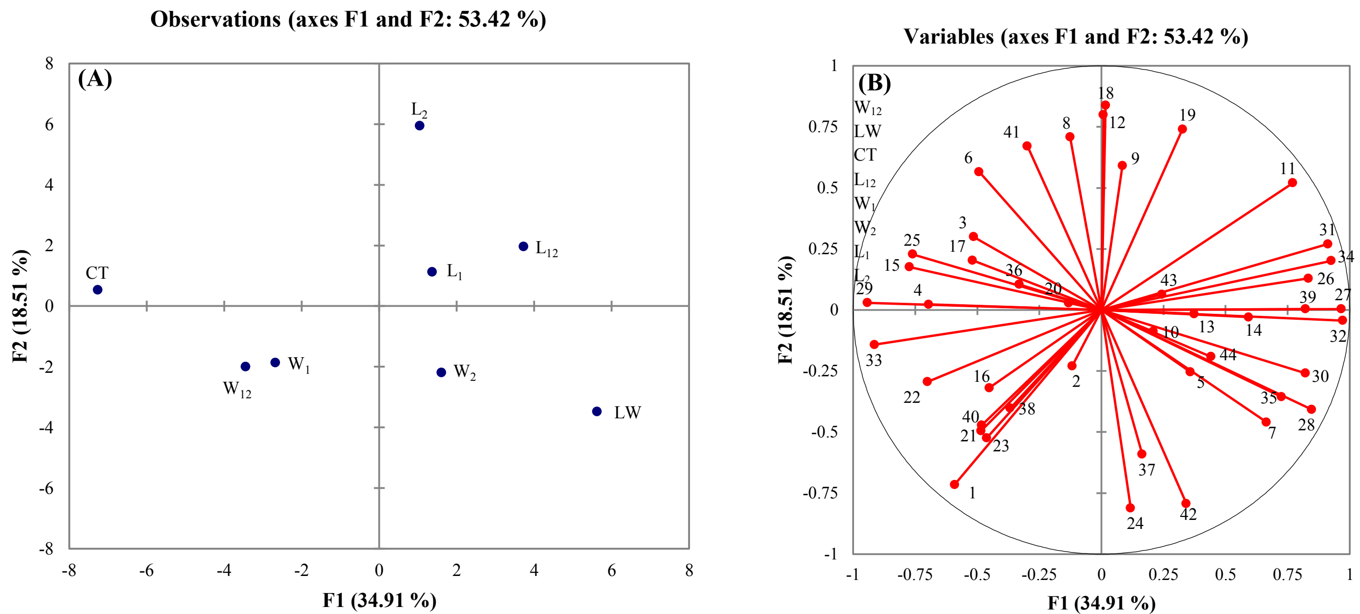

Fig. 6. Loading plot (A) and score plot (B) resulting from principal component analysis on 44 variables determined on sourdoughs and breads. Abbreviations: numbers in the loading plots: 1, m-MRS; 2, PCA; 3, WL; 4, Lactic acid; 5, Acetic acid; 6, Firmness; 7, Height; 8, Crust color; 9, Crumb color; 10, Void fraction; 11, Cell density; 12, Mean cell area; 13, Acids; 14, Alcohol; 15, Aldehydes; 16, Esters; 17, Hydrocarbons; 18, Ketones; 19, Phenol; 20, Terpens; 21, Crust color (sensory analysis); 22, Crumb color (sensory analysis); 23, Crust thickness; 24, Porosity; 25, Alveolation; 26, Alveolation regularity; 27, Odor Intensity; 28, Bread odor; 29, Strange odor; 30, Crumb elasticity; 31, Aroma intensity; 32, Bread aroma; 33, Strange aroma; 34, Sweet; 35, Salty; 36, Acid; 37, Astringent; 38, Bitter; 39, Taste persistency; 40, Crispy crust; 41, Adhesiveness (mouth); 42, Overall assessment; 43, pH; 44, TTA; L_1, *Leuconostoc citreum* PON10079; L_2, *L. citreum* PON10080; L_{12}, *L. citreum* PON10079 + *L. citreum* PON10080; W_1, *Weissella cibaria* PON10030; W_2, *W. cibaria* PON10032; W_{12}, *W. cibaria* PON10030 + *W. cibaria* PON10032; LW, *L. citreum* PON10079 + *L. citreum* PON10080 + *W. cibaria* PON10030 + *W. cibaria* PON10032.

this region has been recognised as an area of high interest for the study, promotion and preservation of the food cultural heritage by the local authorities. Within this products, PPA bread assumes a consistent relevance for Piana degli Albanesi, a town closed to the biggest city (Palermo) of western Sicily, but surrounded by mountains and quite isolated. In this conditions, the inhabitants of this town kept several traditions of the native Albanians, including the typical bread. However, PPA bread producers made a specific request with regards to the development of an *ad hoc* inoculums able to determine the production of breads well appreciated by consumers. In particular, the main reasons for the consumers' dissatisfaction with PPA bread were specifically due to its high acidity and low volume.

With the objective of preserving the traditional production of PPA and ameliorate its quality, our research group performed a microbiological investigation of the local flours and semolinas from the wheat varieties cultivated in Sicily (Alfonzo et al., 2013). In that work, all LAB present at dominating levels were also subjected to several technological tests to select a pool of strains with potential in sourdough fermentation. This because the raw materials for bread making are not subjected to thermal treatments before fermentation and are a source of living microorganisms, including LAB that might become active during sourdough refreshment. In this contest, it is reasonable to isolate autochthonous strains, that are adapted to the production area (environment), the local raw materials (substrates) and the traditional protocol (technology) and provide the typical characteristics that cannot be reproduced elsewhere (Settanni and Moschetti, 2014). Among the 11 strains selected for *in situ* applications during sourdough production for their rapid acidification, optimal FQ, and VOC generation (Alfonzo et al., 2013), *W. cibaria* PON10030 and PON10032 and *L. citreum* PON 10079 and PON10080 showed not only the best behaviour during sourdough fermentation, but they also determined the production of experimental breads characterised by the best quality attributes, included the highest increase of volume (Settanni et al., 2013). Since this strains were able to dominate the sourdough microbiota in controlled conditions at small-scale level, in the present study, they have been tested in different combinations at industrial-scale level in order to determine the more suitable inoculums for the production of the most appreciated PPA bread.

The development of sourdoughs, as well as their refreshments, were performed in a fermenter which was able to control the pH and lower the temperature until refrigeration at the end of fermentation. In this work, the values of pH registered after 16 h of sourdough fermentation ranged between 4.1 and 4.5. These values of pH fall within the common range reported for mature durum wheat sourdoughs produced in southern Italy (Minervini et al., 2012; Pepe et al., 2013; Rizzello et al., 2015; Ventimiglia et al., 2015). In particular, 4.1 represents the value at which the bread makers consider a sourdough ready to be used as leavening agent for PPA bread production.

TTA was also evaluated to follow the acidification kinetics of the sourdoughs. In all trials, TTA values was correlated linearly and inversely with the pHs. In general, the highest TTAs were registered after the fourth refreshments. The levels of TTA reached at the last refreshment (S6) were similar to those displayed by mature sourdoughs produced in southern Italy made from semolina (Minervini et al., 2012; Pepe et al., 2013; Rizzello et al., 2015; Ventimiglia et al., 2015). Furthermore, the analyses of lactic and acetic acid showed levels comparable to those reported for the sourdoughs produced in Sicily (Ventimiglia et al., 2015). The resulting FQs of all sourdoughs at each step of investigation fall in the range considered to influence positively the aroma and the structure of the resulting breads (Spicher, 1983). The sourdoughs of the trial LW, produced with the multi-species strain starter showed FQs included in the optimal range of 2.0–2.7 indicated by Hammes and Gänzle (1998). It is worth noting that the control sourdoughs were characterised

by FQs higher than those calculated in presence of the added selected LAB, showing that the inoculums used for the control trial contained LAB with a limited ability to generate acetic acid and/or a higher percentage of OHo species that produce only lactic acid from the primary metabolism (Salovaara, 1998). Thus, the strains of *L. citreum* and *W. cibaria* used in this study, alone and, better yet, in combination were found to determine the desirable production of lactic and acetic acid concentrations. These results evidence a behaviour of *L. citreum* and *W. cibaria* strains comparable to that of OHe *Lactobacillus* strains. The highest levels of lactic acid detected in the control sourdough might explain the high acidic taste perceived by consumers in PPA. In fact, although organic acids improve the texture giving more elasticity to the dough they also directly influence the acidic sensation (Rizzello et al., 2010).

Except the sourdoughs of the trial L_2 characterised by 8.0 Log CFU/g as level detected by plate count, the final level of LAB at each fermentation step was abundantly above 8.0 Log CFU/g for all other trials including the control trial. The numbers of LAB estimated during propagation are in the range commonly found in mature sourdoughs produced in Sicily (Minervini et al., 2012; Ventimiglia et al., 2015). Furthermore, levels of about 10^9 CFU/g are typically reached by leuconostocs and weissellas inoculated in a mixture of flour and water, even when their source of isolation is different from sourdough (Choi et al., 2012), highlighting the ability of these bacteria to adapt to this ecosystem.

The application of RAPD-PCR technique on the isolates collected from the highest plated dilutions of the sourdoughs at the sixth refreshment showed the dominance of the added strains in all trials carried out with single strain inocula and the dominance of *L. citreum* PON10079 and *W. cibaria* PON10032 in the trials with multiple (dual and quadruple) strain inocula. The explanation of these findings could be that *L. citreum* PON10079 and *W. cibaria* PON10032 showed a faster pH decrease and competition for nutrients than *L. citreum* PON10080 and *W. cibaria* PON10030, respectively, determining their prevalence during cofermentation. Furthermore, a clear codominance of *L. citreum* PON10079 and *W. cibaria* PON10032 was ascertain for the trial LW. These results confirmed the adaptability of the strains selected from semolinas to PPA sourdough production.

Interestingly, yeasts stabilised their levels at 10^6 CFU/g for all trials from the fifth refreshment. Thus at the sixth step of propagation the ratio between LAB and yeasts in all sourdoughs was almost 100:1 that is reported to be optimal for a sourdough with good quality characteristics (Ottogalli et al., 1996).

The final characteristics of the breads showed a different ability of the starter LAB culture to act as leavening agents, since the height of the breads was highly variable. *L. citreum* PON10080 determined the lowest increase in volume, while the breads with the greatest volume resulted from sourdoughs fermented with both strains of leuconostocs and weissellas together. The increase of the specific volume of bread due to the addition of sourdough (Esteve et al., 1994; Crowley et al., 2002) and/or single strains of *Leuconostoc* and *Weissella* genera (Choi et al., 2012) is well documented. This phenomenon is basically due to the CO_2 produced through 6-phosphogluconate/phosphoketolase pathway (heterolactic fermentation) (Axelsson, 1998). The softness of the breads was correlated directly with their height, a common phenomenon registered for breads (Chin et al., 2009; Rose, 2012). The softest breads were those obtained from sourdoughs fermented by *W. cibaria* PON10032 and all strains together, while the hardest breads, even harder than control breads, were those obtained in the trial carried out in presence of *L. citreum* PON10080. Our results are mostly in agreement with those of Choi et al. (2012) who reported a similar softness of sourdough breads produced with *L. citreum* HO12 and *Weissella koreensis* HO20 during storage. Some minor differences were registered among the breads of the different trials for the color of the crumb, void fraction, cell density and mean cell area, confirming that the final characteristics of the breads are influenced by the starter strains and (in the multiple strain combinations) their interactions.

The breads generated nine classes of VOCs, including acids, alcohols, aldehydes, esters, hydrocarbons, ketones, terpenes, furans and phenol. A high complexity of chemicals is generally emitted from sourdough breads (Hansen and Schieberle, 2005; Salim-ur-Rehman et al., 2006). Alcohols, aldehydes and, only for the breads obtained with the dual *L. citreum* strain inoculums, acids constituted the major classes of VOCs. Among the compounds found at the highest concentrations, phenylethyl alcohol, generally detected in breads from spontaneous sourdough processing (Kim et al., 2009), was detected in all breads. Among the other major compounds ethanol, hexanal, 3-methyl-1-butanol, nonanal, ethyl octanoate, acetic acid, furfuraldehyde, ethyl decanoate, furfuryl alcohol, 6-methyl5-hepten-2-ol, benzaldehyde, 2-nonenal and 5-methylfurfural were detected at consistent percentages in the majority of breads. Ethanol and acetic acid produced by OHe and FHe LAB (Axelsson, 1998), are generally found in sourdough breads (Hansen and Hansen, 1996; Hammes and Gänzle, 1998). Acetic acid in small concentrations is a flavour enhancer (Molard et al., 1979). Hexanal concentration has been reported to increase consistently from non-sourdough breads to sourdough breads (Seitz et al., 1998). This compound, and also 2-nonenal are considered key odour compounds of bread (Birch et al., 2013). In this study, the production of 2-nonenal can be directly imputable to *W. cibaria* (Settanni et al., 2013). The production of 3-methyl-1-butanol, responsible for the “fermented” flavour, is common in sourdough breads (Salim-ur-Rehman et al., 2006) and was detected in presence of several sourdough fermented by single strains of LAB (Hansen and Hansen, 1996). High amounts of nonanal are associated to sourdough breads (Seitz et al., 1998). Ethyl octanoate and ethyl decanoate were also observed in fermentations with *Lactobacillus sanfranciscensis* (Guerzoni et al., 2007). 5-methyl-2-furfural is a volatile marker of the baking process (Mildner-Szkudlarz et al., 2011). Benzaldehyde was detected also in PDO Altamura bread which is made with durum flour applying the sourdough technology (Bianchi et al., 2008).

VOCs have different odour activity (Reiners and Grosch, 1998). Thus, a different composition of these compounds determines differences in the sensory characteristics of breads. Although Czerny and Schieberle (2002) stated that LAB influence the flavour compounds already present in flour, the VOC profiles are strongly influenced by the starter strains (Settanni et al., 2013). However, not all VOCs detected by instrumental analysis have a perceptible aroma (Meignen et al., 2001). The compounds that strongly affect bread flavour are mainly organic acids, alcohols, esters and carbonyls (Kirchhoff and Schieberle, 2001; Czerny and Schieberle, 2002).

The panel of judges evidenced several differences among breads. Thus, the inocula used for sourdough production determined breads different for several attributes. Interestingly, the breads from the sourdoughs fermented with the different combinations of leuconostocs and weissellas were characterised by a lesser acidic sensation than control bread, encountering the appreciation of the judges. Lactic acid is the main compound producing a sour taste (Lotong et al., 2000). In particular, the highest score for the overall assessment was reached by the breads of the trial LW carried out with all selected strains together. In general, the enhancement of the desired flavour attributes are observed in presence of OHe LAB (Katina et al., 2006).

All data retrieved from the sourdoughs at the sixth refreshment and the corresponding breads were subjected to the multivariate

analysis to evaluate the differences/variabilities among the trials. Multivariate relationships, especially between consumer hedonic response data and descriptive sensory data are commonly applied to predict consumer liking/preference to food products (Krishnamurthy et al., 2007) including breads (Heenan et al., 2008). The correlation analysis among microbiological, chemical, physical, quality parameters and sensory evaluation showed that there were many significant relationships among them. Both HCA and PCA showed that control breads and the breads obtained from sourdoughs fermented with the different starter LAB inocula were quite distant. From a practical perspective, qualitative and quantitative distances of the individual breads obtained with different inocula provided an explanation of the behaviour of each LAB alone and in combination and allowed the interpretation of all data together. Furthermore, PCAn explained the interdependences between variables and their impact on the classification of data, determining the dimensional reduction of data to useful fingerprint for the breads. Thanks to this approach, the influence of each strain/combination on the overall characteristics of the final breads was easily retrieved from the score plot and a more strict relation was found among the productions carried out in presence of weissellas.

In conclusion, the use of the mixed starter culture composed of *W. cibaria* PON10030 and PON10032 and *L. citreum* PON 10079 and PON10080 determined the production of sourdough breads well appreciated by tasters. The dominance of the added strains over indigenous LAB during propagation of sourdoughs ensured their suitability for industrial PPA bread production.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.05.006.

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