Annals of Microbiology (2019) 69:161–170
https://doi.org/10.1007/s13213-018-1416-1

ORIGINAL ARTICLE

Functional and safety characterization of autochthonous *Lactobacillus paracasei* FS103 isolated from sheep cheese and its survival in sheep and cow fermented milks during cold storage

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Received: 10 July 2018 / Accepted: 30 November 2018 / Published online: 14 December 2018

Abstract

The main objective of this study was to evaluate some probiotic characteristics of *Lactobacillus* spp. isolated from traditional sheep cheese, and to investigate the fermentative ability and viability in sheep and cow milks of a selected potential probiotic *Lactobacillus* (*L.*) strain, i.e., *L. paracasei* FS103. A total of 54 autochthonous *Lactobacillus* isolates were characterized for (i) acidity and bile salt resistance, (ii) tolerance to gastric and intestinal juice models, and (iii) antagonistic activity against pathogens and antibiotic resistance. Potential probiotic *Lactobacillus* has been used in sheep and cow milks for the manufacturing of experimental fermented milks. In these latter, pH value, microbial count, and sensory analysis were carried out. *Lactobacillus* FS103 classified as *L. paracasei* subsp. *paracasei* had a good survival in gastric and intestinal juice models, inhibited the growth of undesirable bacteria, and was susceptible to chloramphenicol, clindamycin, penicillin, amoxicillin, erythromycin, tetracycline, and ampicillin. Moreover, when used to produce experimental sheep and cow fermented milks, *L. paracasei* FS103 was able to acidify both milk types leading to a continuous pH decrease during all fermentation time (24 h). FS103 population remains viable at a level > 10^8 CFU mL^{-1} after 21 days of cold (4 °C) storage. The results of sensory analysis showed that scores related to consistency, taste, and astringent were significantly higher in sheep fermented milk while animal-like was less acceptable compared to cow fermented milk. *Lactobacillus paracasei* FS103 isolated from sheep cheese exhibited potential probiotic properties and suitable features for sheep and cow fermented milks maintaining high vitality during cold storage.

Keywords *Lactobacillus paracasei* · Probiotic characterization · Antimicrobial susceptibility · Sheep fermented milk · Viable microorganisms

Introduction

The genus *Lactobacillus* is the largest group among the lactic acid bacteria (LAB) and *Lactobacillus* species can be found in many different foods such as dairy, meat, vegetables, and bakery products (Corsetti and Settanni 2007; Mangia et al. 2013a, 2016; Xiong et al. 2013).

In the past, *Lactobacillus* strains were widely used in the preparation of fermented dairy products and in food preservation. For some decades, following the isolation of probiotic *Lactobacillus* strains, their use in the food industry is also based on the additional benefits that selected strains are able to confer to the human gastrointestinal tract (GIT) (Maragkoudakis et al. 2006). The main criterion for probiotic selection and application is the survival of the bacterial cells during their passage through the GI tract (FAO/WHO 2006). Probiotic bacteria must also have functional properties such as antimicrobial compound production, immune modulation capacities, and adhesion to gut tissue (Saarela et al. 2002). In addition, specific human health effects, e.g., alleviation of lactose intolerance by probiotics, have been widely documented (see for instance the review paper of Bernardeau et al. 2006). Moreover, with the growing use of antibiotics in human medicine and animal feed industries, it is crucial to evaluate bacteria antibiotic resistance. Even though many *Lactobacillus* members are considered GRAS (generally recognized as safe) and/or included in the QPS (Quality Presumption of Safety) list, strains' safety has to be assessed as they may become vectors of antibiotic resistance genes

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(Aquilanti et al. 2007; Dušková and Karpíšková 2013) and/or producers of toxic metabolites such as biogenic amines (Suzzi and Gardini 2003).

Currently, in the great distribution, several food products containing selected probiotic microorganisms including fermented milk must contain between 10^7 and 10^9 live bacterial cells per milliliter. The daily ingestion of a high concentration of probiotic LAB improves the immune system of the consumer and increases the resistance to diseases, infections, and allergies, as well as prevents certain diseases such as the onset of colon cancer (Cebeci and Gürakan 2003). However, the concentration of probiotic microorganisms in such food products was found to decrease during the shelf life, thus reducing or compromising the health value of fermented milk. This is due to several factors such as the low pH, the production of lactic and acetic acid (Vinderola et al. 2002), and the storage conditions.

Sheep milk, for its high content in proteins, lipids, minerals, and essential vitamins, possesses better nutritional properties than cow milk (Balthazar et al. 2017). The same author highlighted potential of sheep milk as a functional food. In particular, its protective effect on probiotic microorganisms during the process of dairy production has been demonstrated, suggesting that the production of functional dairy products from sheep milk is feasible.

Sardinia (Italy) is the most important Italian region for sheep milk production with about 3 million quintals per year (Laore 2011). Traditionally, most of the sheep milk is transformed into cheeses long and short ripened (Mangia et al. 2013b) which are the ones that most impel the island's economy. In this context, the setting up of sheep fermented milk produced with beneficial and autochthonous microorganisms isolated from traditional cheese, combining innovation and tradition, should represent a new strategy to valorize typical long- and short-ripened cheeses.

Taking into account these considerations, the first objective of the present study was to evaluate some probiotic characteristics of autochthonous *Lactobacillus* isolates and, secondly, to investigate their fermentative ability and viability when inoculated in sheep and cow milks.

Materials and methods

In this study, a total of 54 *Lactobacillus* from traditional Fiore Sardo cheese made from raw sheep milk without starter addition (Mangia et al. 2008) were studied. *Lactobacillus* isolates were refreshed three times in de Man, Rogosa, and Sharpe broth (MRS, Oxoid, Milan, Italy) at 35 °C for 24 h, for activation before safety and probiotic characterization.

Probiotic characterization

Acidity and bile salt tolerance

Probiotic microorganisms should withstand at least pH 3.0 (Fernandez et al. 2003); therefore, this pH value was used for a first screening to limit bacteria number in further tests.

From *Lactobacillus* overnight culture (approximately 10^6 CFU mL^{-1}), 100 μL of bacterial suspension were inoculated in MRS broth tubes (10 mL) acidified to pH 3 using 1 M HCl, and incubated for 3 h at 35 °C. Viable counts were performed at T_0 (starting time) and T_3 (after 3 h) by serial dilution in Ringer's solution (Oxoid) and spread plate technique using MRS agar (Oxoid) as the growing medium; after incubation of MRS plates at 35 °C under anaerobic conditions for 48 h, the results were expressed as the mean log colony-forming units per milliliter. Tolerant *Lactobacillus* strains were retested at pH 2.5 and pH 2 following the same protocol; in this latter test, viable counts were performed at T_3 (after 3 h) and at $T_{1.5}$ (after 1.5 h) respectively.

Acid-resistant *Lactobacillus* strains were subsequently assayed for their ability to resist bile salts (Ox-bile, Oxoid) at 0.6 and 1% concentrations. Aliquots (100 μL) of overnight *Lactobacillus* liquid cultures were transferred into 10 mL of MRS broth containing bile salts and incubated at 35 °C. Viable counts were performed at T_0 (starting time), $T_{1.5}$ (after 1.5 h), and T_3 (after 3 h) as described above.

Tolerance to the gastric and intestinal juice models

Lactobacillus isolates were cultured in the presence of simulated gastric and intestinal juices following the protocol described by Zhou et al. (2009). The gastric juice model was prepared by suspending 10 g of pepsin (Sigma) in 1 L of sterile saline (0.85% NaCl, *w*/*v*) adjusting the pH to 4.68 and 2 with 1 M HCl using a pH meter (Crison Instruments SA, Barcelona, Spain). The intestinal juice model was prepared by suspending pancreatin (Sigma) in phosphate buffer to reach a final concentration of 10 g/L and adjusting the pH to 6.8 with 0.1 M NaOH. The latter test was repeated adding 0.3% (*w*/*v*) of bile salts (Ox-bile, Oxoid). Both gastric and intestinal juices were sterilized by filtration using 0.22-μm filters (Millipore).

The *Lactobacillus* isolates were incubated at 35 °C for 18 h and centrifuged at 2500*g* at 4 °C for 10 min. The collected cells were washed twice with sterile saline before being inoculated to a final concentration of approximately 10^8 CFU mL^{-1} into gastric and intestinal juice models. At 0 and 3 h of incubation, the viable count (log CFU mL^{-1}) was determined by the plate method using MRS medium incubated anaerobically at 35 °C for 48 h.

Antimicrobial activity vs pathogenic bacteria

The antimicrobial activity of *Lactobacillus* isolates was tested against four of the most common foodborne pathogens: *Listeria monocytogenes* (DSMZ 20600), *Salmonella enterica* (DSMZ 13772), *Staphylococcus aureus* (DSMZ 20231), and *Escherichia coli* (DSMZ 30083). All pathogen strains were refreshed in Nutrient Broth (NB, Oxoid, Milan, Italy) except *Listeria monocytogenes* which was refreshed in Brain Heart Infusion medium (BHI, Oxoid, Milan, Italy).

Antimicrobial activity was first assessed using the agar spot test described by Fleming et al. (1985) with minor variations. Briefly, *Lactobacillus* overnight cultures were spotted on MRS agar plates (Oxoid) and incubated for 24 h at 35 °C under anaerobic conditions allowing the spots to grow. Pathogen strains were then inoculated (1%, *v*/*v*) in NB or BHI soft agar (0.7% bacteriological agar) which were poured over the MRS plates containing the lactobacilli spots. Plates were reincubated at 35 °C for 12 h and inhibition zones were measured around lactobacilli spots. Inhibition was considered positive if the inhibition zone diameter was 5 mm or larger. In order to understand whether the antimicrobial activity was due to organic acids, bacteriocins, or hydrogen peroxide production, the well diffusion test was used (Herreros et al. 2005).

Briefly, 1 mL of pathogen (indicator) liquid culture was inoculated in 15 mL of NB or BHI soft agar, poured into petri dishes, and left to dry. Once solidification was achieved, three wells of 5 mm (diameter) were cut into each plate. *Lactobacillus* cell-free supernatant (CFS) was prepared as follows: overnight liquid cultures were first centrifuged at 14,000 rpm for 5 min then filtered through 0.22-μm syringe sterile filter (Millipore); 35 μL of such CFS was added to the first well. The pH of the remaining CFS was then adjusted to 6.5 using 0.1 M NaOH to rule out any potential inhibition due to acidic pH and a volume of 35 μL was then added to the second well. Finally, the neutralized (pH 6.5) CFS was treated with catalase (1 mg mL^{-1}) at 25 °C for 30 min to eliminate possible H_2O_2 effect then filtered and added to the third well. Plates were then incubated anaerobically at 35 °C for 24 h. After incubation, the presence of a clear zone around the wells indicated an antagonistic effect of lactobacilli. All experiments were done in triplicates and the results were expressed as mean ± standard deviation.

Safety characterization

Hemolytic activity

Hemolytic activity of *Lactobacillus* isolates was evaluated on Columbia agar plates supplemented with 5% (*v*/*v*) sheep blood (Oxoid) and incubated at 37 °C for 48 h. Thereafter, the plates were observed and classified based on lysis activities of red blood cells in the medium around and under the colonies. Strains that produced green zones are considered α-hemolysis, clear zones are ß-hemolysis, and no zone are γ-hemolysis. Only strains with γ-hemolysis are considered as safe (Padmavathi et al. 2018).

Antibiotic susceptibility

Ten of the most commonly used antimicrobial agents in human medicine and livestock production were used. Antimicrobial agent susceptibility was assessed by the disc diffusion method (Florez et al. 2005) using MRS medium which is more suitable for lactobacilli growth than Mueller-Hinton medium (Sharma et al. 2017). Previously prepared discs (Oxoid, Milan, Italy) containing chloramphenicol (30 μg), clindamycin (2 μg), penicillin G (10 μg), amoxicillin (2 μg), erythromycin (15 μg), tetracycline (30 μg), ampicillin (10 μg), kanamycin (30 μg), and gentamycin (10 μg) were used. A volume of 100 μL of freshly grown bacterial cultures (10^8 CFU mL^{-1}) was spread on MRS agar plates and allowed to dry. Antibiotic discs were then applied on the MRS plate surface and incubated anaerobically at 35 °C for 48 h. Inhibition zones were measured after incubation and used as an indication of susceptibility (S) or resistance (R) (FEEDAP 2012). Minimal inhibitory concentration (MIC) for resistant *Lactobacillus* isolates was calculated using the broth micro-dilution method (ISO/IDF 2010). Antibiotic stock solutions were first prepared with a concentration of 1024 μg mL^{-1}. Stock solutions were then diluted in LAB susceptibility test medium broth (LSM), to obtain concentrations ranging 0.25 to 1024 μg mL^{-1}. Overnight lactobacilli cultures were then centrifuged at 12,000 rpm per 10 min and re-suspended in sterile saline solution to an optical density at 625 nm (OD_{625}) of 0.2 corresponding to 3×10^8 CFU mL^{-1}. These bacterial suspensions were then diluted 1:500 in LSM broth and 50-μL aliquots were added to each well of a 96-well micro-dilution plate. Each well of the micro-dilution plate contained 50 μL of LSM broth with a given antibiotic concentration as above mentioned. Control wells with no antibiotics were also included. Plates were then incubated at 35 °C for 48 h. After incubation, the bacterial growth was assessed by measuring the OD_{625} in each well and MIC was determined as the lowest concentration of an antibiotic for which bacterial growth was inhibited. MIC values were then compared to MIC breakpoints suggested by FEEDAP (2012).

Sequencing of 16S rRNA gene of *Lactobacillus* isolates

DNA was extracted from 2 mL of overnight *Lactobacillus* culture as described by Georgalaki et al. (2017). Polymerase chain reaction (PCR) was carried out using One Taq Quick-Load 2x Master Mix (New England BioLabs Inc., Ipswich, MA, USA). Amplification of 16S ribosomal DNA fragment (1564 bp) was performed using primers 16S F (5′-GGA GAG

TTA GAT CTT GGC TCA G-3′) and 16S R (5′-AGA AAG GAG GTG ATC CAG CC-3′) (Eurofins Genomics, Ebersberg, Germany). The PCR amplification was carried out in a Mastercycler Gradient thermal cycler (Eppendorf, Germany) according to the following program: initial denaturation at 94 °C for 2 min, amplification for 30 cycles with denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s, and primer extension at 72 °C for 80 s, followed by a final extension at 72 °C for 5 min. Reaction products were screened by electrophoresis in 1% (*w*/*v*) agarose gel and stained with ethidium bromide. For sequence analysis, the PCR products were purified using the Nucleospin gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions and sequenced. Searches in the GenBank database were performed with the BLAST program to determine the closest known relatives of the partial 16S rRNA gene sequences obtained (http://www.ncbi.nlm.nih.gov/BLAST).

The sequences were deposited in the GenBank database using the web-based data submission tool BankIt (http://www.ncbi.nlm.nih.gov/BankIt).

Experimental manufacturing of sheep and cow fermented milks with *Lactobacillus paracasei* FS103

The fermentative ability and viability of selected *L. paracasei* FS103 were assessed in experimental manufacturing trials of sheep and cow fermented milks. Chemical parameters (%) of sheep milk (fat 5.99, proteins 5.00, carbohydrates 4.09) were provided by ARAS Sardegna (Regional Farmer Association of Sardinia, Italy) while pasteurized cow milk was purchased from the market (fat 3.6, proteins 3.3, carbohydrates 4.9). The sheep milk was obtained from Sarda breed, collected 2 h after milking, and kept at 5 °C for 18 h; then, it was partially defatted by centrifugation (3000*g* × 15 min, 4 °C) and treated at 90 °C for 10 min.

Sheep and cow milks at 35 °C were inoculated with *L. paracasei* FS103 overnight culture at the 1% (*v*/*v*) (the final content was about 10^6 CFU mL^{-1}), incubated at 35 °C for 24 h (time fermentation) then stored at 4 °C for 21 days. Samples at 0, 4, 8, and 24 h of fermentation and samples at 12 and 21 days of cold storage for microbiological and physicochemical parameters were evaluated.

To determine *Lactobacillus* viable count, milk or fermented milk samples were 10-fold diluted in Ringer's solution and plated on MRS medium and incubated at 35 °C in anaerobic condition; the pH was measured using a pH meter (Crison Instruments SA, Barcelona, Spain); titratable acidity was assessed in 10 g of milk titrated with 0.1 mol L^{-1} NaOH, phenolphthalein was used as indicator, and acidity was expressed as percentage of lactic acid.

Sensory analysis of experimental fermented milk

Sensory analysis was carried out as reported by Mangia et al. (2014). Both sheep and cow fermented milks at 21 days of storage were evaluated for flavor, consistency, acidity, taste, sweet, astringent, and animal-like attributes by a trained panel of 10 members. A 5-point score system in accordance with their hedonistic preferences (5 excellent, 1 unacceptable) was used.

Data analysis

All the experimental trials were performed in duplicate and, for each trial, analyses were carried in triplicate. Results were expressed as means ± standard deviations. Mean values of microbiological and physicochemical analyses on fermented milk were compared by Student's *t* test and differences were deemed statistically significant at $P < 0.05$.

Results

Acidity and bile salt tolerance

The first screening at pH 3 showed that only six of the *Lactobacillus* isolates ($n = 54$) were able to survive after 3 h

Table 1 Viable counts (means log CFU mL^{-1} ± standard deviation) of 6 *Lactobacillus* isolates grown in MRS broth at pH 3, 2.5, and 2

Lactobacillus	pH 3		pH 2.5		pH 2	
	0 h	3 h	0 h	3 h	0 h	1.5 h
FS20	5.85 ± 0.17	6.10 ± 0.03	6.01 ± 0.11	2.92 ± 0.11	6.13 ± 0.12	–
FS24	6.30 ± 0.05	5.51 ± 0.08	6.12 ± 0.16	1.54 ± 0.06	6.27 ± 0.25	–
FS103	6.30 ± 0.15	5.30 ± 0.08	6.16 ± 0.21	5.81 ± 0.07	6.65 ± 0.03	3.81 ± 0.25
FS109	6.23 ± 0.08	6.41 ± 0.04	6.40 ± 0.06	4.18 ± 0.10	6.89 ± 0.18	–
FS111	6.26 ± 0.02	6.43 ± 0.05	6.07 ± 0.14	2.27 ± 0.12	5.97 ± 0.09	–
FS112	6.25 ± 0.07	5.00 ± 0.06	6.18 ± 0.22	1.11 ± 0.16	6.89 ± 0.04	–

Table 2 Viable counts (means log CFU mL^{-1} ± standard deviation) of *Lactobacillus* isolates grown at bile salt concentration of 0.6 and 1%

Lactobacillus	Bile salts 0.6%		Bile salts 1%	
	0 h	3 h	0 h	1.5 h
FS20	6.00 ± .14	6.31 ± 0.07	6.11 ± 0.11	–
FS24	6.30 ± 0.09	6.37 ± 0.10	6.08 ± 0.16	–
FS103	6.32 ± 0.11	6.26 ± 0.09	6.39 ± 0.12	4.25 ± 0.02
FS109	6.27 ± 0.19	6.47 ± 0.27	6.23 ± 0.03	4.44 ± 0.01
FS111	6.04 ± 0.08	6.25 ± 0.22	6.31 ± 0.07	–
FS112	6.29 ± 0.12	6.28 ± 0.21	6.24 ± 0.24	–

of incubation (Table 1). In more stressful conditions, i.e., at pH 2.5, the viable counts of *Lactobacillus* 20, 24, 111, and 112 decreased substantially (< 3 log CFU mL^{-1}) while the count of *L.* FS103 and FS109 decreased from 6.16 and 6.40 log CFU mL^{-1} to 5.81 and 4.18 log CFU mL^{-1} respectively. *L.* FS103 showed a good tolerance also to pH 2 after 1.5-h exposure, despite a decrease of about 3 log units, while *L.* FS109, as well as the remaining strains, were unable to survive in such harsh conditions. Overall, all *Lactobacillus* isolates which tolerated pH 3 were resistant also to 0.6% bile salts during the 3 h of incubation (Table 2). However, when *Lactobacillus* were tested by increasing the concentration of bile salts to 1%, results showed that most of the tested strains were susceptible to such bile salt concentration except those resisting to pH 2.5; in fact, *L.* FS103 and FS109 had viable counts of 4.25 and 4.44 log CFU mL^{-1} respectively.

Tolerance to the gastric and intestinal juice models

Taking into account the results of acidity (pH 2.5) and bile salt (1%) tolerance, *L.* FS103 and FS109 were further tested to verify their ability to tolerate the harsh conditions of gastric and intestinal juice models.

In the gastric juice model containing the solution of pepsin at pH 4.68, both lactobacilli viable counts remained constant (Table 3). In the same substrate but at pH 2, *L.* FS103 confirmed this acidity tolerance (Table 1) also in the presence of pepsin, even if a decrease of 2 log units of the viable count was observed, while *L.* FS109 was completely inhibited.

From the results shown in Table 3, it is noted that, in the solution containing only pancreatin, viable count remains constant (about 6 log CFU mL^{-1}) for both lactobacilli after 3 h of incubation; when in the substrate containing pancreatin, bile salts were added only viable count of *L.* FS109 isolate decrease of about 1 log unit.

Antimicrobial activity vs pathogenic bacteria

Antimicrobial activity of *L.* FS103 and FS109 isolates was tested against *L. monocytogenes*, *S. enterica*, *S. aureus*, and *E. coli* (Table 4). The results of the agar spot test showed that all strains were able to inhibit *S. enterica*, *S. aureus*, and *E. coli* (diameter > 5 cm) but they did not inhibit at the same level *L. monocytogenes* (diameter < 5 cm). Both lactobacilli showed the best inhibition against *S. enterica* (diameter around 9 mm). When antimicrobial activity was reevaluated with the well diffusion method, both lactobacilli showed inhibition zones around the filtered supernatant spot indicating the presence of inhibitory compounds in the supernatant. *Lactobacillus* isolate antimicrobial activity disappeared when the supernatant was buffered to pH 6 (second well) and catalase was added (third well), indicating that the antimicrobial activity was due to acidic pH (data not shown).

Hemolytic activity and susceptibility to antimicrobial agents

Hemolysis was evaluated and no activity (γ activity) was observed for both *Lactobacillus* isolates (data not shown). *Lactobacillus* isolates were tested for antimicrobial agent susceptibility using both disc diffusion and MIC determination methods. The antibiotic disc diffusion test indicated that *Lactobacillus* isolates were susceptible to chloramphenicol, clindamycin, penicillin, amoxicillin, erythromycin, tetracycline, and ampicillin as they were unable to grow around the antibiotic discs, forming clear inhibition zones (inhibition zones diameters > 20 mm).

Table 3 Survival (means log CFU mL^{-1} ± standard deviation) of *Lactobacillus* isolates in gastric and intestinal juice models

Lactobacillus	Gastric juice model				Intestinal juice model			
	Pepsin (10 g/L) + pH 4.68		Pepsin (10 g/L) + pH 2		Pancreatin (10 g/L)		Pancreatin (10 g/L) + bile salts (0.3%)	
	0 h	3 h	0 h	3 h	0 h	3 h	0 h	3 h
FS103	6.60 ± 0.08	6.41 ± 0.03	6.59 ± 0.11	4.26 ± 0.03	6.68 ± 0.07	6.26 ± 0.12	6.61 ± 0.10	6.10 ± 0.05
FS109	6.46 ± 0.03	6.26 ± 0.55	6.43 ± 0.03	–	6.43 ± 0.31	6.48 ± 0.01	6.00 ± 0.24	5.25 ± 0.07

Table 4 Antimicrobial activity (agar spot test) of *Lactobacillus* isolates against *Listeria* (*L.*) *monocytogenes*, *Escherichia* (*E.*) *coli*, *Salmonella* (*S*). *enterica*, and *Staphylococcus* (*St.*) *aureus*

Lactobacillus	*L. monocytogenes*	*E. coli*	*S. enterica*	*St. aureus*
FS103	4.8 ± 0.5	7.0 ± 1.9	9.2 ± 0.2	6.7 ± 0.9
FS109	3.9 ± 0.3	7.3 ± 1.1	8.4 ± 2.1	6.4 ± 0.5

Results are presented as means ± SD and expressed as inhibition zones diameters (mm). Diameter ≥ 5 mm was considered positive

Both lactobacilli were resistant (inhibition zones diameters < 14 mm) to kanamycin and gentamycin; therefore, antibiotic MIC was determined. Results showed that kanamycin MIC was 32 μg mL^{-1} for *L.* FS103 and 16 μg mL^{-1} for FS109 while gentamycin MIC was 1 μg mL^{-1} for *L.* FS103 and 2 μg mL^{-1} for FS109.

Molecular identification and genotyping of *Lactobacillus* FS103 and FS109

The sequencing of the 16S rRNA gene fragments from *Lactobacillus* FS103 and FS109, lodged in GenBank with MH480514 and MH480515 accession numbers respectively, yielded sequences displaying 99% identity with different 16S rRNA sequences belonging to *Lactobacillus paracasei* subsp. *paracasei*, thus suggesting the affiliation of the strains to such bacterial species.

Experimental sheep and cow fermented milks with *Lactobacillus paracasei* FS103 strains

Given the results of the preliminary probiotic selection, *L. paracasei* subsp. *paracasei* FS103 strains were used in experimental fermented milk manufacturing because it showed better resistance to acidity and better GIT conditions in comparison to *L. paracasei* FS109.

Overall, *L. paracasei* FS103 strain was able to acidify both sheep and cow milks, leading to a continuous decrease of pH during all fermentation time (24 h), especially in sheep milk that showed lower pH value ($P < 0.05$) and higher lactic acid content ($P < 0.05$) compared to cow milk (Table 5). Although significant differences between the two substrates were recorded, *L. paracasei* FS103 grew well in sheep and cow milks, reaching 9 and 8 log CFU mL^{-1} after 8 h of incubation respectively. At the end of fermentation, lactobacilli counts (Table 5) were not different ($P > 0.05$) between cow and sheep milks (about 10^9 CFU mL^{-1}). During the first 12 days of cold storage (Table 6), the acidity values of sheep milk decreased in a less pronounced way compared to cow milk where the pH decreased up to 4.88 and the lactic acid content increased by 0.3%. The same parameters (i.e., pH and lactic acid) remained approximately constant up to 21 days. Even during storage,

Table 5 Acidifying activity (pH and lactic acid) and viable count (means log CFU mL^{-1} ± standard deviation) of *Lactobacillus paracasei* FS103 in the sheep and cow milks during fermentation at 35 °C

	pH				Lactic acid %				Viable count			
	0 h	4 h	8 h	24 h	0 h	4 h	8 h	24 h	0 h	4 h	8 h	24 h
Sheep milk	6.55 ± 0.03^a	6.44 ± 0.45^a	5.15 ± 0.22^a	4.49 ± 0.14^a	0.19 ± 0.12^a	0.23 ± 0.01^a	0.29 ± 0.08^a	0.68 ± 0.07^b	6.24 ± 0.05^a	7.86 ± 0.20^b	9.47 ± 0.06^b	9.71 ± 0.12^a
Cow milk	6.62 ± 0.10^a	6.50 ± 0.32^a	5.71 ± 0.18^a	4.92 ± 0.10^b	0.17 ± 0.09^a	0.20 ± 0.04^a	0.23 ± 0.03^a	0.46 ± 0.03^a	6.81 ± 0.07^b	7.00 ± 0.14^a	8.62 ± 0.23^a	9.51 ± 0.09^a

Different superscript lowercase letters (a, b) on the same row indicate statistically significant differences ($P < 0.05$)

Table 6 Acidifying activity (pH and lactic acid) and viable count (means log CFU mL^{-1} ± standard deviation) of *Lactobacillus paracasei* FS103 in the sheep and cow fermented milks at 12 and 21 days of cold storage (4 °C)

	pH		Lactic acid %		Viable count	
	12 days	21 days	12 days	21 days	12 days	21 days
Sheep fermented milk	4.78 ± 0.01^{a}	4.75 ± 0.11^{a}	0.65 ± 0.03^{a}	0.68 ± 0.02^{a}	9.26 ± 0.26^{a}	8.63 ± 0.64^{a}
Cow fermented milk	4.88 ± 0.01^{b}	5.01 ± 0.22^{a}	$0.76^{b} \pm 0.01^{b}$	$0.75^{b} \pm 0.01^{b}$	9.70 ± 0.23^{a}	8.16 ± 0.06^{a}

Different superscript lowercase letters (a, b) on the same row indicate statistically significant differences ($P < 0.05$)

lactobacilli viable counts remained high in both milks (about 8 log CFU mL^{-1}), being reduced of only 1 log unit after 21 days of storage (Table 6).

Sensory analysis

The results of the sensory analysis (Fig. 1) showed that both fermented milks were excellent for flavor and acidity and acceptable for sweet attributes. The scores related to consistency, taste, and astringent acceptability were significantly higher in sheep fermented milk. On the contrary, animal-like of sheep fermented milk was described by panelists as less acceptable compared to cow fermented milk.

Discussion

The evaluation of bacterial probioticity requires extensive in vitro and in vivo investigation. Thus, many in vitro models can simulate, with good approximation, strain surviving abilities in the GIT and health benefits to the host. One of the main properties of probiotic bacteria is their survival capacity in the GIT mainly in low-pH stomach environment. *Lactobacillus* strains are naturally equipped to produce acidic conditions and to survive for a long time in high acid matrix such as fermented milk. In this study, the behavior of the autochthonous *L. paracasei* strains in the presence of pepsin (gastric juice model) was similar when tested only under acidic conditions; this leads us to suppose that the pepsin has not had any impact on lactobacilli. In this regard, conflicting data by Guo et al. (2009) were reported: pepsin improves probiotic *L. casei* Zhang survival, while after exposure viable count of *L. casei* Shirota dropped. However, it is important to take into account that the survival of ingested bacteria in the stomach is influenced by the buffering capacity of food components as fermented milk and milk substances (Abdel-Daim et al. 2012).

The transit time of food in the small intestine is generally between 1 and 4 h; the concentration of 0.5 and 0.3% of bile salts is suitable for the selection of probiotic bacteria for human consumption (Zhou et al. 2009). In addition to bile salts, other substances such as pancreatin can inhibit the growth of probiotics in the intestinal environment. For these reasons, the intestinal juice (model) was made of a pancreatin solution at pH 6.8, in the absence and presence of bile salts (final

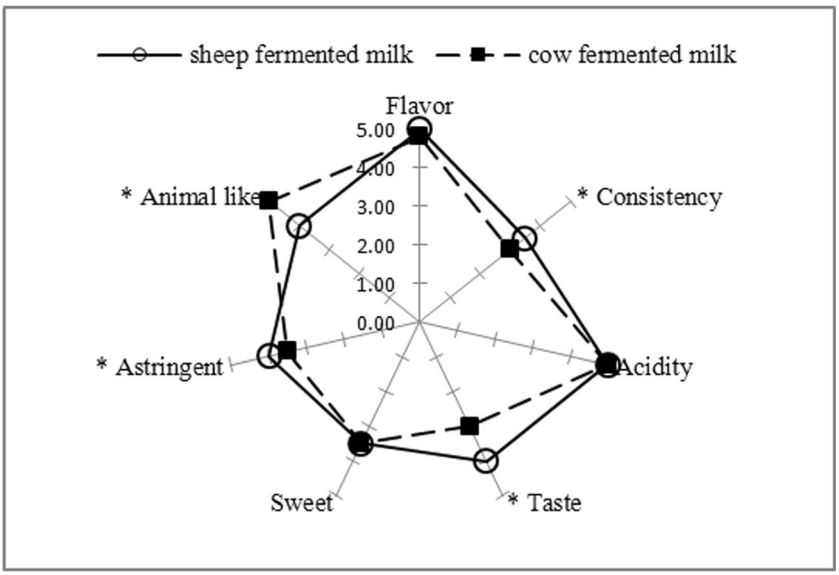

Fig. 1 Sensory attributes of sheep and cow fermented milk at 21 days of cold storage. For each parameter, asterisk denotes significant differences ($P < 0.05$) between sheep and cow fermented milks

concentration 0.3%, *w*/*v*). In this study, *L. paracasei* FS103 in particular showed better tolerance to intestinal juice (pancreatin + bile salts) than gastric juice model (pepsin + pH 2) as already observed by Zhang et al. (2016) on lactobacilli isolated from yak milk cheese.

The ability to inhibit pathogenic bacteria in the host is an important characteristic that probiotic strains should possess to maintain proper balance of gut microflora (Ramasamy et al. 2012). Their antimicrobial activity is often due to the lowered pH, the undissociated organic acids, and production of antimicrobial metabolites, such as hydrogen peroxide and bacteriocins. In this study, the antimicrobial activity of *L. paracasei* strains disappeared when the cell-free culture supernatant was buffered to pH 6 and catalase was added, suggesting that the main mechanism mediating the antimicrobial activity against *E. coli*, *S. enterica*, and *S. aureus* in particular was the lowered pH due to the production of lactic and other organic acids. Several factors may explain the low antimicrobial activity of lactobacilli strains against *L. monocytogenes* such as strain-dependent effects and growth-phase conditions of the pathogenic strain used. Further studies are necessary to understand the behavior of the strains tested.

The outspread of antibiotic-resistant bacteria has been fueled, during the recent years, by the vast use of antibiotics in the food and feed industries. Many studies suggest that the excessive use of antibiotics could turn commensal bacteria into reservoirs of antibiotic-resistant genes; hereafter, bacteria used as probiotics, for humans or animals, should not carry any transferable antibiotic-resistant genes (von Wright 2005). Although lactobacilli display a wide range of natural antibiotic resistance, in most cases not transmissible, as reported for vancomycin antibiotic (Caggia et al. 2015), some *Lactobacillus* strains may transfer their intrinsic antibiotic-resistant genes to the same (or different) species via horizontal gene transfer (Guo et al. 2017). The presence of tetracycline- and erythromycin-resistant genes in *L. paracasei* strains has been previously reported (Comunian et al. 2010) as well as their transfer to different bacterial species (Devirgiliis et al. 2009). In our study, both lactobacilli strains showed susceptibility to most of the tested antibiotics except for kanamycin and gentamycin. In addition, MIC values of both *L. paracasei* strains were lower than microbiological breakpoint defined by FEEDAP (2012). Even though the resistance to aminoglycosides is commonly considered to be intrinsic (i.e., not transferable), further studies should be necessary to assess the potential transferability of kanamycin- and gentamycin-resistant genes. Moreover, a genome-based investigation targeting the presence of possible virulence genes should be desirable to ensure the safe use of *L. paracasei* strains as starter cultures in different food fermentation processes.

In order to be used as a starter culture in fermented milk and to provide health benefits, technological performances of *Lactobacillus strains*, as the ability to acidify milk and the viability during cold storage, should be considered. The acidification trend is a key parameter of the fermentation process, being pH values equal or lower to 4.6 generally considered optimal in yogurt manufacturing (Han et al. 2014). A further increase in acidity during refrigerated storage (i.e., post-acidification) could result in loss of probiotic bacteria (Shah 2000). In this study, different acidifying abilities of *L. paracasei* FS103 on sheep and cow milks during fermentation were observed. In fact, *L. paracasei* FS103 acidified and grew faster in the sheep milk than in cow milk, but in both batches, it showed a greater acidifying activity at the end fermentation period (24 h) compared to probiotic *L. casei* Zhang (Guo et al. 2009).

High viable count of *L. paracasei* FS103 was maintained during the 12 days of storage; after this time, a slight reduction of *Lactobacillus* viability was observed even if the colony-forming units per milliliter remained always higher than 10^8, i.e., the suggested concentration for probiotic bacteria in a product (Shah 2000). Overall, both sheep and cow milks were suitable substrates for lactobacilli survival during cold storage, as already observed by Varga et al. (2014). In addition, sheep fermented milk could be used as carrier because its high fat content may protect the strains through the GIT (Pisano et al. 2008).

Conclusions

This study showed that the autochthonous and potentially probiotic *Lactobacillus paracasei* FS103, isolated from traditional sheep cheese, is able to produce sheep and cow fermented milks characterized by high number of viable lactobacilli during all storage times considered. The setting up of sheep fermented milk with beneficial and autochthonous *L. paracasei*, combining innovation and tradition, could represent a new strategic tool to valorize both new and typical sheep dairy products. Finally, a better awareness about the role of sheep cheese as source of functional and technological *L. paracasei* strains could improve the market of such dairy products and, in addition, increase the growth of dairy food production with specific geographic origins. Certainly, more in-depth investigations are necessary to confirm the technological suitability and probiotic effectiveness of *L. paracasei* FS103 in the manufacturing of different dairy products. Moreover, the transferability of selected antibiotic resistance genes as well as the presence of possible virulence genes in *L. paracasei* FS103 should be ascertained for the safe use of this strain in the food sector.

Acknowledgements The authors are very grateful to Prof. Anna Nudda for valuable comments on the manuscript.

Compliance with ethical standards

The authors declare that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. The authors further confirm that the order of authors listed in the manuscript has been approved by all of us. The authors confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing, we confirm that we have followed the regulations of our institutions concerning intellectual property. The authors understand that the corresponding author is the sole contact for the editorial process. She is responsible for communicating with the other authors about progress, submissions of revisions, and final approval of proofs.

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent N/A

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