Technological and safety properties of lactic acid bacteria isolated from
Spanish dry-cured sausages

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Abstract

Technological and safety-related properties were analyzed in lactic acid bacteria isolated from Spanish dry-cured sausages in order to select them as starter cultures. In relation to technological properties, all the strains showed significative nitrate reductase activity; *Lactobacillus plantarum*, *Lactobacillus paracasei* and 52% of the *Enterococcus faecium* strains showed lipolytic activity and only *Lactobacillus sakei* strains (43%) were able to form biofilms. Related to safety aspects, *E. faecium* strains were the most resistant to antibiotics, whereas, *L. sakei* strains were the most sensitive. In relation to virulence factors, in the *E. faecium* strains analyzed, only the presence of *efaA* gene was detected. The analysis of biogenic amine production showed that most *E. faecium* strains and *L. sakei* AI-142 produced tyramine. In conclusion, *L. paracasei* AI-128 and *L. sakei* AI-143 strains possess the best properties to be selected as adequate and safe meat starter cultures.

51	Keywords
52	Lactic acid bacteria, starter culture, meat products, biogenic amines, biofilms, antibiotic
53	resistance
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1. Introduction

Elaboration of dry-cured sausages is a complex process characterized by deep changes on the main meat components, resulting in the production of specific taste and aroma. This process favours the growth of microorganisms which influence the sensorial and nutritional qualities, safety, and other characteristics of sausages. The fermentation of sausages involves the participation of mainly lactic acid bacteria (LAB), coagulase-negative staphylococci (CNS), and less importantly, yeast and moulds (Ruiz-Moyano, Martín, Benito, Aranda, Casquete & Córdoba 2009). The most frequent LAB species present in fermented sausage processes are *Lactobacillus sakei*, *Lactobacillus curvatus*, and *Lactobacillus plantarum*. However, in some instances, the contribution of enterococci seems to be also relevant (Comi, Urso, Iacumin, Rantsiou, Cattaneo, Cantoni, & Cocolin, 2005).

LAB play an important role in the formation of lactic acid by fermenting carbohydrates, and hence could contribute to the safety of the process. Moreover, the lactic acid production contributes to the formation of the texture and in the acid taste. As a consequence of this process, the muscle protein coagulates, resulting in the slice ability, firmness and cohesiveness found in the final product. The development of curing colour occurs also in acidic conditions when nitric oxide is produced from nitrite and can then react with myoglobin.

Today, modern meat industry has to ensure high quality, reduce variability and enhance organoleptic characteristics in sausages production, which is not feasible using spontaneous fermentation methods. Regarding such situations, the use of selected starter cultures is important to produce the desired flavour and aroma compounds and extend the shelf life of the final product. The indigenous LAB isolated from fermented meats are especially well adapted to the ecological conditions of specific meat fermentations, controlling the ripening processes and inhibiting the growth of spontaneous microorganisms. Autochthonous starter cultures are recommended to achieve the desired fermentation parameters specific for the product type. The identification of the autochthonous microbiota of traditional dry-fermented sausages is of

great interest to standardize the fermentation process and for the selection of strains for their use as starter culture. LAB from the genus *Lactobacillus* in Europe, mainly *L. sakei*, *L. curvatus*, *L. plantarum*, and *Lactobacillus casei*, and from the genus *Pediococcus* in the USA represent the most common starter cultures used in the production of fermented sausages (Rantsiou, Drosinos, Gialitaki, Metaxopoulos, Comi, & Cocolin, 2006).

Generally, LAB from fermented sausages have been traditionally identified based on simple physiological, biochemical, and chemotaxonomic methods. Although valuable from a practical point of view, results obtained by these methods are not always sufficient to characterize strains to species level, mainly within species from genera *Lactobacillus* (Ammor, Rachman, Chaillou, Prévost, Dousset, Zagorec, Dufour, & Chevallier, 2005) and *Enterococcus* (Velasco, Perez, Peña, Dominguez, Cartelle, Molina, Moure, Villanueva, & Bou, 2004).

Despite the main role of LAB in fermented meat products is related with lactic acid production, LAB possess additional relevant characteristics that need to be taken into account in order to select them as starter cultures. The aims of the present study were to taxonomically identify a collection of LAB strains isolated from Spanish dry-cured sausages and analyze activities relevant for their use as starter cultures.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Forty-six LAB strains were analyzed in this study. The strains were previously isolated from dry-cured sausages, and have been molecularly identified in this study by sequencing their 16S rDNA. Dry cured sausages were prepared in local meat factories using traditional techniques and without using microbial starter cultures. They were made with 70–80% of lean pork meat and 20–30% of pork back fat. Ten grams of each sausage sample were homogenized

with 90 mL of a sterile solution of tryptone (0.3%) and NaCl (0.85%), for 2 min, in a Stomacher 400 Lab Blender (Seward Medical, London, UK). Ten fold dilutions were made in the same diluent.

All the strains were grown in MRS medium (Pronadisa, Spain) at 30 °C during 24 hours under microaerobic conditions. The strains were grown also on MRS agar plates (1.5 %) at 30 °C under microaerobic conditions.

2.2. DNA extraction

DNA extraction was carried out from overnight cultures as described by Sambrook, Fritsch and Maniatis (1989). DNA precipitates were resuspended in an appropriate volume of TE solution (10mM Tris-HCl, pH 8.0; 1 mM EDTA).

2.3. Taxonomical identification of LAB strains

LAB strains were identified by PCR amplification and DNA sequencing of their 16S rDNA. The 16S rDNAs were PCR amplified using the eubacterial universal pair of primers 63f and 1387r previously described by Marchesi, Sato, Weghtman, Martin, Fry, Hion, & Wade (1998) (Table 1). The 63f and 1387r primer combination generates an amplified product of 1.3 kb. **PCR was performed using AmpliTaq Gold DNA polymerase (Roche) in 25 µL** amplification reaction mixture by using the following cycling parameters: 10 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 1:30 min at 72 °C. Amplified products were resolved on 0.7% agarose gels. The amplification products were purified on QIAquick spin Columns (Quiagen, Germany) for direct sequencing. DNA sequencing was carried out by using an Abi Prism 377™ DNA sequencer (Applied Biosystems, USA). Sequence similarity

searches were carried out by comparing to sequences from type strains included on the Ribosomal Database (<http://rdp.cme.msu.edu>).

2.4. Technological properties of the strains

2.4.1. Nitrate reductase assay

Nitrate reductase activity was measured using a spectrophotometric method described previously (Miralles, Flores, & Pérez-Martínez, 1996), using MRS medium for bacterial growth. Briefly, an overnight culture (1.5 mL) was harvested by centrifugation and the pelleted cells was resuspended in induction buffer [Bactotryptone (Difco), 10 g/L; KNO₃, 1 g/L; cysteine, 1 g/L pH 7.0] to an OD₅₅₀=1. A fraction of the cell suspension was used for the determination of the dry weight. Anaerobic induction of nitrate reductase activity was achieved by incubating 1mL of cell suspension in Eppendorf tubes covered with a layer of sterile mineral oil during 2 h at 30 °C. As a control for the induction process, 1mL of the cell suspension was kept on ice for 2 h. The cells were harvested by centrifugation and permeabilized in 500 µL reaction buffer (50 mM KNO₃, 50 mM potassium phosphate, 100 mM NaCl, pH 7.0) by the addition of 30 µL of an acetone-toluene mixture (9:1, v/v). The tubes were vigorously shaken in a vortex for 3 min and the reaction was allowed to proceed for 30 min at 30° C. A 100 µL aliquot of the samples was transferred to a new Eppendorf tube containing 1 mL solution A [0.6 mg N-(1-Naphthyl)-ethylenediamine dihydrochloride in 100 mL 5 N acetic acid] and 1 mL solution B (0.8 g sulphanilic acid in 100 mL 5N acetic acid). Absorbance at 540 nm was measured against a control tube. Relative activity was calculated as the ratio: OD₅₄₀ × mg⁻¹ dry weight.

2.4.2. Catalase activity

The catalase activity was determined as described previously (Essid, Ben Ismail, Bel Hadj Ahmed, Ghedamsi, & Hassouna, 2007). The method was adapted to LAB by using MRS medium for bacterial growth. The strains were incubated in MRS to an OD₆₀₀=1, and then 5 mL culture was centrifuged and the resulting pellet was mixed with 1.5 mL of 60 mM H₂O₂ in 20 mM phosphate buffer pH 7.0. A 200 µL-aliquot of each culture was deposited in a 96-well plate. Catalase activity was measured spectrophotometrically at 240 nm after 3 min of incubation at room temperature in a microplate reader (SynergyTMHT, Biotek, EEUU). Results were expressed in arbitrary units (µmoles of degraded H₂O₂/min/mL of cells with OD₆₀₀=1.0).

2.4.3 Proteolytic activity

2.4.3.1 Gelatinase assay

Production of gelatinase was tested on MRS agar plates containing 10 g/L peptone (Pronadisa, Spain) and 30 g/L gelatine (Pronadisa, Spain) as described previously (Cariolato, Andrichetto, & Lombardi, 2008). After 16–18 h of incubation at 37 °C, the plates were placed at 4°C for 5 h before examination for a zone of turbidity around the colonies indicating gelatine hydrolysis.

2.4.3.2. Calcium caseinate agar assay

The proteolytic activity was tested in calcium caseinate agar plates (Pronadisa, Spain) as described previously (Martín, Hugas, Bover-Cid, Veciana-Nogués, & Aymerich, 2006). The strains were grown in MRS broth during 18–20 h and then 10 µL of each culture was placed in

calcium caseinate agar at 37 °C during 3 days. The diameter of the halos formed by the proteolytic strains was measure in mm.

2.4.4. Lipolytic activity

2.4.4.1. "Spirit Blue Agar" assay

The assay with Spirit Blue Agar plus Lipase Reagent (a mixture of tributyrin and polysorbate 80) (Difco) was performed according to the recommendations of the supplier. Lipolytic microorganisms metabolize the lipids present in the medium and halos around the colonies appear.

2.4.4.2. Tween 80 / Tween 20 assay

In order to evaluate lipolytic activity a method previously described by Essid et al. (2007) was also used. An overnight culture of each strain was plated on MRS agar supplemented with 1% of Tween 80 or Tween 20, centrifuged and the pellet resuspended in 20 mM phosphate buffer, pH 7. Each cell suspension (**10 µL**) **was inoculated on a spot at the** surface of the MRS plates containing Tween 80 or Tween 20 (1% v/v). After incubation at 30 °C for 48h, the lipolytic activity was determined by the appearance of a clear halo surrounding the spots which diameter was measured in mm.

2.4.5. Resistance to bile

2.4.5.1. Oxgall method

Resistance to bile was tested according the method described previously by Maragkoudakis, Zoumpopoulou, Miaris, Kalantzopoulos, Pot, and Tsakalidou (2006). LAB cultures were grown in MRS broth supplemented with Oxgall (0.3%) (Difco, France) at 30 °C during 4h. The resistance was determined by the number of viable colonies after incubation during 0 and 4 h, reflecting the time spent by the food in the small intestine.

2.4.5.2. LAPTg broth method

The bile resistance was evaluated on LAPTg broth as previously described (Saavedra, Taranto, Sesma, & de Valdez, 2003). The LAB strains were grown in LAPTg broth (g/L: peptone, 15; tryptone, 10; yeast extract, 10; glucose, 10; Tween 80, 0.1% v/v) and LAPTgO broth (LAPTg containing 0.3% Oxgall) at 37°C. Every hour, for the first 8 h, and after 24 h of incubation, the A_{560} nm was determined using a microplate reader (SynergyTMHT, Biotek. EEUU). The time required for each of them to increase the A_{560} by 0.3 units was recorded. The difference in time (min) between the LAPTg and LAPTgO cultures was considered as the growth delay.

2.5. Presence of relevant safety traits in the strains

2.5.1. Biofilm formation

2.5.1.1. Spectrophotometric method

Biofilm formation was determined according to the method described by Toledo–Arana, Valle, Solano, Arrizubieta, Cucarella, Lamata, Amorena, Leira, Penadés, and Lasa (2001). LAB cultures were grown overnight at 30 °C in MRS broth containing 0.25% glucose.

The culture was diluted 1/20 in fresh MRS broth supplemented with 0.25 % glucose; 200 µL of this suspension was used to inoculate sterile 96-well polystyrene microtitre plates. After 24 h incubation at 37°C, wells were washed with PBS, dried in an inverted position and stained with 1% crystal violet (Merk, Germany) for 15 min. The wells were rinsed with PBS once more and the crystal violet was solubilised in methanol/acetone (80:20, v/v). The A_{595} was determined using a microplate reader (SynergyTMHT, Biotek. EEUU). Biofilm formation was scored as follows: –, non-biofilm forming ($A_{595} \leq 1$); +, **weak** ($1 < A_{595} \leq 2$); ++, **moderate** ($2 < A_{595} \leq 3$); +++, **strong** ($A_{595} > 3$). Each assay was performed in triplicate and repeated three times.

2.5.1.2. Congo Red Agar method

The biofilm formation was evaluated using Congo Red Agar (CRA) (Freeman, Falkiner, & Keane, 1989). The components of the CRA media were MRS broth (37g/L), sucrose (0.8 g/L), agar-agar (10 g/L) and Congo red stain (0.8 g/L). Congo red stain was prepared as a concentrated aqueous solution, autoclaved separately, and added to the media when the agar had cooled to 55 °C. Plates were incubated aerobically for 24 h at 37°C. Biofilm positive strains produced black colonies while biofilm negative strains were pink.

2.5.1.3. PCR method

In *Enterococcus* strains, the biofilm formation ability was also determined by PCR amplification of the *ebpA* gene which is involved in biofilm-associated endocarditis (Nallapareddy, Singh, Sillampaa, Garsin, Hook, Erlandsen, & Murray, 2006). For the PCR reaction the primers Ef1091F and Ef1091R previously described were used (Cobo-Molinos, Hikmate, Ben-Omar, Lucas-López, & Galvez, 2008) (Table 1). PCR was performed using

AmpliTaq Gold DNA polymerase (Roche) in 25 μ L amplification reaction mixture by using the following cycling parameters: 10 min at 94 °C, 35 cycles of 1 min at 94°C, 1 min at 50°C and 1:30 min at 72°C. Amplified products were resolved on 0.7% agarose gels.

2.5.2. Antibiotic susceptibility testing

Susceptibility testing was assayed by the agar overlay disc diffusion test recommended by the National Committee for Clinical Laboratory Standard (NCCLS, 2002). SensiDisc BBL discs (Oxoid, England) were placed onto Mueller–Hinton agar (Difco, France) plates and overlayed with 3 mL of MRS soft agar broth (0.7% agar, p/v) inoculated with 150 μ L of the cultures with a cell concentration corresponding to 0.5 MacFarland turbidity standard. After incubation at 30 °C for 24 h the diameter of inhibition halos around the colonies was measured. Susceptibility or resistance was determined according to the recommendation of NCCLS. The antibiotics discs used were: ampicillin (10 μ g), penicillin G (10 U), vancomycin (30 μ g), amikacine (30 μ g), gentamicin (10 μ g), tetracycline (30 μ g), erythromycin (15 μ g), clindamycin (2 μ g), tobramycin (10 μ g), and rifampicin (5 μ g). In the *Enterococcus* strains the antibiotics tested were: ampicillin (10 μ g), chloramphenicol (30 μ g), erythromycin (15 μ g), penicillin G (10 U), gentamicin (10 μ g), tetracycline (30 μ g), vancomycin (30 μ g), teicoplanin (30 μ g), rifampicin (5 μ g), nitrofurantoin (300 μ g), and ciprofloxacin (5 μ g).

In tetracycline resistant strains the presence of the *tetM* gene, which codifies a protein involved in tetracycline resistance, was determined. The primers used were tetM-F and tetM-R (Gervers, Danielsen, Huys, Swings, J., 2003) (Table 1). PCR was performed using AmpliTaq Gold DNA polymerase (Roche) in 25 μ L amplification reaction mixture by using the following cycling parameters: 10 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C and 1:30 min at 72°C. Amplified products were resolved on 0.7% agarose gels.

2.5.3. Presence of virulence genes in enterococci

In *Enterococcus faecium* strains, the presence of virulence factors previously described was determined by PCR amplification (Table 1). The virulence genes detected were: *asa1*, which encodes the aggregation substance Agg; *esp*, encodes the enterococcal surface protein Esp; *cytA*, involved in cytolysin operon; *hyl*, encodes the hyaluronidase Hyl; *gelE*, the protease GelE with gelatinase activity; *efaA*, the surface A antigen EfaA; *ace*, the collagen adhesine Ace; and *hypR*, encodes HypR, a transcriptional regulator of the oxidative stress response and intracellular survival within macrophages (Diarra, Rempel, Champagne, Masson, Pritchard, & Topp, 2010). PCR was performed using AmpliTaq Gold **DNA polymerase (Roche) in 25 µL** amplification reaction mixture by using the following cycling parameters: 10 min at 94 °C, 35 cycles of 1 min at 94°C, 1 min at 50°C and 1:30 min at 72°C. Amplified products were resolved on 0.7% agarose gels.

2.5.4. Production of biogenic amines

2.5.4.1. TLC method

Biogenic amine production was tested by a TLC method previously described (García–Moruno, Carrascosa, & Muñoz, 2005). Briefly, the cultures were incubated at 30 °C for 7 days in MRS broth containing 0.5% of the corresponding amino acid precursor: L-histidine monohydrochloride (Merk, Germany), L-tyrosine disodium salt (Merk, Germany), L-ornithine hydrochloride (Sigma–Aldrich, Germany), L-lysine monohydrochloride (Merk, Germany). After incubation, the supernatant containing the corresponding biogenic amine was collected. As control, a stock standard solution of each amine (histamine, tyrosine, putrescine and cadaverine) was made by preparing a 2% solution (5% in the case of histamine) in 40%

ethanol. Amines were converted into their fluorescent dansyl derivatives and were incubated in the dark at 55° C during 1h. Amine derivative extracts were applied to TLC plates (silica gel 60 F₂₄, Merk, Germany). The dansylated compounds were separated using a chloroform:triethylamine (4:1) mobile phase. The fluorescent dansyl derivative spots were visualized using a ChemiDoc XRS⁺ (Bio Rad) under UV light exposure.

2.5.4.2. PCR method

PCR was used to detect the genes encoding the decarboxylase enzymes involved in the production of biogenic amines. The primers used in the PCR reactions were described previously: HIS1-F/HIS1-R to amplify a fragment of the histidine decarboxylase gene; TDC-F/TDC-R a fragment of the gene encoding tyrosine decarboxylase; PUT1-F/PUT1-R and PUT2-F/PUT2-R to amplify the two groups of ornithine decarboxylase genes, and finally CAD2-F/CAD2-R to amplify a fragment of the lysine decarboxylase gene present in Gram-positive bacteria (De las Rivas, Marcobal, Carrascosa, & Muñoz, 2006) (Table 1). PCR was performed using AmpliTaq Gold **DNA polymerase (Roche) in 25 µL amplification reaction** mixture by using the following cycling parameters: 10 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C and 1:30 min at 72°C. Amplified products were resolved on 0.7% agarose gels.

3. Results and discussion

3.1. Identification of LAB strains

Identification of LAB species based on biochemical tests has proven to be difficult and time-consuming. These phenotypic methods are limited in terms of both its discriminating ability and accuracy. In this sense, Benito, Serradilla, Ruiz-Moyano, Martín, Perez-Nevado,

and Córdoba, (2008) reported that in LAB strains isolated from Iberian dry-fermented sausages, biochemical identification by API 50 CHL showed different errors at genus and species level, for instance, *Pediococcus acidilactici* was misidentified as *Pediococcus pentosaceus* and *L. plantarum* as *Lactococcus lactis*. For this reason molecular methods have been developed allowing the accurate identification of LAB from meat products (Rantsiou & Cocolin, 2008).

Forty-six LAB strains previously isolated from Spanish dry-cured sausages were analyzed in this study (Table 2). As the characterization of LAB using traditional methods may sometimes be uncertain, these strains have been molecularly identified in this study by sequencing their 16S rDNA. Among the LAB strains, the species most frequently identified were *L. sakei* (20 strains) and *E. faecium* (19 strains), although strains from others LAB species were also found, such as *L. plantarum* (4 strains), *Lactobacillus paracasei* (2 strains) and *Lactobacillus coryniformis* (1 strain).

The isolation of a high number of *L. sakei* strains is in agreement with previous studies on which the high incidence of this species in fermented sausages was reported (Bonomo, Ricciardi, Zotta, Parente, & Salzano, 2008;; Ammor, et al., 2005). Leroy, Verluyten and De Vuyst (2006) reported that, in spontaneously fermented European sausages, lactobacilli constitute the predominant microbiota in fermented sausages. These authors concluded that *L. sakei* and/or *L. curvatus* generally dominate the fermented process being *L. sakei* the most competitive of both. Similarly the high prevalence of *E. faecium* strains in meat products have been previously described (Ruiz-Moyano, Martín, Benito, Pérez-Nevado, Córdoba, 2008). In this sense, Ammor et al. 2005 reported that the dominant species in sausages are usually members of *Lactobacillus* genera, although in certain slightly acidified sausages *Lactobacillus* and *Enterococcus* populations reach similar proportion in agreement with the results obtained in this study. In the same manner, in spontaneously fermented Swiss meat product, a notable

presence of *Enterococcus* species (18%) was reported among the LAB population (Marty, Buchs, Eugster-Meier, Lacroix & Meile, 2012).

3.2. Technological properties

In the present study forty-six LAB strains isolated from Spanish dry-cured sausages were screened in order to select eligible strains as starter culture for fermented meat products. In this sense, different technological and safety properties as nitrate reductase, catalase, proteolytic, and lipolytic activity; bile resistance, biofilm formation, antibiotic susceptibility and biogenic amine production were analyzed (Table 2).

Although CNS are the main bacteria responsible of the red colour in dry-cured sausages, LAB participate in the formation of this typical colour through the spontaneous reduction of nitrates to nitric oxide. Some meat LAB have been reported to possess significative nitrate reductases and nitrite reductase enzymatic activity directly involved in the mechanisms of nitrosomyoglobin formation, thus the screening of the nitrate reductase activity is desirable, even though in LAB these activities are much lower than in CNS (Ammor & Mayo, 2007). In our study, the LAB strains showed variable nitrate reductase activity (Table 2) ranging from 4.14 (*L. plantarum* AI-110) to 35.04 (*L. sakei* AI-107) mM nitrate reduced to nitrite per milligram of dry weight. This variability was observed also amongst strains even belonging to the same species, e.g. in *E. faecium* strains nitrate reductase activity ranged from 4.92 to 15.77, and in *L. sakei* strains from 5.32 to 35.04. Overall, the highest reduction of nitrates was observed in *L. sakei* strains. Bonomo et al. (2008) studied the nitrate activity present in LAB strains isolated from fermented sausages and found that only the strains belonging to *Leuconostoc* genera and *L. sakei* species showed nitrate reductase activity. On the contrary, *P. pentosaceus*, *L. plantarum* and *Lactobacillus brevis* strains were not able to reduce

nitrate. Nevertheless, in our study, *L. plantarum* strains showed intermediate nitrate reductase activity which ranged from 4.14 to 7.15 mM nitrate reduced (Table 2).

Most lactobacilli are able to form hydrogen peroxide by oxidizing lactate and thus increasing rancidity and the discoloration of the final meat product. Some LAB involved in meat fermentation, such as *L. sakei*, *L. plantarum*, *L. pentosus* and *P. acidilactici*, possess weak catalase activity as compared to the constitutive catalase of CNS which is active in meat products hydrolyzing the hydrogen peroxide produced (Ammor & Mayo, 2007). In this study, catalase activity was analyzed using a spectrophotometric method (Table 2). All the analyzed strains showed weak catalase activity in agreement with the reported by Ammor and Mayo (2007).

Several aromatic substances and organic acids are released by protease and lipase activity from microorganisms. Proteolysis and lipolysis influence both texture and flavour development due to the formation of low molecular weight compounds (free fatty acids, peptides, etc.). LAB usually do not possess strong proteolytic or lipolytic activities, although a degree of peptidase and lipase activity has been observed for some LAB meat strains (Fadda, Sanz, Vignolo, Aristoy, Oliver, & Toldrá, 1999). Exopeptidases from meat lactobacilli contribute to the generation of free amino acids involved in the flavour. Lactobacilli and pediococci display low catabolism of branched-chain amino acids, being this the reason they do not play a major role in the formation of aroma compounds (Leroy et al., 2006). Moreover, LAB have only weak proteolytic action on myofibrillar proteins (Sanz, Fadda, Vignolo, Aristoy, Oliver, & Toldrá, 1999). Montel, Masson and Talon (1998) reported that despite *Lactobacillus* species are weakly proteolytic in general, some *L. casei*, *L. plantarum*, *L. curvatus*, and *L. sakei* strains actively contribute to the hydrolysis of the sarcoplasmic proteins, and several peptidase activities have been reported in *L. sakei*, *L. curvatus* and *L. plantarum* isolated from sausages (Fadda, et al.1999). In this sense, in this study none of the strains

analyzed showed proteolytic activity using gelatine and calcium caseinate agar as substrates (Table 2).

In order to analyze the lipolytic activity of LAB strains several assays were performed. Despite the fact that none of the strains could hydrolyze Tween 20 or Tween 80, some of *E. faecium* (52%) and all *L. plantarum* and *L. paracasei* strains analyzed showed lipolytic activity in Blue Spirit. In addition, two *L. sakei* strains, AI-143 and AI-144, also showed lipolytic activity in Blue Spirit (Table 2). In agreement with our results, Montel et al. (1998) reported that *Lactobacillus* species are weakly lipolytic. Occasional descriptions of lipolytic activity from LAB strains have been also reported. For example, Silva-Lopes, Cunha, Clemente, Teixeira-Carrondo, Barreto-Crespo (1999) described that *L. plantarum* DSMZ 12028, isolated from fermented sausage, produces a lipase during the meat fermentation originating free fatty acids.

Even though LAB from dairy products and from intestinal origin are considered to be the main source of probiotics, strains of LAB species found in fermented sausages, such as *L. brevis*, *L. plantarum*, *L. fermentum*, and *P. pentosaceus*, have also been characterized as probiotic (Klingberg, Axelsson, Naterstad, Elsser, & Budde, 2005). The survival ability of isolates in the presence of oxgall bile is an important characteristic for the selection of potentially probiotic strains. Results obtained by the spectrophotometric method showed that, in general, *L. sakei* strains were more sensitive to bile than *E. faecium* strains as their growth retard was higher (Table 2). However, by the direct method which determines the number of viable colonies after incubation in presence of Oxgall, only two *L. plantarum* strains, AI-122 and AI-148, were resistant to bile salt. Several studies described that some lactobacilli, like *L. acidophilus*, *L. casei* and *L. plantarum*, possess hydrolase activity of bile salt (Gilliland & Speck, 1977). When the functional properties of lactobacilli strains isolated from kimchi were studied, only one *L. plantarum* strain survived at high rate in MRS supplemented with 0.5%

oxgall, although *L. sakei* strains showed different survival rates suggesting that survival ability is a strain-specific property (Lee, Yoon, Ji, Kim, Park, Lee, Shin, Holzapfel, 2011).

Taking into account the activities analyzed so far, the strains possessing significative lipolytic activity could be the ones that have the adequate technological characteristics to be selected as starter cultures. Among these strains, *L. paracasei* AI-128, *L. plantarum* AI-122, *L. sakei* AI-144, and the *E. faecium* strains AI-73, AI-74, AI-75, AI-76, AI-77, AI-78, AI-79, AI-83, and AI-89, could be included.

3.3. Safety-related properties

Although LAB are considered GRAS (Generally Regarded As Safe) organisms, it must be kept in mind their potential to transfer antibiotic resistances to pathogenic bacteria or the capacity to produce toxic compounds as biogenic amines. In addition, enterococci are true members of LAB community but they remain controversial because most species harbour a series of virulence factors and have been associated with nosocomial infections, urinary tract infections, bacteraemia, peritonitis, and endocarditis. For these reasons, it is necessary to do an assessment in regards to toxicity and pathogenic determinants as well as the presence of antibiotic resistances.

In nature, bacteria have developed a variety of mechanisms of resistance to environmental stresses, such as the formation of biofilms. In food industry, biofilm formation is undesirable for hygienic and safety reasons due to the possible attachment of food spoilage or pathogenic microorganisms to food or food surfaces. The formation of biofilms by some species of LAB has been reported and several studies have described genes responsible for adhesion or biofilm formation (Lebeer, Keersmaecker, Verhoeven, Fadda, Marchal, & Vanderleyden, 2007). In this study, 43% of *L. sakei* strains showed capacity for biofilm formation. Although some authors consider this as a negative characteristic, others considered

that in food systems, colonization of surfaces by bacteria used as starters could be desirable to inhibit the colonization by pathogenic or spoilage bacteria (Leriche & Carpentier, 2000). Kubota, Senda, Nomura, Tokuda and Uchiyama (2008) when studied the biofilm formation in *L. plantarum*, *L. brevis* and *L. fructivorans* strains, obtained that *L. plantarum* produced biofilm better than the other studied species, and the biofilm produced showed a great resistance to acid and ethanol. Similarly, in our study, *L. plantarum* AI-148 showed the capacity to form biofilm, as well as *L. coryniformis* AI-127 and *L. paracasei* AI-120 strains.

The presence of *ebpA* gene (endocarditis- and biofilm-associated pilus) has been studied in enterococcal isolates in relation with the capacity to form biofilm (Cobo-Molinos et al., 2008). These authors reported the presence of *ebp* genes in enterococcal isolates using specific primers and showed that a 68% of vegetable isolated strains and a 33% of clinical isolates were PCR-positive. In our study using the spectrophotometric method, only two strains were detected as biofilm-positive but were PCR-negative (*E. faecium* AI-79 and AI-89) (Table 2). These apparently contradictory results could be explained due to the variability in *ebp* genes as reported by Cobo-Molinos et al. (2008) in which study the majority of clinical isolates gave positive results using a labelled probe (94.59%) while PCR amplification of *ebp* gene was only positive in 32.43% of clinical isolates. On the hand, other genes involved in biofilm formation were described, for instance, the gene encoding the aggregation substance (Asc10) (Chuang-Smith, Wells, Henry-Stanley, & Dunne, 2010)

Clinical use of antibiotics has achieved a significant reduction in the morbidity and mortality associated with infectious diseases. Their use has been extended to veterinary medicine, where they are employed as therapeutic agents, prophylactics and animal growth promoters, and to agriculture for the control of plant diseases. This has exerted a very strong selective pressure for the appearance of resistant strains. Resistances are not virulence factors by themselves, but infections with resistant microorganisms complicate the course of the diseases and increase the price of their treatment (Ammor, Florez & Mayo, 2007). Antibiotic

resistance determinants contained in starter organisms or naturally occurring LAB may be transferred to commensals or pathogenic bacteria as horizontal transfer of resistance genes occurs in food (Teuber, Meile, & Schwarz, 1999). In this study, the susceptibility of the 46 LAB strains to different antibiotics was tested (Table 2). All non-enterococcal LAB strains analyzed were penicillin G sensitive and only two *L. plantarum* strains were ampicillin resistant. All strains were resistant to vancomycin and 43% resistant to rifampicin. In general, the non-enterococcal strains analyzed were sensitive to erythromycin, tobramycin, gentamicin, clindamycin (75%), and tetracycline (72%); and more resistant to amikacin (only 54% sensitive). In agreement with our results, Ammor et al. (2007) reported that, in general, lactobacilli are usually sensitive to penicillins, most species show a high level of resistance to glycopeptides (e.g. vancomycin), are susceptible to antibiotics inhibiting the synthesis of proteins (e.g. erythromycin, clindamycin and tetracycline), and are more resistant to aminoglycosides (e.g. amikacin, and gentamicin). Nevertheless, resistant strains to these agents have also been identified and the presence of several genes providing such resistances has been studied (e.g., *tet* genes for tetracycline resistance). Furthermore, lactobacilli are usually resistant to most inhibitors of nucleic acid synthesis (e.g. rifampicin) (Charteris, Kelly, Morelli, Collins, 1998).

All the *E. faecium* strains analyzed in this study were sensitive to vancomycin, gentamicin, chloramphenicol, teicoplanin, and ampicillin, but only 32% showed susceptibility to penicillin G. Contrarily, all the strains were resistant to tetracycline, rifampicin, and ciprofloxacin and showed intermedium resistance to erythromycin. Finally, only three strains were resistant to nitrofurantoin (*E. faecium* AI-58, AI-59, and AI-93). In agreement with our results, Diarra et al. (2010) reported that any of the *E. faecium* strains isolated from broiler chickens was resistant to chloramphenicol, and vancomycin. In the present study, all the *E. faecium* strains analyzed were resistant to at least three different classes of antibiotics tested.

Resistance to tetracycline was also evaluated by a molecular method based on the amplification by PCR of the *tetM* gene. Resistance to tetracycline is one of the most common acquired antibiotic resistances in *Enterococcus* strains isolated from food (Teuber, 1999). Huys, D'Haene, Collard, and Swings (2004) reported that tetracycline resistance in *Enterococcus* strains isolated from food is due to the presence of the genes already present in clinical and veterinarian isolates, *tetM*, *tetL*, and *tetS*. In the present study, all *E. faecium* strains analyzed amplified the *tetM* gene (Fig. 1), in agreement with the results obtained by Gervers, et al. (2003) which reported that the *tetM* gene is the most widely distributed *tet* gene, being detected in *Enterococcus*, *Streptococcus*, and *Bifidobacterium*. Nevertheless, in the non-enterococcal tetracycline-resistant strains analyzed, only *L. sakei* AI-123 and AI-144 strains were PCR-positive. However, *L. conyriiformis*, *L. paracasei*, and *L. plantarum* tetracycline-resistant strains were PCR-negative in agreement with the results of Ammor, Florez, van Hoek, de los Reyes-Gavilán, Aarts, Margolles, Mayo (2008) who reported that *L. plantarum* tetracycline-resistant strains were PCR-negative for the amplification of all *tet* genes. Gerver et al. (2003) found that tetracycline-resistant *L. sakei* strains only amplified *tetM* gene while the amplification of *tetO*, *tetS*, *tetK*, and *tetL* was negative. The high level of tetracycline resistance observed in the present study was in agreement with a high detection frequency of *tetM* gene.

Other important safety-related trait in enterococci is the presence of virulence factors, as these have been associated with several important pathologies. Many factors determine the virulence of *Enterococcus* species, for example, its ability to colonize the gastrointestinal tract, to adhere to a range of extracellular matrix proteins, urinary tract epithelia, oral cavity, etc. For this reason, in this study, the presence of genes encoding potential virulence factors was also evaluated. The virulence of enterococci is associated with the presence of several genes, including *asa1* gene, which encodes the aggregation substance Agg, a pheromone-inducible protein that increases the bacterial adherence to renal tubular cells and heart endocardial cells;

543 *esp*, encodes the enterococcal surface protein Esp associated with increased virulence,
 544 colonization and persistence in the urinary tract, and biofilm formation; *cytA*, involved in
 545 cytolysin production which significantly worsens the severity of endocarditis and
 546 endophthalmitis; *hyl*, encodes the hyaluronidase Hyl, homologous to the hyaluronidases of
 547 *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Staphylococcus aureus* which are
 548 believed to contribute to the invasion of the nasopharynx; *gelE*, encodes the protease GelE
 549 with gelatinase activity which has been shown to exacerbate endocarditis; *efaA*, encodes the
 550 surface A antigen EfaA involved in cells adherence; *ace*, encodes the collagen adhesine Ace
 551 involved in cell adherence and biofilm formation; *hypR*, encodes HypR, a transcriptional
 552 regulator of the oxidative stress response and intracellular survival within macrophages. In this
 553 study, all the strains were PCR-negative for all the virulence factors tested, however, in the
 554 case of the *efaA* gene it was amplified in all the strains analyzed (with exception of AI-79
 555 strain). Similarly, Ruiz-Moyano, et al. (2009) reported previously the presence of *efaA* gene in
 556 all the starter *E. faecium* strains screened while the determinants for another enterococcal
 557 adhesins (*esp* and *ace*) could not be detected in any strain. The absence of several virulence
 558 genes in *E. faecium* isolated from food was also described by Fisher and Phillips (2009). These
 559 authors reported that the gene encoding Agg was not present in *E. faecium* isolates, and the *esp*
 560 gene was absent from dairy isolates but present in clinical isolates, suggesting that the *esp* gene
 561 could be associated with pathogenicity. In the case of *cytA* gene, the same authors reported a
 562 higher incidence of this gene in clinical isolates (33%) as compared to 6% in food isolates.
 563 Moreover, any strain amplified the *gelE* gene, thought to provide nutrients to the bacteria by
 564 degrading host tissue and involved in biofilm formation; therefore, the absence of *gelE* gene is
 565 related to the low incidence of biofilm formation found in the strains analyzed in this study.

566 The presence of biogenic amines is a relevant food issue in meat products. In this study,
 567 biogenic amine production was tested by using two different methods, TLC (García-Moruno et
 568 al., 2005) and PCR (de las Rivas et al., 2006). Results obtained are showed in Table 2. Most of

the *E. faecium* strains analyzed produced tyramine (79%) (Fig. 2) and all of them were PCR-positive. The high incidence of tyramine production in these enterococci is in agreement with the results reported by other authors (Komprda, Sládková, Petirová, Dohnal & Burdychová, 2010). In this study, *E. faecium* AI-74, AI-75, AI-79, and AI-87 were PCR-positive for the presence of the tyrosine decarboxylase gene, but were TLC-negative for tyramine production. In this sense, Muñoz-Atienza, Landeta, de las Rivas, Gómez-Sala, Muñoz, Hernández, Cintas and Herranz (2011) similarly reported the non-production of tyramine in enterococci possessing a tyrosine decarboxylase gene due to the absence of gene expression.

In the non-enterococcal LAB strains analyzed, only one *L. sakei* strain showed biogenic amine production, *L. sakei* AI-142, which produced tyramine and was PCR-positive for the presence of the tyrosine decarboxylase gene (Fig. 3). The low capacity for biogenic amine production in the LAB strains analyzed in this study is in agreement with the results reported by Ruiz-Moyano et al (2009). These results are in agreement with the ones reported by other authors (Priyadarshani & Rakshit, 2011), which state that the presence, the activity and the specificity of decarboxylases is strain-specific. Therefore, careful screening for amino acid decarboxylase activity is recommended before selecting LAB as appropriate starter strains in food industry.

In conclusion, in this study the technological and safety-related characterization of LAB allowed to select strains to be used as meat starter. Attending to the safety-related properties, in the final strain selection have been rejected the strains showing capacity to produce biogenic amines (mainly tyramine), to produce biofilms, and to possess a high number of antibiotic resistance determinants (3 or more resistances). As only the presence (or absence) of lipolytic activity was clearly shown by the remaining strains, strains *L. paracasei* AI-128 and *L. sakei* AI-143 strains were finally selected. Other technological properties such as catalase, proteolytic, nitrate reductase, resistance to bile salts, etc. which did not shown a clear variation among the strains analyzed were not considered.

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Figure captions

Fig. 1. PCR amplification of the *tetM* gene involved in tetracycline resistance by using primers tetM-F and tetM-R that give an amplicon of 1513 pb. PCR was performed by using DNA from *E. faecium* strains: AI-59 (lane 1), AI-73 (lane 2), AI-74 (lane 3), AI-75 (lane 4), AI-76 (lane 5), AI-77 (lane 6), AI-78 (lane 7), AI-79 (lane 8), AI-83 (lane 9), AI-85 (lane 10), AI-86 (lane 11), AI-87 (lane 12), AI-88 (lane 13), AI-89 (lane 14). A DNA marker standard (EcoRI/HindIII digested λ DNA) was included in the gel and some of their fragments indicated on the right of the figure.

Fig. 2. Tyramine production by LAB strains using TLC. The strains were grown in MRS containing tyrosine and the tyramine produced during the growth was converted into its fluorescent dansyl derivative and detected. The analyzed strains were *E. faecium* AI-59 (lane 1), *E. faecium* AI-79 (lane 2), *E. faecium* AI-85 (lane 3), *E. faecium* AI-92 (lane 4), *L. sakei* AI-142 (lane 5). Tyramine standard solution (lane 6).

Fig.3. PCR amplification of the tyrosine decarboxylase gene by using primers TDC-F and TDC-R that give an amplicon of 825 pb. PCR was performed by using DNA from *L. sakei* strains: AI-137 (lane 1), AI-139 (lane 2), AI-142 (lane 3), AI-143 (lane 4), AI-144 (lane 5), AI-146 (lane 6), and AI-147 (lane 7). DNA from *E. faecium* AI-57 was used as positive control (lane 8). A DNA marker standard (EcoRI/HindIII digested λ DNA) was included in the gel and some of their fragments are indicated on the left of the figure.

Figure 1

Figure 1 (Landeta et al.)

Figure 2

Figure 2 (Landeta et al.)

Figure 3

Figure 3 (Landeta et al.)

Table 1

TABLE 1. Primers used in this study

Gene	Primer	Sequence ^a	Amplicon size (bp)	references
16S	63f 1387R	CAGGCCTAACACATGCAAGTC GGGCGGWTGGTTACAAGGC	1324	Marchesi et al., 1998
<i>ebpA</i>	Ef1091F Ef1091R	CCGCTCGAGAACTAACAAAAATGATTCGGCTCCAG CCGCTCGAGCCATCTCACGCATTTTATCTTCAACT	1064	Cobo-Molinos et al., 2008
<i>tetM</i>	tetM-F tetM-R	GAYACNCCNGGNCA YRTNGAYTT CACCGAGCAGGGATTTCTCCAC	1513	Gervers et al., 2003
<i>ace</i>	Ace1 Ace2	GGAATGACCGAGAACGATGGC GCTTGATGTTGGCCTGCTTCCG	616	Creti et al. 2004
<i>gelE</i>	GEL11 GEL12	AGTTCATGTCTATTTTCTTCAC AGATGCACCCGAAATAATATA	213	Vankerckhoven et al. 2004
<i>Asa1</i>	ASA11 ASA12	CACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375	Vankerckhoven et al. 2004
<i>efaA</i>	efaA1 efaA2	CGTGAGAAAGAAATGGAGGA CTACTAACACGTCAACGAATG	499	Mannu et al. 2003
<i>esp</i>	ESP 14F ESP 12R	AGATTTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510	Vankerckhoven et al. 2004.
<i>cylA</i>	CYT I CYT I1b	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688	Vankerckhoven et al. 2004
<i>hyl</i>	HYL n1 HYL n2	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCAA	276	Vankerckhoven et al. 2004
<i>hypR</i>	hypR1 hypR2	CGTGGCAAGAAGATTCTTAC GCTAAATATTCTCTTCAGGTG	465	Verneuil et al. 2004
<i>hdc</i>	HIS1-F HIS1-R	GGNATNGTNWSNTAYGAYMGNGCNCA ATNGCDATNGCNSWCCANACNCCRTA	372	De las Rivas et al., 2006

(Cont.)

<i>tdc</i>	TDC-F TDC-R	TGGYTNGTNCNCARACNAARCAYTA ACRTARTCNACCATRTTRAARTCNGG	825	De las Rivas et al., 2006
<i>odc</i>	PUT1-F PUT1-R	TWYMAYGCNGAYAAACNTAYTTYGT ACRCANAGNACNCCNGGNGGRTANGG	1440	De las Rivas et al., 2006
	PUT2-F PUT2-R	ATHWGNTWYGGNAAYACNATHAARAA GCNARNCCNCCRAAYTTNCCDATRTC	624	De las Rivas et al., 2006
<i>ldc</i>	CAD2-R CAD2-F	CAYRTNCCNGGNCAYAA GGDATNCCNGGNGGRTA	1185	De las Rivas et al., 2006

^aY = C or T; R = A or G; W = A or T; S = C or G; M = A or C; D = A, G, or T; H = A, C, or T; B = C, G, or T; N = A, C, G, or T.

Table 2

Table 2. Characteristics of analyzed strains.

Species	Strain	NR ^a	Catalase ^b	Proteolytic ^c		Lipolytic ^d		Biofilm ^e		Bile salt ^f		Biogenic amines ^g								Antibiotic ^h															
												TLC				PCR																			
				Gel	CCA	BS	T20/T80	RC	EM	ebpA	Rt	DM	H	T	P	C	H	T	P	C	Am	Clo	Cli	Ery	Gen	PeG	Tet ⁱ	Van	Tei	Rif	Nit	Cip	Ai	To	
<i>E. faecium</i>	AI-57	9.43	0.17	-	-	+	-	-	-	ND	67	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	I	R	ND	ND	
	AI-58	6.74	0.12	-	-	-	-	-	-	ND	68	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	R	R	ND	ND	
	AI-59	7.13	0.14	-	-	-	-	-	-	ND	70	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R ⁺	S	S	R	R	R	ND	ND	
	AI-73	8.39	0.12	-	-	+	-	-	-	ND	80	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	I	R	ND	ND	
	AI-74	4.92	0.13	-	-	+	-	-	-	ND	36	S	-	-	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	I	R	ND	ND	
	AI-75	11.84	0.14	-	-	+	-	-	-	ND	87	S	-	-	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	I	R	ND	ND	
	AI-76	12.23	0.15	-	-	+	-	-	-	ND	68	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R ⁺	S	S	R	I	R	ND	ND	
	AI-77	15.16	0.17	-	-	+	-	-	-	ND	91	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R ⁺	S	S	R	I	R	ND	ND	
	AI-78	9.58	0.12	-	-	+	-	-	-	ND	103	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R ⁺	S	S	R	I	R	ND	ND	
	AI-79	6.00	0.13	-	-	+	-	-	+	-	79	S	-	-	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	I	R	ND	ND	
	AI-83	15.77	0.15	-	-	+	-	-	-	ND	76	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	I	R	ND	ND	
	AI-85	13.87	0.19	-	-	-	-	-	-	ND	54	I	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	I	R	ND	ND	
	AI-86	10.20	0.14	-	-	-	-	-	-	ND	112	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	S	R	ND	ND	
	AI-87	9.94	0.14	-	-	-	-	-	-	ND	88	S	-	-	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	I	R	ND	ND	
	AI-88	7.95	0.16	-	-	-	-	-	-	ND	100	S	-	+	-	-	-	+	-	-	S	I	ND	I	S	R	R ⁺	S	S	R	S	R	ND	ND	
	AI-89	8.12	0.15	-	-	+	-	-	++	-	107	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	S	R	ND	ND	
	AI-91	11.68	0.16	-	-	-	-	-	-	ND	92	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R ⁺	S	S	R	S	R	ND	ND	
	AI-92	6.15	0.10	-	-	-	-	-	-	ND	98	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R ⁺	S	S	R	S	R	ND	ND	
	AI-93	15.36	0.16	-	-	-	-	-	-	ND	103	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R ⁺	S	S	R	R	R	ND	ND	
<i>L. coryniformis</i>	AI-127	7.61	0.05	-	-	-	-	+	+	ND	153	I	-	-	-	-	-	-	-	S	ND	S	S	S	S	R ⁺	R	ND	S	ND	ND	S	S		
<i>L. paracasei</i>	AI-120	6.86	0.10	-	-	+	-	+	-	ND	109	S	-	-	-	-	-	-	-	S	ND	S	S	S	S	R ⁺	R	ND	S	ND	ND	R	S		
	AI-128	15.49	0.10	-	-	+	-	-	-	ND	206	S	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	S	S		
<i>L. plantarum</i>	AI-110	4.14	0.24	-	-	+	-	-	-	ND	113	S	-	-	-	-	-	-	-	S	ND	R	S	S	S	R ⁺	R	ND	R	ND	ND	S	S		
	AI-116	4.59	0.13	-	-	+	-	-	-	ND	90	S	-	-	-	-	-	-	-	R	ND	S	S	S	S	R ⁺	R	ND	R	ND	ND	S	S		
	AI-122	8.92	0.22	-	-	+	-	-	-	ND	87	R	-	-	-	-	-	-	-	R	ND	R	S	S	S	R ⁺	R	ND	R	ND	ND	S	S		
	AI-148	7.15	0.23	-	-	+	-	+	-	ND	74	R	-	-	-	-	-	-	-	S	ND	R	S	S	S	S	R	ND	R	ND	ND	S	S		
<i>L. sakei</i>	AI-107	35.04	0.13	-	-	-	-	-	+	ND	136	S	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	S	S		
	AI-109	11.31	0.12	-	-	-	-	+	+	ND	76	I	-	-	-	-	-	-	-	R	ND	S	I	S	S	S	R	ND	S	ND	ND	S	S		
	AI-112	17.24	0.33	-	-	-	-	+	+	ND	9	S	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	R	ND	ND	R	S		
	AI-113	18.62	0.10	-	-	-	-	+	+	ND	25	S	-	-	-	-	-	-	-	S	ND	S	I	S	S	S	R	ND	S	ND	ND	S	S		
	AI-115	6.01	0.11	-	-	-	-	+	++	ND	43	S	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	I	S		
	AI-121	5.32	0.21	-	-	-	-	+	++	ND	39	I	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	I	S		
	AI-123	19.64	0.37	-	-	-	-	+	+	ND	48	I	-	-	-	-	-	-	-	S	ND	S	I	S	S	R ⁺	R	ND	R	ND	ND	I	S		
	AI-125	7.01	0.24	-	-	-	-	+	+	ND	53	S	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	I	S		
	AI-129	11.64	0.13	-	-	-	-	-	-	ND	254	S	-	-	-	-	-	-	-	S	ND	S	S	S	S	R ⁺	R	ND	R	ND	ND	S	S		
	AI-132	6.48	0.11	-	-	-	-	-	+	ND	203	S	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	R	ND	ND	I	S		
	AI-134	24.85	0.15	-	-	-	-	+	-	ND	55	S	-	-	-	-	-	-	-	S	ND	R	S	S	S	S	R	ND	R	ND	ND	I	S		
	AI-135	14.69	0.18	-	-	-	-	-	-	ND	526	S	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	S	S		
	AI-136	18.61	0.12	-	-	-	-	+	-	ND	96	S	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	R	S		
	AI-137	8.21	0.39	-	-	-	-	-	-	ND	1357	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	R	S	
	AI-139	23.15	0.10	-	-	-	-	-	-	ND	75	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	S	S	
	AI-142	9.04	0.33	-	-	-	-	-	-	ND	268	I	-	+	-	-	-	+	-	-	S	ND	R	S	S	S	S	R	ND	R	ND	ND	S	S	
	AI-143	5.88	0.13	-	-	+	-	-	-	ND	469	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	R	S	
AI-144	16.57	0.27	-	-	+	-	-	-	ND	422	S	-	-	-	-	-	-	-	-	S	ND	R	S	S	S	R ⁺	R	ND	R	ND	ND	I	I		
AI-146	6.72	0.45	-	-	-	-	-	-	ND	329	I	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	I	S		
AI-147	15.30	0.09	-	-	-	-	-	-	ND	111	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	S	S		

- ^a Nitrate reductase activity.
- ^b Catalase activity.
- ^c Proteolytic activity. (Gel) gelatinase agar, (CCA) calcium caseinate agar
- ^d Lipolytic activity. BS, “**Blue Spirit**” agar; 20T, Tween 20; 80T, Tween 80
- ^e Biofilm production. (RC) Red Congo agar method, (EM) spectrophotometric method: (-) $DO_{595} \leq 1$; (+) $OD_{595} \leq 2$; (++) $OD_{595} \leq 3$; (++++) $OD_{595} > 3$. *ebpA*: *ebpA* gene-PCR amplification. ND: not determined.
- ^f Bile salt resistance. (Rt) retardation in min. (DM) direct method. The number of colonies increases after bile salt treatment: resistant (R) ; the number of colonies keep constant after bile salt treatment: intermedium resistance (I); the number of colonies decrease after bile salt treatment: sensitive (S)
- ^g Biogenic amine production. (H) histamine; (T) tyramine; (P) putrescine; (C) cadaverine by TLC or PCR
- ^h Antibiotic resistance: (Am) ampicillin; (Clo) chloramphenicol; (Cli) clindamycin; (Ery) erythromycin; (Gen) gentamicin; (PeG) penicillin G; (Tet) tetracycline; (Van) vancomycin; (Tei) teicoplanin, (Rif) rifampicin, (Nit) nitrofurantoin, (Cip) ciprofloxacin (Ai) amikacin, and (To) tobramycin. R: resistant; I: intermedium resistance; S: sensitive (NCCLS)
- ⁱ PCR amplification of *tetM* gene in tetracycline resistant strains: (+) positive (-) negative