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ORIGINAL PAPER

Characterization of lactic acid bacteria isolated from sourdoughs for *Cornetto*, a traditional bread produced in Basilicata (Southern Italy)

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Abstract A total of 41 strains of lactic acid bacteria (LAB) isolated from durum wheat sourdoughs used to produce *Cornetto* di Matera bread, were identified by SDS-PAGE of whole cell proteins (WCP) and screened for acid production ability, antimicrobial activity and exopolysaccharide (EPS) production. The isolates were identified as *Lactobacillus plantarum* (49%), *Leuconostoc mesenteroides* (17%), *Lactobacillus curvatus* (15%), *Lactobacillus paraplantarum* (12%), *Weissella cibaria* (5%) and *Lactobacillus pentosus* (2%). Several strains of *Lb. plantarum* and *Leuc. mesenteroides* showed a high acid production ability. The antagonistic activity was tested using an agar-spot deferred antagonism assay against a set of five indicators. The species had different profiles of inhibition. *Lb. plantarum* had the largest spectrum of inhibition, while no isolates of *W. cibaria* and *Leuc. mesenteroides* showed antimicrobial activity. No strains had antimicrobial activity against *Bacillus cereus*. The inhibitory activity of five strains was confirmed to be sensitive to proteolytic enzymes and thus potentially due to bacteriocin production. All *Leuc. mesenteroides* and *W. cibaria* strains produced EPS from sucrose. Some *Lb. plantarum* and *Lb. paraplantarum* strains produced EPS from different sugars in solid media. EPS production in liquid media was different within the species, with the highest production in liquid media containing glucose and maltose. A defined strain starter culture (*W. cibaria* DBPZ1006, *Lb. plantarum* DBPZ1015 and *S. cerevisiae* MTG10) was selected on the basis of technological properties and tested in model sourdough fermentations.

Keywords Characterization · Lactic acid bacteria · Sourdoughs · *Cornetto* di Matera

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Introduction

Sourdough is an important cereal fermentation, involved in bread production, that greatly contributes to the flavor and functional properties of the final product. It is a mixture of wheat or rye flour and water, fermented by an association of lactic acid bacteria and yeasts, whose composition depends on the technology applied for its production (Vogel et al. 1999). Differences in the type of flour, ingredients and technology influence the microbial composition of sourdough and the characteristics of baked goods (Corsetti et al. 2001).

Lactic acid bacteria (LAB) play a major role in sourdough fermentations (De Vuyst and Vancanneyt 2007). Facultative heterofermentative LAB are important for the production of sourdough bread with a good grain and porous crumb and contribute to the sensory quality, while heterofermentative LAB, with their metabolic products, influence the flavor and promote the leavening (Arendt et al. 2007).

In Italy more than 200 types of traditional breads have been classified by the Istituto Nazionale di Sociologia Rurale (INSOR 2000). Sourdoughs are used in more than 30% of bakery products, some of which originate from a very old tradition and differ in the type of flour, ingredients, type of sourdough technology and shelf-life (De Vuyst and Neysens 2005).

Cornetto is a traditional bread produced in small bakeries in Matera (Basilicata, Southern Italy), that recently has obtained the Protected Geographical Indication (PGI) from the European Community (decree 9/6/2007, Gazzetta Ufficiale dell'Unione Europea n. 239, 11/10/2004). According to the standard of identity for PGI, the bread is to be manufactured by using durum wheat flours, NaCl (2.5–3% w/w), water and sourdough as leavening agent. Sourdoughs used for its production can be classified as type Ib (De Vuyst and Neysens 2005): the fermentation is performed at room temperature (20–30°C) with a final pH about 4.0 and is characterized by repeated daily refreshments to keep the mixed culture in an active state.

The objective of this study was the identification and the technological characterization of lactic acid bacteria isolated from sourdoughs used for the production of *Cornetto*, in order to select strains with interesting technological properties that are relevant for their rational exploitation as starters in bread production.

Materials and methods

Strains and culture conditions

Forty-one strains of lactic acid bacteria (LAB), isolated from sourdoughs used for the production of *Cornetto* di Matera and identified by phenotypic tests (Ricciardi et al. 2002), were used in this study.

The strains were routinely propagated by overnight cultivation in MRS broth (Oxoid, Basingstoke, Hampshire,UK) at 30°C.

Lactobacillus paracasei DSM4905, *Lb. plantarum* DSM20174, *Micrococcus flavus* DSM1790 (obtained from Deutsche Sammlüng von Mikroorganismen und Zellkülturen, Braunschweig, Germany), *Listeria innocua* BL86/26 (obtained from the culture collection of the Moorepark Food Research Centre, Fermoy, Co. Cork IRL) and *Bacillus cereus* ATCC9139 (obtained from American Type Culture Collection, commercialized by LGC Promochem s.r.l., Italy) were used as indicators for the deferred antagonism assay (see below). *Lb. paracasei* DSM4905 and *Lb. plantarum* DSM20174 were routinely cultivated in MRS broth at 30°C for 16 h, while *M. flavus* DSM1790, *L. innocua* BL86/26 and *B. cereus* ATCC9139 were routinely cultivated in Tryptone Soya Broth (Oxoid), with 0.6% (w/v) Yeast extract (Oxoid) (TSBYE) at 30°C for 16 h.

All strains were maintained as freeze-dried stocks in 11% (w/v) reconstituted skim milk containing ascorbic acid (0.1% w/v) in the culture collection of Dipartimento di Biologia, Difesa e Biotecnologie Agro-forestali, Università degli Studi della Basilicata, Potenza, Italy.

Identification by SDS-PAGE of whole cell proteins

LAB strains were identified by SDS-PAGE of whole cell proteins as described in Ricciardi et al. (2005). Isolates and reference strains were cultivated in modified APT broth. Cells were harvested by centrifugation (12,000×*g*, 5 min), washed twice in 50 mmol/l Tris/HCl, pH 8.0, and resuspended in 150 µl of the same buffer containing 2 mg of egg white lysozime/ml (Sigma-Aldrich, Milan, Italy). Glass beads (0.15–0.212 mm diameter, Sigma) were added, followed by vortexing at high speed for 3 min. Cells suspensions were incubated at 37°C for 1 h and mixed by three cycles of sonication (10 min for each treatment at power 10%; Sonorex Super 10P, Bandelin, Berlin). After incubation, glass beads and cells debris were removed by centrifugation (12,000×*g*, 5 min) and the supernatants were used for the SDS-PAGE analysis. Protein concentration in the cell lysates was determined by using the Bradford Reagent for Protein Determination (Sigma), as described by the manufacture.

Electrophoretic runs were carried out in a MiniProtean III apparatus (Bio-Rad Laboratories) using the running gel (10% w/v total monomer concentration, T, 2.67% w/v crosslinking monomer concentration, C) and the stacking gel (4% w/v T, 2.67% w/v C) system described in Piraino et al. (2002). Eleven samples (standardized to load 5–10 µg proteins) and two molecular weight standards (Sigma Marker Wide range, Sigma) were applied to each gel. Gels were run at a constant intensity of 20 mA for 3 h using a Power Pack 3,000 unit (Bio-Rad). Gel images were digitized in Diversity Database™ software (Bio-Rad Laboratories Ltd., Watford, Herts, UK) and processed for detection of the protein bands. Gel reports containing sample name, molecular mass and band intensities in lane were exported from Diversity Database™ software and imported in Microsoft Excel™ software (Microsoft Corporation, 2003) for data processing.

Characterization of the isolates

Acid production ability

A sub-culture in Sour Dough Bacteria (SDB) broth (Kline and Sugihara 1971) was obtained from the active stock culture by 1% (v/v) inoculum and incubation overnight at 30°C. The sub-culture was standardized to a final $OD_{650} = 1$ and used to inoculate (5% v/v) sterile SDB broth. The pH was measured on aliquots of the modified SDB broth after 6 and 24 h. The decrease in pH (ΔpH) was calculated for each incubation time as the difference between the values immediately after inoculation (t = 0 h) and the values at the successive time points.

Antimicrobial activity

Inhibitory activity against *Lb. paracasei* DSM4905, *Lb. plantarum* DSM20174, *M. flavus* DSM1790, *L. innocua* BL86/26 and *B. cereus* ATCC9139 was tested using a deferred antagonism assay as described in Parente et al. (2001).

The overlay medium was modified MRS agar (MRS broth buffered at pH 6.5 containing 0.1 mol/l morpholin propanesulphonic acid, Sigma, and 0.6% w/v Bacteriological Agar, Oxoid) for *Lb. paracasei* DSM4905 and *Lb. plantarum* DSM20174, and TSBYE with 0.6% w/v agar for *L. innocua* BL86/26, *M. flavus* DSM1790 and *B. cereus* ATCC9139.

The diameter of the inhibition zones was measured using a calliper. Strains were considered positive when showing an inhibition zone >10 mm diameter.

To evaluate if the inhibition was due to bacteriocin-like inhibitory substances (BLIS), the assay was repeated by spotting 5 µl of trypsin (Sigma), chymotrypsin (Sigma) or pronase (Boehringer) solutions close to the bacterial spots before the overlay. Enzyme solutions was prepared by dissolving 0.2% (w/v) of each enzyme in a 50 mmol/l Tris water solution buffer (pH 7.5) containing 5 mmol/l $CaCl_2$, filtered through a Millex-GV filter (Millipore SpA, Milan, Italy), and spotted next to the inoculum spot. A negative control (sterile buffer without enzymes) was included and negation of the inhibition zone near the enzyme spot was taken as evidence of the presence of BLIS.

EPS production

LAB strains were screened for exopolysaccharides (EPS) production by using a pick test on MRS agar plates containing 2% (w/v) of glucose (MRS) or maltose (mMRS) or 5% (w/v) of sucrose (sMRS) as carbon sources. The plates were incubated at 30°C for 48 h and the strains which produced slimy colonies were recorded as capable of producing EPS.

LAB strains that produced EPS on agar plates were selected and tested for EPS production in liquid MRS medium, supplemented with the same sugars, using the microhaematocrit capillaries method, as described by Ricciardi et al. (1997).

EPS concentration in liquid media (MRS, mMRS, sMRS) was measured by using the Dubois method, after removing simple carbohydrates with desalting gel according to Ricciardi et al. (1998). Eight strains identified as *Lb. plantarum* and *Lb. paraplantarum* showing an EPS concentration >50 mg/l (data not shown) were used for a further experiment in mMRS liquid measuring EPS concentration (Dubois method) and viscosity with a digital viscosimeter (Brookfield DV-I+; Brookfield Engineering Laboratories, Stoughton, MA, USA) with a small sample adapter and a S21C coaxial cylinder spindle at 100 rev/min at 10°C.

Leavening in simulated doughs

Two strains (*Lb. plantarum* DBPZ1015 and *W. cibaria* DBPZ1006) selected on the basis of technological properties were tested in sourdough fermentation, in pure culture and in association with *Saccharomyces cerevisiae* MTG10 isolated from *Cornetto* di Matera sourdoughs (Ricciardi et al. 2002).

Cells were incubated up to the exponential growth phase (ca. 12 h), harvested by centrifugation (4800×*g*, 20 min, 4°C), washed once with sterile 0.85% (w/v) saline solution and resuspended in saline solution to obtain a cell suspensions of 10^8 colony-forming units (cfu)/ml.

The doughs were prepared mixing tap water, commercial wheat flour (type "00"; Divella S.p.A, Bari, Italy) and NaCl (1% w/w) manually for 5 min to obtain a dough yield (DY) of 150. The final concentration of cells was 10^7 cfu/g of dough. After mixing, the doughs were put in sterile cylinders and incubated at 30°C for 24 h. The decrease in pH (ΔpH) and the dough leavening were calculated at intervals of 1 h during incubation time as the difference between the values immediately after inoculation (t = 0 h) and the values at the successive time points. The pH values were measured using a spear tip pH electrode (Orion Research Inc. Runnings Center, Beverly, USA), while the dough leavening was determined measuring the volume increase in sterile cylinders.

Statistical analysis

Statistical analysis of SDS-PAGE patterns of whole cell proteins was carried out as described by Piraino et al. (2002), using a logarithmic transformation of molecular weight (log kDa). Classes (23; class width = 0.050 log kDa) were obtained in the range from 10 kDa (starting class) to 126 kDa (last class); flat range (FR) around the class centre and the membership in the flat range (MFR) were 30% and 99%, respectively, in all cases. Hierarchical cluster analysis (Unweighted Pair Group Method Using Average Linkage, UPGMA) was carried out on the Goodman-Krustal's γ similarity matrix of the profiles.

All statistical analyses were performed using Systat 11.0 for Windows (SPSS, Chicago, IL, USA).

Results and discussion

Identification of lactic acid bacteria from sourdoughs

Forty-one strains of lactic acid bacteria (LAB) isolated from sourdoughs used for the production of the *Cornetto*, were randomly selected among a set of isolates obtained by numerical analysis of phenotypic profiles. In a previous study a total of 407 LAB strains were randomly isolated and characterized using a set of 25 phenotypic tests. 39 clusters were identified at the 80% similarity level using hierarchical cluster analysis. Of the isolates 85% were identified as omofermentative LAB (*Lactobacillus plantarum*, *Lb. paracasei*), 15% as heterofermentative LAB (*Lb. fermentum*, *Weissella* ssp., *W. confusa*) (Ricciardi et al. 2002). In order to obtain a representative subsample of the original composition of the microflora, for each phenotypic cluster, 10% of the isolates were randomly selected and identified using SDS-PAGE of whole cell proteins (WCP) (Ricciardi et al. 2002).

The similarity relationships among the WCP patterns of LAB and related reference or type strains are shown in Fig. 1. Eight major clusters were found at an arbitrary distance value of 0.24 (1-Kruskal γ). Most strains were identified as facultative heterofermentative lactobacilli belonging to *Lactobacillus paraplantarum* (cluster 2), *Lb. plantarum* (cluster 3), *Lb. pentosus* (cluster 4) and *Lb. curvatus* (cluster 6) species. Obligate heterofermentative strains belonging to *Leuconostoc mesenteroides* and *Weissella cibaria* were also identified (clusters 1 and 5, respectively). The strains of *Leuc. mesenteroides* were not identified at subspecies level because the SDS-PAGE pattern of *Leuc. mesenteroides* spp. *mesenteroides* and *Leuc. mesenteroides* spp. *cremoris* were very similar. SDS-PAGE of WCP, when carried out under standardized conditions is considered a highly reproducible technique whose results are comparable with DNA–DNA hybridization (Vandamme et al. 1996). We have found that it provides an excellent classification of lactic acid bacteria (Piraino et al. 2006).

The percentage of obligate heterofermentative species found in our study is lower than that found in other Italian wheat sourdoughs (Corsetti et al. 2001; Gobbetti et al. 1994), in which *Lb. sanfranciscensis* was the dominant bacterial species in association with *Lb. plantarum* or with *Lb. alimentarius.* However, a similar composition in terms both of percentage of facultative and obligate heterofermentative strains and species association, was found in the sourdoughs used for the production of Altamura bread, in which the 88% of the isolates were identified as *Lb. plantarum*, *Lb. casei* and *Lb. paracasei*, 12% as *Lb. brevis* and *Leuc. mesenteroides* (Ricciardi et al. 2005). A study on the bacterial population in Sicilian traditional sourdoughs has also confirmed that *Lb. plantarum* strains may have a high prevalence: among the 45 sourdough LAB, 17 isolates were identified as *Lb. sanfranciscensis*, 14 as *Lb. plantarum*, 4 as *Lb. kimchii/Lb. alimentarius* and 3 as *Lb. casei* (Randazzo et al. 2005). High percentages of facultative heterofermentative LAB (*Lb. plantarum* and *Lb. pentosus* from 68% of facultative heterofermentative species in sourdoughs for *Carasau* bread to 98% in *Moddizzosu* bread) were found in the sourdoughs used to produce some typical breads of Sardinia region (Catzeddu et al. 2006). In this study the structure and the diversity of LAB communities demonstrated that obligate heterofermentative species did not dominate the microflora of traditional sourdoughs, with the exception of some samples of *Zichi* sourdoughs.

The identification of *W. cibaria* strains in our study confirms the results obtained by De Vuyst et al. (2002). *W. cibaria*, a species which is closely related to *W. confusa* (Bjorkroth et al. 2002), was first isolated from Greek traditional wheat sourdough manufactured without the addition of baker's yeasts. The obligate heterofermentative species was associated with strains belonging to *Lb. sanfranciscensis*, *Lb. brevis* and *Lb. paralimentarius* to constitute an exclusive microbial consortium in Greek products. Strains of *W. confusa*, instead, were isolated from organic flours and sourdoughs produced in the Centre and Southern Italy together to strains of *Lb. sanfranciscensis*, *Lb. brevis*, *Lb. alimentarius*, *Lb. plantarum*, *Lb. farciminis* and *Lb. fructivorans* (Corsetti et al. 2003).

Association of *Lb. plantarum*, *Lb. brevis* and *Lb. fermentum*, and association of *Lb. acidophilus* and *Lb. plantarum* species dominate in Russian and Finnish rye sourdoughs, respectively (De Vuyst and Neyssens 2005). LAB belonging to *Lb. brevis* and *Lb. curvatus* species are the most frequently isolated in Portuguese sourdoughs manufactured with maize flours (Rocha and Malcata 1999).

Technological characterization of lactic acid bacteria

In order to select a multiple strain starter culture for the production of *Cornetto*, a preliminary technological characterization of the 41 LAB strains was performed. The strains were characterized on the basis of acid production ability, antimicrobial activity, exopolysaccharides (EPS) production, which are relevant technological properties for bread production.

Figure 2 shows the distribution of acid production ability of the strains after 6 and 24 h of incubation at 30°C in a synthetic medium (panels a and b, respectively). The decrease in pH (ΔpH) ranged from 0.1 to 1.1 at 6 h and from 0.7 to 1.8 at 24 h. A high variability among the species *Lb. curvatus* and *Leuc. mesenteroides* was found at

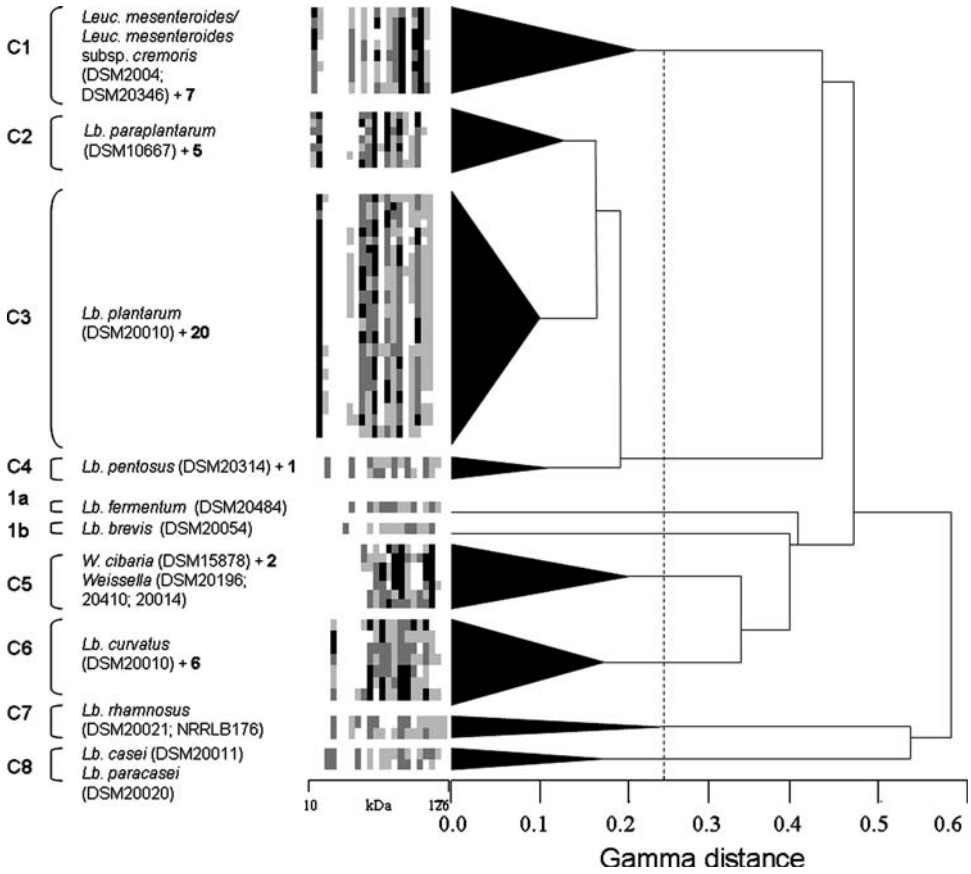

Fig. 1 Abridged dendrogram showing the distance between the 16 reference strains of lactic acid bacteria and the 41 bacterial isolates obtained from Cornetto di Matera sourdoughs. Hierarchical cluster analysis (Unweighted Pair Group Method Using Average Linkage, UPGMA) was carried out on the Goodman-Krustal's γ similarity matrix of the profiles. The electrophoretic fingerprints are shown as processed profiles, by using a grey scale to report the intensities of the 23 classes of molecular mass that were defined in the gel. The number of isolates belonging each cluster is also shown

both 6 and 24 h. On the contrary, the isolates belonging to *W. cibaria* and *Lb. plantarum* group (including *Lb. plantarum*, *Lb. paraplantarum* and *Lb. pentosus* species) showed a similar levels of acid production. *W. cibaria* and some isolates of *Lb. plantarum* and *Lb. paraplantarum* had the highest acid production ability after 6 h of incubation, while *Lb. curvatus* isolates had the lowest values of decrease in pH. At the end of incubation time, the majority of *Lb. plantarum* group isolates and few *Leuc. mesenteroides* isolates had the highest levels of acid production capability.

Dough acidification, due to the production of lactic acid from facultative heterofermentative LAB and lactic and acetic acids from heterofermentative strains, is an important metabolic activity in bread making. Production of organic acids during fermentation to achieve a pH value lower than 5.0 is effective in preventing rope spoilage of wheat bread which is usually caused by strains of *Bacillus* spp., especially *Bacillus subtilis* and *B. licheniformis* (Pepe et al. 2003; Pepe et al. 2004). Lactic and acetic acids produced by LAB during sourdough fermentation also have little direct effects on bread flavor. However, when combined with ethanol and other products of dough fermentation they enhance the perception of aroma (Röcken 1996). In fact, when the molar ratio lactate/acetate (the fermentation quotient, FQ) is in the range of 2.0–2.7, a pleasant odour of bread is perceived. pH decrease is also essential in obtaining the correct rheological and sensory properties of breads (Arendt et al. 2007). Production of organic acids decreases the digestion and absorption of the starch affecting positively the diet of diabetic subjects, cholesterol reduction and glucose tolerance (Pepe et al. 2004). Additionally, Leenhardt et al. (2005) found that acidification of dough due to LAB metabolism improves the nutritional properties of bread promoting the activation of endogenous flour phytases which leads the reduction of phytic acid, an anti-nutritional compound that chelates proteins, amino acids and divalent cations such Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, hindering their adsorption by human intestinal mucosa.

Our data showed that facultatively and obbligately heterofermentative strains generate a significant drop in pH in 6 h of incubation; since the fermentative process for the production of Italian wheat sourdough breads occur in few hours (5–10 h; Gobbetti et al. 1996) some isolates of *W. cibaria* and *Lb. plantarum* group could be selected as producer of antifungal activity and to improve the nutritional properties of bread.

The stability of bread is also due to the production of antimicrobial compounds by sourdough lactic microflora.

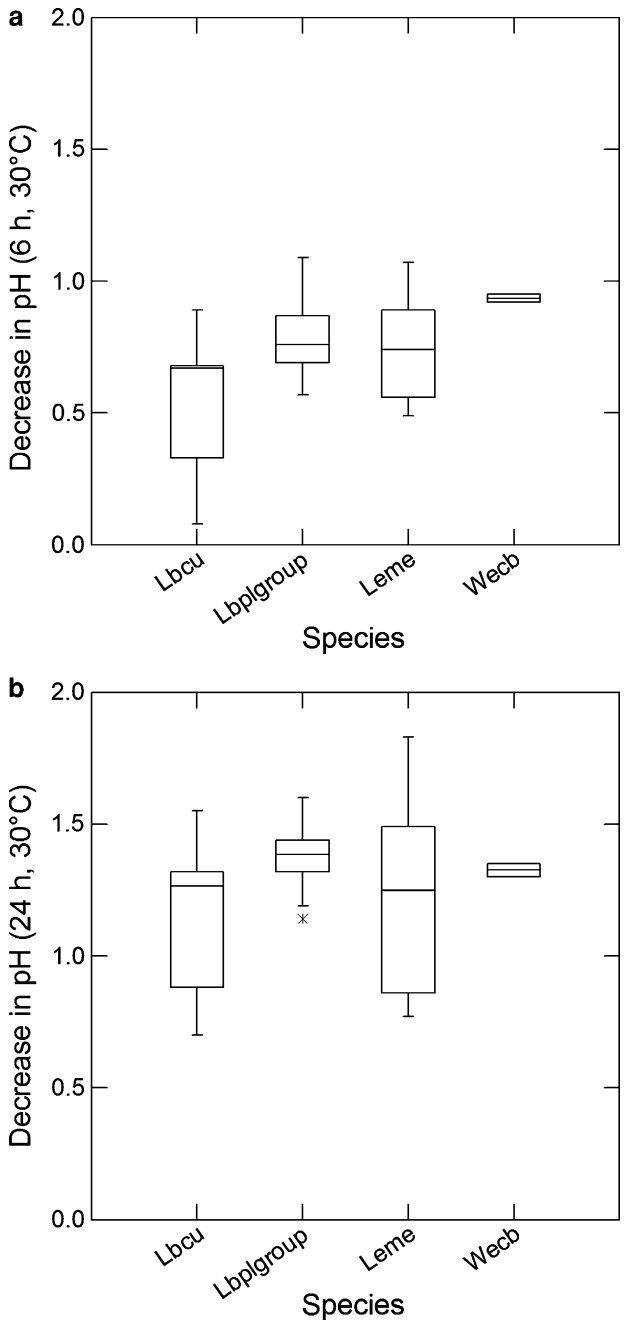

Fig. 2 Box and whiskers plots showing the distribution of decrease in pH after 6 h (**a**) and 24 h (**b**) within strains of LAB isolated from sourdoughs for the production of Cornetto di Matera. *Lb. curvatus* (Lbcu); *Lb. paraplantarum*, *Lb. pentosus*, *Lb. plantarum* (Lbplgroup); *Leuc. mesenteroides* (Leme); *W. cibaria* (Wecb)

To evaluate the antimicrobial activity of the LAB isolates, a deferred antagonism assay was carried out, using as indicators strains that are starters or contaminants in sourdough baked products. Since antimicrobial compounds produced by the sourdough microflora play an important role in the regulation of the complex interactions within the starter microflora and between the starter and contaminant microflora (Messens and De Vuyst 2002), the indicators were chosen to be representative of facultatively heterofermentative bacteria isolated from sourdoughs (*Lb. plantarum* and *Lb. paracasei*) (De Vuyst and Neysens 2005; Ricciardi et al. 2005), of spore formers found in flours (*B. cereus)* or because of high sensitivity to class I or class II bacteriocins (*M. flavus* and *L. innocua*) (Klaenhammer 1993).

W. cibaria and *Leuc. mesenteroides* strains did not show any antimicrobial activity, while *Lb. curvatus* isolates did not show inhibitory activity against *M. flavus* and *B. cereus*. *Lb. plantarum* had the largest spectrum of inhibition with differences among the isolates. Several isolates, in fact, produced inhibition zones against *L. innocua* and *Lb. plantarum*, others against *M. flavus* and *Lb. paracasei*. All the strains exhibited low antagonistic activity against *B. cereus.*

Table 1 shows the results of the six LAB that presented an inhibitory activity of proteinaceus nature (bacteriocin-like inhibitory substance, BLIS). The resistance to proteolytic enzymes was tested using as indicators the strains that showed the widest inhibition diameter zones. Except for *Lb. plantarum* DBPZ1019, all other inhibitory activities were sensitive to the action of the three proteolytic enzymes. All the strains were inhibitory against *Lb. plantarum*, while only two strains (*Lb. curvatus* DBPZ1024 and *Lb. plantarum* DBPZ1019) showed inhibition against *B. cereus*.

Studies on the production of antimicrobial compounds by LAB sourdoughs are important to reduce microbial contamination during fermentation (Pepe et al. 2003; Corsetti et al. 1996) and to explain competition among sourdough microflora, in order to select competitive starters (Torodov et al. 1999). As reported by Messens and De Vuyst (2002) the screening of LAB strains for bacteriocin production is most promising to isolate strains that are

Table 1 Antimicrobial activity spectrum of lactic acid bacteria isolated from Cornetto di Matera sourdoughs

Strains	Indicators [a]					Enzymes [b]
	BC	LI	LBP	LBPL	MF	
Lb. curvatus DBPZ1024	−	+ +	+ +	+ + +	−	T, C, P
Lb. pentosus DBPZ0984	+	+ +	+ +	+ + +	+	T, C, P
Lb. plantarum DBPZ1003	−	+	−	+ +	−	T, C, P
Lb. plantarum DBPZ1012	−	−	+ +	+ +	−	T, C, P
Lb. plantarum DBPZ1021	+ +	+	−	+ +	+ +	T, C, P
Lb. plantarum DBPZ1019	−	+ +	+	+ + +	+ +	C

−, No inhibition zone; +, inhibition zone diameters <5 mm; + +, between 5 and 10 mm; + + +, ≥10 mm

[a] BC, *B. cereus* ATCC9139; LI, *L. innocua* BL86/26; LBP, *Lb. paracasei* DSM4905; LBPL, *Lb. plantarum* DSM20174; MF, *M. flavus* DSM1790

[b] T, trypsin; C, chimotrypsin; P, pronase

adapted to this food ecosystem and that may produce bacteriocins *in situ*. In our study, all the isolates produced inhibition zones against other *Lactobacillus* strains, even if the profiles were strain specific (data not shown), in agreement with Corsetti et al. (1996). In this work, the authors found and isolated a bacteriocin-like inhibitory substance (BLIS) from *Lb. sanfranciscensis* C57. In a later work, Corsetti et al. (2004) confirmed the presence of BLIS in two strains of each *Lb. plantarum* and *Lb. pentosus* isolated from sourdoughs. The BLIS compounds were characterized by a limited inhibitory spectrum and showed no inhibition against *Bacillus* spp., *L. innocua* and yeasts. Settanni et al. (2005) demonstrated that the *in situ* activity of a BLIS produced by *Lc. lactic* ssp. *lactis* M30 during sourdough fermentation influences the sourdough microflora and may support the desired implantation of selected BLIS-insensitive bacteria (*Lb. sanfranciscensis*) useful to confer good characteristics to the dough, inhibiting other LAB frequently emerging (*Lb. plantarum*) as dominant bacteria during sourdough propagation (Corsetti et al. 2004). The production of bacteriocins by *Lb. plantarum* species isolated from sourdoughs was demonstrated by Todorov et al. (1999) which found and characterized the bacteriocin plantaricin ST341 from *Lb. plantarum* ST31. *Lb. amylovorus* DCE 471, an important starter culture of type II rye sourdoughs because of its strong and fast acidifying capability, has been also recognized as producer of bacteriocin (amylovorin) during sourdough fermentation (Messens and De Vuyst 2002).

The production of bacteriocins from sourdough lactobacilli is an important technological trait, and some strains of *Lb. plantarum* tested in our study, showing a wide spectrum of inhibition, could be selected for further experiments in order to select a starter culture to use protective culture, contributing to the safer products and reducing the addition of chemical preservative used by food industry.

The 41 strains of LAB were also tested for the production of EPS on solid and liquid media (Table 2). *Lb. curvatus* strains were not able to produce EPS in solid media, whereas all strains of *Leuc. mesenteroides* and *W. cibaria* produced dextran from sucrose. Some strains of *Lb. plantarum*, *Lb. paraplantarum* and *Lb. pentosus* were able to produce EPS from different sugars in solid media. The isolates of *Lb. plantarum* and *Lb. paraplantarum* showed the highest EPS production when grown in liquid medium containing maltose as carbon source. However, relative high efflux times were observed in several strains of *Lb. plantarum* and two strains of *Lb. paraplantarum* when grown on MRS broth containing glucose or sucrose, respectively, as a carbon source. On the contrary, all strains of *Leuc. mesenteroides* and *W. cibaria* had the highest EPS production from liquid MRS medium containing sucrose.

Eight strains belonging to *Lb. plantarum* and *Lb. paraplantarum* species showing an EPS concentration >50 mg/l (Table 3) were used for a further experiment in mMRS liquid in which EPS concentration and viscosity were measured.

The amount of EPS produced by *Lb. plantarum* and *Lb. paraplantarum* strains ranged from 140 to 297 mg/l. The highest value of viscosity among *Lb. plantarum* group was observed for the strain *Lb. plantarum* DBPZ1014. Viscosity and EPS concentration were not correlated. When *Lb. plantarum* DBPZ0998 or *Lb. plantarum* DBPZ1015 were grown on mMRS, the liquid medium had high viscosity, but the production of EPS was very low; on the contrary, the medium fermented with *Lb. paraplantarum* DBPZ0997 had the lowest value of viscosity but high EPS concentration.

EPS production during sourdough fermentation can potentially affect rheological properties of the dough, as volume, texture and keepability of the bread (Di Cagno et al. 2006; Korakli et al. 2001). EPS produced in situ have been found to have positive effect on the technological properties of dough and bread improving water absorption of the dough, dough rheology and machinability, dough stability during frozen storage, loaf volume and bread staling (Arendt et al. 2007, Tieking and Ganzle 2005).

Korakli et al. (2003) found that *Lb. sanfranciscensis* LTH2590 was able to produce a levan-type fructan from sucrose, which positively affected dough rheology and bread texture, while *Lb. reuteri* LB121 was shown to produce glucan and levan by the same sugar source (van Geel-Schutten et al. 1998). EPS may influence the intestinal flora, because oligofructose and fructans of the levan and inulin types are known to selectively stimulate the growth of bifidobacteria (Tieking et al. 2003) improving the nutritional properties of sourdough fermented products and selectively stimulated the growth of bifidobacteria during cultivation of human faecal microflora in vitro (Di Cagno et al. 2006; Arendt et al. 2007).

Lacaze et al. (2007) found that the dextran produced by *Leuc. mesenteroides* LMGP-16878 isolated from sourdough have multiple advantages in bakery applications; in particular, dextran being a hydrocolloid can bind high amounts of water improving freshness of the end product. Dextran enhances dough stability and gas retention increasing bread volume and crumb softness.

Our results showed that *W. cibaria* and *Lb. plantarum* strains were able to produce EPS in MRS agar containing sucrose according to Di Cagno et al. (2006). In this work the authors found that the strains belonging to *W. cibaria*, *Lb. plantarum* and *P. pentosaceus* species, selected after the screening in solid media, produced amounts of EPS ranging from 153 and 388 mg/l after 1 day of growth on

Table 2 Screening for expolysaccharides production on solid and liquid media

Strains	Substrates[a]					
	Pick test MRS	Pick test mMRS	Pick test sMRS	Flow ratio[b] MRS	Flow ratio[b] mMRS	Flow ratio[b] sMRS
Lb. paraplantarum DBPZ1027	+	+	+	3	3	3
Lb. paraplantarum DBPZ1013	+	+	+	3	12	12
Lb. paraplantarum DBPZ0997	+	+	+	8	12	12
Lb. plantarum DBPZ0990	+	+	+	3	12	8
Lb. plantarum DBPZ0987	+	+	+	7	12	8
Lb. plantarum DBPZ0988	+	+	+	6	12	12
Lb. plantarum DBPZ0993	+	−	−	4	1	1
Lb. plantarum DBPZ0989	+	+	−	1	1	1
Lb. plantarum DBPZ0991	−	+	−	1	2	1
Lb. plantarum DBPZ0992	+	+	−	7	12	1
Lb. plantarum DBPZ0998	+	+	+	7	12	5
Lb. plantarum DBPZ1003	+	+	+	3	12	6
Lb. plantarum DBPZ1011	+	+	+	11	12	4
Lb. plantarum DBPZ1015	+	+	+	8	12	12
Lb. plantarum DBPZ1014	+	+	+	11	12	6
Lb. plantarum DBPZ1012	+	+	−	2	1	1
Leuc. mesenteroides DBPZ1028	−	−	d	1	1	12
Leuc. mesenteroides DBPZ0995	−	−	d	1	1	9
Leuc. mesenteroides DBPZ1005	−	−	d	1	1	12
Leuc. mesenteroides DBPZ0985	−	−	d	1	1	6
Leuc. mesenteroides DBPZ0986	−	−	d	1	1	12
Leuc. mesenteroides DBPZ1016	−	−	d	1	1	12
Leuc. mesenteroides DBPZ1026	−	−	d	1	1	12
W. cibaria DBPZ1017	−	−	d	1	1	2
W. cibaria DBPZ1016	−	−	d	1	1	3

+, Slimy colonies; d, dextran colonies; −, no slimy or dextran colonies

[a] MRS, substrate with glucose; mMRS, substrate with maltose; sMRS, substrate with sucrose

[b] Ratio between flow time of the fermented broth and flow time of the control (maximum flow ratio = 12)

MRS broth with sucrose as carbon source. These levels of EPS production were similar to those obtained in our study by some strains of *Lb. plantarum* (140–297 mg/l) cultivated in MRS broth containing maltose. Considering that in situ production of EPS by *W. cibaria* WC4 e *Lb. plantarum* PL9, the best-producing EPS strains selected by Di Cagno et al. (2006), after 7 h of fermentation in dough reached the final concentration of 2.5 g/kg of dough, our strains could be selected for further study on the production and purification of EPS.

Table 3 Expolysaccharides production and viscosity in liquid medium

Strains	Substrate	Viscosity at 100 rpm (mPa s)	EPS (mg/l)
Lb. paraplantarum DBPZ1013	mMRS	8	251
Lb. paraplantarum DBPZ0997	mMRS	6	244
Lb. plantarum DBPZ0987	mMRS	9	273
Lb. plantarum DBPZ0988	mMRS	7	297
Lb. plantarum DBPZ0992	mMRS	7	228
Lb. plantarum DBPZ0998	mMRS	7	140
Lb. plantarum DBPZ1015	mMRS	8	141
Lb. plantarum DBPZ1014	mMRS	10	242

Selection of a defined strains starter culture

In order to select a defined strain starter culture for the production of the *Cornetto* di Matera, *W. cibaria* DBPZ1006 and *Lb. plantarum* DBPZ1015 were tested in sourdough fermentations in pure culture and in association with a sourdough yeast (*S. cerevisiae* MTG10).

In a previous work (Zotta et al. 2006) we found that the fermentation of doughs started with *Lb. plantarum* DBPZ1015 resulted in a considerable hydrolysis of salt-soluble protein (albumins and globulins) after 6 h fermentations, increasing the low MM proteins fractions. After 24 h of fermentations, the hydrolysis of gliadin and glutenin fractions resulted in new protein fragments of 20 and 57 kDa, respectively. In a later work, Zotta et al. (2007) confirmed the high proteolytic activity of *Lb. plantarum* DBPZ1015 and demonstrated the presence of high β-glucosidase and phosphatase activity for *W. cibaria* DBPZ1006. On the basis of these technological properties (high proteolytic activity of *Lb. plantarum* and the presence of high β-glucosidase and phosphatase activity for *W. cibaria*) and those found in this work (high acidifying capability, EPS production and antimicrobial activity), the two strains were selected as possible defined starter culture to improve the flavour, aroma, texture and nutritional value of bread. The percentage of the volume increase (% Vol) for all doughs is shown in Fig. 3a. All yeasts/LAB associations and the yeasts alone caused an increase in volume around 140% after 2 h of incubation. The doughs started with *S. cerevisiae* in association (D4, D5 and D6) showed the same values of volume increase. The LAB in pure culture had different time courses: as expected, *W. cibaria* was capable to produce a dough with sufficient leavening (143% increased volume) at 24 h of fermentation when used alone (D3) or in association with the yeast (D5), with no additive effect. The yeast in pure colture (D1) showed a similar volume increase (around 154%) at the end of fermentation, while in association with *Lb. plantarum* and *W. cibaria* (D6) the volume increase was only 149%, probably due to a negative effect of the interaction yeast/LAB. In mixed culture, yeast growth might be negatively affected by the rapid pH drop caused by the LAB and the metabolites produced, i.e. lactic and acetic acid (Paramithiotis et al. 2007). It is known that sourdough yeasts and LAB have different kinetics for carbohydrate uptake. Most yeasts found in sourdoughs take up hexoses and maltose by high affinity transport systems, while the disaccharide uptake of LAB is strictly dependent on the external sugar concentration and is less effective (Gobbetti 1998). These results were confirmed by the plate count of the strains in pure and in mixed culture: the yeasts and LAB in mixed cultures had a reduced growth (data not shown).

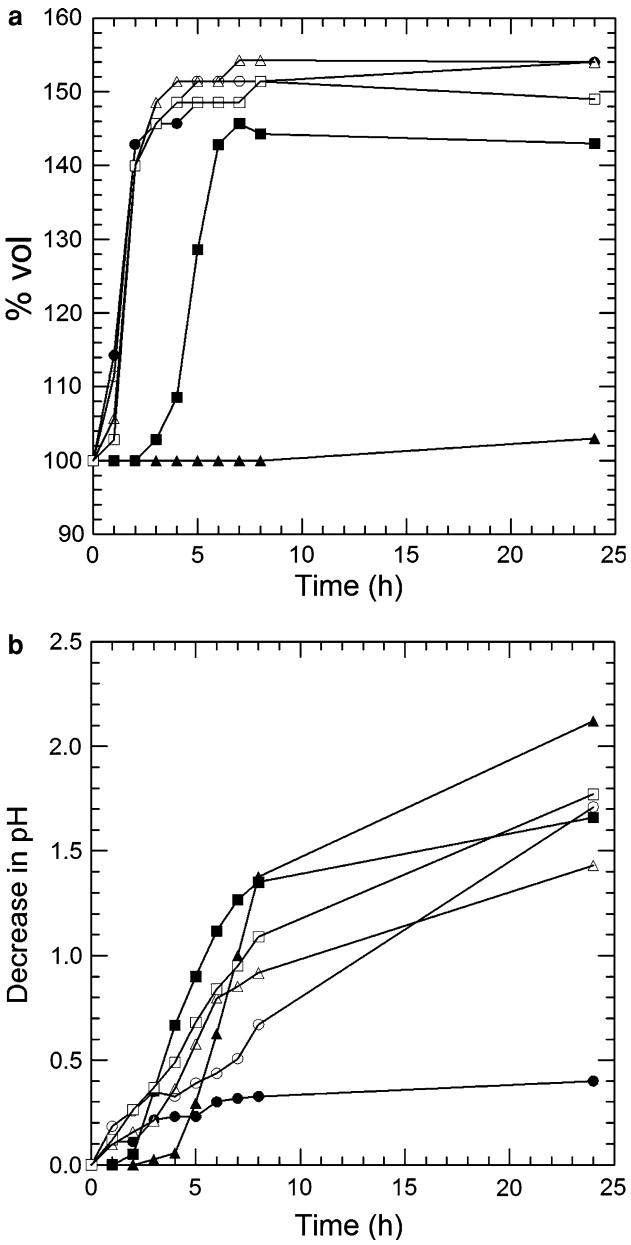

Fig. 3 Acid production and leavening ability in model sourdough fermentation: (**a**) percentage of the volume increase (%Vol) and (**b**) decrease in pH of the doughs during incubation at 30°C. *S. cerevisiae* MTG10 (●, D1); *Lb. plantarum* DBPZ1015 (▲, D2); *W. cibaria* DBPZ1006 (■, D3); *S. cerevisiae* MTG10 + *Lb. plantarum* DBPZ1015 (○, D4); *S. cerevisiae* MTG10 + *W. cibaria* DBPZ1006 (△, D5); *S. cerevisiae* MTG10 + *Lb. plantarum* DBPZ1015 + *W. cibaria* DBPZ1006 (□, D6)

As to the acidification (Fig. 3b), the pH decreased after 24 h of incubation to reach ΔpH values of 2.1 and 1.7, respectively for *Lb. plantarum* in pure culture and in association with *S. cerevisiae*. The interaction yeasts/LAB showed a reduction of the acidification ability: the sourdoughs started with associations of LAB and yeasts showed

a different acid production and acidifying ability, with pH values between 4.49 and 4.71.

Bacterial growth and production of lactic and acetic acids decreased when *S. cerevisiae* was associated with LAB strains probably because of the faster consumption of maltose and glucose by yeast. The imbalance between consumption by yeast and starch hydrolysis by flour enzymes may led to the rapid depletion of soluble carbohydrates during sourdough fermentation which, in turn, decreases LAB acidification. In particular, in a previous study (Zotta et al. 2006) cell counts of facultative heterofermentative LAB were affected by the presence or absence of yeasts to a lesser extent than cell counts of heterofermentative LAB.

Conclusions

In this study lactic acid bacteria isolated from sourdoughs used for the production of *Cornetto* were identified and characterized on the basis of technological properties, in order to select strains to be used as starters in bread production.

Recent studies (Ferchichi et al. 2007; Catzeddu et al. 2006; Randazzo et al. 2005; Ricciardi et al. 2005) showed that facultative heterofermentative species like *Lb. casei, Lb. paracasei, Lb. pentosus, Lb. plantarum* and species of the genera *Leuconostoc* and *Weissella* are important members of sourdough microflora.

On the basis of their technological characteristics (high acidifying capability, antimicrobial activity and EPS production) the mixed culture selected in this work could be used, to formulate a mixed culture for bread production.

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