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Bacterial population in traditional sourdough evaluated by molecular methods

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ABSTRACT

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Aims: To study the microbial communities in artisanal sourdoughs, manufactured by traditional procedure in different areas of Sicily, and to evaluate the lactic acid bacteria (LAB) population by classical and culture-independent approaches.

Methods and Results: Forty-five LAB isolates were identified both by phenotypic and molecular methods. The restriction fragment length polymorphism and 16S ribosomal DNA gene sequencing gave evidence of a variety of species with the dominance of *Lactobacillus sanfranciscensis* and *Lactobacillus pentosus*, in all sourdoughs tested. Culture-independent method, such as denaturing gradient gel electrophoresis (DGGE) of the V6–V8 regions of the 16S rDNA, was applied for microbial community fingerprint. The DGGE profiles revealed the dominance of *L. sanfranciscensis* species.

In addition, *Lactobacillus*-specific primers were used to amplify the V1–V3 regions of the 16S rDNA. DGGE profiles flourished the dominance of *L. sanfranciscensis* and *Lactobacillus fermentum* in the traditional sourdoughs, and revealed that the closely related species *Lactobacillus kimchii* and *Lactobacillus alimentarius* were not discriminated.

Conclusions: *Lactobacillus*-specific PCR-DGGE analysis is a rapid tool for rapid detection of *Lactobacillus* species in artisanal sourdough.

Significance and Impact of the Study: This study reports a characterization of *Lactobacillus* isolates from artisanal sourdoughs and highlights the value of DGGE approach to detect uncultivable *Lactobacillus* species.

Keywords: 16S ribosomal DNA, lactic acid bacteria, LAB-specific PCR-DGGE analysis, sourdough.

INTRODUCTION

Sourdough is still one of the most important way of cereal fermentation (Vogel *et al.* 1999) and for centuries has been the only starter used in artisanal bread making. Artisan bread production, which often employs sourdough processes, provides a wide regional variety of breads and speciality products. Sourdough is a mixture of wheat or rye flour and water that is fermented with indigenous lactic acid bacteria (LAB) and yeasts (Gänzle *et al.* 1998; Vogel *et al.* 1999), and it is used as an inoculum for sourdough bread and for bread making. Based on usual principle used in artisanal or industrial processes, sourdoughs have been grouped into three types, based on fermentation time, temperature, propagation frequency and baker's yeast addition (Böcker *et al.* 1995; Vogel *et al.* 1996). Italian traditional wheat sourdoughs belong to type I, produced with traditional techniques and characterized by daily propagation at room temperature (20–30°C). Because of their artisan and region-depending handling, sourdoughs are an immense source of diverse LAB and yeast species and strains (De Vuyst *et al.* 2002). *Lactobacillus sanfranciscensis* and *Lactobacillus brevis* are the predominant LAB in Type I sourdoughs (Vogel *et al.* 1999), and *Lactobacillus fructivorans*, *Lactobacillus fermentum*, *L. brevis* and *Lactobacillus farciminis* were also

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found in some of these sourdoughs (Salovaara 1998; Vogel *et al.* 1999).

As the growth rate and yield of micro-organisms are governed by a multitude of ecological factors such as temperature, ionic strength and microbial products, LAB from traditional sourdoughs cannot be properly identified by cultivation-dependent approach, which could underestimate the microbial diversity (Muyzer *et al.* 1993; Hugenholtz *et al.* 1998). Different molecular methods were already used in order to evaluate the microbial biodiversity of the sourdough, such as whole-cell protein, cell-wall composition, random amplified polymorphic DNA (RAPD) and other PCR-based methods, that are useful tools to discriminate the diversity at species and strain level (Corsetti *et al.* 2003).

Recently, LAB population dynamics during fermentation process in propagated sourdoughs have been monitored by LAB-specific polymerase chain reaction denaturing gradient gel electrophoresis analysis (PCR-DGGE) system (Meroth *et al.* 2003). PCR-DGGE detects the 90–99% most numerous species of a community without discriminating living from dead cells or cells in a noncultivable state during whole-food fermentation processes, as demonstrated for the fermentation of maize dough pozol (Ampe *et al.* 1999; Ben Omar and Ampe 2000), malt whisky (Beek and Priest 2001), Italian sausages (Cocolin *et al.* 2001) and Ragusano cheese (Randazzo *et al.* 2002).

The aim of the present work was to study bacterial and *Lactobacillus* communities of traditional sourdoughs by using a combination of culture-dependent and culture-independent techniques. The latter included a DNA extraction protocol directly from sourdough, PCR-DGGE of rRNA genes (rDNA), using bacterial universal primers and *Lactobacillus*-specific primers.

MATERIALS AND METHODS

Sourdough sampling

Nine artisanal sourdough samples, collected in different areas of Sicily, were produced according to the most common traditional procedure locally used. Grape juice or honey was added to durum wheat flour kneaded with warm water and the dough was incubated for 16 h at room temperature. The first sourdough was again mixed with flour, tepid water and incubated for another 16 h at the same temperature. Then the sourdough was daily propagated by back slopping for several days and the ripe sourdough was used as inoculum for bread making.

Isolation of micro-organisms

The artisanal sourdough samples were taken aseptically, transferred to the laboratory and subjected to microbiological analyses within 4 h. The samples were homogenized with a Stomacher Lab-Blender 400 (Brinkmann, Westbury, NY, USA) for 5 min and serially diluted in sterile physiological solution (0·9% NaCl). Aliquots of samples were plated into DeMan-Rogosa-Sharpe (MRS) agar (Oxoid, Basingstoke, UK) and into sourdough bacteria (SDB) agar (Kline and Sugihara 1971), and incubated at 32°C for 48–72 h. Forty-five colonies were randomly picked from agar plates with different samples and each colony was purified by streaking three times. All purified isolates were stored in liquid cultures with 20% glycerol at −80°C until use.

Physiological and biochemical characterization of isolates

All isolates were tested for Gram reaction, catalase activity, spore formation, CO_2 production from glucose and ability to grow in MRS broth at 10 and 45°C in stationary tubes. Cell morphology was observed with a phase contrast microscope. Carbohydrate fermentation patterns were determined using API 50CHL kit (bioMérieux, Marcy-l'Etoille, France) and the isolates were identified using the bioMérieux SA software. Ability to ferment maltose at different concentrations (10 and 20 g l^{-1}) was determined in liquid medium with the following composition: peptone (10 g l^{-1}); yeast extract (5 g l^{-1}); tween 80 (1 ml l^{-1}); dipotassium phosphate (2 g l^{-1}); sodium acetate $3H_2O$ (5 g l^{-1}); diammonium citrate (2 g l^{-1}); magnesium sulphate $7H_2O$ (0·20 g l^{-1}); manganese sulphate $4H_2O$ (0·05 g l^{-1}); bromocresol purple (0·17 g l^{-1}); maltose (10 and 20 g l^{-1} respectively) and demineralized water. Change of the indicator's colour after 2 and 4 d indicated maltose fermentation to acids.

Reference strains

The following strains were used as reference in genotypic characterization: *Lactobacillus casei* DSM 20011^T, *Lactobacillus alimentarius* DSM 20249^T, *Lactobacillus pentosus* DSM 20314^T, *Lactobacillus plantarum* DSM 20174^T, *L. sanfranciscensis* DSM 20451^T, *Lactobacillus mindensis* DSMZ 20184^T, *Lactobacillus pontis* DSMZ 8475^T, *Lactobacillus panis* DSMZ 6036^T, *L. brevis* DSMZ 20054^T, *Lactobacillus reuteri* DSMZ 20016^T. Strains K1 and CR6A, previously identified as *Lactobacillus kimchii* and *L. fermentum*, respectively, were also used in this study. The strains were grown in MRS medium and the plates were incubated at 32°C under anaerobic conditions using AnaeroGen kit (Oxoid).

Total DNA extraction from pure isolates

Cell cultures (1·5 ml), in the late exponential growth phase, were centrifuged at 8000 *g* for 10 min and the cell pellets were washed and resuspended in 0·5 ml of TE-buffer (10 mmol l^{-1}

Tris–HCl, 1 mmol l^{-1} EDTA; pH 8·0). The suspension was transferred in a 2-ml screw-capped tube containing 0·3 g of sterile zirconium beads (diameter, 0·1 mm), homogenized in a bead-beater (Biospec Products, Bartlesville, OK, USA) at 5000 *g* for 3 min and cooled on ice. The homogenate was centrifuged at 13 000 *g* for 5 min and the supernatant fluid was stored at −20°C until use.

PCR conditions

The PCR amplification for PCR or restriction fragment length polymorphism (RFLP) analysis was carried out with *Taq* DNA polymerase kit (Invitrogen, Carlsbad, CA, USA) using the universal PCR primers 7-f and 1510-r to amplify the bacterial 16S rDNA (Lane 1991). Reaction mixtures consisted of 20 mmol l^{-1} Tris–HCl (pH 8·4), 50 mmol l^{-1} KCl, 3 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} each of deoxynucleoside triphosphates, 1·25 U of *Taq* polymerase, 5 pmol of each primer and 1 μg ml^{-1} of DNA, in a final volume of 50 μl. The samples were amplified in a Perkin–Elmer Applied Biosystem GenAmp PCR System 9700 (Foster City, CA, USA) programmed as follows: initial denaturation of DNA for 5 min at 94°C; 35 cycles each consisting of 30 s at 94°C, 30 s at 56°C and 40 s at 68°C; and extension of incomplete products for 7 min at 68°C. PCR products were quantified by electrophoresis on a 1·2% (w/v) agarose gel containing ethidium bromide, and where necessary were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

The universal PCR primers U968-GC-f and L1401-r were used to amplify the V6–V8 regions of eubacterial 16S rDNA (Nubel *et al.* 1996) for DGGE analysis. The 40-nucleotide GC rich sequence at the 5′-end of primer U968-GC improves the detection of sequence variations of amplified DNA fragments by subsequent DGGE (Muyzer *et al.* 1993).

The PCR to investigate the *Lactobacillus*-specific sourdough bacterial community by DGGE was performed with primer 7-f and Lab-0677-r. The PCR product was used as template in a nested PCR by using the primer Bact0124-CG-f and Uni-0515-r (Heilig *et al.* 2002) and the following programme: predenaturation at 94°C for 5 min; 35 cycles of denaturation 94°C for 30 s, 56°C for 20 s, and 68°C for 40 s; and finally 7 min at 68°C.

RFLP analysis of 16S rDNA

To examine the microbial biodiversity and to select unique strains prior to sequence analysis, RFLP analysis of 16S rDNA of the PCR products was performed using *Hae*III, *Alu*I and *Msp*I restriction enzymes (MBI Fermentas, Hannover, CA, USA), followed by electrophoresis of the products on a 2% (w/v) agarose gel in 1X TBE buffer (89 mmol l^{-1} Tris–borate, 89 mmol l^{-1} boric acid, 2 mmol l^{-1} EDTA; pH 8·0) containing ethidium bromide. Gel was visualized as described before.

Sourdough DNA extraction

Ten grams of sourdough samples, provided from Modica and Ispica areas (sample A and B respectively) were resuspended into 90 ml of sterile physiological solution and homogenized for 180 s in a Stomacher. A total of 25 ml of suspension were placed into a 50-ml sterile tube containing glass pearls (diameter, 3 mm; Tamson, Zoetermeer, the Netherlands) and mixed in a vortex mixer for 5 min. Then, the supernatant was transferred to clean tubes and centrifuged for 10 min at 10 000 rpm at 4°C. The cell pellets were resuspended in 1 ml of TE buffer and centrifuged at 8000 rpm for 10 min. The supernatant fluid was removed and the remaining 100 μl and pellet were mixed and transferred to a 2-ml screw-cap tubes containing 0·3 g of zirconium beads and 150 μl of phenol-TE (phenol equilibrated with TE) (Life Technologies, Carlsbad, CA, USA). The samples were treated at 5000 rpm for 180 s in a bead beater. After the addition of 150 μl of CI solution, consisting of chloroform and isoamyl alcohol at a 24 : 1 (v/v) ratio, the tubes were vortexed briefly and centrifuged at 13 000 rpm for 5 min. DNA was obtained from the lysate by using phenol–chloroform–isoamyl alcohol extraction and ethanol precipitation procedures, as previously described (Randazzo *et al.* 2002).

DGGE analysis

The PCR products, generated with primers U968-GC-f and L1401-r and Bact 0124-CG-f and Uni-0515-r were separated by DGGE, according to the specification of Muyzer *et al.* (1993) by using the Dcode System (Bio-Rad, Laboratories, CA, USA) with the following modifications. Polyacrilamide gels (dimensions 200 × 200 × 1 mm) consisted of 8% (w/v) polyacrylamide (37·5 : 1 acrylamide–bisacrylamide) and 0·5X TAE buffer (2 mol l^{-1} Tris base, 1 mol l^{-1} glacial acetic acid, 50 mol l^{-1} EDTA; pH 8·0). Optimal separation of the PCR products for the species within these sourdough samples was achieved with a 30–60% urea–formamide denaturant gradient. A 100% denaturant corresponded to 7 mol l^{-1} urea and 40% (w/v) formamide.

Electrophoresis was performed at a constant voltage of 85 V and a temperature of 60°C for 16 h. The DNA bands were visualized by silver staining and developed as previously described (Sanguinetti *et al.* 1994).

DNA sequence analysis

The amplified PCR products obtained from 10 bacterial isolates (F3, K2, K4, K6, H3, H4, I3, I5, G4 and L5),

purified with the Qiaquick PCR purification kit (Qiagen), were directly sequenced using primer 1100 reverse (Lane 1991) 5′-GGGTTGCGCTCGTTG-3′ and Sequenase sequencing Kit (Amersham, Slough, UK) to obtain partial sequence of the 16S rRNA genes. Products of sequencing reactions were analysed with the automatic LI-COR DNA sequencer 4000L (Lincoln, NE, USA) and corrected manually. Homology searches of the 16S rDNA sequences were performed using the BMC Nucleic Acid Sequence Search Service available in the internet (http://searchlauncher.bcm.tmc.edu).

RESULTS

Physiological and biochemical characterization of isolates

A total of 45 LAB were randomly chosen from MRS and SDB agar plates for phenotypic and biochemical characterization. All 45 isolates were considered LAB-based on their positive-Gram reaction, nonmotility, absence of catalase activity and spore forming, and their rod or coccal-shape. Forty-two isolates grew at 15°C and 19 at 45°C. Thirty-four isolates produced gas from glucose

Table 1 Phenotypical characterization of lactic acid bacteria isolates

		Growth at (°C)			Maltose fermentation (g l^{-1})	
Strains	Source	15	45	CO_2 from glucose	10	20
MBB	Bronte (CT)	+	+	–	+	+
MCA, MCB, MCC and MCD	Carlentini (SR)	+	+	–	+	+
SCA	Carlentini (SR)	+	–	+	+	+
SCB	Carlentini (SR)	+	–	+	+	+
MR1A and MR1B	Raddusa (CT)	+	+	–	+	+
SR1A	Raddusa (CT)	+	+	+	+	+
SR1B and SR2A	Raddusa (CT)	+	–	+	+	+
SR1C and SR3C	Raddusa (CT)	+	+	+	+	+
SR2B	Raddusa (CT)	+	–	+	+	+
SR3A and SR3B	Raddusa (CT)	+	+	–	+	+
MSA	Sila	+	+	+	–	–
MSB	Sila	+	+	+	–	+
MSC	Sila	+	–	+	+	+
SSA	Sila	+	+	+	–	–
SSB	Sila	+	–	+	–	+
SSC	Sila	+	–	+	–	–
MGA	Grammichele (CT)	+	–	+	+	+
MGB	Grammichele (CT)	+	+	+	–	–
MGC	Grammichele (CT)	+	–	+	–	+
SGB and SGC	Grammichele (CT)	+	–	+	–	–
SM1A1 and SM1B	Modica (RG)	–	–	+	+	+
SM1A2	Modica (RG)	+	–	+	+	+
SM2A	Modica (RG)	+	+	+	+	+
SM2B	Modica (RG)	–	+	+	–	–
SM2C	Modica (RG)	+	–	+	–	–
H3	Modica (RG)	+	–	+	–	–
H4	Modica (RG)	+	–	+	–	–
I3	Modica (RG)	+	–	+	+	+
I5	Modica (RG)	+	–	+	+	+
F3 and F4	Rosolini (SR)	+	–	–	–	–
K4	Ispica (RG)	+	–	+	+	+
K2	Ispica (RG)	+	–	+	+	–
K6	Ispica (RG)	+	–	+	+	+
G4	Catania	+	–	+	–	–
L5	Catania	+	+	+	+	+

indicating a heterofermentative metabolism (Table 1). Regarding biochemical traits, all strains were positive for the following characters: hydrolysis of aesculin; fermentation of L-arabinose, ribose, galactose, glucose, mannose, mannitol, amygdalin, sucrose, trealose and gentibiose (data not shown). The heterofermentative strains SR1A, SR1B, SR2A and K2 were assigned to the *Lactobacillus paracasei* species, the strains MR1A, MR1B and L5 to *L. casei*, the strains SCB and SM2B were not identified, and the remaining strains were identified as *L. pentosus*. The percentage of identification obtained by using the bioMérieux SA software was always low (48–51%). Thirteen strains did not ferment maltose at tested concentrations. Twenty-nine strains were able to ferment maltose at 10 g l^{-1} and 31 isolates at 20 g l^{-1}.

RFLP of the 16S rDNA of isolates

The RFLP of 16S rDNA of the 45 isolates was carried out (Table 2) and the different restriction profiles were compared with type strains in order to aid identification of the strains at the species level. Six unique types were distinguished by RFLP, and five of them were identified as *L. pentosus*, *L. casei*, *L. kimchii*/*L. alimentarius*, *L. sanfranciscensis* and *L. plantarum*. The sixth unique type comprised the isolates SCB and SM2B, which could not be identified because of their unique profiles. Molecular weight of fragments generated by digestion enzymes are presented in Table 2.

Identification of bacterial isolates by 16S rDNA sequence analysis

Ten strains (F3, K2, K4, K6, H3, H4, I3, I5, G4 and L5) were chosen for partial sequencing analysis of the 16S rDNA (Table 3). The data demonstrated the highest identity (98–99%) with 16S rDNA sequences of *L. sanfranciscensis*, *L. casei*, *Lactobacillus paraplantarum*, *L. pentosus* and *L. kimchii* species in GenBank database confirming the RFLP results.

PCR-DGGE fingerprinting of sourdough samples and isolates

The 16S rDNA amplicons from the five type strains (*L. sanfranciscensis* DSM 20451^T, *L. plantarum* DSM 20174^T, *L. pentosus* DSM 20314^T, *L. casei* DSM 20011^T, *L. alimentarius* DSM 20249^T), generated with primers U968GC-f and L1401-r, were compared with the microbial community DGGE patterns of artisanal sourdough samples, provided from Modica area (Fig. 1). The most intense amplicon a (lane 1, Fig. 1), present in sourdough sample, showed identical position of *L. sanfranciscensis* 20451 type

Table 2 Restriction fragment length polymorphism (RFLP) results and molecular weight of fragments generated by restriction enzymes

Isolates	RFLP results	Molecular weight of fragments (bp)		
		*Hae*III	*Msp*I	*Alu*I
MBB, MCA, MCB, MCC, MCD, SCA, SR1C, SR3C, SR2B, SR3A, SR3B, SSA, MGA, H4 and K6	*Lactobacillus pentosus*	580 450 300 150	600 380 200 150	650 280 250 210 110
MR1A, MR1B and L5	*Lactobacillus casei*	600 450 290 120	550 490 190	650 250 200 190 180
SCB and SM2B	Unique profile	580 450 320 150	600 380 200 150	650 250 220 110
SR1A, SR1B, SR2A and K2	*Lactobacillus kimchii*/ *Lactobacillus alimetarius*	600 450 290 120	550 490 190	600 450 310 200 190
MSA, MSB, MSC, SSB, SSC, MGB, MGC, SGB, SGC, SM1A1, SM1B, SM1A2, SM2A, SM2C, I3, I5 and K4	*Lactobacillus sanfranciscensis*	1100 250 120	610 590 240	600 250 200 130
H3, F3, F4 and G4	*Lactobacillus plantarum*	580 450 300 150	600 380 200 150	620 280 250 200 120

Table 3 Identification of isolates by partial sequence of the 16S rDNA

Isolates	References of ribosomal database project	Identity (%)
F3	*Lactobacillus paraplantarum*	99
K2	*Lactobacillus kimchii*	99
K4	*Lactobacillus sanfranciscensis*	98
K6	*Lactobacillus pentosus*	99
H3	*Lactobacillus paraplantarum*	99
H4	*Lactobacillus pentosus*	99
I3	*Lactobacillus sanfranciscensis*	97
I5	*Lactobacillus sanfranciscensis*	98
G4	*Lactobacillus paraplantarum*	98
L5	*Lactobacillus casei*	99

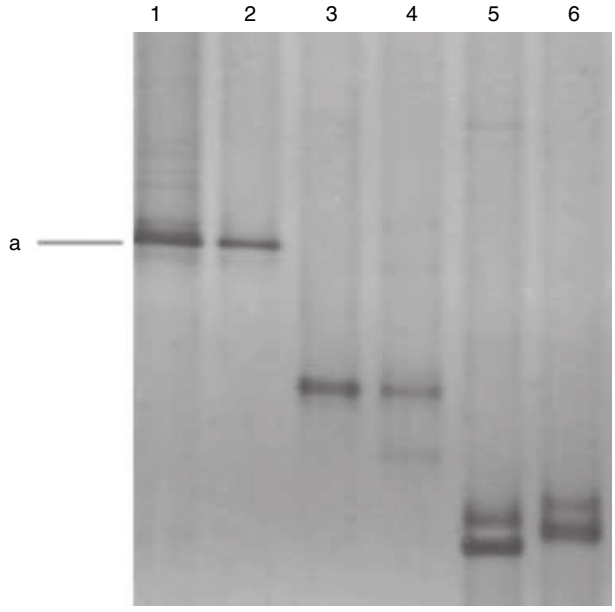

Fig. 1 Denaturing gradient gel electrophoresis profiles of amplified rDNA regions with universal primers, obtained from sourdough sample and from different lactic acid bacteria species. Lane 1: traditional sourdough sample provided from Modica area; lane 2: *Lactobacillus sanfranciscensis* (DSM 20451^T); lane 3: *Lactobacillus plantarum* (DSM 20174^T); lane 4: *Lactobacillus pentosus* (DSM 20314^T); lane 5: *Lactobacillus casei* (DSM 20011^T); lane 6: *Lactobacillus alimentarius* (DSM 20249^T)

strain (lane 2, Fig. 1). Weak bands were also present but were not labelled through type strains used in this study.

To characterize the *Lactobacillus* community of the two sourdough samples provided from Modica and Ispica areas (sample A and B respectively), PCR-DGGE with primers Bact-0124-CG-f and Univ-0515-r was performed with DNA extracted from sourdoughs (lanes 1 and 16, Fig. 2), from nine reference strains (lanes 3, 5, 6, 7, 8, 10, 11 and 13, Fig. 2), from four wild strains (lanes 2, 4, 12 and 15), isolated form the sourdough samples, and from the isolate K1 (lane 4) previously identified as *L. kimchii*. Analyses of sourdoughs A and B showed similar profiles, with intense bands at the same position, suggesting similarity in the *Lactobacillus* group composition. Band-a in the sourdough samples (lanes 1 and 16, Fig. 2) showed the same migration of *L. alimentarius* and *L. kimchii* species (lanes 13 and 14, Fig. 2). Band-b generated in the sourdoughs (lanes 1 and 16, Fig. 2) showed the same position as the isolate K4, identified as *L. sanfranciscensis* (lane 2, Fig. 2). The band-c was likely to be *L. fermentum*, as its position corresponded to that of the strain *L. fermentum* CR6A.

DISCUSSION

In the present study, the microbial population of traditional sourdough samples, produced by using wheat flour under traditional manufacture, was revealed by classical and culture-independent methods. Investigation by culturing techniques showed that among the 45 sourdough LAB isolates, 17 isolates were identified as *L. sanfranciscensis*, 14 as *L. pentosus*, five as *L. plantarum*, four as *L. kimchii*/*L. alimentarius* and three as *L. casei*. Strains belonging to *L. fermentum*, *L. brevis* and *L. paralimentarius* species were not found, although these species are frequently isolated in European sourdoughs (Vogel *et al.* 1999; De Vuyst *et al.* 2002). The dominance of *L. sanfranciscensis*, *L. pentosus* and

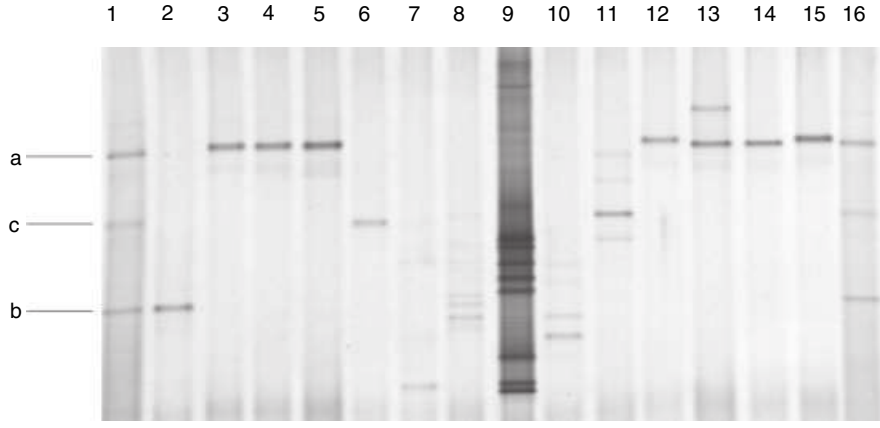

Fig. 2 Denaturing gradient gel electrophoresis profiles of amplicons, generated by PCR with the *Lactobacillus*-specific primer, of traditional sourdough samples, *Lactobacillus* isolates and *Lactobacillus* type strains; Lane 1: traditional sourdough (sample B) provided from Ispica area; lane 2: K4 isolate; lane 3: *Lactobacillus plantarum* (DSM 20174^T); lane 4: K6 isolate; lane 5 *Lactobacillus brevis* DSM 20054^T; lane 6: *Lactobacillus fermentum* CR6A; lane 7: *Lactobacillus reuteri* DSMZ 20016^T; lane 8: *Lactobacillus pontis* DSMZ 8475^T; lane 9: Marker; lane 10: *L. pontis* DSMZ 6036^T; lane 11: *Lactobacillus mindensis* DSMZ 20184^T; lane 12: H4 isolate; lane 13: *Lactobacillus alimentarius* DSMZ 20249^T; lane 14: K1 isolate; lane 15: H3 isolate; 16: traditional sourdough (sample A) provided from Modica area

L. plantarum, confirmed previous studies (Spicher 1983; Gobbetti *et al.* 1994; Ottogalli *et al.* 1996; Gobbetti 1998) which have reported *L. plantarum* as the facultatively heterofermentative species mainly associated with *L. sanfranciscensis*. Recently, the association has been outlined of *L. sanfranciscensis* with *L. alimentarius* in the sourdough samples of Italian wheat and rye sourdoughs (Corsetti *et al.* 2001), while other LAB species were frequently isolated from Russian (Kazanskaya *et al.* 1983), Finnish (Salovaara and Katumpää 1984), Swedish and German rye sourdoughs (Spicher and Lönner 1985; Spicher 1987).

In the present study, molecular identification, based on RFLP analysis of the 16S rDNA, depicted a unique profile for *L. sanfranciscensis* and *L. pentosus* isolates. This could be due to the high degree of intraspecific homogeneity of the 16S rDNA region. In fact the analyses, investigating this part of the ribosomal operon, have been proved to be reliable to identify the species but not suitable to type the strains as reported by Foschino *et al.* (2001), and to discriminate among phylogenetically related species, such as between *L. alimentarius* and *L. kimchii*. The fermentation profiles of the presumptive *L. kimchii* or *L. alimentarius* strains showed that the four strains examined were able to ferment all soluble carbohydrates contained in the wheat flour (maltose, sucrose, glucose and fructose) and were assigned to *L. alimentarius* species, according to previous study (Corsetti *et al.* 2001). Furthermore, because of the absence of fermentation of D-xylose and melezitose, the isolates have been allotted to *L. alimentarius*, as previously reported (Yoon *et al.* 2000). In contrast, the wild strain K2 has been assigned to the *L. kimchii* species by 16S rDNA sequence analysis.

As phenotypic characterization was ambiguous, giving the growth of few isolates belonging to the *L. sanfranciscensis* species at 45°C and a maltose-negative fermentation, the presence of the species was confirmed by sequence analysis. Moreover, the PCR-DGGE approach showed the dominance of *L. sanfranciscensis* in traditional sourdoughs. The polymorphism among *L. sanfranciscensis* strains revealed that no evident relation was found between phenotypic traits of strains and geographical areas of isolation. In addition, using LAB-specific PCR-DGGE systems, we confirmed the presence, in the composition of the two traditional sourdoughs, of the three LAB species *L. sanfranciscensis*, *L. fermentum* and *L. alimentarius*/*L. kimchii* by comparison of the PCR amplicon migration distances in DGGE gel with those of references strains. Most of the bands of the genetic fingerprint of the sourdough population could be allotted by using a second primer pair (Bact-0124-CG-f and Univ-0515-r) in a nested PCR-DGGE analysis, without the need for further sequence analysis of PCR fragments (Meroth *et al.* 2003). As the migration distance of PCR amplicons of *L. alimentarius* and *L. kimchii* species appeared to be identical in the DGGE gel, further investigations like DNA–DNA hybridization or sequence analysis of the fragments are required to clearly reveal the presence of the species in the traditional sourdoughs.

With regard to the effects of ecological factors on the development of the microbiota in sourdough samples, culturing approach could not permit to isolate strains belonging to *L. fermentum* species, which were detected by PCR-DGGE analysis, in contrast with previous reported studies (Meroth *et al.* 2003).

Comparing the profiles of sourdough samples with those generated from type strains, no *L. mindensis*, *L. panis* and *L. pontis* strains were found although these species showed more that one fragment in DGGE, because of slightly different copies of the 16S rRNA gene present in the genome.

Differences in wheat and rye flour, other ingredients and traditional technology are the main factors, which may influence the sourdough microbial ecosystem, which still represents a source of unidentifiable and, perhaps, new sourdough LAB species. In conclusion, the present study proved to be a preliminary approach to better understand the dominant LAB of traditional sourdoughs, which could be involved in the sensorial properties of sourdough bread.

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Oggetto: dichiarazione sul contributo dell'autore

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DICHIARA

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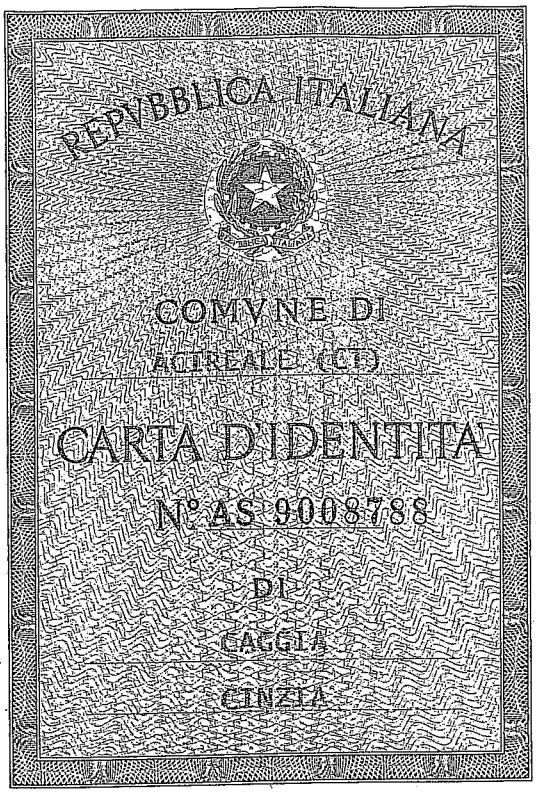
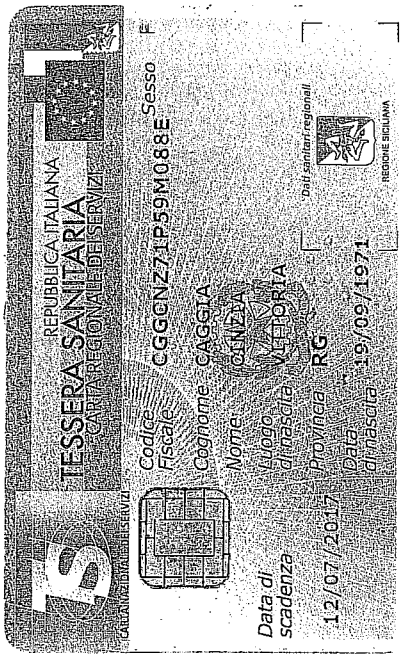
Catania, 24/10/2016

In fede





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