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Diversity and technological potential of lactic acid bacteria of wheat flours

Antonio Alfonzo, Giusi Ventimiglia, Onofrio Corona, Rosalia Di Gerlando, Raimondo Gaglio, Nicola Francesca, Giancarlo Moschetti, Luca Settanni*

Department of Agricultural and Forestry Science, University of Palermo, Viale delle Scienze 4, 90128 Palermo, Italy

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ABSTRACT

Lactic acid bacteria (LAB) were analysed from wheat flours used in traditional bread making throughout Sicily (southern Italy). Plate counts, carried out in three different media commonly used to detect food and sourdough LAB, revealed a maximal LAB concentration of approximately 4.75 Log CFU g^{-1}. Colonies representing various morphological appearances were isolated and differentiated based on phenotypic characteristics and genetic analysis by randomly amplified polymorphic DNA (RAPD)-PCR. Fifty unique strains were identified. Analysis by 16S rRNA gene sequencing grouped the strains into 11 LAB species, which belonged to six genera: *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Weissella*. *Weissella cibaria*, *Lactobacillus plantarum*, *Leuconostoc pseudomesenteroides* and *Leuconostoc citreum* were the most prevalent species. The strains were not geographically related. Denaturing gradient gel electrophoresis (DGGE) analysis of total DNA of flour was used to provide a more complete understanding of the LAB population; it confirmed the presence of species identified with the culture-dependent approach, but did not reveal the presence of any additional LAB species. Finally, the technological characteristics (acidifying capacity, antimicrobial production, proteolytic activity, organic acid, and volatile organic compound generation) of the 50 LAB strains were investigated. Eleven strains were selected for future *in situ* applications.

1. Introduction

Cereal based foods have been key components of human diets for thousands of years. They remain a main source of nutrition, particularly in developing and overpopulated countries (Blandino et al., 2003). Indeed, the history of several cultures is directly defined by cereals and, consequently, many populations are identified by the cereals they eat with Mediterranean people referred to as "wheat people" (Gifford and Baer-Sinnot, 2007). Historically, millennia B.C. wheat was among the most important crops grown in the Mediterranean basin. Following mass migrations, its cultivation underwent a huge expansion, resulting in its production worldwide (Toderi, 1989).

Wheat is critical in the Mediterranean diet: it provides approximately one-third of the daily protein and energy requirements (~2400 kcal) for an adult (Cannella and Piredda, 2006). For this reason, wheat surpasses other cereals in terms of the number of hectares dedicated to its cultivation worldwide (Gifford and Baer-Sinnot, 2007).

Wheat kernels are naturally contaminated with microorganisms (Corsetti et al., 2007), which may then be present in flours. The microorganisms that contaminate cereals are generally concentrated in the outer layers of kernels, and they tend to stay in fractions rich in bran during milling. Consequently, flour obtained from milling should theoretically contain a lower bacterial load than caryopses, but the subsequent conditioning phase can increase flour's microbial content (Berghofer et al., 2003). Among the microbial populations associated with wheat kernels, protechnological lactic acid bacteria (LAB) are often detected (Corsetti et al., 2007) and are also found in wheat flours (Russo et al., 2010). Because raw materials are not commonly subjected to thermal treatments before the fermentation stage, they provide a source of living, active LAB during the production of baked goods. This is especially important for baked goods prepared with sourdough: in this mixture of flour and water, LAB enable the production of lactic and acetic acid (Salovaara, 1998). LAB that contribute to wheat flour fermentation can also come from the equipment used in the milling and/or production process (Berghofer et al., 2003).

* Corresponding author. Tel.: +39 091 23896043; fax: +39 091 6515531.
E-mail addresses: luca.settanni@unipa.it, settanniluca@yahoo.it (L. Settanni).

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Although a number of studies have identified and characterized LAB of mature sourdoughs, only a few have analysed the microbial ecology of raw materials used in the production of baked goods.

Understanding the microbial composition during fermentation at the species and strain level is particularly important for the correct management of sourdough. Microorganisms within flours may be essential in ensuring sourdough contains the correct proportion of bacteria and yeasts as well as the optimal ratio between heterofermentative and homofermentative species of LAB. The overall sourdough microbial composition is especially important in the early stages of development, when typical sourdough LAB are not present at high levels and the acidity does not inhibit the undesired microbiota.

Investigation of LAB can be difficult, especially in raw materials subjected to uncontrolled conditions. Although wheat flour contains all of the nutrients required for the growth of several microorganisms, including the nutritionally fastidious LAB, its low water content does not permit LAB growth. Indeed, within flours, several LAB species are found in a dormant state (Corsetti et al., 2007), from which their subsequent culturing in specific media is not guaranteed, even if optimal incubation conditions are applied. Therefore, to reveal the complete microbial population within flours, total DNA needs to be investigated using culture-independent methods.

To characterise the LAB populations within wheat flours that are commonly used to make traditional Sicilian breads, the specific objectives of this study were to: (i) enumerate, isolate and genetically differentiate (at the strain level) LAB found in flour samples collected from throughout Sicily; (ii) perform bacterial image analysis on all flour samples using a culture-independent tool; (iii) evaluate the technological capabilities of LAB by examining their acidifying abilities in a flour extract system, proteolytic activity, production of antibacterial and antifungal compounds, and generation of chemical compounds.

2. Materials and methods

2.1. Sample collection

Thirteen wheat (*Triticum durum* and *Triticum aestivum*) flour samples (Table 1) were collected from several bakeries throughout Sicily (southern Italy) from January to May 2012, immediately before the mature sourdough was refreshed. Samples were chosen for their typical characteristics and/or origin. Many of the flours are unique to local Sicilian milling, while others are also commercially available outside Sicily. Flour samples were transferred into sterile Stomacher bags and placed into a portable cooler. Once in the laboratory, all samples were refrigerated until analysed.

2.2. Microbiological analysis

Each sample (15 g) was suspended in Ringer's solution (135 mL) (Sigma–Aldrich, Milan, Italy), homogenised in a stomacher (BagMixer® 400; Interscience, Saint Nom, France) for 2 min at maximum speed, and then serially diluted. Decimal dilutions were plated on three agar media: MRS (Oxoid, Milan, Italy) (generic for rod LAB) pour plated and incubated anaerobically at 30 °C for 48 h; M17 (Oxoid) (generic for coccus LAB) pour plated and incubated anaerobically at 30 °C for 48 h; Sour Dough Bacteria (SDB) (Kline and Sugihara, 1971), specific for sourdough LAB, spread plated and incubated aerobically at 30 °C for 48 h. To avoid fungal growth, cycloheximide (10 mg mL^{-1}) was added to all media. Microbiological counts were performed in triplicate.

Statistical analyses were conducted using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Microbial data were analysed using a generalised linear model (GLM) that included the effects of sample; the Student "*t*" test was used for mean comparison. The *post-hoc* Tukey method was applied for pairwise comparison. Significance level was $P < 0.05$.

2.3. LAB isolation and phenotypic grouping

Four colonies of Gram-positive [Gregersen KOH method (Gregersen, 1978)] and catalase negative bacteria of differing morphologies were isolated following their growth on the three media from the LAB count experiments (from the highest plated dilution), to represent the diversity of LAB, and subsequently transferred into the corresponding broth media. The isolates were then purified with successive sub-culturing. Isolates used in subsequent analyses were stored at −80 °C in broth containing 20% (v/v) glycerol.

Phenotypic characterisation was carried out to obtain an initial grouping of isolates. The cell morphology type of LAB isolates was determined using an optical microscope. Rod- and coccus-shaped LAB cultures were further grouped based on cell disposition. Specifically, rod LAB were sub-grouped according to their growth at 15 and 45 °C and ability to produce CO_2 from glucose. The final grouping test was carried out with the same growth media used for isolation, but with citrate removed because its fermentation by certain LAB can result in gas formation. Furthermore, M17 contained glucose in place of lactose, and SDB was prepared with glucose instead of maltose. For the assay, LAB were inoculated into test tubes sealed with H_2O agar (2%, w/v). After incubation for 48 h, LAB were scored positive for CO_2 production if a rising of the agar cap was detected, indicating the LAB had an obligate heterofermentative metabolism. LAB strains that scored negative in the assay were

Table 1
Samples of wheat flours used to produce traditional sourdough breads in Sicily.

Sample	Species	Flour	Commercial name	Company	Geographical origin
F2	*T. durum*	Durum wheat semolina	Local grain	Salvia	Partinico (PA)
F3	*T. durum*	Durum wheat semolina	Linea Blu – Sicilian grain	Landro S.n.c. company – Manata Santo & C.	Misilmeri (PA)
F4	*T. aestivum*	Soft wheat flour	Nazionale Archilli	Grandi Molini Italiani	Pachino (SR)
F5	*T. aestivum*	Soft wheat flour	Mister Sprint	Molitoria Sanpaolo S.p.A.	Siracusa
F6	*T. durum*	Whole meal durum wheat semolina	Linea Rossa	Landro S.n.c. company – Manata Santo & C.	Misilmeri (PA)
F7	*T. aestivum*	Soft wheat flour	Terra	Selezione Casillo S.r.L.	Corato (BA)
F9	*T. durum*	Durum wheat semolina	Local grain	Molini Gattuso	Castronovo di Sicilia (PA)
F10	*T. durum*	Durum wheat semolina	Divella	F. Divella S.p.A.	Rutigliano (BA)
F11	*T. durum*	Durum wheat semolina	Local grain	Granaio del Sole	Palermo
F23	*T. durum*	Durum wheat semolina	Local grain	Molino Ivana	Roccapalumba (PA)
F26	*T. durum*	Durum wheat semolina	Local grain	Molino Ancona	San Giovanni Gemini (AG)
F30	*T. durum*	Durum wheat semolina	Local grain	Molini Roccasalva Giorgio & C.	Modica (RG)
F33	*T. durum*	Durum wheat semolina	Local grain	Molini Grillo & C. S.a.S.	Marsala (TP)

inoculated into test tubes containing the optimal growth media prepared with a mixture of pentose carbohydrates (xylose, arabinose, and ribose; 8 g L^{-1} each) in place of glucose. Strains that grew in this media were defined as having a facultative heterofermentative metabolism, and strains that were unable to grow were defined as having an obligate homofermentative metabolism. For coccus isolates, sub-grouping also included the examination of growth at pH 9.2 and in the presence of 6.5% (w/v) NaCl.

2.4. Genotypic characterization of LAB

Genomic DNA for PCR assays was prepared from flour isolates after their overnight growth in broth media at 30 °C. Cells were harvested, and DNA was extracted using an InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Crude cell extracts were used as templates for PCR.

Strain differentiation was performed with random amplification of polymorphic DNA (RAPD)-PCR analysis in a 25-μL reaction mix using the single primers M13, AB111, and AB106 as previously described by Settanni et al. (2012). Resultant PCR products were separated by electrophoresis on a 2% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France), stained with SYBR® Safe DNA gel stain (Molecular Probes, Eugene, OR, USA), and subsequently visualised by UV transillumination. The GeneRuler 100 bp Plus DNA Ladder (M-Medical S.r.l, Milan, Italy) was used as a molecular size marker. RAPD patterns were analysed using Gelcompar II software, version 6.5 (Applied-Maths, Sint-Martens-Latem, Belgium).

Genotypic characterisation of LAB containing different RAPD-PCR profiles was carried with 16S rRNA gene sequencing. PCR reactions were performed as described by Weisburg et al. (1991). DNA fragments were visualised and amplicons of approximately 1600 bp were purified with a QIA-quick purification kit (Qiagen S.p.A., Milan, Italy), and subsequently sequenced by Primm s.r.l. (Milan, Italy) using the same primers employed for PCR amplification above. The resultant sequences were compared to sequenced bacteria with a BLAST search using the GenBank/EMBL/DDBJ database.

2.5. Culture-independent detection of LAB in flour samples

Total DNA was extracted from 500 mg of each flour sample using a FastDNA® Pro Soil-Direct Kit (MP Biomedicals, CA, USA) with the manufacturer's instructions. Bacterial DNA was amplified by PCR with the primer pair GC-HDA1/HDA2 (Walter et al., 2000) as reported by Giannino et al. (2009). Resultant amplicons were run in polyacrylamide gels (20 cm × 20 cm × 1 mm) using the Phor-U2 system (Ingeny, Leiden, The Netherlands). Gels were prepared with 6% (w/v) polyacrylamide [acrylamide/bisacrylamide (37:1)] in a 1 × TAE [40 mM Tris, 20 mM acetic acid, and 1 M EDTA (pH 8.0)] buffer with a 30–60% denaturing gradient [100% denaturant corresponding to 7 M urea and 40% (v/v) deionised formamide] increasing in the direction of electrophoresis. Electrophoresis was performed at 60 °C with 1 × TAE for 14 h, following which gels were stained in an $AgNO_3$ (0.2%, w/v) solution (Niessen et al., 2005) and captured with a Gel Doc 2000 (Bio-Rad). The specificity of the primers for flour/sourdough LAB was confirmed using the DNA extracted from *Lactobacillus plantarum* ATCC 14917^T, *Lactobacillus sanfranciscensis* LMG 17498^T, and *Leuconostoc mesenteroides* DSM 20343^T.

The observed major bands were then excised from gels and cloned into the pGEM®-T Easy Vector (Promega, Milan, Italy) according to the manufacturer's instructions. Ligation products were subsequently transformed into *Escherichia coli* JM109 high-efficiency competent cells, which were plated onto Luria–Bertani (LB) agar (Oxoid) containing 100 μg mL^{-1} ampicillin (Sigma–Aldrich), 80 μg mL^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 0.5 mM IPTG (isopropyl β-D-thio-galactopyranoside; Eppendorf, Milan, Italy). Recombinant white colonies were screened with colony-PCR in a 25-μL reaction mix using the vector specific primers SP6 (5′-ATTTAGGTGACACTATA-GAATAC-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′) and the following amplification program: initial denaturation at 94 °C for 5 min with 4 °C for 4 min, then 35 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 50 s, followed by a final extension step at 72 °C for 5 min. Insert integrity was confirmed by nested-amplification following the amplification program described above, using purified colony-PCR amplicons as templates and HDA1 and HDA2 primers. The PCR products were separated by electrophoresis in a 1.5% (w/v) agarose gel and stained with SYBR® Safe DNA gel stain. Sequencing was performed by Primm s.r.l, and the sequences were compared with a BLAST search using the GenBank/EMBL/DDBJ database.

2.6. Technological capacity characterization of LAB

2.6.1. Acidification activity

To evaluate the kinetics of acidification by LAB *in vitro*, sterile flour extract (SFE) liquid broth was prepared as follows: 200 g of wheat flour (Il Molino Chiavazza, Casalgrasso, Italy) was suspended in distilled H_2O (1 L) and sterilised by autoclaving at 121 °C for 20 min; the flour was then precipitated and removed; and the supernatant was used as liquid broth in subsequent experiments. Overnight LAB cultures, grown in MRS, M17, or SDB according to the isolation procedure, were harvested by centrifugation at 5000 × *g* for 5 min, washed with Ringer's solution, and, to standardise bacterial inocula, resuspended in the same solution to an optical density at 600 nm of 1.00, corresponding to approximately 10^9 CFU mL^{-1}, as measured with a 6400 Spectrophotometer (Jenway Ltd., Felsted, Dunmow, UK). The acidifying capacity of LAB was subsequently assayed during their incubation in 20 mL of SFE at 30 °C, with 1% (v/v) of the solution consisting of the cell suspension. pH measurements were taken at 2 h intervals for the first 8 h of incubation, and then at 24, 48, and 72 h after inoculation.

2.6.2. Proteolytic activity

The extracellular protease activity of LAB was determined on agar plates, with the method described by Vermelho et al. (1996). Bovine serum albumin (BSA) and gelatine (Sigma–Aldrich) were used as protease substrates; they were incorporated into each medium at 1% (w/v).

2.6.3. Antimicrobial activity

The antibacterial activity of flour LAB was determined against *Lactobacillus sakei* LMG2313, *Listeria innocua* 4202, and *Listeria monocytogenes* ATCC 19114, strains that are highly sensitive to bacteriocins (Corsetti et al., 2008; Hartnett et al., 2002). Inhibitory activity was first evaluated with the agar-spot deferred method, and strains displaying antimicrobial properties were further subjected to the well diffusion assay (WDA) (Schillinger and Lücke, 1989), with the method modifications described by Corsetti et al. (2008). All tests were carried out in triplicate. Resultant active supernatants were treated with proteinase K (12.5 U mg^{-1}), protease B (45 U mg^{-1}), and trypsin (10.6 U mg^{-1}), each diluted to 1 mg mL^{-1} in phosphate buffer (pH 7.0), for 2 h at 37 °C, following which any remaining activity was quantified with the WDA (Settanni et al., 2005). Enzymes were purchased from Sigma–Aldrich.

The antifungal activity of flour LAB was evaluated using the method previously described by Villani et al. (1993), with the modifications reported by Alfonzo et al. (2012). *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Cladosporium cladosporioides*,

Table 2
LAB concentrations in flour samples collected from bakeries located throughout Sicily.

Sample	LAB counts (Log CFU g^{-1})		
	MRS	M17	SDB
F2	2.92 ± 0.04	4.49 ± 0.16	4.08 ± 0.12
F3	3.48 ± 0.09	4.64 ± 0.19	3.45 ± 0.08
F4	1 ± 0.02	4.28 ± 0.14	1.78 ± 0.22
F5	<1	2.56 ± 0.09	2.72 ± 0.04
F6	3.70 ± 0.06	4.48 ± 0.17	3.70 ± 0.14
F7	<1	3.45 ± 0.11	1.06 ± 0.11
F9	3.6 ± 0.09	4.75 ± 0.17	3.57 ± 0.11
F10	1.48 ± 0.07	4.01 ± 0.19	3.48 ± 0.13
F11	<1	4.09 ± 0.21	2.98 ± 0.23
F23	<1	3.58 ± 0.18	<2
F26	4.36 ± 0.18	2.93 ± 0.12	4.26 ± 0.18
F30	1.85 ± 0.09	4.62 ± 0.21	<2
F33	3.48 ± 0.13	4.16 ± 0.18	4.40 ± 0.22
Significance	$P < 0.001$	$P < 0.001$	$P < 0.001$

Abbreviations: MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; M17, medium 17 agar for mesophilic coccus LAB; SDB, sourdough bacteria agar for typical sourdough LAB.
Results indicate mean values ± S.D. of three plate counts.

Penicillium verrucosum, and *Rhizopus stolonifer* fungi were used as indicators of LAB antimicrobial activity.

2.6.4. Lactic and acetic acid determination

Strains containing the most technologically relevant attributes, in particular those that rapidly and greatly decreased SFE pH, were analysed for their ability to produce lactic and acetic acid, following 8 h of fermentation. Ten millilitres of each resultant sample of acidified SFE (aSFE) was first homogenised by a stomacher with 90 mL distilled H_2O, and then aliquots of 10 mL were added to 5 mL of a 0.1 mM $HClO_4$ solution. These mixtures were centrifuged at 4000 × *g* for 15 min at 15 °C, after which the supernatants were acidified to pH 3.0 ± 0.1 with 1 mM $HClO_4$ and brought to a final volume of 25 mL with distilled H_2O. These solutions were left in ice for 30 min and subsequently filtered through 0.45-μm cellulose filters (Millipore). High performance liquid chromatography (HPLC) analyses of filtered samples (20 mL) were performed with a Perkin–Elmer Series 200 instrument (Milan, Italy) equipped with a quaternary pump, a micro vacuum degasser, and a variable-wavelength detector operating at $\lambda = 220$ nm. Chromatographic separation was performed using a reverse phase C18 capped column (Econosphere™ C18, 5 μm, 250 × 4.6 mm i.d., part no. 70066; Lokeren, Belgium), with a flow rate of 0.5 mL min^{-1} and column temperature of 35.0 ± 0.1 °C. Isocratic mobile phase conditions were met with 0.5 mM aqueous $HClO_4$ solution for 20 min. PerkinElmer software specific to the HPLC instrument (TotalChrom Workstation 2008 rev. 6.3.2) was used to acquire and process data. Analyses were carried out in triplicate, with results expressed as mean ± standard deviation.

2.6.5. Volatile organic compound production

Strains displaying high acidification capabilities were also examined for their generation of volatile organic compounds (VOCs). VOCs within SFEs were identified using the solid phase micro extraction (SPME) isolation technique after 8 h of fermentation by LAB. In a vial, 5 mL of aSFEs were heated to 60 °C, during which the headspace was collected with DBV-Carboxen-PDMS fibres (Supelco, Bellefonte, PA) for 40 min. The SPME fibre was directly inserted into a Finnegan Trace MS for GC/MS (Agilent 6890 Series GC system, Agilent 5973 Net Work Mass 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 no 122–7032). The GC temperature was 40 °C for the first 2 min (during splitless injection), then from 40 °C to 60 °C, increasing 4 °C min^{-1}, 60 °C for 2 min, from 60 °C to 190 °C, increasing 2 °C min^{-1}, from 190 °C to 230 °C, increasing 5 °C min^{-1}, and finally 230 °C for 15 min. The GC injector was at 250 °C, the Fid at 250 °C, the transfer line at 230 °C, with carrier helium being transported at a rate of 1 mL min^{-1}, and EM at 70 eV. Mass spectra were recorded by electronic impact at 70 eV using the ion source temperature of 200 °C. All compounds of *m/z* 33–495 atomic mass units (amu) were detected with this scan mode. Individual peaks were identified by comparing their retention indices to those of control samples and by comparing their mass spectra with those within the NIST/EPA/NIH Mass Spectral Library database (Version 2.0d, build 2005). Volatile compounds were expressed as relative peak areas (peak area of each compound/total area) × l00. All solvents and reagents were purchased from WWR International (Milan, Italy). Chemical and physical tests were performed in triplicate, with the results expressed as mean ± standard deviation.

3. Results

3.1. Microbiological analyses

Viable counts of LAB identified within flour samples are reported in Table 2. The differences among LAB concentrations were statistically significant ($P < 0.001$) for all media tested, although the highest counts, in the range 2.56–4.64 Log CFU g^{-1}, were reported for M17 medium. Indeed, for the flour sample F23, LAB were only detected in M17. With the exception of only a few cases, the number of LAB estimated in SDB was higher than that in MRS. The latter medium actually provided the lowest bacterial concentration results and the lowest number of LAB culture-positive flour samples. In general, with the exception of F30, flour samples containing high levels of LAB on M17 also grew LAB in the other media.

Table 3
Phenotypic grouping of LAB.

Characters	Clusters								
	I ($n = 25$)	II ($n = 5$)	III ($n = 28$)	IV ($n = 3$)	V ($n = 8$)	VI ($n = 5$)	VII ($n = 6$)	VIII ($n = 22$)	IX ($n = 22$)
Morphology	Rod	Rod	Rod	Coccus (tetrads)	Coccus (short chain)	Coccus (short chain)	Coccus (short chain)	Coccus (short chain)	Coccus (short chain)
Growth:									
15 °C	+	+	+	+	+	+	+	+	+
45 °C	+	−	−	+	+	−	−	+	+
pH 9.6	n.d.	n.d.	n.d.	+	+	+	−	+	−
6.5% NaCl	n.d.	n.d.	n.d.	+	+	−	+	+	−
CO_2 from glucose	+	+	−	−	−	−	+	+	+
Growth in presence of pentose carbohydrates	n.d.	n.d.	+	+	+	−	n.d.	n.d.	n.d.

n.d. not determined.

3.2. Isolation and grouping of LAB

From 13 flour samples, 138 colonies were collected, subjected to microscopic inspection, and separated into 78 cocci and 60 rods. After Gram-stain characterisation and catalase testing, 66 cocci and 58 rods were still considered putative LAB cultures (Gram-positive and catalase-negative). Despite the growth of other colonies from sample F23, no probable LAB were recovered from it on M17. In fact, all isolates collected from this flour sample were identified as *Staphylococcus* spp. (data not shown).

All cultures were tested for their ability to grow at 15 °C and 45 °C and to produce CO_2 from glucose. Coccus LAB were additionally evaluated for growth at pH 9.6 and in the presence of 6.5% (w/v) NaCl. Based on these characteristics, the 124 LAB cultures were separated into 9 groups (Table 3), 3 for rods and 6 for cocci. Groups III, I, VIII, and IX contained the highest number of isolates, between 22 and 28. Unequivocal determination of the type of fermentative metabolism of LAB required evaluation of isolate growth in the presence of pentose sugars. This experiment revealed that group VI isolates had an obligate homofermentative

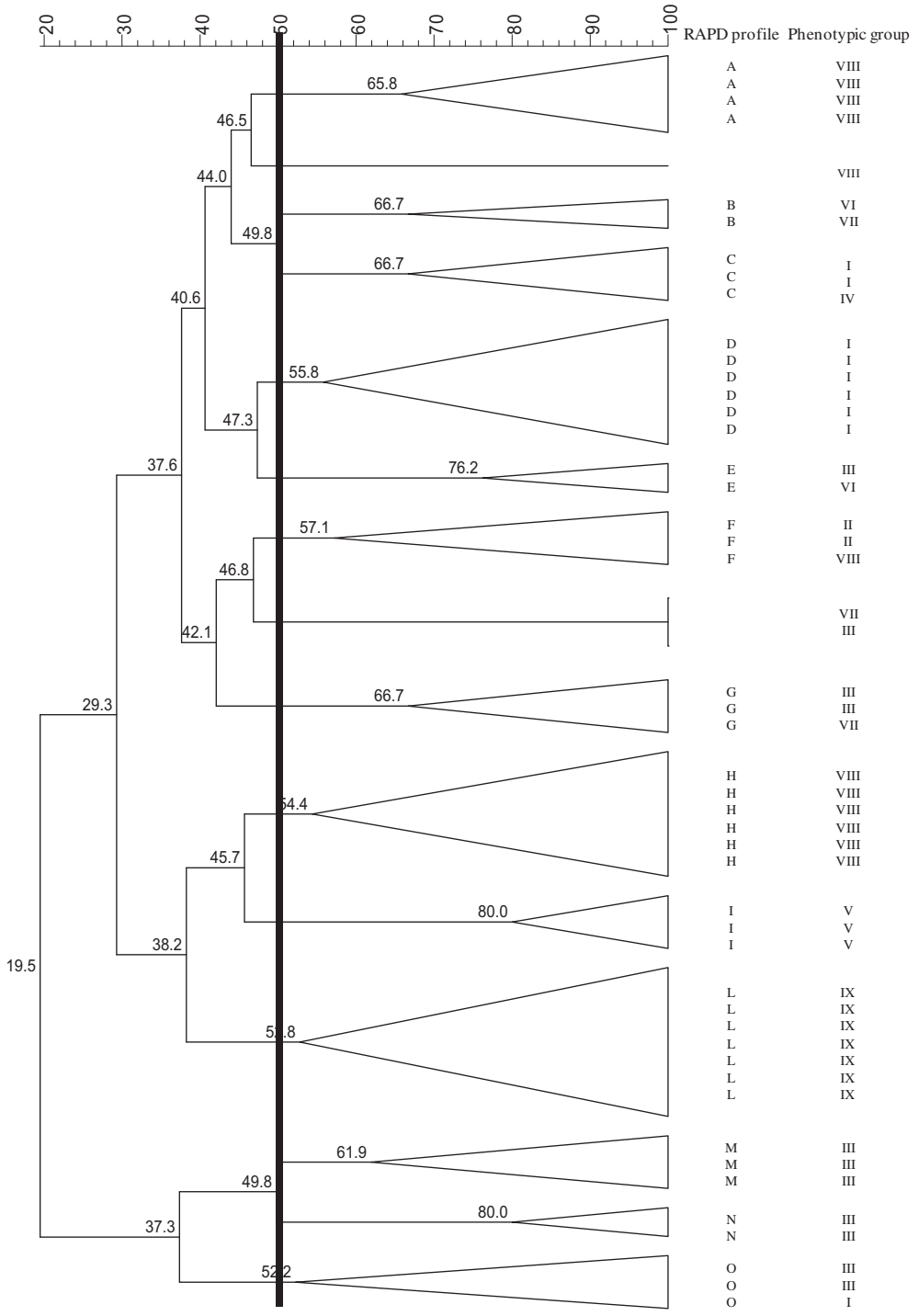

Fig. 1. Dendrogram obtained from combined RAPD-PCR patterns of LAB strains from flour samples generated with three primers. Upper line indicate the percentage of similarity.

metabolism and that group III, IV, and V isolates had a facultative heterofermentative metabolism.

3.3. Genetic differentiation of LAB

All 124 isolates were subjected to RAPD analysis with primers M13, AB106, and AB111. The unique patterns ($n = 50$) were used to construct a dendrogram (Fig. 1). To obtain the optimal molecular grouping that corresponded to phenotypic differentiation, a similarity level of 50% was chosen. The analysis revealed 13 main clusters: 3 (C, D, and O) for the isolates in phenotypic group (PG) I; 1 (F) for PG II isolates; 5 (E, G, M, N, and O) for PG III isolates; 1 (C) for PG IV isolates; 1 (I) for PG V isolates; 2 (B and E) for PG VI isolates; 2 (B and G) for PG VII isolates; 3 (A, F, and H) for PG VIII isolates; and 1 (L) for PG IX isolates. With the 50% similarity level, 3 strains remained ungrouped, one each from PG VIII, VII, and III. Only strains of PG V and IX were grouped into a single RAPD cluster. Interestingly, in several cases, strains belonging to the same PG were divided in 2 or more RAPD clusters that were quite distant from one another, in particular, strains of PG I, III, VII, and VIII.

3.4. Identification of LAB

All 50 strains were identified by 16S rRNA gene sequencing. A BLAST search revealed that the sequences had an identity of at least 97% with those available within the NCBI database, which is the minimum value of similarity expected between 16S rRNA genes of strains belonging to the same species (Stackebrandt and Goebel, 1994). More recently, however, Stackebrandt and Ebers (2006) argued that 'a 16S rRNA gene sequence similarity range above 98.7–99% should be mandatory for testing the genomic uniqueness of a novel isolate', thereby overturning the old standard of 97%. For this reason, species within the *L. plantarum* group were also analysed with multiplex PCR as described by Torriani et al. (2001),

Table 4
Identification of wheat flour LAB strains.

Strain	No. of isolates	Cluster				
		Phenotypic group	RAPD profile	Species	Flour source	Acc. no.
PON10030	3	I	C	*Weissella cibaria*	F6	KC416977
PON10032	3	I	D	*W. cibaria*	F6	KC416978
PON10050	3	I	D	*W. cibaria*	F6	KC416979
PON10056	2	I	C	*W. cibaria*	F5	KC416980
PON10069	2	I	O	*W. cibaria*	F2	KC416981
PON100337	2	I	D	*W. cibaria*	F33	KC416982
PON100338	2	I	D	*W. cibaria*	F33	KC416983
PON100339	3	I	D	*W. cibaria*	F33	KC416984
PON10029	3	I	D	*W. cibaria*	F6	KC416985
PON10052	2	I	G	*Weissella confusa*	F6	KC416986
PON100100	2	II	F	*Lactobacillus sanfranciscensis*	F11	KC416987
PON100336	3	II	F	*Lb. sanfranciscensis*	F33	KC416988
PON10013	3	III	N	*Lactobacillus plantarum*	F2	KC416989
PON10014	3	III	M	*Lb. plantarum*	F2	KC416990
PON10016	2	III	N	*Lb. plantarum*	F2	KC416991
PON100272	4	III	M	*Lb. plantarum*	F26	KC416992
PON100274	3	III	M	*Lb. plantarum*	F26	KC416993
PON100348	3	III	O	*Lb. plantarum*	F33	KC416994
PON100351	2	III	O	*Lb. plantarum*	F33	KC416995
PON10022	1	III	–	*Lactobacillus sakei*	F3	KC416996
PON10067	3	III	E	*Lb. sakei*	F3	KC416997
PON10097	2	III	G	*Lb. sakei*	F10	KC416998
PON10098	2	III	G	*Lb. sakei*	F10	KC416999
PON10015	3	IV	C	*Pediococcus pentosaceus*	F2	KC417000
PON10062	2	V	I	*Enterococcus mundtii*	F4	KC417001
PON10063	3	V	I	*E. mundtii*	F4	KC291251
PON10085	3	V	I	*E. mundtii*	F9	KC417002
PON10057	3	VI	B	*Lactococcus lactis*	F7	KC417003
PON10072	2	VI	E	*Lc. lactis*	F2	KC417004
PON10031	4	VII	B	*Leuconostoc mesenteroides*	F6	KC417005
PON10064	2	VII	–	*Ln. mesenteroides*	F4	KC417006
PON10024	2	VIII	F	*Leuconostoc pseudomesenteroides*	F3	KC417007
PON10066	1	VIII	–	*Ln. pseudomesenteroides*	F3	KC417008
PON10086	2	VIII	A	*Ln. pseudomesenteroides*	F9	KC417009
PON100315	2	VIII	A	*Ln. pseudomesenteroides*	F30	KC417010
PON100349	2	VIII	A	*Ln. pseudomesenteroides*	F33	KC417011
PON100350	2	VIII	A	*Ln. pseudomesenteroides*	F33	KC417012
PON10021	2	VIII	H	*Leuconostoc citreum*	F3	KC417013
PON10051	2	VIII	H	*Ln. citreum*	F6	KC417014
PON10055	2	IX	L	*Ln. citreum*	F5	KC417015
PON10059	2	IX	L	*Ln. citreum*	F7	KC417016
PON10061	3	IX	L	*Ln. citreum*	F4	KC417017
PON10065	2	IX	L	*Ln. citreum*	F3	KC417018
PON10068	3	VIII	H	*Ln. citreum*	F3	KC417019
PON10077	3	VIII	H	*Ln. citreum*	F9	KC417020
PON10078	2	VIII	H	*Ln. citreum*	F9	KC417021
PON10079	1	IX	L	*Ln. citreum*	F9	KC417022
PON10080	4	IX	L	*Ln. citreum*	F9	KC417023
PON10087	3	VIII	H	*Ln. citreum*	F9	KC417024
PON100273	4	IX	L	*Ln. citreum*	F26	KC417025

Table 5
Distribution of LAB species among flour samples.

LAB species	Flour samples												
	F2	F3	F4	F5	F6	F7	F9	F10	F11	F23	F26	F30	F33
E. mundtii			■				■						
Lb. plantarum	■										■		■
Lb. sakei		■						■					
Lb. sanfranciscensis									■				■
Lc. lactis	■					■							
Ln. citreum		■	■	■	■	■	■				■		
Ln. mesenteroides			■		■								
Ln. pseudomesenteroides		■					■					■	■
P. pentosaceus	■												
W. cibaria	■			■	■								■
W. confusa					■								

while *Weissella cibaria* and *Weissella confusa* were discriminated with species specific PCR as reported by Fusco et al. (2011). This approaches allowed for the classification of all strains at the species level (Table 4); all were confirmed to be LAB. The species encompassing the most LAB strains were *Leuconostoc citreum* ($n = 13$ strains), *W. cibaria* ($n = 9$), *Lb. plantarum* ($n = 7$), and *Leuconostoc pseudomesenteroides* ($n = 7$). No correlation was found between the species and strains identified and their geographical origin. Species distribution among the flour samples (Table 5) revealed that samples F2, F6, and F33 were richest in terms of the number of LAB species, whereas samples F10, F11, and F30 contained only *Lb. sakei*, *Lb. sanfranciscensis*, and *Ln. pseudomesenteroides*, respectively.

3.5. Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE patterns obtained from direct amplification of the V3 region located within 16S rRNA genes (Fig. 2) identified the presence of LAB species in all flour samples. The samples with the highest number of DGGE bands were F9, F10, and F11; F2–F7 produced only two bands. The major bands (Table 6) confirmed the detection of the genera to which most isolates belonged to (*Lactobacillus*, *Enterococcus*, *Leuconostoc*, and *Weissella*), but only two species, *Lb. plantarum* and *Ln. citreum* (bands 3 and 6, respectively), were clearly identified.

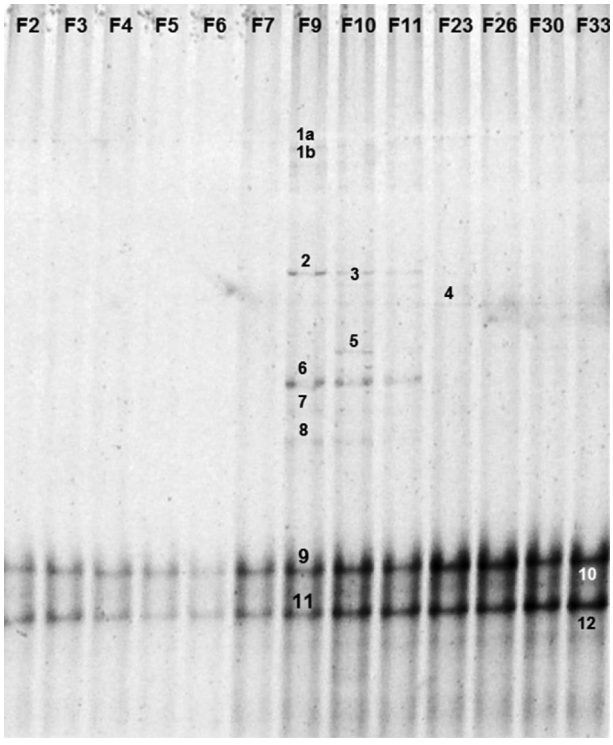

Fig. 2. DGGE profiles of 16S rRNA gene V3 regions obtained from wheat flour samples collected throughout Sicily. DGGE fragments have been enumerated from 1 to 12 and the corresponding identities are reported in Table 6.

3.6. Evaluation of the technological attributes of LAB

3.6.1. Acidification activity

The results of the acidification kinetics of SFE by the 50 LAB strains are shown in Fig. 3. Eleven LAB strains (*Ln. citreum* PON10021, PON10079, and PON10080; *W. cibaria* PON10030, PON10032, and PON100337; *Ln. pseudomesenteroides* PON10024 and PON100315; *Ln. mesenteroides* PON10031; *Lb. plantarum* PON100274; and *Lb. sakei* PON10098) were able to decrease the SFE pH below 5.0 after 6 h. At 24 h, almost all of the strains acidified the medium to below pH 4.0.

3.6.2. Protease activity

The flour LAB strains preferentially hydrolysed gelatine over BSA: after incubation on medium containing gelatine, a clear halo was present for 35 strains, whereas only 17 strains generated the same result on medium supplemented with BSA (Table 7). However, *W. cibaria* PON10029, *Ln. pseudomesenteroides* PON10024, and PON100315, which were positive for protease production in the presence of BSA, did not hydrolyse gelatine.

3.6.3. Antimicrobial activity

Flour LAB only displayed antimicrobial activity against the bacterial indicators tested; none of the 5 fungal strains used were

Table 6
DGGE fragment sequence similarities.

Bands	Closest relative (%)	Acc. no.
1a	SF	
1b	SF	
2	Uncultured *Enterococcus* spp. (99)	KC416968
3	*Lactobacillus plantarum* (98)	KC416969
4	Uncultured *Lactobacillus* spp. (99)	KC416970
5	SF	
6	*Leuconostoc citreum* (99)	KC416971
7	Uncultured *Lactobacillus* spp. (99)	KC416972
8	Uncultured *Weissella* spp. (98)	KC416973
9	Uncultured *Weissella* spp. (98)	KC416974
10	Uncultured *Weissella* spp. (98)	KC416975
11	Uncultured Lactobacillales bacterium (99)	KC416976
12	SF	

SF, sequencing failed.

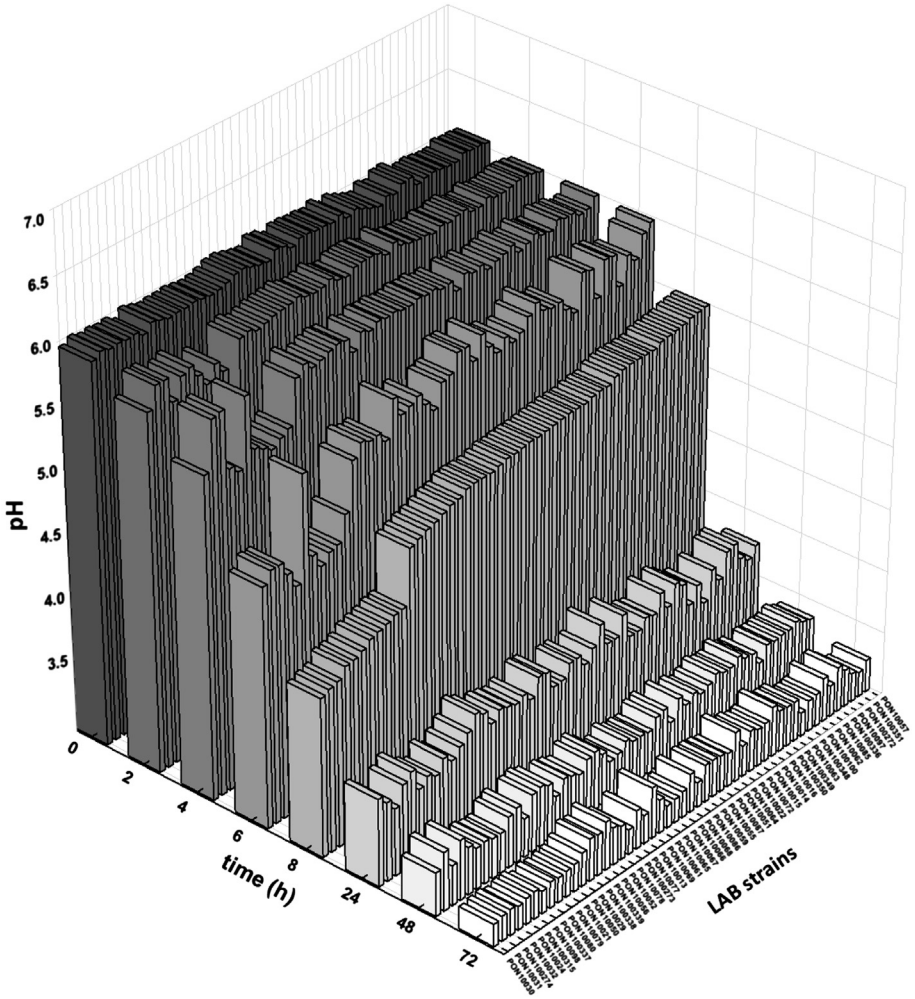

Fig. 3. Kinetics of acidification of wheat flour LAB. Histograms are in the order of increasing pH at 8 h.

inhibited following antagonistic tests (data not shown). Nineteen strains produced active substances (Table 7). The species to which the highest number of antibacterial compound-producing strains belonged were *Lb. plantarum* and *Ln. citreum*. Interestingly, both of the *Lb. sanfranciscensis* strains isolated from flour showed anti-*Listeria* activity. All excreted antibacterial compounds were hydrolysed by proteolytic enzymes (data not shown), indicating their proteinaceous nature, a general characteristic of bacteriocins (Jack et al., 1995). However, because the amino acid and nucleotide sequences of these substances have not yet been characterised, they shall be referred to as bacteriocin-like inhibitory substances (BLIS) (Corsetti et al., 2008).

3.6.4. Chemical analysis of fermented flour extracts

Additional chemical evaluations were only performed for strains with described, relevant technological traits. Thus, organic acid production and VOC generation were measured from the 11 strains that rapidly acidified SFE (see Section 3.6.1.).

The concentrations of the organic acids that were identified are reported in Table 8. Lactic acid was produced in the range of 0.33–0.59 mg g^{-1}, with the lowest amounts corresponding to *W. cibaria* PON10030 and the highest to *Ln. citreum* PON10021. Acetic acid production was lowest from *Ln. citreum* PON10021 at 0.02 mg g^{-1} and highest from *W. cibaria* PON10032 at 0.11 mg g^{-1}.

VOC generation, established from chromatographic analysis, is reported in Table 9. Within the headspace of aSFEs, 18 compounds were identified: 2 acids, 7 alcohols, 5 aldehydes, 2 esters, 1 ketone, and 1 heterocyclic compound. Benzyl alcohol, whose concentration was also high in control broth, was the VOC present in the highest quantity in most aSFEs, although hexanoic acid was the major VOC produced from *Ln. pseudomesenteroides* PON10024 and *Ln. mesenteroides* PON10031. Moreover, those two strains, along with *Ln. citreum* PON10021, produced high amounts of decanal, and *Ln. pseudomesenteroides* PON100315 produced a high amount of 2-ethylhexanol.

4. Discussion

LAB are the most important microbial populations during sourdough production. Thus, several papers have described the isolation and physiological, genotypic, and technological characterisation of LAB from sourdough matrices that are used throughout the world to produce breads and other fermented baked goods (Corsetti and Settanni, 2007). However, within the published literature, little information is available on LAB associated with the raw materials used in bakeries. To provide a more complete understanding of the indigenous LAB present in raw materials used for sourdough production, in the present study, LAB of 13 flour samples belonging to the main flour species used to produce bread in Sicily (*T. aestivum* and *T. durum*) were analysed.

The majority of the flour samples were obtained from local Sicilian wheat cultivations, while others were purchased outside

Table 7
Proteolysis and inhibitory activity of wheat flour LAB.

Strain	Extracellular protease activity[a]		Antibacterial activity[b]		
	Substrate		Indicator strains		
	BSA	Gelatine	*L. monocytogenes* ATCC 19114	*L. innocua* 4202	*Lb. sakei* 2313
W. cibaria PON10030	−	+	−	−	−
W. cibaria PON10032	+	+	−	−	−
W. cibaria PON10050	−	−	−	−	−
W. cibaria PON10056	−	+	−	−	−
W. cibaria PON10069	−	+	−	−	−
W. cibaria PON100337	+	+	1.87 ± 0.15	1.97 ± 0.15	2.03 ± 0.12
W. cibaria PON100338	+	+	−	−	−
W. cibaria PON100339	−	+	−	−	−
W. cibaria PON10029	+	−	−	−	−
W. confusa PON10052	−	+	−	−	−
Lb. sanfranciscensis PON100100	−	+	1.27 ± 0.15	−	−
Lb. sanfranciscensis PON100336	−	−	2.33 ± 0.12	2.00 ± 0.20	−
Lb. plantarum PON10013	−	+	1.47 ± 0.12	1.67 ± 0.06	2.63 ± 0.06
Lb. plantarum PON10014	+	+	1.37 ± 0.06	2.03 ± 0.12	1.93 ± 0.12
Lb. plantarum PON10016	+	+	1.97 ± 0.12	1.47 ± 0.12	2.02 ± 0.08
Lb. plantarum PON100272	−	−	−	−	−
Lb. plantarum PON100274	−	+	1.73 ± 0.15	1.77 ± 0.12	2.37 ± 0.06
Lb. plantarum PON100348	−	−	−	−	1.07 ± 0.12
Lb. plantarum PON100351	−	−	−	−	−
Lb. sakei PON10022	−	+	−	1.37 ± 0.06	1.60 ± 0.00
Lb. sakei PON10067	−	−	−	−	−
Lb. sakei PON10097	+	+	−	−	−
Lb. sakei PON10098	+	+	−	1.40 ± 0.10	2.23 ± 0.25
P. pentosaceus PON10015	+	+	1.47 ± 0.06	±	1.63 ± 0.15
E. mundtii PON10062	−	+	−	−	−
E. mundtii PON10063	−	+	−	−	−
E. mundtii PON10085	−	+	−	−	−
Lc. lactis PON10057	−	+	−	−	−
Lc. lactis PON10072	−	−	−	−	−
Ln. mesenteroides PON10031	+	+	1.43 ± 0.15	1.33 ± 0.02	−
Ln. mesenteroides PON10064	−	+	−	−	−
Ln. pseudomesenteroides PON10024	+	−	−	1.50 ± 0.20	−
Ln. pseudomesenteroides PON10066	−	+	−	−	−
Ln. pseudomesenteroides PON10086	−	+	−	−	−
Ln. pseudomesenteroides PON100315	+	−	1.63 ± 0.15	1.97 ± 0.06	2.63 ± 0.25
Ln. pseudomesenteroides PON100349	−	−	−	−	−
Ln. pseudomesenteroides PON100350	−	+	−	−	−
Ln. citreum PON10021	+	+	1.60 ± 0.00	1.60 ± 0.00	2.23 ± 0.06
Ln. citreum PON10051	−	+	−	−	−
Ln. citreum PON10055	−	−	−	−	−
Ln. citreum PON10059	+	+	−	−	−
Ln. citreum PON10061	−	+	2.57 ± 0.15	2.03 ± 0.12	−
Ln. citreum PON10065	−	−	−	−	−
Ln. citreum PON10068	−	+	−	−	−
Ln. citreum PON10077	+	+	2.03 ± 0.15	−	−
Ln. citreum PON10078	−	−	−	−	−
Ln. citreum PON10079	+	+	−	−	−
Ln. citreum PON10080	+	+	1.47 ± 0.12	1.77 ± 0.12	2.60 ± 0.00
Ln. citreum PON10087	−	+	−	−	−
Ln. citreum PON100273	−	−	2.03 ± 0.12	−	−

Abbreviations: BSA, bovine serum albumin; *E.*, *Enterococcus*; *L.*, *Listeria*; *Lb.*, *Lactobacillus*; *Ln.*, *Leuconostoc*; *Lc.*, *Lactococcus*; *P.*, *Pediococcus*; *W.*, *Weissella*.
+, Positive for protease production; −, negative for protease production or, in case of antibacterial tests, no inhibition found; ±, little inhibition.
[a] The test was repeated twice.
[b] Width of the inhibition zone (mm). Results indicate mean ± S.D. of three independent experiments.

the regional boundaries. Notation of the collection is important for evaluation of the geographical relatedness among microbial populations. The samples were analysed with culture-dependent (isolation and culture of microorganisms) and culture-independent (analysis of total DNA within each sample) techniques. This approach allows for the optimal study of microbiota within complex matrices characterised by conditions that do not favour bacterial growth, such as the low water content of flour in the case of LAB growth. LAB may be present within flour in a dormant state; thus, they may not be detected by culture-dependent methods. Furthermore, it is conventional to employ a polyphasic (phenotypic and genetic) study approach. This type of combined detection system is generally reported to retrieve the most complete information on the presence, concentration, species, and strain composition of LAB within complex food matrices (Corsetti and Settanni, 2007).

Study samples were analysed for culturable LAB using three different media: two generic media for food LAB, MRS and M17, which are used to count rod and coccus LAB, respectively, and one specific medium for sourdough LAB, SDB, whose pH (5.6) is lower than those of other media, thus facilitating the growth of LAB adapted to acidic environments. SDB was therefore used to detect LAB with technological potential for sourdough production processes. The flours harboured until 10^4 LAB CFU g^{-1}, although the

Table 8
Organic acids produced in sterile flour extract broth by flour LAB.

Strains	Lactic acid (mg g^{-1})	Acetic acid (mg g^{-1})
Control	0.01 ± 0.00	0.00
Lb. plantarum PON100274	0.35 ± 0.02	0.00
Lb. sakei PON10098	0.34 ± 0.06	0.00
Ln. citreum PON10021	0.59 ± 0.08	0.02 ± 0.01
Ln. citreum PON10079	0.42 ± 0.04	0.09 ± 0.03
Ln. citreum PON10080	0.41 ± 0.05	0.08 ± 0.02
Ln. mesenteroides PON10031	0.52 ± 0.03	0.09 ± 0.01
Ln. pseudomesenteroides PON10024	0.49 ± 0.09	0.07 ± 0.02
Ln. pseudomesenteroides PON100315	0.44 ± 0.05	0.06 ± 0.02
W. cibaria PON10030	0.33 ± 0.06	0.06 ± 0.01
W. cibaria PON10032	0.47 ± 0.05	0.11 ± 0.03
W. cibaria PON100337	0.53 ± 0.07	0.08 ± 0.01
Statistical significance	$P < 0.001$	$P < 0.001$

Abbreviations: SFD, sterile flour dough; nSFD, non sterile flour dough; wnFDS, non sterile water non sterile flour; *Lb.*, *Lactobacillus*; *Ln.*, *Leuconostoc*; *W.*, *Weissella*; n.r., not found; n.d., not determined.
Results indicate mean values ± SD of three determinations.

three media gave slightly different results, with the highest counts obtained with M17. M17, however, was not highly specific for LAB. In fact, despite the presence of colonies on M17 agar plates of sample F23, none were found to be Gram-positive and catalase-negative. The low specificity of M17 for flour LAB may explain the high counts present on M17, and is likely attributed to it containing lactose, a sugar not found in flour, as the main carbon source. With the exception of sample F26, the levels of LAB detected on SDB were higher than those of MRS, in accordance with the findings of Picozzi et al. (2005), but opposite to those reported by Vera et al. (2009), who quantitatively examined sourdough lactobacilli on agar media. Moreover, in our study, some flour samples were negative for LAB on both SDB and MRS. Although flour and raw materials used in bread making in general have not been substantially investigated for LAB presence, Galli and Franzetti (1987) reported that wheat flours from Italian bakeries contain 10^4 LAB CFU g^{-1}, while maximal values of 10^3 LAB CFU g^{-1} were reported with soft wheat flour from organic cultivations in central Italy (Marche region) by Corsetti et al. (1998). Thus, our results are in agreement with those previously estimated for this kind of raw material. In general, the levels of LAB within wheat flours are higher than those in flours used in non-conventional bread making (amaranth, chickpea, rice, corn, quinoa, and potato) to generate products for celiac sprue affected persons, which showed a maximum of 2.06 Log LAB CFU g^{-1}(Corsetti et al., 2007).

Colonies that were likely LAB were isolated and subjected to phenotypic grouping as well as molecular typing by RAPD-PCR, the latter a technique commonly applied, alone or in combination with other methodologies, to detect LAB strains within food matrices (Corsetti and Settanni, 2007). Nine phenotypic groups and 50 unique strains, grouped into 13 RAPD clusters, were revealed, indicating a certain LAB biodiversity within wheat flour.

The 50 strains belonged to 11 LAB species: *Enterococcus mundtii*, *Lb. plantarum*, *Lb. sakei*, *Lb. sanfranciscensis*, *Lc. lactis*, *Ln. citreum*, *Ln. mesenteroides*, *Ln. pseudomesenteroides*, *Pediococcus pentosaceus*, *W. cibaria*, and *W. confusa*. Previous studies conducted on raw materials associated with bread production, such as wheat grains and flours (Galli and Franzetti, 1987; Corsetti et al., 1998, 2007), concluded that LAB species isolated from unprocessed raw materials were different from those commonly associated with sourdough fermentation. On the contrary, some of the LAB species we identified within our flour samples are typically found in mature sourdoughs at high cell concentrations, in particular *Lb. sanfranciscensis* and *Lb. plantarum* (Corsetti and Settanni, 2007), but also *W. cibaria* (De Vuyst et al., 2002; Valmorri et al., 2006; Iacumin et al., 2009).

In fact, the species mostly frequently isolated in our work belonged to the genus *Leuconostoc*: *Ln. citreum*, which is commonly present in sourdoughs (Björkroth and Holzapfel, 2006; De Vuyst and Neysens, 2005; Hemme and Foucaud-Scheunemann, 2004; Robert et al., 2009), followed by *Ln. mesenteroides*, which exerts positive effects during the production of sourdough-based baked products (Coppola et al., 1998; Robert et al., 2006).

There was no correlation between LAB species and/or strains and the geographical origin of the flour samples. Likewise, in a previous study evaluating the influence of several factors on the

Table 9
Analysis of volatile organic compounds emitted from flour extract broth inoculated with flour LAB.

Chemical compounds[a]	Samples[b]											
	Control	10021	10024	100274	10030	10031	100315	10032	100337	10079	10080	10098
Hexanal	5.9 ± 0.7	10.0 ± 1.0	11.0 ± 0.6	14.1 ± 1.3	10.7 ± 1.2	9.6 ± 0.6	6.6 ± 0.9	15.7 ± 0.6	8.0 ± 0.5	10.0 ± 1.4	10.4 ± 0.9	12.7 ± 1.2
2-Pentylfuran	2.6 ± 0.3	4.6 ± 0.2	3.5 ± 03	9.2 ± 1.4	10.5 ± 1.0	19.2 ± 2.3	10.4 ± 1.0	17.5 ± 0.6	6.6 ± 0.8	14.3 ± 0.6	10.9 ± 0.6	12.3 ± 1.6
1-Pentanol	n.d.	0.7 ± 0.2	1.2 ± 0.0	0.9 ± 0.2	1.0 ± 0.1	0.9 ± 0.3	1.3 ± 0.0	0.4 ± 0.1	n.d.	0.7 ± 0.1	0.7 ± 0.1	0.60 ± 0.1
2-Heptenal	n.d.	0.6 ± 0.0	1.8 ± 0.3	1.1 ± 0.1	0.9 ± 0.2	1.3 ± 0.3	n.d.	0.7 ± 0.1	n.d.	1.8 ± 0.1	0.7 ± 0.2	0.8 ± 0.1
1-Hexanol	7.9 ± 0.6	8.6 ± 1.0	10.5 ± 0.7	10.9 ± 0.6	8.1 ± 0.4	11.6 ± 0.5	18.3 ± 0.6	12.7 ± 2.0	6.3 ± 0.2	10.7 ± 0.5	8.8 ± 1.1	10.4 ± 1.0
Nonanal	2.3 ± 0.3	13.1 ± 0.8	10.7 ± 1.2	5.3 ± 0.5	7.5 ± 0.7	11.3 ± 1.3	20.9 ± 1.9	8.9 ± 0.6	2.9 ± 0.8	3.9 ± 0.1	5.4 ± 1.0	6.8 ± 0.8
Acetic acid	n.d.	0.7 ± 0.3	1.3 ± 0.1	0.4 ± 0.1	1.0 ± 0.1	0.8 ± 0.5	0.4 ± 0.1	1.1 ± 0.2	0.6 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	0.6 ± 0.1
1-Octen-3-ol	1.5 ± 0.3	4.8 ± 1.0	6.2 ± 1.1	5.8 ± 0.5	5.3 ± 0.6	4.8 ± 0.9	6.1 ± 0.9	6.6 ± 0.7	2.3 ± 0.3	5.0 ± 0.8	5.1 ± 0.7	5.6 ± 0.5
1-Heptanol	n.d.	1.7 ± 0.1	2.1 ± 0.2	2.2 ± 0.3	1.2 ± 0.3	2.0 ± 0.1	n.d.	1.9 ± 0.7	0.9 ± 0.0	2.0 ± 0.2	1.7 ± 0.1	1.9 ± 0.4
6-Methyl-5-hepten-2-ol	1.0 ± 0.4	2.1 ± 0.3	1.8 ± 0.1	1.8 ± 0.5	1.8 ± 0.1	1.4 ± 0.3	n.d.	2.6 ± 0.9	0.8 ± 0.2	1.4 ± 0.3	2.2 ± 0.3	1.1 ± 0.5
Decanal	8.8 ± 1.3	39.8 ± 1.8	28.1 ± 0.9	3.7 ± 0.4	8.2 ± 1.5	29.1 ± 1.5	0.7 ± 0.1	5.4 ± 0.7	4.3 ± 0.4	5.3 ± 0.9	4.1 ± 0.7	9.4 ± 0.6
2-Ethylhexanol	3.2 ± 0.5	12.7 ± 0.7	4.1 ± 0.7	3.4 ± 0.4	4.2 ± 1.0	4.8 ± 0.3	29.3 ± 2.9	2.7 ± 0.3	1.3 ± 0.1	2.9 ± 0.5	2.9 ± 0.2	4.1 ± 0.6
Benzaldehyde	8.3 ± 0.8	10.0 ± 0.7	10.1 ± 0.9	10.0 ± 1.2	8.1 ± 1.1	9.2 ± 0.6	10.3 ± 1.2	7.1 ± 0.9	6.7 ± 0.7	9.8 ± 1.1	8.7 ± 0.9	9.9 ± 1.4
3,5-Octadien-2-one	1.9 ± 0.1	2.0 ± 0.3	2.3 ± 0.6	1.2 ± 0.0	0.8 ± 0.3	2.4 ± 0.6	1.1 ± 0.5	1.2 ± 0.0	0.5 ± 0.0	1.4 ± 0.1	0.7 ± 0.0	1.4 ± 0.4
1-Octanol	1.0 ± 0.3	2.9 ± 0.5	3.5 ± 0.4	3.9 ± 0.8	2.6 ± 0.5	3.2 ± 0.5	5.7 ± 0.7	3.3 ± 0.6	1.7 ± 0.2	4.0 ± 0.6	2.8 ± 0.5	3.6 ± 0.2
Methylnaphthalene	1.5 ± 0.7	0.9 ± 0.5	2.6 ± 0.3	3.2 ± 0.7	1.4 ± 0.5	1.6 ± 0.3	1.1 ± 0.4	0.8 ± 0.3	1.3 ± 0.3	2.8 ± 0.4	2.2 ± 0.0	1.8 ± 0.5
Hexanoic acid	8.9 ± 1.1	3.3 ± 0.6	65.4 ± 1.4	12.8 ± 1.5	16.6 ± 1.3	63.2 ± 2.5	16.6 ± 1.7	20.4 ± 1.2	9.1 ± 1.7	12.7 ± 1.9	13.0 ± 0.8	23.8 ± 1.9
Benzyl alcohol	27.9 ± 2.8	51.0 ± 2.8	47.9 ± 1.8	50.5 ± 1.2	41.7 ± 1.2	39.3 ± 1.5	30.8 ± 1.3	33.3 ± 1.9	32.4 ± 1.4	46.6 ± 1.2	44.3 ± 3.0	47.2 ± 2.9

Results indicate mean values of three measurements and are expressed as relative peak areas (peak area of each compound/total area) × l00 ± SD.
n.d., not detected.
[a] The chemicals are shown following their retention time.
[b] Flour extract fermented with: *Ln. citreum* PON1002; *Ln. pseudomesenteroides* PON10024; *Lb. plantarum* PON100274; *W. cibaria* PON10030; *Ln. mesenteroides* PON10031; *Ln. pseudomesenteroides* PON100315; *W. cibaria* PON10032; *W. cibaria* PON100337; *Ln. citreum* PON10079; *Ln. citreum* PON10080; *Lb. sakei* PON10098.

diversity of LAB within traditional Belgian sourdoughs, Scheirlinck et al. (2007) found no significant correlation between LAB species and the type of flour, province of flour origin, or technological characteristics of the strains.

The apparent lack of correspondence between grain or flour and sourdough LAB communities is generally explained by the fact that although typical sourdough LAB may be present in raw materials, it is present as dormant (non-cultivable) flora (Corsetti et al., 2007). Therefore, we applied DGGE analysis to bypass the limitations of conventional cultivation techniques for the detection of microbial communities (Vaughan et al., 2002). The resultant DGGE findings did not fully agree with those obtained using the culture-dependent approach. Some LAB species were detected only with DGGE, and others isolated by culturing were not detectable by DGGE. This discordance is not surprising because DGGE analyses solely the total DNA of a given matrix (Muyzer et al., 1993) and thus does not distinguish between viable and nonviable microorganisms. On the other hand, it is reasonable to conclude that when bacteria isolated by plating are not detected with DGGE analysis based on 16S rRNA gene amplification, they are not a major component of the microbial community investigated (Shinohara et al., 2011). Taken together, the present study confirmed previous reports stating that a combined approach consisting of culture-dependent and -independent tools produces the best strategy for detection of microbial communities within complex matrices (Carraro et al., 2011).

All 50 different LAB strains were investigated for the presence of characteristics that are technologically relevant for sourdough fermentation. Eleven strains belonging to *Ln. citreum*, *Ln. pseudomesenteroides*, *Ln. mesenteroides*, *Lb. plantarum*, *Lb. sakei*, and *W. cibaria* showed good acidifying abilities *in vitro*: they rapidly decreased the pH of flour extract broth. Rapid acidification during sourdough fermentation is a technological characteristic of LAB that is of paramount importance (Corsetti and Settanni, 2007). The majority of flour LAB showed extracellular protease activity, a finding relevant to their use in bread making, because proteolysis affects the rheology and staleness of breads (Corsetti et al., 2000). LAB only displayed antimicrobial activity against bacterial indicators; no fungal strain was inhibited by LAB supernatants. Nineteen strains produced active substances of proteinaceous nature. Thus, a high number of strains isolated from the flour samples were BLIS producers. This confirms previous observations that raw materials used in bread making can harbour higher numbers of bacteriocinogenic LAB than fermented sourdough and the suggestion that their adaptation to a particular environment may allow them to persist during fermentation without the need of antagonistic strategies (Corsetti et al., 2008).

Further chemical analyses were carried out only on strains that displayed characteristics technologically relevant to sourdough production. Some strains had a fermentation quotient (FQ; lactic/acetic acid molar ratio) of 1.5–4, which is considered to positively affect the aroma and structure of final products (Spicher, 1983). Analysis of the volatile fraction of the broths identified 18 chemicals, including compounds whose presence is associated with the metabolism of LAB and that are able to beneficially affect aroma and taste in the final bread (Hansen and Hansen, 1996). Furthermore, compounds present at high amounts in sourdough breads, such as nonanal and benzaldehyde (Seitz et al., 1998), were detected at consistent levels for most strains.

In conclusion, flours used to produce sourdough bread in Sicily were found to harbour LAB of high technological potential with respect to sourdough bread production. Studies that evaluate the role of 11 of these identified strains during the bakery production of typical Sicilian sourdough-based baked goods are currently being prepared.

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