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Selection criteria of lactic acid bacteria to be used as starter for sweet and salty leavened baked products

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ABSTRACT

One hundred lactic acid bacteria from Italian sourdoughs were characterized for technological features and selected for their tolerance to stress conditions commonly encountered within the production of leavened fermented bakery products. Specifically, lactic acid bacteria belonging to the family Lactobacillaceae were evaluated for the capability to withstand acidic conditions (pH 3.5, 4.0, 4.5, 5.5, 6.5) and to cope with different concentrations of NaCl (2, 3, 4, 5, 6, 8%), ethanol (2, 4, and 6%) and sucrose (20 and 30%) during 48 h fermentation. Strains were also tested for urease, amylase, proteolytic activities and for the capability to produce exopolysaccharides.

The strains had a wide diversity in stress response pattern. *Fructilactobacillus sanfranciscensis* PE4, *Furfurilactobacillus rossiae* PS48, *Levilactobacillus brevis* PA6 and two strains of *Leuconostoc pseudomesentereoides* (PW2 and PD4) showed the highest survival to stress treatments and interesting technological properties (i.e. amino acid and exopolysaccharides production). Several strains exhibited high robustness also to strongest stress conditions, suggesting their potential use for applications in bakery industry.

This study confirms the diversity of lactic acid bacteria to stress treatments and proposes suitable criteria for selection of competitive strains to be used in versatile way for production of different salty and sweetened leavened bakery products.

1. Introduction

Sourdough-based fermentation positively affects the quality, tasty and healthy aspects (Siepmann, Ripari, Waszczynskyj, & Spier, 2017) of many salty and sweet leavened bakery goods, including traditional typical breads (Minervini et al., 2012; Reale, Di Renzo, Boscaino, et al., 2019), crackers (Chavan & Chavan, 2011), pizza (Pepe, Villani, Oliviero, Greco, & Coppola, 2003) and regional and artisan sweet cakes as "Panettone", "Pandoro" and "Colomba" (Lattanzi et al., 2013).

To date, the dough fermentation can be started by a) "spontaneous fermentation" due to the indigenous microbiota occurring in the raw materials and in the processing environment (Yu, Wang, Qian, Zhang, & Qi, 2018); b) "backslopping" method, i.e. by adding "mature mother dough" of the previous fermentation to start the fermentation (Harth, Van Kerrebroeck, & De Vuyst, 2016); c) liquid, dried or lyophilized sourdoughs obtained from commercial suppliers (De Vuyst & Neysens, 2005; Reale, Di Renzo, Preziuso, et al., 2019); d) starter cultures that include one or more selected strains of lactic acid bacteria (LAB) and/or yeasts (Dimitrellou, Kandylis, Kourkoutas, Koutinas, & Kanellaki, 2009). Nowadays, the industry offers several commercial formulations of baker's yeasts and LAB, whose choice depends on the type of dough and production technology (Leroy & De Vuyst, 2004; Reale et al., 2013).

The first three methods are undoubtedly reliable to obtain tasty and flavor products, but they require time and skilled bakers to properly propagate the mother dough and reach a stable and effective microbial community, useful to ensure the quality of final products.

For these reasons, the use of selected starter is growing interest in the bakery sector. Commercial cultures, however, may have several limitations because of different variables to consider during microbial selection step.

Often, the selection of strains to be used in industrial-scale processes is limited to a small number of technological parameters, such as rapid growth and acidification in dough, and other important criteria (e.g. tolerance to niche-associated stress, sensory and nutritional features) are overlooked.

The adaptation to the dough environment may be useful to ensure

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Table 1
Genetic identification and characterization of lactic acid bacteria and preliminary screening on the basis of acidification and growth capability.

Sourdough	Total number isolates	Identification by 16 S rRNA sequencing (Species and n. of identified)	Biotyping by RAPD-PCR° (n. of selected strains)	Selection on the basis of high acidification * (n. of strains)	Selection on the basis of high growth § (n. of strains)	Selected strains
PA	8	*Levl. brevis (4)*	*Levl. brevis (4)*	2	1	*Levl. brevis* PA6
		Liml. fermentum (4)	*Liml. fermentum (2)*	1	–	
PB	7	*Coml. paralimentarius (3)*	*Coml. paralimentarius (3)*	3	1	*Coml. paralimentarius* PB6
		Levl. brevis (4)	*Levl.brevis (2)*	–	–	
PD	6	*Leuc. pseudomesenteroides (3)*	*Leuc. pseudomesenteroides (3)*	2	1	*Leuc. pseudomesenteroides* PD4
		not identified *(3)*	—	–	–	
PE	7	*Lacp. plantarum (3)*	*Lacp. plantarum (3)*	3	1	*Lacp. plantarum* PE7 *Fl. sanfranciscensis* PE4
		Fl. sanfranciscensis (4)	*Fl. sanfranciscensis (3)*	2	1	
PF	5	*Lacp. plantarum (5)*	*Lacp. plantarum (4)*	2	1	*Lacp. plantarum* PF1
PG	6	*Coml. paralimentarius (4)*	*Coml. paralimentarius (2)*	1	1	*Coml. paralimentarius* PG4
		not identified *(2)*	—	–	–	
PI	7	*Lacp. plantarum (4)*	*Lacp. plantarum (4)*	3	1	*Lacp. plantarum* PI1
		Furl. rossiae (3)	*Furl. rossiae (2)*	–	–	
PN	8	*Lacp. paraplantarum (4)*	*Lacp. paraplantarum (4)*	3	1	*Lacp. paraplantarum* PN2
		Fl. sanfranciscensis (4)	*Fl. sanfranciscensis (1)*	–	–	
PSB	16	*Leuc. mesenteroides (2)*	*Leuc. mesenteroides (2)*	2	1	*Leuc. mesenteroides* PSB66 *Furl. rossiae* PSB30, 34, 48, 60, 62, 64 Fl. *sanfranciscensis* PSB51, 52, 53, 55, 57
		Furl. rossiae (8)	*Furl. rossiae (7)*	7	6	
		Fl. sanfranciscensis (6)	*Fl. sanfranciscensis (5)*	5	5	
PT	8	*Lacp. plantarum (5)*	*Lacp. plantarum (4)*	1	1	*Lacp. plantarum* PT4
		Furl. rossiae (2)	*Furl. rossiae* (2)	1	–	
		not identified (1)	–	–	–	
PW	10	*Leuc. pseudomesenteroides* (6)	*Leuc. pseudomesenteroides* (5)	2	1	*Leuc. pseudomesentaroides* PW2
		Lacp. plantarum (2)	*Lacp. plantarum* (2)	1	–	
		not identified (2)	–	–	–	
PX	7	*Coml. paralimentarius* (6)	*Coml. paralimentarius* (4)	2	1	*Coml. paralimentarius* PX1
		Lacp. paraplantarum (1)	*Lacp. paraplantarum* (1)	1	–	
PZ	5	*Lacp. paraplantarum* (4)	*Lacp. paraplantarum* (3)	2	1	*Lacp. paraplantarum* PZ2
		Lacc. casei (1)	*Lacc. casei* (1)	–	–	
	100	92 identified	73 deduplicated strains	46	25	25

° Strains with the same RAPD-profile (clones) were discarded.
* The strains were selected for the ability to acidify MRS broth reaching pH < 4.5 after 24 h.
§ Strains with a ΔOD595nm > 0.6 in the first 8 h of incubation, were selected for further evaluation.

the fitness of starter cultures; the inability to survive and to be metabolically active during prolonged sourdough propagations may result in fermentation failure and may impaired the quality of baked products. The study of stress response diversity in LAB may be of practical relevance for the selection of strains and formulation of more competitive starter.

So, the aim of this study was the selection of robust LAB strains to be used as starter cultures in the production of leavened bakery products. For this purpose, (i) one-hundred presumptive LAB were isolated from Italian sourdoughs, identified and characterized by genetic approach; (ii) strains were screened firstly on the basis of growth and acidification rate and then for the ability to cope with the main stresses encountered during sourdough fermentation, such as low pH and high concentrations of salt, sucrose and ethanol; (iii) strains were characterized also for urease, amylase and proteolytic activities and for the capability to produce exopolysaccharides (EPS).

2. Material and methods

2.1. Bacterial strains

One-hundred lactic acid bacteria (LAB) isolated from traditional Italian sourdoughs (Campania region) were subjected to genetic identification by 16 S rRNA gene sequencing and biotyped by RAPD-PCR analysis as described by Reale et al. (2011). All the strains were maintained as frozen (−80 °C) stocks in reconstituted 11% (w/v) Skim Milk (Oxoid, Milan, Italy) containing 0.1% (w/v) ascorbic acid (RSM) in the Culture Collection of the Institute of Food Science, National Research Council, Avellino, Italy.

2.2. Screening for growth performances and acidifying capability

Identified and deduplicated strains (73 LAB) were preliminary screened on the basis of growth performances and acidifying capability.

In detail, LAB were cultivated in MRS broth pH 6.8 (MRS, Oxoid, Milan, Italy) for 16 h at 28 °C. Pre-cultures were harvested by centrifugation (10,000×*g* for 10 min), washed twice in sterile NaCl 0.85% (w/v) and inoculated in MRS broth at final concentration of 1×10^6 cfu/mL. Samples were incubated for 24 h at 28 °C, and optical density at 595 nm (OD_{595nm}) and pH values were measured during growth at 2-h intervals for the first 10 h, and then after 24 h of cultivation. Strains with a $\Delta OD_{595nm} > 0.6$ in the first 8 h of incubation, were selected for further evaluation. Values of ΔpH after 24 h were calculated to compare the acidifying capability of the strains. All experiments were done in triplicate and average values were taken.

2.3. Screening for tolerance to acid, salt, sucrose and ethanol stresses

Strains (see Table 1) with the best growth and acidification features were selected and tested for their capability to cope with the main stresses encountered during the production of salty and sweet leavened bakery products. For the growth trials, LAB were cultivated in MRS broth pH 6.8 for 16 h at 28 °C, harvested by centrifugation (13,000×*g* for 10 min), washed twice in 20 mmol/L Na-phosphate buffer pH 7.0 (PB7), standardized at $OD_{595nm} = 0.6$ and inoculated (10% v/v) in 180 μL of MRS broth (96-well microplate experiment), properly modified to reach the following stress conditions: a) acid treatment, in MRS broth at pH 3.5, 4.0, 4.5, 5.5 and 6.5; b) sodium chloride treatment, in MRS broth pH 6.8 with 2, 3, 4, 5 and 6% (w/v) NaCl; c) sucrose treatment, in MRS broth pH 6.8 with 20 and 30% (w/v) sucrose; d) ethanol treatment, in

Table 2
EPS production, amylasic and ureasic activities of the twenty-five lactic acid bacteria.

Strain	Label	EPS production from			Amylasic activity		Ureasic activity
		glucose°	maltose°	sucrose°	zone of clearance *	enzyme activity (μkatal/mL) §	
Levl. brevis PA6	Lbr_PA6	–	+/–	–	+	3.75 ± 0.09	–
Coml. paralimentarius PB6	Cpar_PB6	–	–	–	+	5.81 ± 0.09	–
Leuc. pseudomesenteroides PD4	Leucpm_PD4	–	–	dextran	+	2.94 ± 0.23	–
Lacp. plantarum PE7	Lpla_PE7	–	–	+	+	4.20 ± 0.05	–
Fl. sanfranciscensis PE4	Fsan_PE4	–	–	+	+	4.40 ± 0.04	–
*Lacp. plantarum*PF1	Lpla_PF1	–	–	+	+	3.25 ± 0.07	–
Coml. paralimentarius PG4	Cparalim_PG4	–	–	–	+	3.30 ± 0.02	–
Lacp. plantarum PI1	Lpla_PI1	+	+	+	+	3.85 ± 0.07	–
Lacp. paraplantarum PN2	Lparapl_PN2	–	–	–	–	nd	–
Leuc. mesenteroides PS66	Leucmes_PS66	–	–	–	–	nd	–
*Furl. rossiae*PS30	Fros_PS30	–	–	–	–	nd	–
Furl. rossiae PS34	Fros_PS34	–	–	–	+	4.10 ± 0.07	–
Furl. rossiae PS48	Fros_PS48	–	–	–	+	2.79 ± 0.05	–
Furl. rossiae PS60	Fros_PS60	+	+	–	+	2.99 ± 0.16	–
Furl. rossiae PS62	Fros_PS62	+	+	–	–	nd	–
Furl. rossiae PS64	Fros_PS64	–	–	–	–	nd	–
Fl. sanfranciscensis PS51	Fsan_PS51	–	–	–	–	nd	–
Fl. sanfranciscensis PS52	Fsan_PS52	–	–	–	+	5.16 ± 0.14	–
Fl. sanfranciscensis PS53	Fsan_PS53	–	–	–	–	nd	–
Fl. sanfranciscensis PS55	Fsan_PS55	–	–	–	+	2.54 ± 0.11	–
Fl. sanfranciscensis PS57	Fsan_PS57	–	–	–	+	2.84 ± 0.12	–
Fl. plantarum PT4	Lpla_PT4	–	–	+	+	2.54 ± 0.11	–
Leuc. pseudomesenteroides PW2	Leucpm_PW2	–	–	dextran	+	5.71 ± 0.14	–
Coml. paralimentarius PX1	Cparalim_PX1	–	–	–	+	3.50 ± 0.05	–
Lacp. paraplantarum PZ2	Lparapl_PZ2	–	+	–	+	3.60 ± 0.11	–

° symbols±mean production/non production of EPS; * symbols±mean the presence of the halo of clarification of the medium indicating potential amylase activity; § enzymatic activity was determined on the samples showed potential amylase activity; *nd* = not determined.

MRS broth pH 6.8 with 2, 4 and 6.0% (v/v) ethanol. Microplates were incubated for 48 h, at 28 °C in anaerobiosis (AnaeroGen bags, Oxoid). After 0 (control samples), 6, 24 and 48 h of incubation, OD_{595nm} was measured by using a microplate reader (Benchmark, BioRad). For all treatments, the results were expressed as ΔOD_{595nm} at 6, 24 and 48 h, i.e. as difference between OD_{595nm} at 6 or 24 or 48 h and OD_{595nm} at time zero. All tests were done in triplicate and average values were taken.

2.4. Screening for exopolysaccharides (EPS) production

EPS production was evaluated as described by Ruas-Madiedo and de los Reyes-Gavilàn (2005). Briefly, LAB were inoculated on modified MRS agar plate containing Ruthenium Red (0.08 g/L) and glucose 20 g/L (G-MRS) or maltose 20 g/L (M-MRS) or sucrose 50 g/L (S-MRS) as carbon source. After 48 h incubation at 28 °C, the ruthenium red stained the bacterial cell wall, producing pink colonies for non-ropy strains and white colonies for ropy strains. The tests were done in duplicate.

2.5. Screening for urease, amylase and proteolytic activities

Twenty-five selected strains (see Table 2) were tested for urease activity as reported in Zotta, Ricciardi, and Parente (2007). Amylase activity was evaluated as described in Padmavathi, Bhargavi, Priyanka, Niranjan, and Pavitra (2018). Briefly, the bacterial strains were inoculated in Petri dishes containing modified MRS media (0.5% peptone, 0.7% yeast extract, 0.2% NaCl, 2% starch, and 1.5% agar; % as w/v) supplemented with 0.25% (w/v) of starch. After incubation for 48 h at 28 °C, the clarification halo observed by adding Gram's iodine reagent, indicated potential amylase activity. The activity of α-amylase in strains that generated a clear zone on agar medium, was evaluated by an enzymatic assay as described by Tavea, Bert Fossi, Takop, and Ndjouenkeu (2016).

Proteolytic activity was evaluated by measuring the concentration of total free amino acids (trinitrobenzenesulfonic acid, TNBS method; Adler-Nissen, 1979) during fermentation in model doughs. The latter were prepared by mixing water and wheat flour to obtain dough yield (DY) of 160. Doughs were inoculated with 10^7 ufc/g of each strains and incubated for 24 h at 28 °C. Free amino acid content was measured after 6, 24 and 48 h of fermentation. A calibration curve was prepared using leucine (Leu, Sigma) as standard (range 0.0–1.0 mmol/L of Leu), and results were expressed as milligrams of Leu/kilogram of dough. The assays were performed in triplicate and average values were taken.

2.6. Statistical analyses

Statistical and graphic analyses were performed using SYSTAT 13.0 for Windows (Systat Software Inc., Richmond, CA, USA), while the Matrix Hierarchical Cluster Analysis (normalized data, Euclidean distance, Average linkage UPGMA method) was obtained with the PermutMatrix program v. 1.9.3 (LIRMM, France).

3. Results and discussion

3.1. Identification and preliminary screening

One-hundred presumptive lactic acid bacteria (LAB) were isolated from traditional sourdoughs to ensure the adaptation and survival to this ecological niche. Results of genetic identification were showed in Table 1. Ninety-two LAB belonged to several species of the family Lactobacillaceae, while for 8 isolates the identification was not conclusive. After that, the collection was de-duplicated by removing 19 isolates whose RAPD-PCR profiles were identical and, therefore, the remaining 73 strains were identified as *Levilactobacillus brevis* (6 strains), *Limosilactobacillus fermentum* (2), *Companilactobacillus paralimentarius* (9), *Lactiplantibacillus plantarum* (17), *Fructilactobacillus sanfranciscensis* (9), *Furfurilactobacillus rossiae* (11), *Lactiplantibacillus paraplantarum* (8), *Lacticaseibacillus casei* (1), recently re-classified by Zheng et al. (2020), *Leuc. mesenteroides* (2) and *Leuc. pseudomesenteroides* (8).

After identification and biotypization, the 73 strains were subjected to a preliminary characterization on the basis of acidification and growth capability during 24 h fermentation. Out of 73, twenty-five strains were shortlisted on the basis of the best acidifying properties (ability to acidify the MRS medium at pH < 4.5 after 24 h fermentation) and growth performances (OD_{595nm} > 0.600 in the first 8 h of

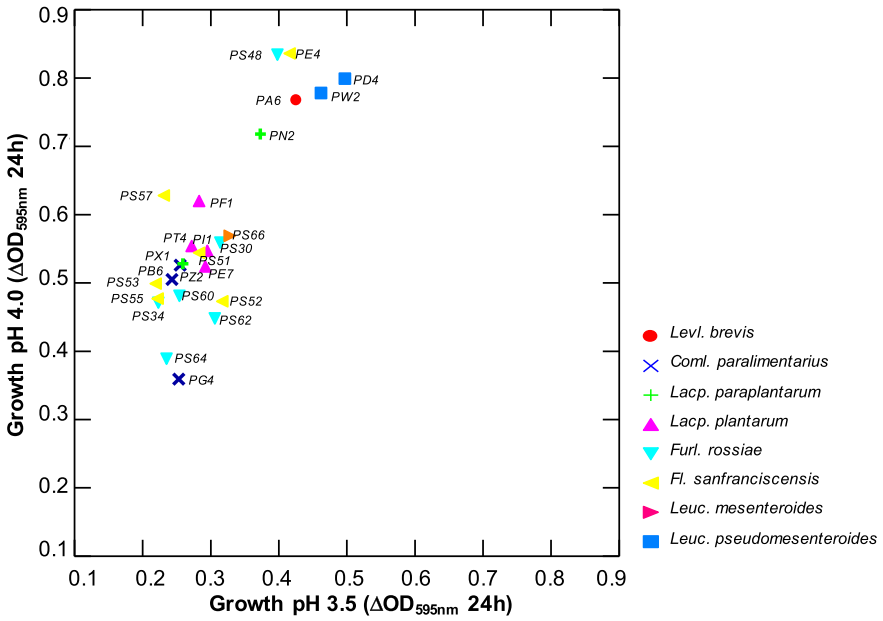

Fig. 1. Scatterplot showing the distribution of the 25 LAB strains on the basis of growth capability (ΔOD_{595nm}) at pH 3.5 and 4.0 after 24 h incubation.

incubation).

3.2. Screening for stress tolerance

The selected twenty-five strains were analyzed for the ability to grow after 6, 24 and 48 h at low pH and in presence of different concentrations of NaCl, sucrose and ethanol. For all stresses, the greatest differences in strain behavior were recorded after 24 and 48 h of incubation (Fig. S1-Suppl. material).

3.2.1. Effect of acid condition

The acidification is main consequence of LAB metabolism in sourdoughs. The pH of a mature sourdoughs ranges from 3.8 to 4.5, depending on several endogenous and exogenous factors (Catzeddu, 2019), and it could exert a selective pressure on the evolution of LAB microbiota. For this reason, the evaluation of acid stress response is crucial to select competitive starter.

Most of strains analyzed in this study were able to cope with low pH values, even if a large variability was found after 24 and 48 h of stress exposure (Figs. S1–A). As expected, the control sample (pH 6.5) had the highest growth levels at both 24 and 48 h.

The distribution of strains on the basis of ΔOD_{595nm} after 24 h at the more representative pH conditions of mature sourdoughs, i. e pH 3.5 and 4.0, were analyzed and reported in Fig. 1.

Two main groups were clearly evident: the first one included the more robust strains, the second comprised the majority of strains with an intermediate stress tolerance. *Leuconostoc pseudomesenteroides* PD4 and PW2 showed the highest survival to pH 3.5 conditions, while *Furl. rossiae* PS48 and *Fl. sanfranciscensis* PE4 had the highest robustness to pH 4.0. The strains *Furl. rossiae* PS64 and *Coml. paralimentarius* PG4, on the,

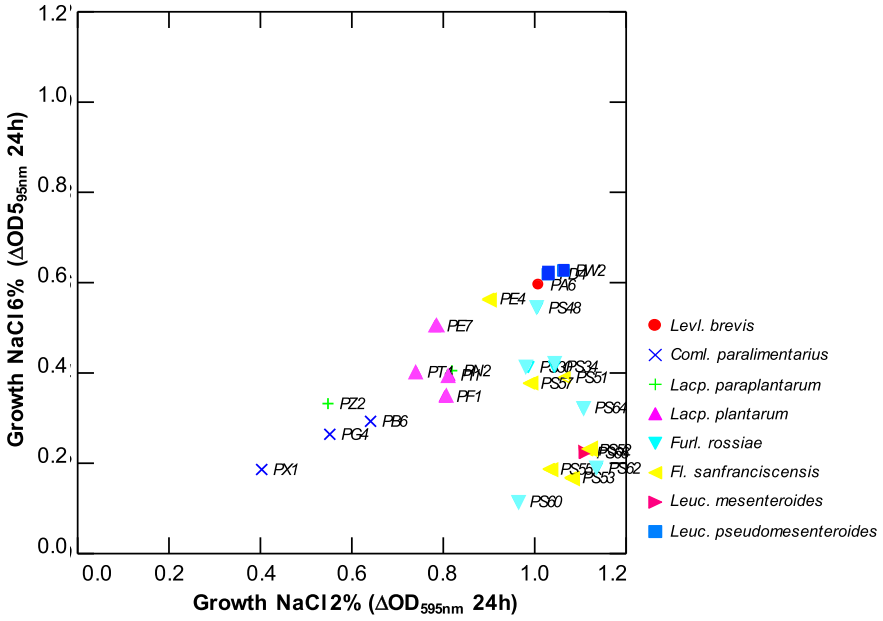

Fig. 2. Scatterplot showing the distribution of the 25 LAB strains on the basis of growth capability (ΔOD_{595nm}) in MRS supplemented with NaCl 2% (w/v) and 6% (w/v) after 24 h incubation.

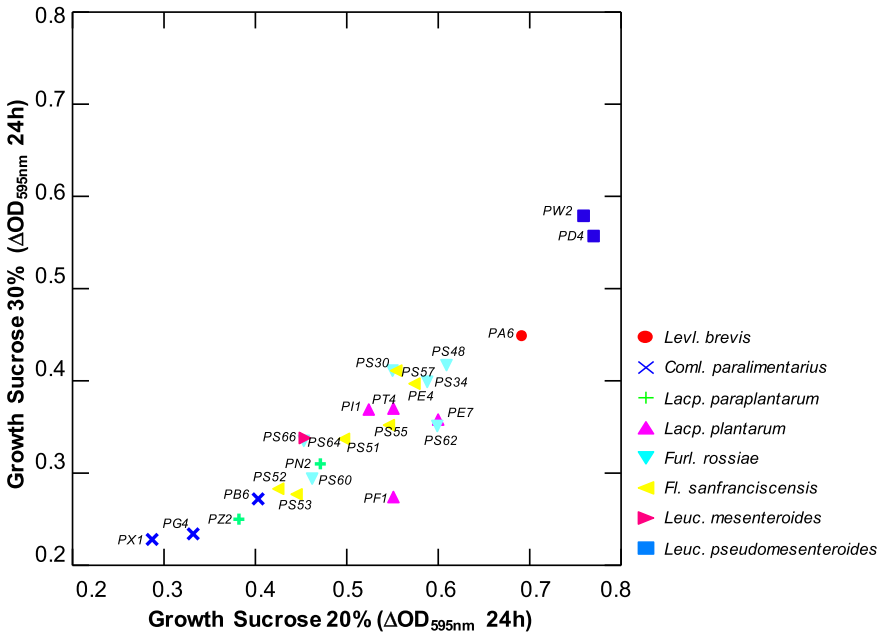

Fig. 3. Scatterplot showing the distribution of the 25 LAB strains on the basis of growth capability (ΔOD_{595nm}) in MRS supplemented with sucrose 20% (w/v) and 30% (w/v) after 24 h incubation.

contrary, were the most sensitive ones.

These results suggested that the above strains (PD4, PW2, PS64, PG4) may be successfully used for sourdough production for their ability to survive at low pH values, a condition that usually occurs in the last stage of fermentation process.

During sourdough fermentation, the acidification affects the activities of several microbial and flour-associated enzymes (Zotta, Piraino, Ricciardi, Mcsweeney, & Parente, 2006; Zotta et al., 2007). Drop in pH favors the inactivation of amylases, inhibiting the excessive starch degradation (Corsetti & Settanni, 2007), and improve quality of bread, especially when flour lacking gluten are used. Acidification induces proteolytic activities and modifies the hydration capability of gluten proteins, increasing volume, texture and flavor of bakery products (Di Renzo, Reale, Boscaino, & Messia, 2018; Spier, Rapacci, Dutcosky, & Tedrus, 2007). Moreover, microbial and indigenous phytases are activated at low pH values, reducing phytate content and increasing the nutritional values of products (Reale, Konietzny, Coppola, Sorrentino, & Greiner, 2007; Zotta et al., 2007). For this reason, the selection of LAB showing robustness to low pH (i.e. pH 4 and pH 3.5) is essential and has been recommended in this study.

3.2.2. *Effect of salt addition*

Salt is an ingredient that is almost always present in the formulation of bread and other bakery products. The use of salt in leavened baked goods generally refers to sodium chloride (Pagani, Bottega, & Mariotti, 2013). The amount of salt added in the dough may strongly affect the activities of lactic acid bacteria and for this reason the evaluation of osmotic stress response in selection step is necessary.

The ability of 25 LAB strains to grow at different salt concentrations and incubation time was shown in Figs. S1–B. As for acid condition, a large variability was found after 24 and 48 h, whereas after 6 h incubation the differences were less evident. Growth behavior at 2%, 3% and 4% NaCl was similar at both 24 and 48 h of incubation (median value of ΔOD_{595nm} was about 1, 0.9 and 0.8, respectively). Salt concentration >5% significantly impaired the strain survival (median ΔOD_{595nm} value < 0.6).

The distribution of strains on the basis of growth capability after 24 h incubation at 2% NaCl (percentage usually used in bakery products such as bread and pizza) and at 6% NaCl (percentage of NaCl used to discriminate the highest tolerant strain) concentrations was showed in Fig. 2.

Most of strains showed a good correlation among ΔOD_{595nm} values at 2% and 6% NaCl; other strains, on the contrary, exhibited a good growth ($\Delta OD_{595nm} > 1.0$) only at the lowest salt concentration (2% NaCl). At 6% NaCl, the highest tolerant strains were *Fl. sanfranciscensis* PE4, *Levl. brevis* PA6, *Furl. rossiae* PS48 and the two strains of *Leuconostoc pseudomesenteroides*, while the more sensitive were *Lacp. paraplantarum* PZ2 and some *Lacp. plantarum, Furl. rossiae* and *Coml. paralimentarius* strains (Fig. 2).

Similar results were obtained by Reale et al. (2015) that highlighted that all 184 LAB strains studied were able to grow in presence of 2% NaCl and exhibit a low growth in presence of 6% NaCl ($0.2 < \Delta OD_{595nm} < 0.6$) after 24 h incubation.

D'Angelo et al. (2017) found that different strains belonging to the genus *Leuconostoc* spp. revealed a good resistance to technological stresses such as acidic (pH 4.0), alkaline (pH 9.8) and osmotic (NaCl 4%) conditions. Other LAB identified as *Limosilactobacillus reuteri* (formerly known as *Lactobacillus reuteri)*, *Pediococcus acidilactici* and *Enterococcus faecium* showed a high tolerance to 6.5% NaCl concentration (Reuben, Roy, Sarkar, Alam, & Jahid, 2019).

These studies suggest that a wide intraspecies variability exists among LAB in response to NaCl stress, and that the selection of strains to be used as starter culture in low, moderate or high salt containing food preparation needs of appropriate trials. Although most of sourdoughs are usually prepared without or very low salt concentration, in many processes amounts of 2–5% NaCl are added during sourdough production (Gänzle, Ehmann, & Hammes, 1998; Spicher & Stephan, 1993).

3.2.3. *Effect of sucrose addition*

Sucrose is one of the main ingredients in sweet breads and leavened cakes, and its addition significantly affects energy production and microbial metabolism during dough fermentation (Nagodawithana & Trivedi, 1990). Sugar content depend on product recipe, and some sweet doughs may contain up to 30% generating an osmotic stress that may impair the leavening capability and enzymatic activities of yeasts and LAB (Struyf et al., 2017; Zhang et al., 2016).

In this study, the sucrose tolerance of 25 LAB strains were investigated. The response to different sucrose concentrations is shown in

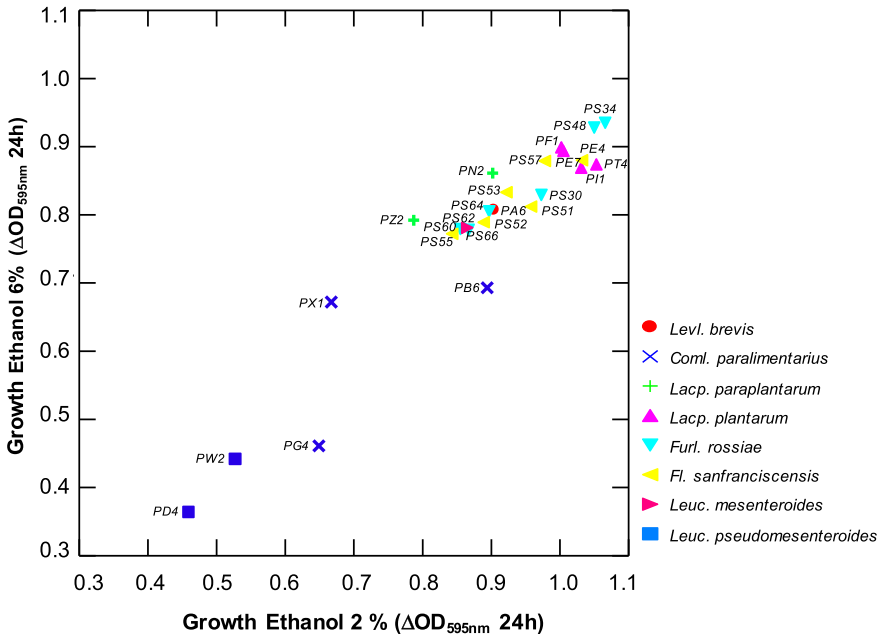

Fig. 4. Scatterplot showing the distribution of the 25 LAB strains on the basis of growth capability (ΔOD_{595nm}) in MRS supplemented with ethanol 2% (v/v) and 6% (v/v) after 24 h fermentation.

Fig. 1S–C. Highest variability was found at 24 and 48 h of incubation. Strains exhibited moderate ability to grow in 20% sucrose (median value of $\Delta OD_{595nm} \cong 0.6$), but survival was significantly impaired at 30% (median value of $\Delta OD_{595nm} \cong 0.3$).

The distribution of the strains on the basis of ability to grow after 24 h incubation in presence of 20% (percentage usually used for production of sweet fermented dough) and 30% sucrose (percentage used to select highly tolerant sucrose strains) is shown in Fig. 3. A good correlation was found among growth behavior at both 20% and 30% of sucrose concentration, and *Leuc. pseudomesenteroides* PW2 and PD4 showed the highest sugar tolerance. Furthermore, these strains were able to produce dextran from sucrose (see Table 2), which can have interesting applications as texturing agents or prebiotics.

The other strains belonging to the species *Furl. rossiae*, *Lacp. plantarum* and *Fl. sanfranciscensis* exhibited a moderate growth ability in presence of 20 and 30% sucrose. The species *Coml. paralimentarius* and *Lacp. paraplantarum*, instead, were the most sensitive to both sucrose concentrations.

Consistently with our data, Vilanova, Dìez, Quirino, and Alava (2015) found that species of the genus *Leuconostoc* are sucrose-resistant bacteria and that supplementation of dough with sucrose or dried fruits (e.g. apricots, figs, raisins) may promote selection of sugar-tolerant microorganisms, driving the assembly of microbiota during sourdough formation.

Sucrose tolerance in LAB starter is of great importance because different sugar-rich ingredients, such as fruits, vegetables, yoghurt and honey, may be used to produce sourdoughs (Ripari, Gänzle, & Berardi, 2016). Also, the sourdoughs used for manufacturing traditional bread such as Pane di Matera PGI and Coppia Ferrarese are prepared by adding macerated ripe fruits or grape must, respectively, to flour and water (Gobbetti, Minervini, Pontonio, Di Cagno, & De Angelis, 2016). Apples, grapes and sugarcane are commonly used in the preparation of Brazilian sourdoughs (Aplevicz, Mazo, Ilha, Dinon, & Sant'Anna, 2014), while sugar syrup is used as sweetener in many modern bakeries of Baltic countries that produce rye bread (Valjakka, Kerojoki, & Katina, 2003).

Based on these considerations, the selection of strains in response to sugar tolerance is crucial to ensure the performances of starter cultures in fermentation processes leading production of sweet baked goods.

3.2.4. *Effect of ethanol*

During sourdough fermentation, several microbial metabolites (i.e. organic acids, CO_2, ethanol, hydrogen peroxide, aroma compounds) may accumulate in dough. Among them, ethanol results from the sugar conversion pathways of both yeasts and heterofermentative lactic acid bacteria. As showed by Van der Meulen et al. (2007) during daily back-slopping propagation, the concentration of ethanol could reach until 1.5 g ethanol/Kg dough. However, during prolonged sourdough fermentation, or during long-storage of sourdough, lactic acid bacteria must survive and kept metabolically active under different stress conditions, including also higher ethanol content.

In this study, the ability of the strains to grow in MRS broth supplemented with different concentrations of ethanol was investigated and results are shown in Figs. S1–D. Comparable growth behavior was observed at 2% and 4% of ethanol concentrations with a median value of ΔOD_{595nm} of about 0.9), while the higher stressor levels (6%) impaired the survival only slightly with a median value of $\Delta OD_{595nm} < 0.8$.

Distribution of tolerance ability (Fig. 4) demonstrated that most of strains had satisfactory resistance even to the highest ethanol level (6%). The most tolerant strains were *Furl. rossiae* PS34, S48, *Lacp. plantarum* PI1, PT4, PF1 and *Fl. sanfranciscensis* SB57. The most sensitive strains belonged to the species *Coml. alimentarius* and *Leuc. pseudomesenteroides*.

Our data were in agreement with those of other authors (Gold, Meagher, Hutkins, & Conway, 1992) that demonstrated a significant growth decrease for all of the strains in presence of 6% (v/v) ethanol compared with the OD at 0% ethanol after 48 h of incubation. Pittet, Morrow, and Ziola (2011), moreover, reported that LAB strains belonging to the genera *Lactobacillus* (old classification), *Leuconostoc* and *Pediococcus* are generally ethanol-tolerant microorganisms having a higher resistance to ethanol than most bacteria.

Although fermented baked products had less than 0.5% ethanol, during sourdough fermentation ethanol may accumulate, especially when heterofermentative strains are dominant; in these cases, ethanol, together with *n*-hexanal and ethyl acetate, is the most produced compound (Aponte et al., 2014). Also, Weckx et al. (2010), evaluated that the main metabolites from carbohydrate fermentation during rye sourdough fermentations are lactic acid and ethanol that reach concentrations up to 10.5 ± 0.5 g/kg and 9.0 ± 0.5 g/kg, respectively. In brief, regarding ethanol tolerance, all strains tested in the present study,

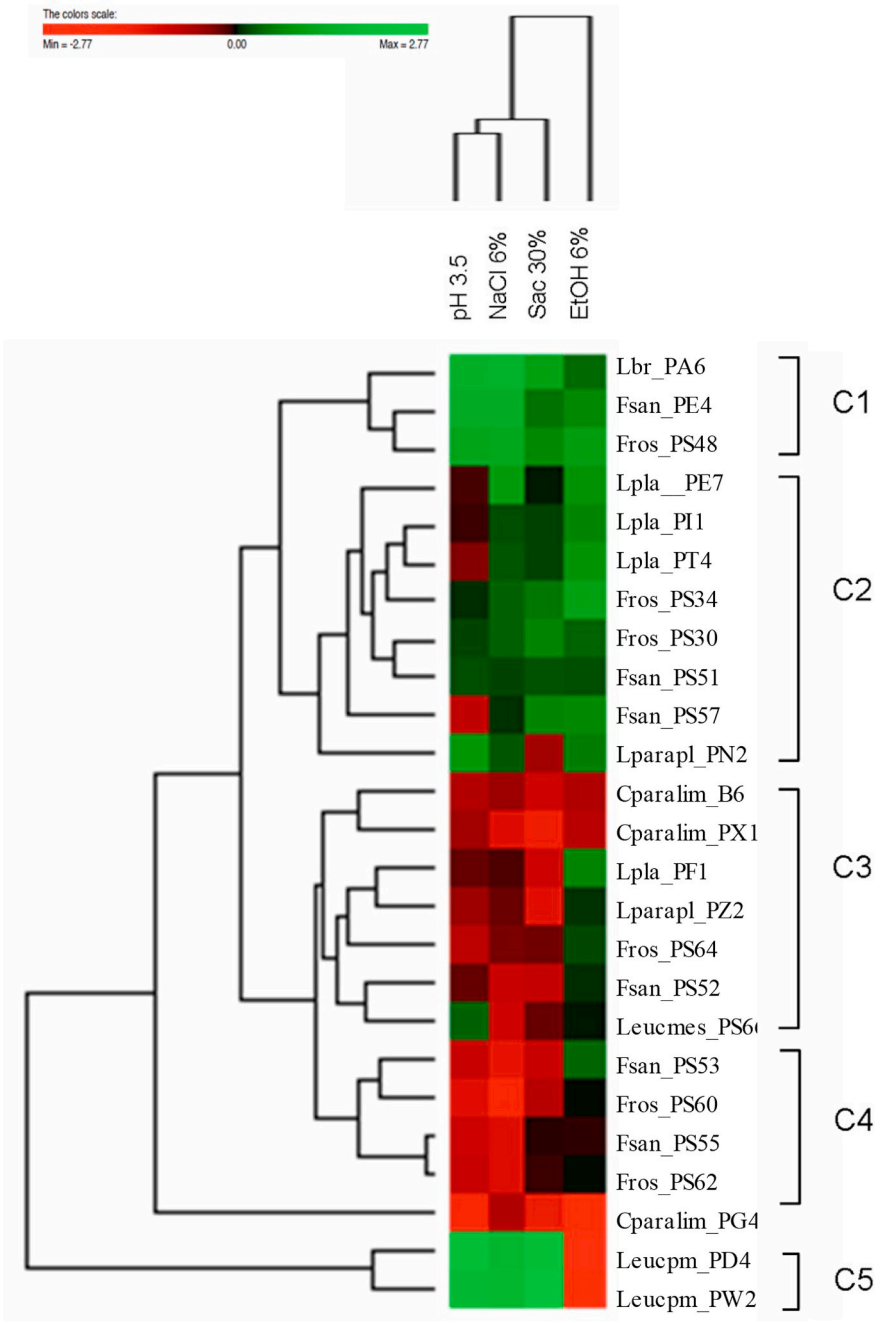

Fig. 5. Matrix Hierarchical Cluster Analysis of the 25 LAB strains (row dendrogram) in response to different stress factors. Column dendrogram: increase of optical density (ΔOD_{595nm}) at pH 3.5, in presence of 6% (w/v) NaCl, 30% (w/v) sucrose and 6% (v/v) ethanol. Labels report strain species (Lbr: *Levilactobacillus brevis*; Fros: *Furfurilactobacillus rossiae*; Fsan: *Fructilactobacillus sanfranciscensis*; Lpla: *Lactiplantibacillus plantarum*; Lparapl: *Lactiplantibacillus paraplantarum*; Cparalim: *Companilactobacillus paralimentarius*; Leucmes: *Leuconostoc mesenteroides*; Leucpm: *Leuc. pseudomesenteroides*) and number. Color scale: from red (negative data; minimum value is −2.83) to green (positive data, maximum value is +2.83), indicates the difference from the mean in standard deviation units. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

appeared suitable as starter for sourdough production, since the ethanol concentration in doughs usually does not exceed 1%. Moreover, many of them were able also to cope with stronger stress conditions (4% and 6% ethanol) that may verify when sourdoughs are stored for much time, or are not properly fed, or the fermentation is too longer.

3.2.5. Selection of high tolerant strains

To identify strains with high tolerance to more than one stress and, therefore, suitable for different types of leavened bakery products, a Matrix Hierarchical Cluster Analysis (MHCA, Fig. 5) was performed on the 25 selected strains, using as variables data (a z-value transformation was applied), the tolerance to pH 3.5, 6% NaCl, 30% sucrose and 6% ethanol, that were the conditions that mostly allowed to discriminate strains on the basis of growth ability.

Classification generated five main clusters according to the different levels of stress tolerance. Cluster C1 included the strongest three strains (*Levl. brevis* PA6, *Furl. rossiae* PS48, *Fl. sanfranciscensis* PE4) that exhibited the highest robustness to all stresses. Cluster C5 included two strains of *Leuc. pseudomesenteroides* (PD4, PW2) highly tolerant to pH 3.5, NaCl 6% and 30% sucrose, but sensitive to 6% ethanol. Cluster C2 comprised 7 strains sensitive to acidic conditions, but more resistant to NaCl, sucrose and ethanol. Clusters C3 and C4 grouped strains highly sensitive to all stress conditions.

These results confirmed the high intraspecific variability, to overall stress conditions, in different species associated to sourdough fermentation (e.g. *Fl. sanfranciscensis*, *Furl. rossiae*), suggesting that screening procedures are of practical relevance for the selection and development of starter cultures.

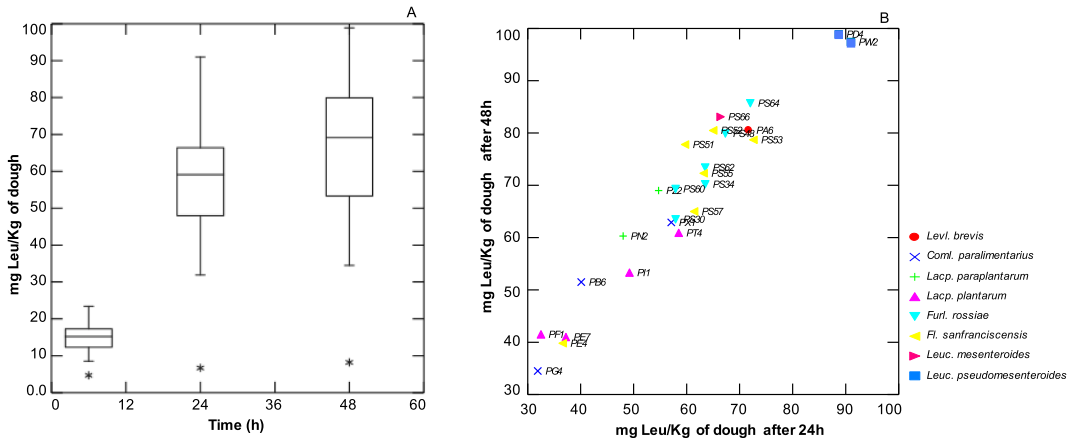

Fig. 6. A) Box and whiskers plot showing the total free amino acid (FAA) contents in 25 model doughs after 6 h, 24 h and 48 h fermentation. The symbol * indicates the total FAA values in the un-inoculated control doughs, during fermentation; B) Scatterplot showing the total FAA contents in the model doughs inoculated with the 25 LAB strains after 24 h and 48 h fermentation.

3.3. Screening for technological properties

3.3.1. EPS production and enzymatic activities

The 25 strains were also characterized for EPS production and amylasic and ureasic activities. Very few strains showed the ability to produce EPS from glucose, maltose and sucrose; specifically, all strains of *Leuc. pseudomesenteroides* and *Lacp. plantarum*, two strains of *Furl. rossiae*, and 1 strain of the species *Levl. brevis, Lacp. paraplantarum* and *Fl. sanfranciscensis* were recorded as EPS-producers.

The use of EPS-producing LAB could be particularly advantageous for food industries, as microbial EPS are involved in several mechanisms, such as prebiosis and probiosis and tolerance to stresses associated to food processes (Caggianiello, Kleerebezem, & Spano, 2016). EPS produced by LAB affect the rheological properties, texture, and mouthfeel of several foods, such as yogurt and bread, since they play a role of viscosifiers, stabilizers and emulsifiers (Galle & Arendt, 2014; Zannini, Waters, Coffey, & Arendt, 2016). Arendt, Ryan, and Dal Bello (2007) suggested that EPS produced by sourdough LAB could be a valid and cheaper alternative to replace the more expensive vegetal hydrocolloids, while Galle et al. (2012) highlighted an improved quality of gluten-free sorghum bread produced with EPS-producing LAB. Katina et al. (2009) showed that production of dextran by *Weissella confusa* significantly increased the viscosity of the sourdoughs, providing mild acidic wheat bread with a greater volume and softness of the loaf.

None of the strains exhibited ureasic activity, whereas 17 strains exhibit positive amylase activity (Table 2).

3.3.2. Proteolytic activity

The total free amino acid content during dough fermentation is reported in Fig. 6A. After 6 h fermentation all strains showed a reduced proteolytic level, while after 24 h and, mainly after 48 h, most of strains increased the total free amino acids production. The control sample, an un-inoculated dough (* in the graph), did not show any change in total amino acids contents within 48 h incubation.

Our results are in agreement with Zotta et al. (2006) that highlighted that during sourdough fermentation the level of amino acids was low after 6 h of incubation and doubled after 24 h, suggesting a period of adaptation of bacterial strains to sourdough environment followed by a strong demand for assimilable nitrogen.

Fig. 6B reports the distribution of the 25 strains on the basis of total free amino acid production after 24 and 48 h fermentation. The strains with the highest proteolytic activity were *L. pseudomesenteroides* PD4 and PW2 able to produce about 100 mg Leu/Kg of dough after 48 h incubation. Also, *Levl. brevis* A6, *Furl. rossiae* PS64, PS48, *Leuc. mesenteroides* PS66, and *Fl. sanfranciscensis* PS53 and PS52 produced after 48 h high amount of total free amino acids, higher than 80 mg Leu/Kg of dough. Our results confirmed that proteolytic activity was strain specific, indicating differences in the enzyme systems of the LAB, according to previous studies (Di Cagno et al., 2002).

It should be noted that some of the most proteolytic strains (i.e. *Levl. brevis* PA6, *Furl. rossiae* PS48, *Leuc. pseudomesenteroides* PD4 and PW2) were also those with the best stress tolerance pattern, confirming their suitability for sourdough productions.

4. Conclusions

The aim of this study was the selection of LAB strains with promising potential for the production of different types of sourdough-based products. Selection criteria were based on the capability to cope with different sourdough-associated stresses and on the basis of several technological features useful for sourdough production. Our work confirmed the intraspecific variability of LAB strains, and the need to perform a careful selection to identify competitive strains, able to survive and propagate during the overall fermentation process. High concentrations of sucrose, ethanol and NaCl were the main factors that allowed ranking sensitive and tolerant strains. Some members of *Leuc. pseudomesenteroides*, *Levl. brevis* and *Fl. sanfranciscensis* may be used to formulate a starter culture for both salty and sweet doughs, because of their high robustness to different stress conditions.

CRediT authorship contribution statement

Anna Reale: Conceptualization, Methodology, Data curation, Writing - review & editing, Supervision. **Teresa Zotta:** Data curation, Writing - review & editing. **Rocco G. Ianniello:** Formal analysis. **Gianfranco Mamone:** Validation, Funding acquisition. **Tiziana Di Renzo:** Investigation, Resources.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.110092.

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