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TECHNOLOGICAL CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM SHEEP MILK FOR POTENTIAL USE AS NON-STARTER CULTURES

Caracterização tecnológica de bactérias ácido lácticas isoladas de leite de ovelha para uso potencial como culturas não-iniciadoras

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ABSTRACT

Sheep milk has different physicochemical properties, which means that its derived products have a high added value. Some of these properties are conferred by lactic bacteria which play important roles in the raw milk. This study aimed to analyze five lactic bacteria that were previously identified and evaluated for parameters such as their acidification, proteolysis, diacetyl and exopolysaccharides production, potential antimicrobial activity and safety parameters. The 16S rDNA gene identification via sequencing showed an agreement with the data obtained by MALDI-TOF/MS. The bacteria were identified as *Lactococcus lactis* MRS1, *Lactococcus lactis* MRS2, *Lactococcus lactis* MRS5, *Lactococcus lactis* MRS6, and *Enterococcus faecalis* M173. All isolates showed the same acidification profile, maintaining a pH of 4.5 from 6 h of incubation, under the conditions employed. Proteolytic activity, coexistence capacity, and production of exopolysaccharides were observed in all the isolates tested. Diacetyl production was only evident in the isolates Lactococcus lactis MRS1 and *Lactococcus lactis* MRS2. Regarding the presence of antimicrobial activity, *Lactococcus lactis* MRS6 and Enterococcus faecalis M173 isolates inhibited all tested cultures. In the evaluation of the safety parameters, none of the isolates presented high-level resistance to clinically important antibiotics and did not present gelatinase production and hemolytic activity. These results provide important information on the potential bacteria that could become valuable additives in sheep milk-derived products, in a condition of starters or adjunct cultures.

Keywords: *Lactococcus*; *Enterococcus*; technological properties.

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RESUMO

O leite de ovelha possui diferentes propriedades físico-químicas, o que significa que seus derivados têm alto valor agregado. Parte dessas propriedades é conferida por bactérias lácticas que desempenham importantes atividades no leite cru. Esta pesquisa teve como objetivo analisar cinco bactérias lácticas previamente identificadas e avaliadas quanto a seus parâmetros de acidificação, proteólise, produção de diacetil e exopolissacarídeos, potencial atividade antimicrobiana e parâmetros de segurança. A identificação do gene 16S rDNA por sequenciamento mostrou concordância com os dados obtidos por MALDI-TOF/MS. As bactérias foram identificadas como *Lactococcus lactis* MRS1, *Lactococcus lactis* MRS2, Lactococcus lactis MRS5, *Lactococcus lactis* MRS6 e *Enterococcus faecalis* M173. Todos os isolados apresentaram o mesmo perfil de acidificação, mantendo o pH em 4,5 a partir de 6 horas de incubação, nas condições empregadas. Atividade proteolítica, capacidade de coexistência e produção de exopolissacarídeos foram observadas em todos os isolados testados. A produção de diacetil foi evidente nos isolados *Lactococcus lactis* MRS1 e *Lactococcus lactis* MRS2. Em relação à presença de atividade antimicrobiana, os isolados de *Lactococcus lactis* MRS6 e *Enterococcus faecalis* M173 inibiram todas as culturas testadas. Na avaliação dos parâmetros de segurança, nenhum dos isolados apresentou resistência de alto nível a antibióticos clinicamente importantes e não apresentaram produção de gelatinase e atividade hemolítica. Estes resultados fornecem uma informação importante sobre as potenciais bactérias a serem exploradas para a aplicação em produtos derivados de leite de ovelha, em condições de cultura starter ou adjuntas.

Palavras-chave: *Lactococcus*; *Enterococcus*; propriedades tecnológicas.

INTRODUCTION

At approximately 18 million head, Brazil is considered the eighteenth biggest sheep producer in the world (FAO, 2015). The dairy sheep industry is a young industry in Brazil, so its production and processing remain limited. It is estimated that the country processes around 509,000 liters of sheep milk per year, which corresponds to approximately 526 tons and only 0.0019% of the total milk processed in the country. Between 2004 and 2010, sheep milk production increased by 5.5% per year (ROHENKOHL, 2011). Compared to the composition information of cow milk, sheep milk stands out for its higher total of solid content, nutrients, dry extract, fat and proteins such as casein (MCKUSICK *et al*., 2002; BALTHAZAR *et al*., 2017). These characteristics make it attractive for the elaboration of dairy products and justify its exceptional productivity in cheese making, as well as accentuated sensorial differences and coagulation properties (MCKUSICK *et al*., 2002; BARLOWSKA *et al*., 2011).

Despite the importance for the dairy industry, few studies have focused on autochthonous lactic microbiota in sheep milk. Most of the studies identified lactic acid bacteria (LAB) *Enterococcus*, *Lactococcus*, *Lactobacillus* and *Leuconostoc* as the most prevalent genera (CHANOS; WILLIAMS, 2011; IRANMANESH; EZZATPANAH, 2015). In general, the LAB constitute a heterogeneous group of Gram-positive bacteria, generally non-sporulating, characterized by bacilli or coccus morphology, catalase-negative, aerobic or facultatively anaerobic, acid-tolerant, and

usually producing lactic acid as the major metabolic end-product of carbohydrate fermentation (CARR *et al*., 2002; LIU *et al*., 2011).

This group of bacteria has a high biotechnological potential in food production, especially in fermented dairy products due to their ability to produce desirable substances, that can be used for both a technological point of view as well as for food safety studies (LIU *et al*., 2011; GÄNZLE, 2015). In this context, this research aimed to identify and describe BAL from sheep milk and evaluate its technological properties, presenting new cultures with potential to enhance dairy sheep industry development.

MATERIALS AND METHODS

Origin, identification and selection of lactic acid bacteria (LAB)

Twenty-four bacteria previously isolated from sheep milk from Cabanha Dedo Verde (Viamão, RS, Brazil), belonging to the Microbiology Laboratory – ICBS/UFRGS collection, were studied. These bacteria were grown in Man Rogosa and Sharpe medium (MRS) (Oxoid Ltd., Basingstoke, England) as well as M17 (Oxoid) and stored in 20% (v/v) glycerol at -20 °C. For the identification and selection as LAB, microorganisms were analyzed for their morphotinorial and biochemical properties through Gram staining and by the catalase test, respectively. Only cultures that presented Gram-positive and catalase negative, characteristics common to BAL, were selected.

Identification of LAB by Matrix Associated Laser Desorption-Ionization – Time of Flight (MALDI-TOF/MS)

The MALDI-TOF/MS was used to identify cultures selected by the parameters previously described (SAUGET *et al*., 2016). Briefly, an MRS agar (Oxoid) culture was used, and a colony of each isolate was inoculated into 1.5 mL microtubes, with 300 µL of Milli-Q water and 900 µL of absolute ethanol. Subsequently, the samples were centrifuged at a speed of ≥ 13,000 rpm for 2 min., the supernatant was discarded, and the pellet dried at room temperature. Then 70% formic acid (30 µL) and pure acetonitrile (30 µL) were added to the pellet, followed by vortex homogenization and a new centrifugation step for 2 min. at a speed ≥ 13,000 rpm. All material was collected in a pellet. Then, 1 µL of supernatant was pipetted onto a stainless-steel plate and dried at room temperature, followed by addition of 1 µL of a matrix consisting of α-cyano-4-hydroxycinnamic acid solution (HCCA). The analyses were performed by MALDI-Biotyper (MBT OC) 4.0 software, at the Institute of Basic Health Sciences of the Federal University of Rio Grande do Sul.

Identification of LAB by 16S rDNA sequencing

DNA extraction from selected isolates was carried out, and a polymerase chain reaction (PCR) amplification of 16S rDNA was performed in an Applied Biosystems Thermal Cycler 2720 (Thermo Scientific, Madison, WI, USA) followed by sequencing bases. The PCR primers were FC27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R530 (5'-CCGCGGCTGCTGGCACGTA-3') (GONTANG *et al*., 2007). The reaction was carried out totalizing 25 µL, with 100 ng of DNA, 1.5 mM of magnesium chloride ($MgCl_2$, Quatro G, Pesquisa e Desenvolvimento, Porto Alegre, RS, Brazil), 0.4 µM of each primer (Invitrogen, Life Technologies, Carlsbad, California, USA), 1 U Taq DNA polymerase (Quatro G), 1 X PCR buffer (Quatro G), 200 µM dNTP (Ludwig Biotecnologia, Alvorada, RS, Brazil), and miliQ sterile water to complete the reaction volume.

Cycling conditions consisted of an initial 5 min. denaturation step at 94 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 58 °C, 1 min at 72 °C, and a final 10 min extension step at 72 °C. The sequencing of samples was performed at ACTGene Análises Moleculares (Biotechnology Center, UFRGS, Porto Alegre, RS, Brazil) using the ABI-Prism 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing data were collected using the Data Collection 2 program (Applied Biosystems). The results were analyzed by the Chromas software version 2.6.4 (Technelysium Pty Ltd South Brisbane, QLD, Australia) and compared to the database of the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/).

Acidification capacity

Aliquots (1% v/v) of revitalized strains were transferred to tubes containing MRS broth (Oxoid), whole and skimmed UHT milk (Nestlé, São Paulo, SP, Brazil), and incubated at 30 °C. The acidification capacity was determined by means of pH evaluation at time 0 and after 6, 12, 24 and 48 h of incubation, using a pH test tape (RIBEIRO *et al*., 2014). Data were expressed as the mean of duplicate.

Proteolytic activity

Proteolytic activity was determined following the method of Franciosi *et al*. (2009). Two microliters of revitalized strains were spotted onto the surface of a Plate Count Agar (PCA, HiMedia, Mumbai, MH, India) composed of 10% of skim milk (w/v, Nestlé) and incubated at 30 °C for 4 days. As a positive control, the strain *Pseudomonas aeruginosa* ATCC 27853 was used and the experiment was carried out in triplicate. Proteolytic activity was indicated by a clear zone around the colonies.

Diacetyl production

For diacetyl production, revitalized strains (1% v/v) were inoculated in 10 mL of skim milk (10% w/v, Nestlé) and incubated at 30 °C for 24 h. Then, 1 mL of sample was combined with 0.5 mL of α-naphthol (1% w/v, Merck Millipore, Darmstadt, HE, Germany) and potassium hydroxide (KOH, 16% w/v, Grupo Química Industrial, Rio de Janeiro, RJ, Brazil) and incubated at 30 °C for 10 min. The diacetyl production was indicated by the formation of a red ring at the top of the tubes (FRANCIOSI *et al*., 2009).

Production of exopolysaccharides (EPS)

The strains of LAB were tested for the production of EPS by the agar method with red congo (FREEMAN *et al*., 1989). Strains were plated by streaking and incubated at 37 °C for 24 h. As a positive control, the *Staphylococcus epidermidis* ATCC 35984 strain was used and the production of EPS was indicated by the black coloration of the colonies with a crystalline appearance.

Coexistence among isolates of LAB

To analyze the coexistence capacity between the isolates, the bacteria were seeded on plates containing MRS agar (Oxoid). Each LAB isolate was individually seeded in a single line at the center and afterward, the other isolates were seeded perpendicularly at an angle of 90° to the central isolate. The antagonism was verified through the formation of a zone of inhibition between the isolates and/or retraction in the bacterial growth (GUO *et al.*, 2010).

Evaluation of the production of antimicrobial activity by LAB

The antimicrobial activity was per-

formed according to the *spot-on-the-lawn* test (LEWUS et al., 1991), with modifications. Each BAL isolate was seeded in duplicate as a bite on plates containing MRS agar (Oxoid) and incubated at 30 °C for 24 h. The indicator microorganisms *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644 and *Pseudomonas aeruginosa* ATCC 27853 were inoculated into Tryptone Soy Broth (TSB, Oxoid) and incubated at 37 °C for 24 h. Subsequently, plates were overcoated with an overlay of semi-solid TSB agar (0.6% agar, Oxoid) containing 1% (v/v) of an indicator culture with a concentration of approximately 10^5 CFU/mL. The plates were incubated at 37 ºC for 24 h and the antimicrobial activity of the LAB against the indicator microorganisms was verified by the formation of inhibition halos around the bite of the LAB and measurement of the diameter in millimeters (mm).

Safety Parameters

The susceptibility profile of LAB isolates was evaluated for the following antibiotics: amoxicillin + clavulanic acid, ampicillin, cephalexin, cefotaxime, chloramphenicol, erythromycin, streptomycin, gentamicin, rifampicin, tetracycline, and vancomycin. The BAL cell suspension was adjusted to the value of 0.5 of the McFarland scale and seeded with a sterile swab on plates containing Muller-Hinton Agar (Oxoid). Then, disks with each antimicrobial were placed on the surface of the plates and incubated at 37 °C for 48 h. To evaluate the gelatinase production, the cultures were inoculated with a needle into tubes containing gelatin agar (Oxoid) and incubated at 37 °C for 48 h. The tubes were considered positive when the liquefaction of the medium was verified. As a positive control, the bacterium *Pseudomonas aeruginosa* ATCC 27853 was used. Hemolysis of erythrocytes was observed on Columbia agar (Oxoid) plates supplemented with 5% sheep blood, where the bacterial suspensions were scored and incubated for 24 h at 37 °C. After this time, plaques were analyzed for hemolysis, and *Staphylococcus aureus* ATCC 25938 strain was used as a positive control.

RESULTS AND DISCUSSION

Identification of lactic acid bacteria by mass spectrometry and 16S rDNA sequencing

From the 24 isolates derived from sheep milk, five were characterized as LAB, Gram-positive cocci and catalase negative. Relative to the identification by MALDI-TOF, it was used the predicted scores as parameters to infer the species or genera identification of the isolates previously selected by biochemical and morphological analysis. This methodology states that scores between 0.000 and 1.699 indicate an unreliable identification, between 1,700 and 1,999 as a probable identification of genera, scores between 2,000 and 2,299 as a safe identification at the genus level and a score between 2,300 and 3,000 indicates a reliable level for species identification. The analyzed bacteria presented the scores listed in Table 1.

Due to the score presented by M173 isolate (2.376), it can be inferred that its description at species level is reliable, allowing its identification as *E. faecalis*. The MRS1, MRS2, and MRS5 isolates presented safe scores at the genera level, all belonging to the *Lactococcus* genus and probably belonging to the lactis species. For the MRS6 isolate, the score obtained indicates a likely genus, possibly *Lactococcus*. Using MALDI-TOF/MS methodology, García-Cayuela *et al*. (2017) identified *L. lactis* strains in samples of traditional cheeses made of raw goat and sheep milk. This reinforces the use of this technique as a notable tool for the identification of *L. lactis* species and subspecies.

The identification of isolates by amplifying 16S rDNA (Table 1) confirmed the results at the genera and species levels obtained via MALDI-TOF/MS. Considering the results, the most frequent genus obtained were *Lactococcus*. Descriptions of *L. lactis* and *E. faecalis* in sheep milk have also been presented by Medina *et al*. (2001), Delavenne *et al*. (2012), Iranmanesh and Ezzatpanah (2015), and Kopčáková *et al*. (2018).

Acidification capacity

The strains were tested for acidification capacity in MRS broth, whole and skimmed UHT milk at 0, 6, 12, 24 and 48 h. The results showed the same profile for all isolates, maintaining pH at 4.5 after 6 h of incubation. Rapid pH drop is very important during cheese production, contributing to changes in cheese texture and for the control of undesirable microorganisms. According to Beresford *et al*. (2001), the starter cultures should be capable of reducing milk pH to 5.3 or less after 6 h at 30-37 °C. Studies have shown that most of the strains of BAL are initially slow in acid production (FRANCIOSI *et al*., 2009; MORANDI *et al*., 2011; DAL BELLO *et al*., 2012). However, in the present study, all isolates produced high levels of acidification after 6 h. Data presented by Perin *et al*. (2017) from isolates of raw goat milk, indicate a higher pH reduction in *Lactococcus* when compared to *Enterococcus* strains, showing high acidification profiles in milk after 6 h for *Lactococcus* and 24 hrs for *Enterococcus*.

Proteolytic activity, diacetyl and exopolysaccharides production

The proteolytic activity was tested for all five isolates through observation of an opaque zone around the isolates in milk agar plates and variations in the diameter (mm) of halos. The activity was observed in all isolates (Table 2). These results are in agreement with those reported by Tulini *et al*. (2016), in which strains of *L. lactis* were isolated from buffalo's milk and cheese with cow's milk and goat's milk, as well as strains of *E. faecalis* isolated from cow and buffalo's milk showed proteolytic characteristics. Perin *et al*. (2017) found proteolytic activity in *E. faecalis* and *L. lactis* strains, where a more proteolytic activity of *E. faecalis* was observed, due to the incidence of larger proteolytic halos when compared to *L. lactis*. Proteolytic activity is an essential property for starter cultures. The proteolytic system of LAB favors growth in milk to degrade caseins and peptides, aroma precursors and taste formation in the fermentation of dairy products and maturation of cheeses (TULINI *et al*., 2016).

The production of diacetyl was verified

Table 1 – The relation between bacterial isolates identified by MALDI-TOF/MS and 16S rDNA sequencing

	MALDI-TOF/MS		16S rDNA sequencing	
Isolate	Genera/species	Scores	Genera/species	Identification number[a]
MRS1	*Lactococcus lactis*	2.214	*Lactococcus lactis*	MH681536
MRS2	*Lactococcus lactis*	2.165	*Lactococcus lactis*	MH681537
MRS5	*Lactococcus lactis*	2.042	*Lactococcus lactis*	MH681538
MRS6	*Lactococcus lactis*	1.891	*Lactococcus lactis*	MH681539
M173	*Enterococcus faecalis*	2.376	*Enterococcus faecalis*	MH681540

[a] GenBank accession number for nucleotide sequence.

Table 2 – Technological characteristics attributed to LABs isolated from sheep milk

Isolate	Proteolytic activity[a]	Diacetyl production[b]	EPS production[b]
L. lactis MRS1	14,67	+	+
L. lactis MRS2	16,34	+	+
L. lactis MRS5	22,34	-	+
L. lactis MRS6	21,00	-	+
E. faecalis M173	16,34	-	+

[a] halos diameter (mm).
[b] (+) positive result; (-) negative result.

in the isolates *L. lactis* MRS1 and MRS2 (Table 2), with this characteristic commonly presented in *L. lactis* isolates (FRANCIOSI *et al*., 2009; DAL BELLO *et al*., 2012; DOMINGOS-LOPES *et al*., 2017; PERIN *et al*., 2017). The production of diacetyl is directly related to this type of strain. Since not all LABs have the capacity to metabolize the citrate, this behavior differs between species and subspecies. Diacetyl is a volatile compound generated as the final product in the conversion of citrate to pyruvate. It confers a buttery aroma to fermented dairy products and it is a common feature for LAB, such as *L. lactis* subsp. *lactis* biovar *diacetylactis* and some strains of *Enterococcus* (KEMPLER; MCKAY, 1981; SMIT *et al*., 2005; FRANCIOSI *et al*., 2009).

The production of EPS by LABs is an important characteristic for modifying the texture and viscosity of dairy products. In the present study, all the isolates presented black and crystalline colonies, indicating that they were positive for the EPS production (Table 2). Ribeiro *et al*. (2014) and Perin *et al*. (2017) did not verify the production of EPS for *L. lactis* and *E. faecalis* strains. Costa *et al*. (2010) found that the application of *L. lactis* strains producing EPS in Cheddar cheese resulted in a higher yield in the production, without negatively affecting the taste of the cheese. Also, favorable texture characteristics were attributed to the cheese, being described as soft, whereas cheeses in the absence of EPS were described as brittle. In this way, the generation of EPS is considered an important resource for LABs that are applied to dairy products, since soft and creamy products have considerable appeal to consumers. Therefore, this is a relevant feature in the selection of initial strains and secondary cultures (CAGGIANIELLO *et al*., 2016; LYNCH *et al*., 2018).

Coexistence among isolates of LAB

The results demonstrated that all LAB isolates can coexist without interfering in the development of each other. This is an important feature, since it allows the application of different isolates in foods, providing distinct and desirable characteristics, enabling the improvement of a technological performance. Ribeiro *et al*. (2014) demonstrated that BALs from artisanal Pico cheese can coexist, but *L. lactis* inhibited the growth of all *Enterococcus* isolates. Observations by Leroy and De Vuyst (2004) suggest that the main application of LAB is as pure or mixed starter cultures. The production of fermented foods, especially dairy products, is based on the use of these cultures which initiate the rapid acidification of the raw material. They may also contribute to the flavor, texture and nutritional value of fermented foods and therefore can be used as adjunct cultures.

Table 3 – Halos diameter (mm) of LAB inhibition against indicator cultures. The data are present as means

Isolate	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*L. monocytogenes* ATCC 7644	*S. aureus* ATCC 25923
L. lactis MRS1	3.0	0	0	0
L. lactis MRS2	8.5	0	0	10
L. lactis MRS5	11.5	0	9	8
L. lactis MRS6	21.5	20	11	12.5
E. faecalis M173	26.5	21	10	9

Evaluation of the production of antimicrobial activity by LAB

In the evaluation of the antimicrobial activity, *L. lactis* MRS6 and *E. faecalis* M173 showed activity in all tested indicator cultures (Table 3). All the *L. lactis* MRS1, MRS2 and MRS5 strains presented activity against *E. coli* ATCC 25922. Inhibition against *L. monocytogenes* ATCC 7644 was observed by *L. lactis* MRS5 strain, as well as, *S. aureus* ATCC 25923 inhibition by *L. lactis* MRS2 and MRS5. Variations in the halos diameters can be justified by the diversity and variety of antimicrobial substances that can be produced. These compounds can act by competitive exclusion and synthesis of antagonistic substances such as organic acids, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, and bacteriocins (GÁLVEZ *et al*., 2007; BALI *et al*., 2016).

These results demonstrate a potential application of the analyzed strains as bioprotective cultures. Inhibition of pathogenic bacteria by LAB is a positive feature since LAB starter cultures could confer an aspect of functionality, which contribute to a microbial safety or offer organoleptic, technological, nutritional, or health advantages (LEROY; DE VUYST, 2004; BINTSIS, 2018).

Safety Parameters

Regarding antibiotic susceptibility profile, the LAB isolates showed different sensibility to the antibiotics tested (Table 4). All isolates were sensitive to amoxicillin + clavulanic acid, ampicillin, cephalexin, cefotaxime, chloramphenicol, erythromycin, streptomycin and gentamicin. However, the *L. lactis* MRS1 and MRS2 isolates were resistant to rifampicin, and the *L. lactis* MRS5 and MRS6 isolates presented intermediate resistance to this antibiotic. Relative to tetracycline, *L. lactis* MRS1 and *E. faecalis* M173 isolates demonstrated intermediate resistance. This was also observed with *L. lactis* MRS5, however, relative to Vancomycin. Dal Bello *et al*. (2012) also found *L. lactis* isolates susceptible to chloramphenicol, gentamicin, rifampicin, and vancomycin. Domingos-Lopes *et al*. (2017) reported the resistance profile of enterococcal strains to chloramphenicol, cefotaxime, tetracycline, rifampicin, erythromycin, and chloramphenicol. In contrast, resistance to clinically important antibiotics, such as amoxicillin/clavulanic acid and vancomycin, has not been found.

These results point to antibiotic-resistance by LAB isolates of dairy origin, which can be attributed, in part, to the extensive use of these compounds in animal's

Table 4 – Antibiotic susceptibility profile of LABs isolated from sheep milk. The data are presented as the mean of inhibition halos (mm)

Antibiotic	*L. lactis* MRS1	*L. lactis* MRS2	*L. lactis* MRS5	*L. lactis* MRS6	*E. faecalis* M173	R[a]	S[b]
Amoxicillin + ac. Clavulanic	62	42	42	38	61	≤ 13	≥ 18
Ampicillin	44	40	38	38	44	≤ 13	≥ 17
Cephalexin	44	36	34	38	30	≤ 14	≥ 18
Cefotaxime	56	40	38	43	44	≤ 22	≥ 26
Chloramphenicol	36	36	30	32	44	≤ 12	≥ 18
Erythromycin	40	40	35	38	38	≤ 13	≥ 23
Streptomycin	24	26	22	24	17	≤ 11	≥ 15
Gentamicin	30	35	33	32	26	≤ 12	≥ 15
Rifampicin	16	15	17	17	32	≤ 16	≥ 20
Tetracycline	18	40	49	31	18	≤ 14	≥ 19
Vancomycin	24	26	16	24	30	≤ 14	≥ 17

[a] (R) resistant.
[b] (S) sensitive.
Source: SENSIFAR AND MULTIFAR-CEFAR®, 2015.

and human's care in recent years. Although resistance to antibiotics per se is not harmful, the spread of antibiotic resistant genes to other organisms is one of the most important safety issues. Concerning the use of bacteria in a variety of food products, the food chain is considered one of the main routes for the transmission of antibiotic-resistant bacteria (PESAVENTO *et al.*, 2014; SHARMA *et al*., 2014). In general, due to food safety reasons, the absence of antibiotic-resistance in LAB is of great importance for its use as starter cultures or co-cultures (FRANCIOSI *et al.*, 2009; SHARMA *et al*., 2014).

Regarding the results of hemolytic activity and gelatinase production, all LAB isolates showed negative results. Relative to hemolytic activity, Ribeiro *et al*. (2014) also found negative results in *L. lactis* and *E. faecalis* isolates. The inability to produce gelatinase is gratifying since gelatinase is a proteolytic enzyme mainly associated with *Enterococcus* and may be related to virulence profile. The degradation of the host tissue by the bacteria may provide nutrients for their survival and also contribute to the formation of biofilm (FRANZ *et al.*, 2011).

CONCLUSIONS

The technological characterization of *L. lactis* and *E. faecalis* isolates from sheep milk showed a favorable potential to using them in the industry as a starter culture. A variability of technologically relevant characteristics was found among the isolates and may be the basis for the selection of specific strains to be used as starter or adjunct cultures. Selected isolates with acidifying ability, proteolytic activity, diacetyl and exopolysaccharides production have the potential to be used as adjuvant cultures in the manufacture of cheeses. In addition, the cultures meet safety criteria, since no isolates presented high resistance to clinically important antibiotics and did not present gelatinase production and hemolytic activity. Technological characteristics associated with LABs isolated from sheep milk are little reported in the literature, which highlights the importance of the present study,

showing that these isolates are promising candidates for application as adjuvant cultures in cheese production.

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