



## Selection of lactic acid bacteria from home-made sourdoughs for resistance to the main almond skin polyphenols

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### ABSTRACT

The aim of this research was to identify lactic acid bacteria (LAB) that could be used as starter strains in sourdough made with almond peel powder to create novel fermented functional bakery products. Physicochemical characteristics and LAB were analyzed from ten home-made sourdoughs used at the family level in western Sicily (Italy). The pH (3.57–4.80) and TTA (8.20–14.20 mL 0.1 N NaOH/10 g) were inversely correlated. LAB dominated all sourdoughs, and the highest cell densities (6.74–9.28 log cfu/g) were detected on the medium Sour Dough Bacteria, except for one sample. Among LAB groups, the lowest counts (4.92–6.19 log cfu/g) were obtained on M17 used for LAB cocci. Yeasts were lower than LAB from two to four orders of magnitude with values between 3.93 and 6.11 log cfu/g. Twenty strains belonging to the species *Enterococcus gilvus*, *Fructilactobacillus sanfranciscensis*, *Lactocaseibacillus paracasei*, *Lactiplantibacillus pentosus*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Lentilactobacillus buchneri*, *Lentilactobacillus diolivorans*, *Leuconostoc citreum*, *Leuconostoc holzapfelii*, *Levilactobacillus brevis*, *Pediococcus pentosaceus*, and *Weissella cibaria* were identified. Eleven out of the 20 strains identified showed the ability to grow in presence of 16 polyphenols characterizing the peel of almonds. Eight of these LAB strains dropped pH below 4.5 after 6–8 h of fermentation, and two strains showed a strong inhibitory effect against *Listeria monocytogenes* ATCC19114 with inhibition diameters around the wells >19 mm. Thus, all acidifying strains were selected to produce sourdough bakery goods enriched with almond skin powder.

### 1. Introduction

Sourdough has been experiencing a renewed era of application. Despite the long history of use, the interest in this leavening agent is still on the rise [1]. In the southern part of Italy, sourdough propagation is a common home practice [2]. In the past, sourdough propagation in Sicily was a weekly home practice performed by housewives and women who had been engaged in this activity since childhood [3]. Sourdough was considered a family treasure and was even given as a dowry from mothers to daughters.

Sourdough contains lactic acid bacteria (LAB) and yeasts, but LAB populations are the most important groups that exert several positive properties of sourdough [4]. Sourdough LAB provide several advantages over baker's yeast in terms of organoleptic, nutritional and safety

aspects of the final bakery products [5]. Therefore, research groups and R&D units of industrial companies continuously select sourdough LAB to find strains with desired properties [6]. Recently, the National Recovery and Resilience Plan (NRRP) has aimed to design nature-based sustainable solutions, explore microbial biodiversity, improve a clean and circular economy, and sustain human health [7]. In this context, enriching cereal-based foods with fruit and vegetable wastes and by-products rich in polyphenols and using selected LAB strains as leavening agents represent a good strategy to improve the functional value of bakery products [8]. However, it is well known that phenolic compounds present in grape and oregano essential oils could negatively influence the development of starter LAB [9,10]. To our knowledge, no studies regarding the ability of LAB to grow in the presence of the main polyphenols characterizing almond by-products are present in literature.

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The purpose of this research is to select LAB that are resistant to almond peel polyphenols to develop *ad hoc* starter cultures for future applications in functional sourdough bread production in the presence of almond skin in powder form.

The specific objectives of this study were to: (i) isolate, characterize, and identify LAB from home-made semolina sourdoughs; (ii) evaluate the resistance of LAB to growth in the presence of almond peel polyphenols; and (iii) determine the bread technological potential of LAB. This study is part of a project aimed at producing novel healthy bakery products by reusing almond skin.

## 2. Materials and methods

### 2.1. Sample collection

The sourdoughs analyzed in this study were primarily produced from semolina alone and rarely in combination with flour (Table 1). These sourdoughs were sampled in western Sicily, specifically among the provinces of Agrigento, Palermo and Trapani, and were all propagated at home level without the addition of baker's yeast in any step of production. The collection was performed at the moment of the daily refreshment when the acidity level was at the maximum level. Two aliquots of each sourdough, representing the technical repetitions, were collected on the same day, and a given sourdough was sampled twice, at a 1-week distance, to obtain two independent replicate productions. All sourdoughs were aseptically collected by means of a portable Bunsen's burner and a sterile stainless steel spoon. Almost 100 g of sourdough were placed into sterile BagLight® 400 bags (Interscience, Saint Nom, France) and transported into a portable cooler. Once in the laboratory of Food and Agricultural Microbiology of Palermo University (Italy), all sourdough samples were immediately analyzed.

### 2.2. Physicochemical analysis and microbiological investigation

The pH values were determined using a pH-meter HI98165 (Hanna Instruments, Villafranca Padovana, Italy). Each sourdough (10 g) was suspended in 90 mL of distilled H<sub>2</sub>O, homogenized by means of a Bag-Mixer® 400 (Interscience) at the highest speed for 2 min, and subjected to total titratable acidity (TTA) measurement using 0.1 N NaOH (results expressed as mL of 0.1 N NaOH).

Another 10 g aliquot of each sourdough was homogenized as previously described, but Ringer's solution (Sigma-Aldrich, Milan, Italy) was used instead of distilled H<sub>2</sub>O. The initial concentrated cell suspension was serially diluted (1:10) in Ringer's solution, and the highest dilutions were plated on the following media: plate count agar (PCA) incubated for 72 h at 30 °C for total mesophilic microorganisms (TMM); mMRS [11] and M17 incubated at 30 °C for 48 h for LAB rods and cocci, respectively; Sour Dough Bacteria (SDB) [12] incubated at 30 °C for 48 h for sourdough LAB; San Francisco Medium (SFM) [13] incubated for 48 h at 30 °C for presumptive *Fructilactobacillus sanfranciscensis*; yeast

peptone dextrose (YPD) incubated at 25 °C for 48 h for yeasts. mMRS, M17, SDB and SFM were supplemented with cycloheximide (10 mg/mL) to inhibit fungal growth, while YPD was added with chloramphenicol (0.1 mg/mL) to inhibit bacterial growth. mMRS and M17 plates were inserted into hermetically sealed jar added with the AnaeroGen AN25 system (Oxoid, Milan, Italy) to create anaerobic conditions during incubation. PCA, M17 and YPD were purchased from Oxoid. Microbiological analyses were performed in duplicates.

### 2.3. Isolation, typing, and identification of LAB

Presumptive LAB were isolated at the highest cell densities from all media used for plate count (mMRS, M17, SDB and SFM) of this bacterial population. To collect the highest LAB biodiversity, at least four colonies sharing the appearance in terms of shape, size, colour, edge, characteristics of the surface and elevation were isolated. All isolates were preliminary characterized by Gram determination applying the method described by Gregersen [14] and catalase test determined as reported by Koneman et al. [15]. Since all LAB species are characterized by a single thick peptidoglycan layer and are not able to convert oxygen peroxide into water and oxygen, only Gram-positive cultures negative for catalase expression were purified by successive sub-culturing and stored in glycerol stocks at −80 °C.

A phenotypic grouping was performed to analyze the morphological, physiological, and biochemical characteristics of LAB per group. To this purpose, all presumptive LAB isolates were microscopically investigated to distinguish their cell morphology type and the arrangement of the cells. Furthermore, they were evaluated for the growth at 15 and 45 °C, NH<sub>3</sub> production from arginine, aesculin hydrolysis, metabolism type by testing the ability to produce CO<sub>2</sub> from glucose and acid production from several carbohydrates [16].

A representative group of isolates (approximately 30%) within each phenotypic group was genetically characterized. LAB cultures were grown overnight in their optimal growth media at 30 °C and their genomic DNAs were extracted from pelleted cells with the InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) following manufacturer's instructions. Crude cell extracts were directly used as templates for PCR. A random amplification of polymorphic DNA (RAPD)-PCR analysis was performed to differentiate the isolates at strain level. To this purpose, three single primers (M13, AB111, and AB106) were used as previously described by Settanni et al. [17] and the resulting amplicons were separated and visualized as reported by Ventimiglia et al. [18]. RAPD profiles were analyzed by Gelcompar II software, version 6.5 (Applied-Maths, Sint-Martens-Latem, Belgium).

All different strains were identified at the species level by 16S rRNA gene sequencing performing PCR reactions with the primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3')/rD1 (5'-AAGGAGGTGATCCAGCC-3') as described by Weisburg et al. [19]. The molecular size of about 1600 bp for the PCR products was confirmed on agarose gels and the amplicons were purified using the QIAquick purification kit (Qiagen S.p.a.,

**Table 1**  
Characteristics of sourdoughs.

Samples	City (province) <sup>a</sup>	Final product	Years from first propagation	Type of flour	DY	Fermentation temperature (°C)	Fermentation duration (h)
S1	Gibellina (TP)	Bread	5	Wholemeal flour	375	ambient	24
S2	Alcamo (TP)	Bread	10	Durum semolina	160	ambient	48
S3	Naro (AG)	Bread	2	Durum semolina	250	fridge	120
S4	Canicatti (AG)	Bread/Pizza	4	Flour 50%/semolina 50%	225	ambient	12
S5	Favara (AG)	Bread/Pizza	20	Durum semolina	175	ambient	12
S6	Altofonte (PA)	Bread	10	Durum semolina	170	ambient	24
S7	Altavilla (PA)	Bread/Pizza	>50	Flour 50%/semolina 50%	230	ambient	8
S8	Palermo (PA)	Bread	7	Tender flour	300	ambient	12
S9	Marsala (TP)	Bread/Pizza	>100	Durum semolina	250	ambient	8
S10	Licata (AG)	Bread/Pizza	30	Durum semolina	180	ambient	12

<sup>a</sup> Province codes: AG, Agrigento; PA, Palermo; TP, Trapani. Abbreviations: S, sourdough; 1–10, home 1–home 10; DY, dough yield (weight of dough/weight of flour × 100).

Milan, Italy) and sequenced using the same primers used for PCR amplification at AGRIVET (University of Palermo, Italy). The sequences were compared with those available in the GenBank/EMBL/DBJ [20] and EzTaxon-e databases [21].

#### 2.4. Almond peel polyphenol resistance test

All LAB strains were tested for their ability to grow in the presence of 16 polyphenols characterizing the peel of almonds [22], which were purchased in purified form from Extrasynthese (Genay, France). To this purpose, the following polyphenols: catechin, epicatechin, eryodictiol, eryodictiol-7-O-glucoside, hyperoside, isorhamnetin, isorhamnetin-3-O-glucoside, isorhamnetin-3-O-rutinoside, kaempferol, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, morin, naringenin, naringenin-7-O-glucoside, quercetin and rutin, were tested. Briefly, pure LAB cultures from  $-80^{\circ}\text{C}$  glycerol stocks, were propagated in their optimal growth media for 24 h at  $30^{\circ}\text{C}$  and then washed in Ringer's solution, after centrifugation at 5000 rpm for 5 min. The washed cells of each LAB were inoculated at 1% (v/v) in 96-well microplates containing sterile semolina extract (SSE), prepared as described by Alfonzo et al. [23], to a final concentration of 0.5 mg/mL of each polyphenol. This concentration is considered the necessary amount of polyphenols to exert the antioxidant activity [24]. The bacterial growth was measured over time (2 h intervals for 48 h), using a P-800 ScanReady Spectrophotometer (Life Real Biotechnology Co., Ltd, Hangzhou, China). The optical density (OD) was measured at 0, 2, 4, 6, 8 and 24 h after inoculation. SSE inoculated only with each LAB strain was used as a positive control, while SSE alone as a negative control. This test was carried out in duplicate.

#### 2.5. Technological characterization of LAB

Only the LAB resistant to the main almond peel polyphenols were characterized for some of the technological properties useful during sourdough bread production: acidification capacity and antimicrobial activity.

The acidification kinetics was assayed as reported by Alfonzo et al. [25] using SSE as growth medium. LAB cultures were propagated, washed and inoculated in SSE as reported above. The pH decrease was monitored at 0, 2, 4, 6, 8 and 24 h after inoculation.

The inhibitory capacity of LAB was first tested by the agar-spot deferred method (ASDM) reported by Corsetti et al. [11] who modified the protocol of Schillinger and Lücke [26]. The indicator strains, sensitive to the inhibitory substances produced by the LAB tested were *Listeria innocua* 4202, *Listeria monocytogenes* ATCC19114 and *Lactobacillus sakei* LMG2313. The strains showing an inhibitory activity, were further tested by the well diffusion assay (WDA) of Schillinger and Lücke [26] against the same indicator strains. WDA was performed with cell-free LAB supernatants obtained after centrifugation of the broth cultures at 5000 rpm for 5 min, addition of 0.1 N NaOH until pH 6.5, addition of catalase (1 mg/mL), and filtration through 0.20  $\mu\text{m}$  sterile filters (ClearLine® Dominique Dutscher, Brumath, France). In order to evaluate the protein nature of the active substances, all supernatants were further treated with proteinase K, protease B and trypsin as described by Gaglio et al. [27]. The inhibitory activity was evaluated after incubation at  $37^{\circ}\text{C}$  for 18 h and was considered positive only in case of a definite clear halo around the wells. All measurements regarding acidification capacity and antimicrobial activity were duplicated.

#### 2.6. Statistical analyses

Microbiological data were subjected to One-Way Variance Analysis (ANOVA) using XLStat software version 7.5.2 for Excel (Addinsoft, New York, USA). The Tukey's test was applied for pairwise comparison. Statistical significance was attributed to p values of  $p < 0.05$  and marked with different letters.

### 3. Results

#### 3.1. Characteristics of sourdoughs

Table 2 reports the main physicochemical characteristics evaluated for the home-made sourdoughs produced from semolina. The pH value was quite variable, ranging from 3.57 to 4.80, and inversely correlated with TTA. In particular, a linear correlation was observed between the two parameters. TTA ranged between 8.20 and 14.20 mL 0.1 NaOH.

The presence of the main populations affecting the fermentation process are reported in Table 3. TMM were estimated to provide a general overview of the microbial community present in a viable state. This group was above  $10^7$  cfu/g in all samples, reaching the highest levels (8.96 log cfu/g) in two samples (S5 and S10). Except for S1, the levels of TMM were almost superimposable on LAB estimated on SDB. The levels of LAB cocci on M17 were lower than those of the other LAB groups. This observation was particularly evident for the sample S3 that showed a level of LAB cocci almost four orders of magnitude lower than those estimated on mMRS and SDB. Yeasts ranged from 3.93 to 6.11 log cfu/g. The yeast/LAB ratio was 1:100 for the sourdoughs S3, S8 and S9, while for the majority of the samples, it was at least 1:1000.

#### 3.2. Composition of dominant populations

After isolating the dominant LAB populations, they were grouped based on morphological, physiological, and biochemical traits. The study revealed a total of 13 groups (Table 4), with groups I–VIII consisting of rod-shaped bacteria and groups IX–XIII consisting of cocci. All rods and most cocci were arranged in short chains, while group XIII cocci appeared in tetrads. All bacteria grew at  $15^{\circ}\text{C}$ , and only four groups, one per rods (VI) and three for cocci (X, XI and XIII), showed development at  $45^{\circ}\text{C}$ . Three out of the five groups for cocci grew at pH 9.2 and in the presence of 6.5% NaCl. The capacity to hydrolyze arginine and aesculin was quite common among the isolates. Except bacteria of group VIII, all other bacteria produced acids from pentose carbohydrates. The majority of rods (groups I–VI) showed an obligate heterofermentative metabolism, while only groups XI and XII showed this trait among cocci.

RAPD-PCR was used to differentiate the different strains of presumptive LAB. To this end, all isolates sharing a given RAPD profile were considered to represent the same strain. The dendrogram in Fig. 1 shows 20 different strains. Thus, the dominant LAB populations of the 10 sourdoughs analyzed included only a few strains. The 20 strains were subjected to the 16S rRNA gene sequencing, which allowed the identification of 13 LAB species, one per phenotypic group. The six obligate heterofermentative LAB groups were identified as five species: *Lentilactobacillus brevis*, *Lentilactobacillus diolivorans*, *Lentilactobacillus*

**Table 2**  
Physicochemical characteristics of sourdoughs.

Samples	pH	TTA
S1	4.24 $\pm$ 0.03 c	11.80 $\pm$ 0.20 c
S2	4.03 $\pm$ 0.01 d	13.20 $\pm$ 0.30 b
S3	4.80 $\pm$ 0.08 a	8.20 $\pm$ 0.10 g
S4	4.07 $\pm$ 0.04 d	10.90 $\pm$ 0.40 d
S5	3.85 $\pm$ 0.05 e	10.10 $\pm$ 0.30 e
S6	3.60 $\pm$ 0.01 f	14.20 $\pm$ 0.10 a
S7	3.88 $\pm$ 0.03 e	12.70 $\pm$ 0.40 b
S8	4.38 $\pm$ 0.01 b	9.90 $\pm$ 0.00 e
S9	4.30 $\pm$ 0.01 bc	9.10 $\pm$ 0.10 f
S10	3.57 $\pm$ 0.07 f	13.10 $\pm$ 0.30 b
Statistical significance	***	***

Results indicate mean values  $\pm$  S.D. of four determinations (carried out in duplicate for two independent sampling). Data within a column followed by the same letter are not significantly different according to Tukey's test. p value: \*\*\*p < 0.001. Abbreviations: S, sourdough; 1–10, home 1-home 10; TTA, total titratable acidity.

**Table 3**  
Microbiological characteristics of sourdoughs.

Samples	Microbial loads					
	PCA	mMRS	M17	SDB	SFM	YPD
S1	7.77 ± 0.21 cde	7.87 ± 0.19 ab	6.19 ± 0.27 b	6.74 ± 0.25 c	6.34 ± 0.27 d	4.80 ± 0.13 de
S2	7.34 ± 0.15 e	5.51 ± 0.28 d	6.01 ± 0.19 b	7.13 ± 0.24 c	7.05 ± 0.19 d	3.93 ± 0.17 f
S3	8.46 ± 0.20 ab	8.20 ± 0.12 ab	4.27 ± 0.22 e	8.02 ± 0.17 b	7.21 ± 0.13 c	6.11 ± 0.18 a
S4	8.05 ± 0.10 bcd	6.85 ± 0.13 d	6.03 ± 0.15 b	7.96 ± 0.24 b	7.10 ± 0.30 c	4.99 ± 0.14 cd
S5	8.96 ± 0.27 a	7.59 ± 0.31 bc	5.55 ± 0.18 bcd	9.05 ± 0.22 a	8.19 ± 0.15 ab	5.34 ± 0.16 bc
S6	8.09 ± 0.18 bc	6.77 ± 0.18 d	5.10 ± 0.30 d	8.27 ± 0.19 b	8.02 ± 0.11 c	4.22 ± 0.13 f
S7	7.99 ± 0.21 bcd	6.54 ± 0.27 d	4.92 ± 0.20 de	8.19 ± 0.25 b	7.35 ± 0.20 c	4.44 ± 0.29 ef
S8	8.33 ± 0.20 bc	8.52 ± 0.25 a	7.61 ± 0.10 a	8.39 ± 0.19 b	7.33 ± 0.21 bc	6.01 ± 0.17 a
S9	7.49 ± 0.17 de	7.07 ± 0.33 cd	5.34 ± 0.23 cd	7.19 ± 0.21 c	6.90 ± 0.10 d	4.88 ± 0.19 cde
S10	8.96 ± 0.23 a	7.90 ± 0.21 ab	5.97 ± 0.33 bc	9.28 ± 0.14 a	8.00 ± 0.15 a	5.72 ± 0.16 ab
Statistical significance	***	***	***	***	***	***

Units are log cfu/g. Results indicate mean values ± SD of two determinations. Results indicate mean values ± S.D. of six plate counts (carried out in duplicates for two independent sampling). Data within a column followed by the same letter are not significantly different according to Tukey's test. p value: \*\*\*p < 0.001. Abbreviations: S, sourdough; 1–10, home 1-home 10; PCA, plate count agar for total mesophilic count; mMRS, De Man-Rogosa-Sharpe modified agar for mesophilic rod LAB; M17, medium 17 agar for mesophilic coccus LAB; SDB, sourdough bacteria agar for sourdough LAB; SFM, San Francisco medium for sourdough LAB; YPD, yeast peptone dextrose agar for yeasts.

*buchneri*, *Fructilactobacillus sanfranciscensis* and *Weissella cibaria*, since *Lvb. brevis* was included in groups I and II. The facultative hetero-fermentative LAB (group VII) were *Lactiplantibacillus plantarum*, while the obligate homofermentative ones were identified as *Lacticaseibacillus paracasei*. Despite the phenotypic characterization, only group X included enterococci, particularly the species *Enterococcus gilvus*. The other cocci were identified as *Lactococcus lactis*, *Leuconostoc citreum*, *Leuconostoc holzapfelii* and *Pediococcus pentosaceus*.

3.3. Screening of LAB for growth with polyphenols

The 20 LAB strains were tested for their ability to grow in the presence of 16 commercial polyphenols that characterize the peel of almonds (Table 5). Only eleven strains (*E. gilvus* SD135, *F. sanfranciscensis* SD22, *Lc. lactis* SD128, *Lpb. pentosus* SD130, *Lpb. plantarum* SD96, *Lnb. diolivorans* SD4, *Ln. citreum* SD142, *Ln. holzapfelii* SD148, *Lvb. brevis* SD46, *P. pentosaceus* SD144 and *W. cibaria* SD123) showed the ability to grow in SSE added with a final concentration of 0.5 mg/mL of each polyphenol.

3.4. LAB and fermentation properties

The acidification activity of the LAB strains resistant to the main almond skin polyphenols is graphically reported in Fig. 2. The eleven strains showed a different SSE acidification kinetics. Considering the first 8 h of fermentation, only eight strains (*F. sanfranciscensis* SD22, *Lpb. pentosus* SD130, *Lpb. plantarum* SD96, *Lnb. diolivorans* SD4, *Ln. citreum* SD142, *Ln. holzapfelii* SD148, *Lvb. brevis* SD46 and *W. cibaria* SD123) showed an interesting pH decrease at around 4.5 or lower. After 24 h of fermentation, except *Lvb. brevis* SD52 and *Lcb. paracasei* SD 161, all LAB dropped SSE pH below 5.0. Among the 11 LAB tested, nine strains showed an inhibitory halo around the indicator bacteria applying the ASDM (results not shown), but only five strains inhibited at least one of these sensitive strains after the test performed via WDA (Fig. 3). Barely one LAB, *P. pentosaceus* SD144, inhibited all three indicator strains. The highest inhibitory activity against *L. monocytogenes*, was registered for *W. cibaria* SD123 and *P. pentosaceus* SD144 with diameters of the clear area around the wells of 21.7 and 19.2 mm, respectively.

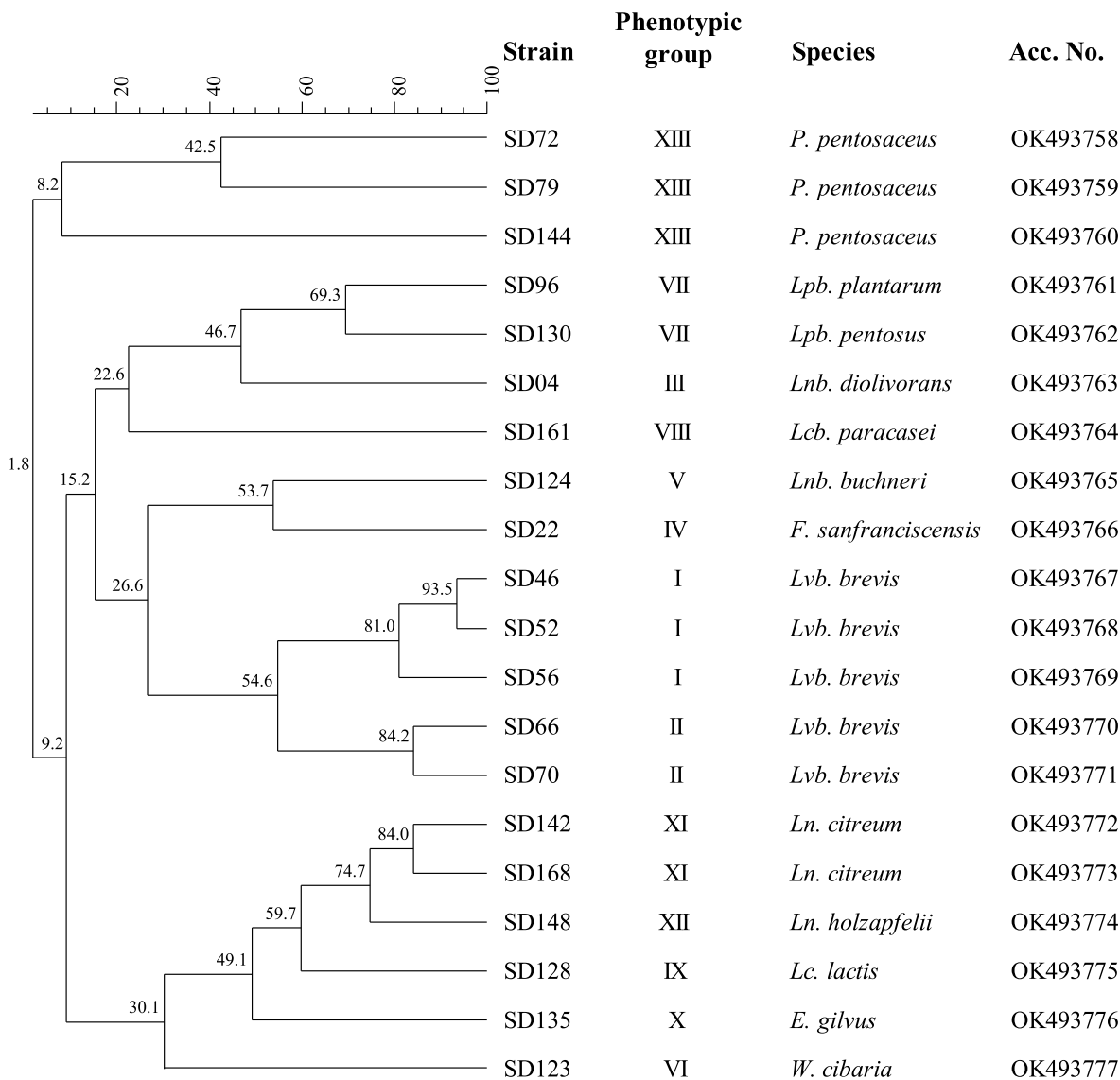
4. Discussion

Recently, many bakery producers have become interested in using plant by-product antioxidants to enrich bread due to the increasing

**Table 4**  
Phenotypic grouping of lactic acid bacteria.

Characters	Clusters												
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Morphology <sup>a</sup>	R	R	R	R	R	R	R	R	C	C	C	C	C
Cell arrangement <sup>b</sup>	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	sc	t
Growth:													
15 °C	+	+	+	+	+	+	+	+	+	+	+	+	+
45 °C	–	–	–	–	–	+	–	–	–	+	+	–	+
pH 9.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	+	+	+	+
6.5% NaCl	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	+	+	–	+
Hydrolysis of:													
arginine	+	+	+	–	+	+	–	–	+	–	+	–	+
aesculin	+	–	–	–	+	+	+	–	+	+	+	–	+
Acid production from:													
arabinose	+	+	+	–	+	+	+	–	–	–	+	+	+
ribose	+	+	+	+	+	–	+	–	+	+	–	–	+
xylose	+	+	–	–	–	+	+	–	–	–	–	–	+
fructose	+	+	+	–	+	+	+	–	+	+	+	+	+
galactose	+	–	–	+	+	–	+	–	+	+	–	+	+
sucrose	+	+	–	+	–	+	+	–	+	+	+	–	–
CO <sub>2</sub> from glucose	+	+	+	+	+	+	–	–	–	–	+	+	–

<sup>a</sup> R, rod; C, coccus.  
<sup>b</sup> sc, short chain; t, tetrads. Abbreviation: n.d., not determined.



**Fig. 1.** Dendrogram obtained from combined RAPD-PCR patterns generated with three primers of the lactic acid bacteria strains identified. Abbreviations: *E.*, *Enterococcus*; *F.*, *Fructilactobacillus*; *Lcb.*, *Lactocaseibacillus*; *Lpb.*, *Lactiplantibacillus*; *Lc.*, *Lactococcus*; *Lnb.*, *Lentilactobacillus*; *Ln.*, *Leuconostoc*; *Lvb.*, *Levilactobacillus*; *P.*, *Pediococcus*; *W.*, *Weissella*.

consumer demand for functional foods [28]. In southern Italy, particularly in Sicily and Apulia, semolina fermented by sourdough technology is used to make the majority of traditional and typical breads [23,29]. Sourdoughs are an important source of starters LAB biodiversity [30]. Although the inhibitory action of polyphenols on the growth of dairy LAB has been investigated [31], little is known about the effect of the main almond by-product polyphenols on the sourdough LAB. Therefore, this study evaluated the biodiversity and the technological potential of LAB from 10 home-made sourdoughs collected in western Sicily that are resistant to the main almond skin polyphenols. The aim is to develop *ad hoc* starter cultures for applications in novel fermented functional bakery products. All sourdoughs were evaluated for their general characteristics by the measuring pH, TTA and plate counts.

The samples analyzed in this study showed pH and TTA values similar to those registered for semolina sourdoughs produced in Sicily at both artisanal and industrial level [18,25,32]. The levels of TMM were comparable to those developed on SDB, a medium used to estimate specifically sourdough LAB [12], indicating the ability of these bacteria to dominate the microbial community of the home-made semolina Sicilian sourdough samples. Lower levels of culturable LAB were

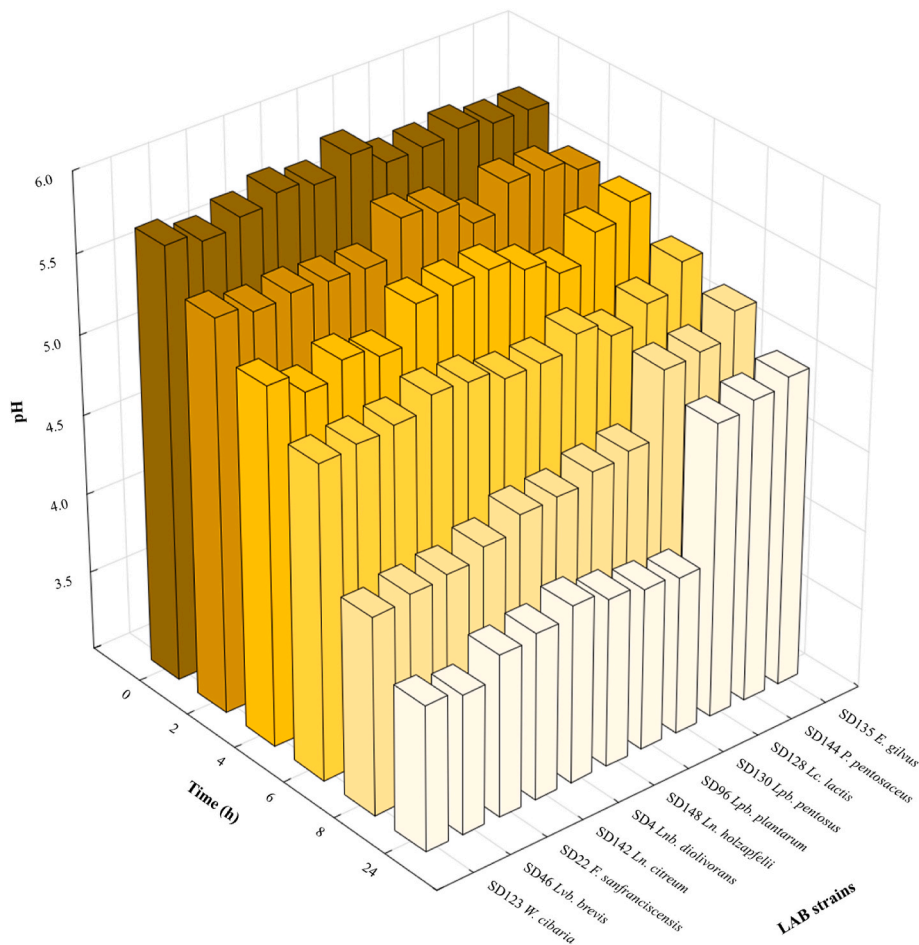
detected on M17, a generic medium generally used to monitor coccus LAB [25]. This finding is not surprising because LAB cocci are found in raw materials [33,34] and play their major role during the first steps of sourdough development [35], but they are found at high levels in mature sourdoughs [36]. However, their importance along with rod LAB has been highlighted by several authors [37,38]. Yeasts are also important in sourdough ecosystems [39], not only for the leavening performances [40], but also for their contribution to the aromatic properties of sourdoughs [41]. In this work, yeasts were found in all sample at cell densities ranging between  $10^4$  and  $10^6$  cfu/g. These levels are comparable to those found in other Sicilian sourdoughs made from flour and semolina [18]. Only three sourdoughs (S3, S8 and S9) had a yeast/LAB ratio of 1:100, which represents the optimal value for type I sourdoughs with good characteristics [42–44]. The other samples were characterized by a yeast/LAB ratio of 1:1000, indicating a great dominance of LAB. The presumptive LAB were isolated from the home-made sourdoughs and subjected to phenotypic grouping. Thirteen phenotypic groups were revealed, eight of which were rod. This result is not surprising since typical LAB associated with sourdoughs are rods [45]. Six out of the eight groups for rods showed an obligate heterofermentative



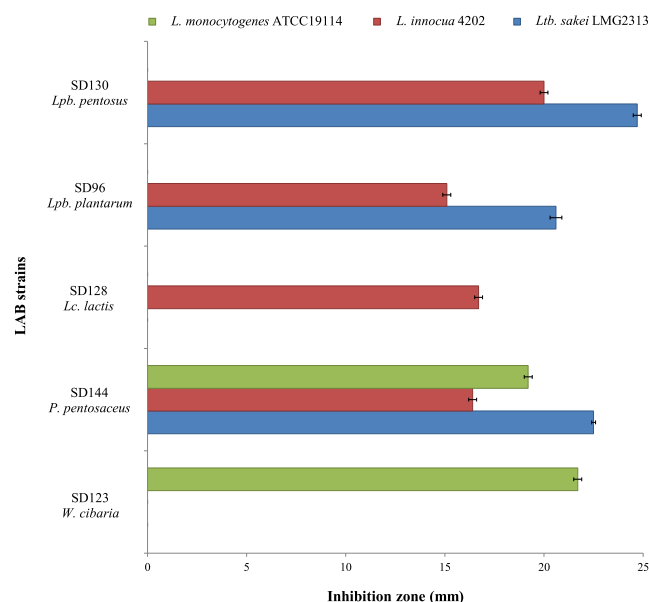
**Table 5**  
Ability of home-made sourdough lactic acid bacteria strains to grow in presence of commercial polyphenols.

Strains	Species	Polyphenols <sup>a</sup>															
		C	EC	E	E7Glu	HyS	I	I3Glu	I3R	k	K3Glu	K3R	M	N	N7Glu	Q	R
SD135	<i>E. gilvus</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD22	<i>F. sanfranciscensis</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD161	<i>Lcb. paracasei</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD130	<i>Lpb. pentosus</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD96	<i>Lpb. plantarum</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD128	<i>Lc. lactis</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD124	<i>Lnb. buchneri</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD4	<i>Lnb. diolivorans</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD142	<i>Ln. citreum</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD168	<i>Ln. citreum</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD148	<i>Ln. holzapfelii</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD46	<i>Lvb. brevis</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD52	<i>Lvb. brevis</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD56	<i>Lvb. brevis</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD66	<i>Lvb. brevis</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD70	<i>Lvb. brevis</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD72	<i>P. pentosaceus</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD79	<i>P. pentosaceus</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD144	<i>P. pentosaceus</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SD123	<i>W. cibaria</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

<sup>a</sup> C, catechin; EC, epicatechin; E, eriodictyol; Er7Glu, eriodictyol-7-O-glucoside; HyS, hyperoside; I, isorhamnetin; I3Glu, isorhamnetin-3-O-glucoside; I3R, isorhamnetin-3-O-rutinoside; K, kaempferol; K3Glu, kaempferol-3-O-glucoside; K3R, kaempferol-3-O-rutinoside; M, morin; N, naringenin; N7Glu, naringenin-7-O-glucoside; Q, quercetin; R, rutin. Abbreviations: *E.*, *Enterococcus*; *F.*, *Fructilactobacillus*; *Lc.*, *Lactococcus*; *Lcb.*, *Lacticaseibacillus*; *Lpb.*, *Lactiplantibacillus*; *Lnb.*, *Lentilactobacillus*; *Ln.*, *Leuconostoc*; *Lvb.*, *Levilactobacillus*. Symbols: black square indicate the ability of strains to grow in presence of each almond peel polyphenol.



**Fig. 2.** Drop of pH of sterile semolina extract inoculated with lactic acid bacteria resistant to almond peel polyphenols. Histograms are in the order of decreasing pH at 8 h. Abbreviations: *E.*, *Enterococcus*; *F.*, *Fructilactobacillus*; *Lpb.*, *Lactiplantibacillus*; *Lc.*, *Lactococcus*; *Lnb.*, *Lentilactobacillus*; *Ln.*, *Leuconostoc*; *Lvb.*, *Levilactobacillus*; *P.*, *Pediococcus*; *W.*, *Weissella*.



**Fig. 3.** Inhibitory activity of lactic acid bacteria resistant to almond peel polyphenols against *Listeria innocua*, *Listeria monocytogenes* and *Latilactobacillus sakei*. Abbreviations: *L.*, *Listeria*; *Lpb.*, *Lactiplantibacillus*; *Lc.*, *Lactococcus*; *Ltb.*, *Latilactobacillus*; *P.*, *Pediococcus*; *W.*, *Weissella*.

metabolism, confirming the presence of a constitutive phosphoketolase [46,47]. RAPD analysis clearly showed the presence of 20 different strains, which were subjected to 16S rRNA gene sequencing resulting in the identification of 13 LAB species belonging to ten genera (*Enterococcus*, *Fructilactobacillus*, *Lactocaseibacillus*, *Lactococcus*, *Lactiplantibacillus*, *Lentilactobacillus*, *Leuconostoc*, *Levilactobacillus*, *Pediococcus* and *Weissella*). Among rod-shaped LAB, the species *Lvb. brevis*, *Lpb. plantarum* and *F. sanfranciscensis* are the most characteristic of type I sourdoughs [48] and are commonly found in sourdoughs produced in Sicily [18,49]. *Weissella cibaria* is a LAB typically associated with sourdoughs of Sicily [18], easily associated with raw materials (tender flour and durum semolina) obtained from wheat varieties cultivated in this southern Italian region and used in bread making [25,33,34]. Regarding cocci LAB, *Lc. lactis* was identified, generally detected in unprocessed semolinas [33] rather than in mature sourdoughs. *Enterococcus gilvus*, *Ln. citreum*, *Ln. holzapfelii* and *P. pentosaceus* were also identified. Except for *Ln. holzapfelii*, which was first isolated during coffee fermentation [50], the other three species (*E. gilvus*, *Ln. citreum* and *P. pentosaceus*) are easily found in sourdoughs [51,52]. Furthermore, *Ln. citreum* and *W. cibaria* showed very interesting properties during sourdough propagation [53] and are being applied at industrial level without lactobacilli to produce the typical Sicilian “pagnotta di Piana degli Albanesi” bread [32].

To select LAB to be used as starter strains in sourdough processed with almond peel powder to obtain functional breads, all strains were grown in the presence of the main polyphenols characterizing the peel of almonds. Among the 20 LAB isolated from home-made sourdoughs, eleven strains were resistant to all polyphenols tested. These strains were evaluated for their technological properties useful in sourdough productions. To this purpose, acidification and production of antimicrobial substances were evaluated because starter LAB must perform rapid acidification [54] and contribute to prolonging the shelf-life of sourdoughs [55] in future applications in industrial sourdough fermentations. Eight strains showed a rapid drop of pH, while two strains were also bacteriocin-like inhibitory substances (BLIS) producer against *L. monocytogenes*, indicating a potential application in biopreservation.

## 5. Conclusions

Traditional fermented foods are a valuable source of microbial diversity that can be used to isolate strains with technological performances to develop starter cultures for industrial use. However, home-made foods have a limited diffusion and are not routinely explored for starter strain selection. In this study, LAB diversity was investigated in 10 semolina sourdoughs provided by private producers with the aims of developing an *ad hoc* starter culture to be used in sourdough processed with almond peel powder to obtain functional breads. The levels of the two main communities active during fermentation were almost comparable to those revealed in sourdoughs propagated in traditional bakeries and industrial facilities, but the expected high biodiversity was not confirmed. Only 13 LAB species were found to dominate the sourdoughs investigated. However, all LAB species identified are typically (*F. sanfranciscensis*, *Lpb. plantarum*, *Lvb. brevis*) or frequently (*P. pentosaceus*, *Ln. citreum* and *W. cibaria*) isolated from sourdough ecosystems. Eleven strains showed the ability to grow in the presence of almond peel polyphenols, and eight of them (*F. sanfranciscensis* SD22, *Lpb. pentosus* SD130, *Lpb. plantarum* SD96, *Lnb. diolivorans* SD4, *Ln. citreum* SD142, *Ln. holzapfelii* SD148, *Lvb. brevis* SD46 and *W. cibaria* SD123) also showed pH drop kinetics compatible with industrial production. Studies are underway to better evaluate the role of these eight strains during the sourdough-based baked goods productions enriched with almond skin powder.

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## CRedit authorship contribution statement

**Enrico Viola:** Investigation, Formal analysis. **Giuliana Garofalo:** Investigation, Formal analysis. **Gabriele Busetta:** Investigation, Formal analysis. **Maria Supper:** Investigation, Formal analysis. **Antonio Alfonso:** Software, Investigation, Formal analysis. **Marco Tolone:** Software, Investigation, Formal analysis. **Nicola Francesca:** Resources, Project administration. **Giancarlo Moschetti:** Validation. **Francesco Sottile:** Resources, Project administration, Funding acquisition. **Raimondo Gaglio:** Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation, Conceptualization. **Luca Settanni:** Writing – review & editing, Writing – original draft, Validation, Methodology, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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