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PAPER

LACTIC ACID BACTERIA AND YEASTS FROM WHEAT SOURDOUGHS OF THE MARCHE REGION

BATTERI LATTICI E LIEVITI DA MADRI ACIDE DI FARINA DI GRANO TENERO DELLA REGIONE MARCHE

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ABSTRACT

The need for a greater diversification of baked products has given rise to the on-going search for yeast and lactic acid bacteria (LAB) strains with optimal baking potential. Thirty-six yeasts and 118 LAB, isolated from nine type I sourdoughs that were sampled in bakeries located in the Marche region (central Italy), were molecularly and phenotypically characterized. The polyphasic approach used revealed the biodiversity of the microbial communities in-

RIASSUNTO

La necessità di ampliare e diversificare l'offerta di prodotti lievitati da forno ha portato alla ricerca di ceppi di lieviti e batteri lattici con spiccate attitudini panificatorie. Trentasei lieviti e 118 batteri lattici, isolati da 9 madri acide di tipo I campionate in panifici situati sul territorio della regione Marche (Italia centrale), sono stati caratterizzati a livello molecolare e fenotipico. L'approccio polifasico utilizzato ha evidenziato la biodiversità presente nella comunità

- Key words: baking industry, lactic acid bacteria, starter cultures, wheat sourdough, yeasts -

vestigated and two yeasts and ten LAB cultures with the potential to be used in sourdough bread-making processes were identified.

microbica oggetto di studio ed ha permesso di identificare 2 colture di lievito e 10 di batteri lattici potenzialmente utilizzabili nei processi di panificazione.

INTRODUCTION

Yeasts and lactic acid bacteria (LAB) are common inhabitants of sourdoughs that originate from flour and bakery environments (DE VUYST and NEYSENS, 2005) as well as from the vegetable matter that can be added to the initial mixtures of flour and water (FOSCHINO *et al.*, 2004). A very complex ecological system is established, where different microbial species co-exist and interact in a dynamic equilibrium (GAROFALO *et al.*, 2008). This natural consortium is constantly renewed in a recycling mode under strict recipe and ripening conditions (HAMMES and GÄNZLE, 1998), and it is responsible for the so-called "sourdough fermentation" that leads to leavened baked goods with distinctive tangy or sour tastes (GÄNZLE *et al.*, 1998).

Type I sourdoughs are known to be colonized mainly by *Lactobacillus sanfraciscensis* and *Lact. pontis* (VOGEL *et al.*, 1999). Recently, DE VUYST and NEYSENS (2005) proposed a new classification of type I sourdoughs that encompasses: i) sourdoughs fermented by pasty pure starter cultures, defined as type Ia; ii) sourdoughs prepared through multiple-stage fermentation processes that contain spontaneously developed mixed cultures, defined as type Ib; and iii) sourdoughs fermented at high temperatures, and typically used in tropical regions, classified as type Ic.

In recent years, sourdough-based bread-making has been the object of renewed interest, with the ever-increasing consumer demand for tasty, more natural and healthier foods (PAGANI *et al.*, 2007). This consensus is explained by the numerous benefits of sourdough fermentation; namely, improved dough properties, crumb structure and bread texture; increased bread volume and flavour; and slower staling and prolonged mould-free shelf-life (ARENDT *et al.*, 2007). An examination of the current scientific literature shows that most of these benefits are greatly influenced by the particular yeast and lactic acid bacteria (LAB) strains that carry out the fermentation.

Commercial starters, made up of selected strains, as either single or mixed cultures, entered the market a few decades ago. The need for a greater diversification of baked products has given rise to the on-going search for strains with peculiar traits and baking potential (VOGEL *et al.*, 2002; DI CAGNO *et al.*, 2008). Although useful for maintaining the advantages of the biological fermentation process, while providing baked goods with a stable quality, the use of selected strains in sourdough biotechnology is still limited due to a generally low persistence of starters during propagation. Accordingly, the evaluation and further selection of candidate starter strains is a multi-step process that focuses on ecological, functional and technological aspects. Key criteria for this selection are based on general aspects, including origin and identity, technical aspects (growth properties *in vitro* and during processing, survival and viabil-

ity during transport, storage, etc.) and eventual functional aspects (i.e. beneficial features).

On the basis of these premises, an in-depth investigation was carried out on the microbial ecology of nine type-I wheat sourdoughs that were sampled from one semi-industrial and eight artisan bakeries of the Marche region (central Italy) using a culture-dependent approach. The main technological traits of a selected pool of yeasts and LAB were further investigated, as a basis for a preliminary selection of promising strains that could be exploited by the local baking industry for sourdough fermentation processes.

MATERIALS AND METHODS

Micro-organisms

Nine yeast (Table 1) and seven LAB (Table 2) reference strains were purchased from: (i) the Deutsche Sammlung von Mikrorganismen und Zellkulturen (DSMZ, Braunschweig, Germany); (ii) the American Type Culture Collection (ATCC, Manassas, VA, USA); (iii) the Industrial Yeasts Collection of the Department of Applied Biology, University of Perugia (DBVPG, Perugia, Italy); (iv) the National Collection of Yeast Cultures (NCYC, Norwich, UK); and (v) the Centraalbureau voor Schimmelcultures Fungal and Yeast Collection (CBS, Utrecht, The Netherlands). All of the strains were revitalized as indicated by the culture suppliers.

Sourdough sampling and pH measurements

Nine mature type I sourdoughs that had been propagated daily by back-slopping at room temperature with wheat flour were sampled in one semi-industrial (referred to as C) and eight artisan bakeries (referred to as A, B and D to I) located in the Marche region (central Italy) (Table 3). The length of the sourdough fermentation time and temperature varied considerably among the nine bakeries, according to the particular cycle of production. During ripening, the sourdoughs were bound tightly with canvas (bakeries A and C) or left to ripen in plastic containers (bakeries B, and D to I).

In the semi-industrial bakery C, compressed bakers' yeast had never been used before. In bakeries A, G and H, it was routinely used in the production lines that are different from those considered in the present study, while in bakeries B, D, E, F and I, the sourdoughs sampled had been prepared with trace amounts of this leavening agent. The compressed bakers' yeast used by bakeries A, B, and D to I were sampled and used for the isolation of the industrial yeast strains, referred to as the controls in both the molecular typing and the technological characterization. All of the samples were kept under refrigerated conditions and analysed within 24 h. Only the sourdough samples collected from bakeries A, C, G and H were used further for the isolation of sourdough yeasts because their preparations did not include the addition of compressed bakers' yeast.

The pH measurements of the sourdough samples were carried out in duplicate with a model 300 pH meter (Hanna Instruments, Padova, Italy) equipped with an HI2031 solid electrode (Hanna Instruments).

Microbial counting and isolation of yeasts and LAB

Approximately 10 g of each sourdough sample were diluted in 90 mL of a sterile peptone water solution (1 gL^{-1} peptone, 8.5 gL^{-1} NaCl) and homogenised for 2 min at 260 rpm using a Stomacher apparatus (400 Circulator, PBI International, Milan, Italy). The homogenates were serially diluted and aliquots (100

Table 1 - Yeast reference strains used for the construction of the RFLP pattern database.

Species	Reference strain	Amplicon size (bp)	Restriction fragments (bp)		
			CfoI	*HaeIII*	*HinfI*
Issatchenkia orientalis	DSM3433	510	210-180-80	380-90	200-150-130
Pichia angusta	NCYC495	750	300-240-120-90	540-210	300-400
Candida milleri	DBVPG6753	790	330-330-120	790	350-200-120-70
Pichia anomala	DBVPG6613, DBVPG4357, DBVPG6781	650	550	650	310-310
Saccharomyces cerevisiae	CBS4054, CBS1171	880	370-350-140	330-230-180-140	365-365-140
Starmerella bombicola	DBVPG3827	475	220-190	325-80	235-235

Table 2 - Lactic acid bacteria reference strains and GenBank 16S rRNA gene sequences used for the construction of the ARDRA pattern database.

Species	GenBank Acc. no.	Strain	Amplicon size (bp)	Restriction fragments (bp)		
				AluI	*FokI*	*HaeIII*
Lactobacillus alimentarius	M58804	DSM20249	360	95-251	122-243	314
Lact. paralimentarius	AJ417500; AJ422034	N.A.	360	69-96-182	123-222	314
Lact. panis	X94230	DSM6035	360	183-237	191-229	78-120-191
Lact. pontis	X76329; AJ422033	DSM8475	360	114-226	122-232	51-78-191
Lact. sanfranciscensis	X76331; X76327	DSM20451	360	95-217	122-254	126-221
Lact. fructivorans	X76330	N.A.	360	52-114-179	121-253	104-220
Lact. lindneri	X95421	N.A.	360	67-96-179	123-254	347
Weissella confusa	AB023241	ATCC14434; DSM20196	360	57-115-188	360	308
W. viridescens	AB023236	DSM20410	360	57-115-188	360	308
W. kimchii	AF515221; AF312874	N.A.	360	75-304	358	327

N.A. not available.

Table 3 - Type I sourdough samples collected in one semi-industrial and eight artisan bakeries of the Marche region (central Italy).

Bakery	Location (District)	Ripening time (h)	Ripening temperature (°C)	pH*	Log cfu g^{-1}	
					Yeasts	LAB
A	Ancona	20	20	3.80±0.02	7.0	8.3
B	Pesaro-Urbino	22	Troom	3.15±0.01	7.4	8.6
C	Pesaro-Urbino	24	Troom	3.85±0.01	7.7	9.2
D	Ancona	4-5	20-25	3.80±0.01	7.6	8.4
E	Macerata	15-17	8-10	4.20±0.02	8.0	8.5
F	Macerata	24	Troom	4.27±0.02	7.4	8.6
G	Ascoli Piceno	20	Troom	4.45±0.01	6.9	8.6
H	Ascoli Piceno	48	25	3.80±0.02	7.1	9.0
I	Ascoli Piceno	7-8	25	3.90±0.01	8.0	9.0

* Mean value ± standard deviation.

μL) of each dilution were streaked onto the agar media listed below.

The yeasts were counted and isolated on Wallerstein Laboratory Nutrient (WLN) agar (Oxoid, Basingstoke, UK) with 250 mg L^{-1} chloramphenicol added (GOBBETTI *et al.*, 2000) and incubated at 25°C for 96 h. Up to three colonies were selected according to each morphology and colour on the WLN plates, and they were streaked to purity onto the same medium. For each isolate, the cell morphology was examined using a light microscope under oil-immersion (100x). The yeast isolates were stored frozen (-80°C) in a mixture of glycerol and YPD (Oxoid) (1:1).

The LAB were counted on: (i) De Man, Rogosa and Sharp (MRS) agar (DE MAN *et al.*, 1960), modified by the addition of 1% maltose and 5% fresh yeast extract (mMRS) (GOBBETTI *et al.*, 1996) (ii) sourdough medium (SDB) (KLINE and SUGIHARA, 1971); and (iii) GM17 agar (HORN *et al.*, 1999). To inhibit the yeast growth 250 mg L^{-1} cycloheximide were added to these three media. The incubations were carried out at 30°C under anaerobic conditions (Anaerogen GasPak System, Oxoid, Basingstoke, UK) for 48-72 h. After counting, LAB colonies were randomly selected and picked from the last dilution plates; the bacterial isolates were tested for Gram and catalase reactions, and stored frozen (-80°C) in a mixture of glycerol and mMRS (1:1).

Molecular identification of yeasts and LAB

The yeast isolates were initially identified by restriction fragment length polymorphism (RFLP) analysis. To widen the existing 5.8S-ITS restriction pattern database that is available for the identification of food-borne yeasts, *in-silico* restriction of nucleotide sequences retrieved form the GenBank DNA database was performed with three endonucleases *Cfo*I, *Hae*III and *Hinf*I and the TACG software, which is available at http://bioweb.pasteur.fr/seqanal/interfaces/tacg.html.

In the experimental RFLP analyses, the nine reference yeast strains listed in Table 1 were used as controls. The DNA was extracted from YPD broth cultures as described by MAKIMURA *et al.* (1999). Aliquots (1 μL) of the template DNA were amplified according to ESTEVE-ZARZOSO *et al.* (1999). Twenty μL of the PCR products were digested separately in 30 μL reaction volumes with 2 U of *Cfo*I, *Hae*I-

II and *Hinf*I (Roche Diagnostics, Germany). The digests were analysed by electrophoresis in 2.5% (w/v) agarose gels at 3.5 V cm^{-1} constant voltage for 3 h. A 50-bp DNA size marker (Amersham Biosciences, Amersham, UK) was used for size standards.

The LAB isolates were initially identified by amplified rDNA restriction analysis (ARDRA). An *in-silico* restriction analysis similar to that performed with the 5.8S-ITS nucleotide sequences was carried out on the 16S rRNA gene sequences retrieved from the GenBank DNA database and ascribed to LAB species commonly found in flour and sourdough.

In the experimental ARDRA assays, seven reference strains (listed in Table 2) were used as controls. The bacterial DNA was extracted from the SDB broth cultures as described by DE LOS REYES-GAVILÀN *et al.* (1992). Quantity and purity of the DNA were assessed by optical reading at 260 and 280 nm, respectively (SAMBROOK *et al.*, 1989). An approximately 360-bp portion of the 16S rRNA gene was amplified using universal primers for eubacteria, and separately digested with *Fok*I, *Hae*III and *Alu*I (Roche Diagnostics, Mannheim, Germany), as previously described (AQUILANTI *et al.*, 2007).

The gels were stained with ethidium bromide and photographed under UV light. The electronic images of the gels were visualized with the ImageMaster VDS apparatus (Amersham Pharmacia), captured with LISCAP software v.1.0 (Amersham Pharmacia), and stored as TIFF files. The digitalized images were normalized with the 50-bp DNA size marker (Amersham Pharmacia), and analyzed with GelCompar, v. 4.0 (Applied Maths, Kortrijk, Belgium).

The isolates were grouped by comparing their restriction patterns to those included in published RFLP (GUILLAMÓN *et al.*, 1998; DLAUCHY *et al.*, 1999; ESTEVE-ZARZOSO *et al.*, 1999; ARIAS *et al.*, 2002; GULLO *et al.*, 2003; PULVIRENTI *et al.*, 2004) and ARDRA (AQUILANTI *et al.*, 2007) databases, as well as those of the databases built specifically for the present study. The diagnosis of species was verified by sequencing of the amplicons (600-800 bp for yeasts, 360 bp for LABs) from one or more isolates selected within each RFLP and ARDRA group. The PCR products were purified using micro-columns (GFX purification kit, Amersham Biosciences), according to the manufacturer's instructions, and sent to MWG Biotech (Milan, Italy) for sequencing. The closest relatives of the sequences obtained were determined through searches within the GenBank DNA database using the BLAST algorithm (ALTSCHUL *et al.*, 1990).

The 54 bacterial isolates assigned to *Lact. plantarum sensu latu* underwent *recA* multiplex PCR assay, as described by TORRIANI *et al.* (2001), for a finer discrimination at the species level. The 11 bacterial isolates for which the genotype-based identification led to an ambiguous diagnosis underwent the following phenotype-based tests: fermentation of sucrose, melibiose, L-arabinose, trehalose, raffinose and lactose (evaluated using a miniaturized assay in microtiter plates) and hydrolysis of arginine, in modified MRS broth medium (AQUILANTI *et al.*, 2007).

Molecular typing of yeasts

Three µL of the total genomic DNA extracted from the sourdough and compressed bakers' yeast isolates were used in the PCR reactions, as described by MAKIMURA *et al.* (1999). Inter-delta sequences were amplified as described by CIANI *et al.* (2004).

Technological characterization of the isolates

The LAB were sub-cultured in SDB medium and incubated at 30°C for 24

h, while the yeasts were sub-cultured in YPD broth (Oxoid) and incubated at 25°C for 24 h.

For the assessment of the LAB acidifying activities and the CO_2 production from yeasts and LAB, an inoculum standardisation was required. Accordingly, stationary phase cells were harvested by centrifugation at 4,200 x *g*, washed twice, and resuspended in sterile distilled water. The cell suspensions were diluted to an optical density at 620 nm (OD_{620}) of 1.25 which, according to CORSETTI *et al.* (1998), corresponded to yeast and LAB counts of 10^7 and 10^9 cfu mL^{-1}, respectively. The standardised suspensions were used as a 4% (v/v) inoculum in the first two assays, following.

LAB acidifying activity

The acidifying activity of the 118 LAB isolates were determined in SDB broth (initial pH, 5.6), incubated at 30°C under aerobic conditions. After 6 and 24 h of fermentation, 1 mL samples were aseptically withdrawn for pH assessment using a model 9224 pH meter (Hanna Instruments, Padova, Italy) equipped with a microtube electrode (Hamilton, Reno Nevada, USA). For the 34 pre-selected LAB isolates ascribed to species of technological interest for the baking industry, the amount of lactic acid and acetic acid produced after a 24 h incubation was determined spectrophotometrically, using two commercially available enzymatic kits (Kit Nos. 11112821035 and 0148261, respectively; Boehringer Mannheim/ R-Biopharm, Darmstadt, Germany).

Yeast and LAB CO_2 production

The amount of CO_2 produced by 36 sourdough yeasts and 34 pre-selected heterofermentative LAB isolates was evaluated by inoculating the cell suspensions into 100 mL flasks containing 70 mL SDB broth. After the inoculation, the flasks were aseptically sealed with Müller valves, which allowed the CO_2 to escape the system (CIANI and ROSINI, 1987). The flasks were weighed immediately after inoculation and after 24 h incubation at 25°C for yeasts, and at 30°C for LAB, using an analytical balance (Analytical Plus, Ohaus, New Jersey). The CO_2 produced was expressed as the weight loss of the fermented broths after this 24 h fermentation.

Yeast and LAB amylase activity

The amylase activities of 36 sourdough yeasts and 34 pre-selected LAB isolates were qualitatively determined using the method described by SEELEY *et al.* (1995). Starch hydrolysis was revealed by the appearance of clear halos surrounding the colonies exposed to a 0.25% iodine solution (DUNGA *et al.*, 2006). For the LAB isolates that could hydrolyse starch, the amylase activities were quantitatively determined as follows: twenty µL of the standardised cell suspensions were inoculated into 180 µL of 0.1 mol L^{-1} phosphate buffer (pH 7.0) containing 10 g L^{-1} soluble starch (Sigma-Aldrich, Milan, Italy). After incubation at 30°C for 3 h, the residual concentrations of starch were determined using an enzymatic kit (Kit No. 207748 Boehringer Mannheim/R-Biopharm), according to the manufacturer instructions.

Statistical analysis

Three replicates of each technological assay were performed. Arithmetic means and standard deviations were calculated. One-way analysis of variance (ANOVA) and the Tukey Kramer honestly significant difference (HSD) based on three replicates were carried out using the JMP software package (version 3.15, S.a.s. Institute Inc. Carry, NC, USA), with the following variables: VAR1, species, VAR2, isolates within the same species; VAR3, acidifying activity at 6 h fermentation; VAR4, acidifying activity at 24-h fermentation; VAR5, lactic-acid production;

VAR6, acetic-acid production; VAR7, CO_2 production; and VAR8, amylase activity.

RESULTS

Sourdough pH measurements, microbial counting and isolation

Nine mature type I sourdough samples were collected from nine bakeries in the Marche region (central Italy). For the sourdough acidification, the pH values ranged from 3.15 to 4.45 (Table 3).

The viable microbial counts showed that the sourdough samples contained yeasts and LAB in ratios that varied from about 1:100 (samples collected from bakeries C, G and H) to 1:10 (samples collected from bakeries A, B, D to F) (Table 3). The yeast cell numbers ranged from log 6.9 to log 8.0 cfu g^{-1}, while those of the LAB varied from log 8.3 to log 9.2 cfu g^{-1}.

The yeasts were isolated only from the sourdough samples collected from bakeries A, C, G and H, the refreshment of which did not include the direct addition of compressed bakers' yeast, while the LAB were isolated from all of the sourdough samples. The isolation campaign yielded 36 sourdough yeast and 118 bacterial isolates. Eleven yeast cultures were isolated the compressed bakers' yeast, that is commonly used in bakeries A, B, D to I. Remarkable morphological differences in colony colour, elevation, surface and edge were seen in the yeast colonies grown on WLN agar (data not shown). All of the bacterial isolates were Gram positive and catalase negative.

Yeast and LAB molecular identification

For the RFLP analysis, the amplification of the 5.8S-ITS region from the reference strains and the isolates yielded PCR products that were characterized by a high degree of length variation. The sizes of the PCR amplicons and of the *Cfo*I, *Hae*III and *Hin*fI digests are shown in Table 2. For all of the yeast reference strains, the experimental patterns were comparable to those obtained through the *in-silico* simulation. For the 36 yeast isolates, 33 were characterized by restriction patterns identical to those produced by the two *S. cerevisiae* reference strains, CBS1171T and CBS4054. The remaining three isolates were ascribed to a different group, since they showed restriction profiles that were not comparable to those collected in the present and other published databases (FOSCHINO *et al.*, 2004; ARIAS *et al.*, 2002; GULLO *et al.*, 2003; PULVIRENTI *et al.*, 2004). Consequently, the 5.8S-ITS amplicons had to be sequenced in order to have an unequivocal identification of these isolates. The alignment of these sequences with those published for the species *Candida humilis* (Acc. no. AY188851) resulted in identities that exceeded 98%, thus confirming the identification of these yeasts. For the ARDRA analysis, PCR products of the expected sizes were obtained from both the LAB reference strains and the isolates. The sizes of the *Fok*I, *Hae*III and *Alu*I digests are shown in Table 2. The restriction profiles generated by the LAB reference strains were consistent with those obtained through the *in-silico* simulation (Table 2). A large proportion of the most abundant LAB species in the sourdoughs was readily differentiated from each other. On the other hand, two species, *Weissella confusa* and *W. viridescens*, could not be differentiated after digestion with *Fok*I, *Hae*III and *Alu*I.

Restriction profile comparisons, partial sequencing of the 16S rRNA gene, and *recA* multiplex PCR assays allowed 107 isolates to be unambiguously identified at the species level. For the remaining 11 isolates, further phenotype-based analyses were performed, thus allowing their definitive assignment to species.

The microbial map of the nine sourdoughs is shown in Fig. 1. A high degree of biodiversity was seen across the ecosystems investigated. *Lact. plantarum* and

Bacteria	Bakery A	B	C	D	E	F	G	H	I	No. isolates
Lact. plantarum	■(6)	■(3)	■(6)	■(6)	■(4)	■(3)	■(6)	■(9)	■(11)	54
Lact. curvatus	■(2)	■(2)		■(3)						7
Lact. sakei		■(1)								1
Lact. rhamnosus			■(1)							1
Lact. helveticus								■(2)		2
Lact. gallinarum									■(1)	1
Lact. sanfranciscensis	■(3)	■(4)					■(1)			8
Lact. paralimentarius			■(7)			■(3)		■(1)	■(1)	12
Lact. brevis						■(5)				5
Lact. casei ssp. carnosus	■(1)									1
Lact. paracasei ssp. paracasei							■(1)	■(1)		2
Lact. fermentum					■(1)	■(2)				3
Lact. casei sensu latu	■(1)									1
S. salivarius		■(1)		■(1)						2
Leuc. citreum	■(1)	■(1)								2
Leuc. pseudomesenteroides							■(9)	■(1)		10
W. confusa				■(1)	■(2)	■(1)				4
W. cibaria				■(1)						1
E. faecalis									■(1)	1
									Total	**118**
Yeasts										
S. cerevisiae	■(6)		■(10)				■(7)	■(10)		33
C. humilis	■(3)									3
									Total	**36**

Fig. 1 - Microbial map of the nine sourdoughs from the Marche region (central Italy) under study. For each sourdough sample, the number of isolates ascribed to yeast and LAB species are reported in brackets.

Saccharomyces cerevisiae were present in all of the sourdough samples, followed by *Lact. paralimentarius* (4 samples), *Lact. curvatus* (3 samples), *Lact. sanfranciscensis* (3 samples) and *W. confusa* (3 samples).

Yeast molecular typing

The amplification of the inter-delta sequences from the 36 sourdough and 11 bakers' yeast isolates allowed the identification of seven fingerprints, referred to as α to η (Fig. 2). The first four amplification patterns (α, β, γ, δ) were obtained from the sourdough isolates, while the latter three (ε, ζ, η) were obtained from the industrial strains. Comparative evaluation of these fingerprints showed the genetic diversity of the sourdough isolates (Table 4). For nine isolates, the amplification of the inter-delta sequences did not allow the visualization of any PCR products. This is not unusual for *S. cerevisiae* (EGLI *et al.*, 1998; CIANI *et al.*, 2004; MARINANGELI *et al.*, 2004). Regarding the sources of the isolates, the sourdough sample collected in bakery C was characterised by the highest strain

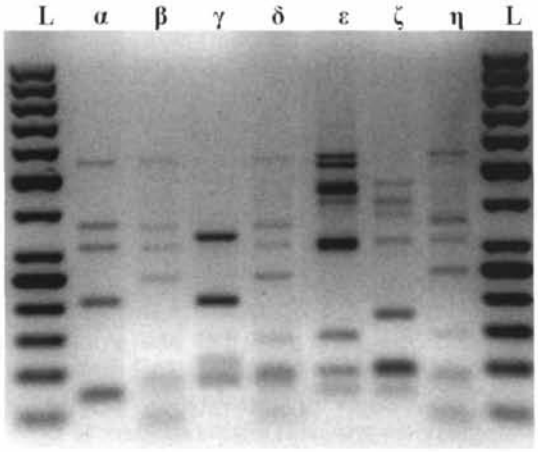

Fig. 2 - Inter-delta amplification profiles generated by PCR from the 36 sourdough and 11 industrial *Saccharomyces cerevisiae* isolates.
L- Molecular weight marker (100 bp DNA Ladder, M-Medical, Milan, Italy).

Table 4 - Technological characterization and molecular typing of the 36 sourdough yeast isolates.

Bakery	Isolate	Species	Amyl.	CO_2 (g L^{-1})			Inter-delta fingerprint
A	PL11	*C. humilis*	-	0.01	o	(0.00)	N.D.
	PL12		+	0.20	o	(0.00)	N.D.
	PL13		-	0.50	n	(0.01)	N.D.
	PL15	*S. cerevisiae*	-	1.35	l	(0.01)	*
	PL16		++	3.17	ef	(0.01)	*
	PL17		++	3.18	ef	(0.02)	*
	PL18		+	2.30	hi	(0.01)	*
	PL19		++	3.13	f	(0.02)	*
	PL20		++	2.54	g	(0.03)	*
C	PL31		+	3.63	de	(0.01)	*
	PL32		-	3.72	de	(0.02)	α
	PL33		+++	4.50	b	(0.04)	α
	PL34		+/-	2.67	fg	(0.02)	α
	PL35		++	N.D.	N.D.	N.D.	α
	PL36		+	3.63	de	(0.03)	*
	PL37		-	4.08	c	(0.04)	*
	PL38		++	N.D.	N.D.	N.D.	α
	PL39		-	N.D.	N.D.	N.D.	α
	PL40		+	3.29	e	(0.02)	β
G	PL76		+/-	N.D.	N.D.	N.D.	γ
	PL77		+/-	0.65	n	(0.00)	γ
	PL78		+/-	N.D.	N.D.	N.D.	γ
	PL79		+++	2.69	fg	(0.01)	δ
	PL80		+++	2.77	fg	(0.01)	δ
	PL82		++	3.25	e	(0.02)	δ
	PL83		+++	2.38	gh	(0.02)	δ
H	PL94		+++	4.13	b	(0.05)	α
	PL95		+++	N.D.	N.D.	N.D.	α
	PL96		++	2.73	gh	(0.01)	α
	PL97		+++	4.70	a	(0.04)	α
	PL98		+++	N.D.	N.D.	N.D.	α
	PL99		+/-	2.49	g	(0.01)	α
	PL100		+/-	2.16	hi	(0.02)	α
	PL101		+/-	3.76	de	(0.02)	α
	PL102		+/-	N.D.	N.D.	N.D.	α
	PL103		+++	N.D.	N.D.	N.D.	α

For CO_2 production, the mean values are expressed in g L^{-1} of gas released after 24 h. Standard deviations (±) are reported in brackets, while different letters denote significant differences ($P<0.05$). * no amplification; N.D. Not Determined; - no hydrolysis; + low hydrolysis; ++ medium hydrolysis; +++ high hydrolysis.

diversity (two fingerprints and three isolates that did not generate any amplification products), while apparently genetically homogeneous populations of *S. cerevisiae* were seen for the sourdough samples from bakery A and H.

Technological characterization

LAB acidifying activity

The LAB acidification kinetics were assessed after 6 and 24 h of fermentation (data not shown). For each sour-

dough sample, the isolate showing the highest acidifying activity among those ascribed to the same species was chosen and taken through further technological assays. This preliminary screening allowed 34 isolates to be pre-selected (Table 5). Although the ability to rapidly decrease the pH of the SDB medium significantly varied among the isolates, the majority of them showed pH values lower than 5.00 after only 6 h of fermentation. Among these, the isolates *Lact. plantarum* PB11 and *Lact. sakei* PB14 stood out as having significantly greater acidifying velocities.

Production of L/D-lactic acid and acetic acid

The amounts of L/D-lactic acid and acetic acid produced by the 34 pre-selected LAB isolates are shown in Table 5. As it has emerged from the statistical evaluation of these two chemometric parameters, the isolate *Lact. plantarum* PB11 differed significantly from the others; it had the highest lactic acid production (6.28 gL^{-1}). On the other hand, the isolates *Lact. plantarum* PB210 and PB46, as well as *Lact. sanfranciscensis* PB211 and *Leuc. citreum* PB220, had the highest acetic acid production values.

CO_2 production

The CO_2 production from the 36 sourdough yeasts and 34 pre-selected heterofermentative LAB was evaluated (Tables 4 and 5). For the majority of the LAB, the amount of CO_2 produced was between 0.20 and 1.90 g L^{-1}, although it ranged from 3.5 g L^{-1} (detected for *Lact. plantarum* PB11) to 0.2 g L^{-1} (detected for *Lact. paralimentarius* PB94). For the yeasts, the isolates belonging to *Candida humilis* showed CO_2 production between 0.01 and 0.50 g L^{-1}, while for those ascribed to *S. cerevisiae*, this ranged from 0.65 to 4.70 g L^{-1}. Within this latter group, values comparable to those shown by the 11 *S. cerevisiae* strains isolated from the bakers' yeast (2.6-5.0 gL^{-1}) were seen in 17 cultures. Two isolates (*S. cerevisiae* PL97 and PL33) showed the best performance, releasing 4.70 and 4.50 g L^{-1} CO_2, respectively. Both these genera had high variabilities as noted by ANOVA (Table 5).

Starch hydrolyses

The 36 sourdough yeasts and 34 pre-selected LAB were first screened on MRS-starch plates for qualitative assessment of their amylolytic abilities. For the yeasts, the ability of the isolates ascribed to *S. cerevisiae* to hydrolyse starch varied from (-) to (+++), while a negative amylolytic ability was seen for all of the isolates belonging to *Candida*, except for the isolate PL12 (Table 4). For the LAB isolates that showed a positive activity (from + to +++), a quantitative determination of the starch hydrolysis was conducted (Table 5). Among the LAB, only nine isolates were able to hydrolyse starch. When the amounts of hydrolysed starch, which ranged from 1.10 to 2.13 g L^{-1}, was statistically evaluated, a wide inter- and intra-specific variability was seen (Table 5). While several *Lact. plantarum* isolates were characterized by a high amylolytic capacity, the *W. confusa* PB150 isolate had the best performance.

Definition of LAB and yeast biotypes

By considering the species, the source of isolation and the results of the technological characterization (plus the molecular fingerprinting, when available), almost all of the 36 yeast and 34 pre-selected LAB isolates were bio-diverse, except for two clones (*S. cerevisiae* PL79 and PL80) identified among the yeasts.

DISCUSSION

The industrialization of the baking process has led to an increased demand for defined single-strain and multi-strain culture preparations for standardised sourdough fermentations (GOB-

Table 5 - Technological characterization of the 34 LAB isolates from type I sourdoughs of the Marche region (central Italy).

Bakery	Isolate	Species	Acidification			Amy. activity	Starch	Primary metabolites (gL^{-1})		
			6 h	24 h			hydrolysed (gL^{-1})	Lactate	Acetate	CO_2
A	PB5	*Lact. casei sensu latu*	3.69 c (0.01)	3.27 b (0.01)		-	N.D.	3.20 de (0.02)	0.06 b (0.00)	1.10 d (0.01)
	PB1	*Lact. curvatus*	4.24 i (0.02)	4.12 g (0.01)		-	N.D.	3.33 d (0.03)	0.05 b (0.00)	1.20 cd (0.01)
	PB210	*Lact. plantarum*	4.04 g (0.01)	3.48 cd (0.01)		+	1.12 m (0.01)	2.62 de (0.04)	0.13 a (0.02)	0.92 d (0.01)
	PB211	*Lact. sanfranciscensis*	5.27 q (0.02)	3.82 f (0.01)		+++	1.73 f (0.01)	2.37 f (0.01)	0.13 a (0.03)	0.95 d (0.02)
	PB3	*Leuc. citreum*	3.81 ef (0.01)	3.54 cd (0.01)		+++	1.94 c (0.02)	2.33 ef (0.01)	0.10 ab (0.01)	1.00 d (0.01)
B	PB115	*Lact. curvatus*	4.13 h (0.02)	3.71 e (0.01)		-	N.D.	5.32 ef (0.01)	0.09 ab (0.01)	1.90 b (0.02)
	PB11	*Lact. plantarum*	3.43 a (0.01)	3.14 a (0.05)		-	N.D.	6.28 a (0.02)	0.10 ab (0.02)	3.50 a (0.01)
	PB14	*Lact. sakei*	3.52 b (0.01)	3.25 b (0.01)		-	N.D.	3.33 de (0.04)	0.08 ab (0.00)	1.12 d (0.01)
	PB223	*Lact. sanfranciscensis*	4.05 g (0.03)	3.46 cd (0.03)		-	N.D.	2.11 ef (0.01)	0.07 ab (0.01)	0.86 d (0.01)
	PB220	*Leuc. citreum*	4.52 l (0.02)	3.68 e (0.08)		-	N.D.	2.02 fg (0.02)	0.13 a (0.01)	0.70 e (0.01)
C	PB23	*Lact. paralimentarius*	3.93 c (0.01)	3.54 cd (0.01)		-	N.D.	2.88 de (0.04)	0.06 b (0.00)	1.20 cd (0.04)
	PB233	*Lact. rhamnosus*	4.26 l (0.01)	3.65 e (0.09)		-	N.D.	3.00 de (0.06)	0.01 b (0.00)	1.45 c (0.02)
D	PB33	*Lact. curvatus*	3.92 g (0.01)	3.65 e (0.01)		+++	1.47 h (0.03)	2.10 ef (0.01)	0.05 b (0.00)	0.75 e (0.02)
	PB24	*Lact. plantarum*	3.68 cd (0.05)	3.21 ab (0.02)		-	N.D.	2.96 de (0.01)	0.07 ab (0.00)	1.10 d (0.03)
	PB242	*Lact. plantarum*	3.84 f (0.02)	3.50 cd (0.03)		-	N.D.	1.45 g (0.01)	0.03 b (0.00)	0.50 e (0.02)
	PB243	*Lact. plantarum*	3.83 f (0.03)	3.44 cd (0.01)		+++	1.83 e (0.05)	2.19 ef (0.01)	0.10 ab (0.01)	0.80 d (0.01)
	PB32	*W. cibaria*	3.62 d (0.03)	3.52 cd (0.01)		-	N.D.	2.92 ef (0.01)	0.06 b (0.00)	1.11 d (0.03)
E	PB46	*Lact. plantarum*	3.75 de (0.01)	3.28 b (0.01)		++	1.46 h (0.02)	2.12 ef (0.03)	0.13 a (0.02)	0.92 d (0.02)
	PB150	*W. confusa*	4.86 n (0.02)	4.29 h (0.02)		+++	2.13 a (0.03)	2.62 ef (0.04)	0.10 ab (0.02)	1.10 d (0.01)
F	PB 55	*Lact. brevis*	3.88 f (0.01)	3.46 cd (0.01)		-	N.D.	2.43 ef (0.01)	0.12 a (0.01)	0.90 d (0.01)
	PB162	*Lact. fermentum*	4.23 i (0.04)	3.45 cd (0.02)		-	N.D.	3.10 de (0.01)	0.08 ab (0.01)	1.15 cd (0.03)
	PB264	*Lact. paralimentarius*	4.06 h (0.03)	3.66 e (0.02)		-	N.D.	2.71 ef (0.02)	0.05 b (0.00)	1.10 d (0.03)
	PB268	*Lact. plantarum*	4.14 h (0.03)	3.42 c (0.03)		+	1.10 n (0.01)	2.02 gf (0.01)	0.12 a (0.01)	0.65 e (0.01)
G	PB284	*Lact. paracasei* ssp. *paracasei*	4.04 g (0.01)	3.56 d (0.02)		-	N.D.	1.24 g (0.08)	0.03 b (0.00)	0.47 e (0.01)
	PB277	*Lact. plantarum*	3.84 f (0.08)	3.44 c (0.07)		-	n.d.	4.20 c (0.01)	0.10 ab (0.02)	1.50 c (0.00)
	PB287	*Lact. plantarum*	5.17 p (0.01)	3.71 e (0.01)		+++	1.98 b (0.04)	3.32 de (0.02)	0.05 b (0.01)	0.45 e (0.01)
	PB276	*Lact. sanfranciscensis*	4.13 h (0.03)	3.50 cd (0.02)		+++	1.72 g (0.01)	3.52 d (0.06)	0.05 b (0.01)	1.35 cd (0.01)
	PB172	*Leuc. pseudomesenteroides*	4.84 h (0.04)	3.75 e (0.10)		-	N.D.	2.61 ef (0.01)	0.08 ab (0.01)	1.10 d (0.02)
	PB288	*Leuc. pseudomesenteroides*	5.09 op (0.01)	3.80 ef (0.01)		-	N.D.	1.78 fg (0.01)	0.04 b (0.01)	0.65 e (0.01)

Continued tab. 5.

Bakery	Isolate	Species	Acidification						Amy. activity	Starch	Primary metabolites (gL^{-1})							
			6 h			24 h				hydrolysed (gL^{-1})	Lactate			Acetate			CO_2	
H	PB294	*Lact. paracasei* ssp. *paracasei*	5.05	o	(0.01)	3.43	c	(0.03)	-	N.D.	1.31	g	(0.07)	0.06	b	(0.01)	0.46	e (0.00)
	PB94	*Lact. paralimentarius*	4.02	g	(0.04)	3.50	cd	(0.01)	-	N.D.	1.03	g	(0.01)	0.04	b	(0.00)	0.20	e (0.01)
	PB193	*Lact. plantarum*	5.38	r	(0.01)	3.36	bc	(0.04)	-	N.D.	2.89	ef	(0.01)	0.06	b	(0.01)	1.10	d (0.01)
	PB202	*Lact. plantarum*	4.97	o	(0.01)	3.33	b	(0.02)	-	N.D.	2.90	ef	(0.06)	0.04	b	(0.00)	1.00	d (0.01)
	PB295	*Leuc. pseudomesenteroides*	4.02	g	(0.01)	3.48	cd	(0.02)	-	N.D.	2.56	ef	(0.01)	0.05	b	(0.01)	1.10	d (0.01)

For the quantitative assays, mean values are expressed as: pH measured after 6 and 24 h; gL^{-1} of hydrolyzed starch; gL^{-1} of lactate and acetate produced after 12 h; gL^{-1} of CO_2 produced after 24 h. Within each dataset, standard deviations (±) are reported in brackets, while different letters denote significant differences ($P < 0.05$).
N.D.: not detected.
-: no hydrolysis; +: low hydrolysis; ++: medium hydrolysis; +++: high hydrolysis; ++++: very high hydrolysis.

BETTI *et al.*, 1995). The strain selection for industrial applications relies greatly on the evaluation of distinctive microbial activities for improved sourdough bread quality (GOBBETTI and GÄNZLE, 2007). Each sourdough is a unique ecosystem, where a balanced population of yeasts and LAB interact in a dynamic equilibrium. Accordingly, the selection of the resident sourdough strains to be used as starters requires prior knowledge of the complexity and structure of this ecosystem. Indeed, the successful use of starters largely depends on competition with autochthonous strains as well as on strain robustness during sourdough back-slopping.

In the present study, the microbial ecology of nine wheat flour sourdoughs sampled in the Marche region was assessed in order to identify candidate yeast and LAB starter strains for the local baking industry. High loads of yeasts and LAB were seen in all of the sourdough samples analysed. These data are in agreement with those reported for wheat flour sourdoughs (DE VUYST and NEYSENS, 2005). The viable counts for the yeasts were lower than those recorded for the LAB for all of the sourdoughs under study.

With the restriction analysis of the 5.8S-ITS rRNA region, the results of this study fully confirm the usefulness of this analysis as a routine, fast technique for identifying yeasts from sourdough. However the published restriction pattern databases must be continuously updated. Indeed, new and atypical profiles can be generated by both reference and wild strains, as has been shown by the RFLP trials. The production of these atypical profiles could result from single mutations in the 5.8S-ITS region, which, in turn, are known to be responsible for the loss or gain of restriction sites. Accordingly, an intra-specific variability in the large subunit rDNA sequences of yeast species was previously seen with *C. lipolytica* (KURTZMAN and

ROBNETT, 1997) and *Clavispora lusitaniae* (LACHANCE *et al.*, 2003).

As far as the yeast ecology of our sourdough samples is concerned, *S. cerevisiae* was shown to dominate, and it was the only species detected in three out of four bakeries; namely, C, G and H. This finding confirms the high competitiveness of this species in wheat flour sourdough ecosystems (ROCHA and MALCATA, 1999; SUCCI *et al.*, 2003; PULVIRENTI *et al.*, 2004). Interestingly, in all four of the sourdoughs evaluated, the colony morphology of isolates of *S. cerevisiae* was different on WLN agar. According to CAVAZZA *et al.* (1992), this finding suggests the presence of a heterogeneous *S. cerevisiae* population. The presence of a biodiverse pool of *S. cerevisiae* strains among our isolates was confirmed by the visualization of different inter-delta amplification patterns. The δ1 and δ2 primers were previously used to successfully investigate the genetic polymorphism of yeasts from wheat flour sourdoughs (PULVIRENTI *et al.*, 2004). Interestingly, none of our yeast isolates showed fingerprints that were similar to those yielded by the *S. cerevisiae* strains isolated from the four compressed bakers' yeasts. This finding confirms the presence of a wild heterogeneous population of *S. cerevisiae* in the sourdough sample collected from bakery C, where the compressed bakers' yeast had never been used; at the same time, it suggests the exclusive presence of a wild *S. cerevisiae* community in the sourdough samples collected from bakeries A, G and H, where the compressed bakers' yeast was used in production lines different from those that were considered in the present study.

While *C. humilis* was previously identified as one of the prevalent species in wheat flour (GULLO *et al.*, 2003) and sourdoughs (FOSCHINO *et al.*, 2004), in the present study it was only isolated from one bakery. This suggests that this species was poorly adapted to the sourdough ecosystems analysed.

Similar to the RFLP, the restriction analysis of the amplified 16S rRNA gene proved to be a useful tool for identifying the sourdough LAB species. One of the main advantages of the techniques that rely on the restriction analysis of DNA sequences is that comprehensive restriction pattern databases can be built up and easily updated when new species, or even new profiles, are described. Given the availability of sequences of sufficient quality, the prediction of restriction patterns of deposited nucleotide sequences by applying computer-aided digestion helps to increase the discrimination power of these techniques.

Similar to other ecological investigations carried out on wheat sourdoughs (DE VUYST and NEYSENS, 2005), in the samples studied the LAB species with heterofermentative metabolism were prevalent. Remarkable differences in species richness were also found; in two sourdoughs considered (collected from bakeries C and E), only three species were isolated, while in the remaining samples, mixed LAB populations were detected that included up to six species. This high heterogeneity is probably due to the differences in the technological parameters (SALOVAARA, 1998) and/or to contamination from the raw materials and bakery environments (DE VUYST and NEYSENS, 2005).

Among the facultative heterofermentative species, *Lact. plantarum* was the most frequently isolated, while within the obligate heterofermentative group, *W. confusa* and *Lact. sanfranciscensis* prevailed; each was isolated from three sourdoughs. The frequent isolation of *Lact. plantarum* in wheat flour sourdoughs, as well as the association with *Lact. sanfranciscensis* and *Lact. brevis*, has been described (GOBBETTI *et al.*, 1994; GOBBETTI, 1998) and tentatively ascribed to the high adaptation of these micro-organisms to plant materials (e.g. maize and rye) (ROCHA and MALCATA, 1999).

Although type I sourdoughs are dominated by *Lact. sanfraciscensis* and *Lact. pontis* (VOGEL *et al.*, 1999) the composition of the microbial communities in the sourdoughs analyzed was closer to those usually detected in type-III sourdoughs, according to the classification proposed by VOGEL *et al.* (1999). A similar discrepancy was detected by RICCIARDI *et al.* (2005) in type-I sourdoughs used to manufacture Altamura bread in Apulia (southern Italy).

Based on the new classification of type-I sourdoughs proposed by DE VUYST and NEYSENS (2005), all of the sourdoughs analyzed in this study fall within the type-Ib group because they were prepared through multiple steps and characterized by extremely heterogeneous LAB populations, including species groups that were previously isolated from sourdoughs, i.e. *Lact. casei*, *Lact. plantarum*, *Lact. sakei*, *Lact. sanfranciscensis*, *Lact. gallinarum* (VOGEL *et al.*, 1999; EHRMANN and VOGEL, 2005). To our knowledge, representatives of the *Lact. salivarius* species have never been isolated from sourdoughs, but lactobacilli ascribed to *Lact. salivarius* were found in the samples collected from bakeries B and D.

The selection of micro-organisms with suitable functional properties and the establishment of optimized and controlled processing conditions in breadmaking are the key issues for the production of breads with the level and uniformity of quality currently required by the European consumer. Overall, the ecological investigations performed here have allowed both wild yeasts and LAB with baking potential to be found.

It is known that a good yeast strain for the sourdough process must possess definite properties, such as fast leavening of the dough and high amylolytic activity (LINKO *et al.*, 1997). With regard to the pool of *S. cerevisiae* isolates considered in this study, there was a high heterogeneity in both CO_2 production and amylolytic activity.

For the LAB, the high acid production during the first hours of the sourdough fermentation process is a desired trait of starter strains used in baking applications (CLARKE *et al.*, 2002). Based on this parameter, two isolates decreased the pH more rapidly, namely *Lact. plantarum* PB11 and *Lact. sakei* PB14, and are thus potential starter cultures.

Other microbial traits affect the stability of leavened baked goods, such as the type of organic acids produced during sourdough fermentation (DAESCHEL *et al.*, 1987). Lactic acid accounts for a more elastic gluten structure (LORENZ, 1983), while acetic acid positively affects the sensory properties of baked goods, and has anti-ropiness and anti-mould activities. To date, many efforts have been made to increase the concentration of acetic acid in sourdoughs, especially through the selection of starter strains ascribed to obligately heterofermentative species, like *Lact. sanfranciscensis* (GOBBETTI, 1998). When the amount of lactic acid and acetic acid produced by the isolates in this study were compared, the best performances were seen for *Lact. plantarum* PB11, PB46 and PB210, *Leuc. citreum* PB220 and *Lact. safranciscensis* PB211.

The amount of fermentable carbohydrates in flour largely depends on the extent of starch hydrolysis performed by the endogenous and microbial amylases and xylanases (MARTINEZ-ANAYA, 1996). Accordingly, a further criterion for selecting LAB starter strains is a high amylolytic ability, because this increases the availability of energy sources for non-amylolytic LAB and yeasts (SANNI *et al.*, 2002). Based on the results of the agar plate assays, the *Lact. plantarum* PB287 and *W. confusa* PB150 strains would provide the greatest hydrolysis of soluble starch.

The CO_2 released by the lactic acid bacteria has been reported to enhance

yeast metabolism, and consequently the yeast-leavening ability (GOBBETTI *et al.*, 1995). Accordingly, *Lact. plantarum* PB11 and PB 277, as well as *Lact. curvatus* PB115 could be candidates for starter strains, since they had the greatest gas production, together with *S. cerevisiae* PL97 and PL33. Although *Lact. plantarum* is a facultative heterofermentative species, which is not expected to produce high amounts of CO_2, under aerobic conditions, it can ferment lactate to acetate and CO_2 through a pathway which involves NAD+dependant and/or NAD+independent LDH, pyruvate oxidase and acetate kinase (MURPHY et al., 1985).

The overall results obtained in this study demonstrate the high biodiversity of the sourdough ecosystem and the effective possibility of exploiting this source of wild yeasts and LAB to find strains with baking potential. The yeast and LAB strains selected in the present study were included in a defined multispecies starter culture, which was used to prepare sourdoughs destined for in bread-making with barley flour (ZANNINI *et al.*, 2009).

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