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Isolation, enumeration, molecular identification and probiotic potential evaluation of lactic acid bacteria isolated from sheep milk

[*Isolamento, enumeração, identificação molecular e avaliação do potencial probiótico de bactérias ácido-lácticas do leite de ovelha*]

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RESUMO

Espécies de bactérias ácido-lácticas foram identificadas em nível molecular em leite das raças ovinas Lacaune, Santa Inês e suas mestiças, e o seu potencial probiótico *in vitro* foi avaliado. As espécies identificadas foram *Enterococcus faecium* (56,25%), *E. durans* (31,25%) e *E. casseliflavus* (12,5%). Nenhuma outra espécie de bactéria ácido-láctica, como *Lactobacillus* sp., foi identificada. A maioria dos enterococos isolados foi resistente ao pH gástrico (2.0) e a 0,3% de oxgall. Todos os enterococos testados foram resistentes à ceftazidima, oxacilina e estreptomicina e sensíveis à clindamicina, eritromicina e penicilina. A resistência à ciprofloxacina, gentamicina, tetraciclina e vancomicina variou entre as amostras. Todos os enterococos testados inibiram fortemente (P<0,05) *Escherichia coli* e *Listeria monocytogenes*, inibiram moderadamente *E. faecalis* e *Staphylococcus aureus* e não inibiram *Pseudomonas aeruginosa*, *Salmonella enterica* var. Typhimurium e uma amostra de *E. durans* isolada de leite de ovelha. Quatro amostras de *E. faecium*, uma de *E. durans* e uma de *E. casseliflavus* apresentaram o melhor potencial probiótico.

Palavras-chave: leite de ovelha, enterococos, potencial probiótico

ABSTRACT

Lactic acid bacteria species were molecularly identified in milk from Lacaune, Santa Inês and crossbred sheep breeds and their in vitro *probiotic potential was evaluated. The species identified were* Enterococcus faecium (56.25%), E. durans *(31.25%) and* E. casseliflavus *(12.5%). No other lactic acid bacteria species, such as lactobacilli, was identified. Most of the isolated enterococci were resistant to gastric pH (2.0) and to 0.3% oxgall. All tested enterococci were resistant to ceftazidime, oxacillin and streptomycin and sensible to clindamycin, erythromycin and penicillin. The resistance to ciprofloxacin, gentamicin, tetracycline and vancomycin varied among tested species. All tested enterococci strongly inhibited (P<0.05)* Escherichia coli *and* Listeria monocytogenes, *moderately inhibited* E. faecalis *and* Staphylococcus aureus *and did not inhibit* Pseudomonas aeruginosa, Salmonella enterica *var. Typhimurium and also one* E. durans *sample isolated from sheep milk. Four samples of* E. faecium, *one of* E. durans *and one of* E. casseliflavus *presented the best probiotic potential.*

Keywords: sheep milk, enterococci, probiotic potential

INTRODUCTION

Sheep milk represents only 1.3% of total global milk production, but is mostly used for cheese making, such as Roquefort, Pecorino and Serra da Estrela, with a better yield and higher value-added since its solids ratio is higher than other ruminant milks (Tsakalidou and Odos, 2012).

It is important to determine the sheep milk microbiota in order to understand the transformations that occur in cheese through its maturation. Studies from Abeijón *et al.* (2006)

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and Madrau *et al*. (2006) found predominance of the *Enterococcus* genus in sheep milk and determined their role in proteolysis during cheese maturation. Despite of that, there is not a focus on the probiotic potential of these microorganisms, such as their ability to resist gastric acid and biliary salts in order to reach their final destination – small or large intestine - in viable counts, and their antagonistic potential against important pathogenic microorganisms in the gastrointestinal tract (Silva *et al*., 2013). It is also important to evaluate antimicrobial susceptibility regarding probiotic properties because of the possible transference of antimicrobial resistance to pathogenic microorganisms from probiotic microorganisms (Lund and Edlund, 2001). Probiotic products are mainly represented by dairy products such as yogurts and fermented milks (Maragkoudakis *et al*., 2006).

Thus, the objective of this study was to determine the probiotic potential of microorganisms isolated from sheep milk for possible future use as probiotics in several dairies improving flavor and safety.

MATERIAL AND METHODS

Sheep milk was obtained at a small farm situated near Jaboticatubas city, Minas Gerais state, Brazil. Twenty samples were obtained from Lacaune, Santa Inês (a Brazilian breed) and their crossbreds. Dilutions were made up to 10^{-5} and were spread into Petri dishes containing MRS (*Man Rogosa and Sharpe*, *Difco*, USA) agar – incubated for 96h at 37^0C - and M17 (*Difco*) agar – incubated for 96h at 32^0C. Enumeration was made in Petri dishes containing from 20 to 200 CFU. Morphologically distinct colonies were submitted to Gram test, and the ones that had rod or round shape and were Gram positive were selected for further identification (adapted from IDF, 1983).

DNA from selected cultures was first obtained through treatment with LiCl (1M) for pellets obtained from each activated culture.Then they were incubated at 37^0C with constant mixing for 1h. New pellets were obtained and suspended in 1mL of protoplast buffer (50mM Tris HCl pH 8.0; 10mM EDTA and 10mg de lysozyme mL^{-1}) with incubation at 37^0C with constant mixing for 1h. Total DNA was finally obtained with Wizard SV Genomic and DNA Purification System (*Promega*, USA), following the manufacturer indication.

All DNA samples were submitted to PCR reaction according to Tisala-Timisjarvi and Alatossava (1997). Primer 27F (5' AGAGTTTGATCCTGGCTCAG 3') was used as forward and 1492R (5' GGTTACCTTGTTACGACTT 3') as reverse to amplify the 16S rDNA gene of each sample. The program used was: 95^0C for the first 150sec, 35 cycles of 94^0C for 30sec, 55^0C for 60sec, 72^0C for 60sec and finally 72^0C for 10min, according to Moreira *et al.* (2005). Each sample was purified by Wizard SV Gel and PCR Clean-up System (*Promega*), according the manufacturer's instructions.

Each 16S rDNA sample was sequenced through Sanger's method with MegaBace 1000 (*GE HealthCare*, UK) according to Reysenbach *et al.* (2000). Results were submitted to the BLAST algorithm from GenBank located on the NCBI website.

Selected microorganisms were activated twice – 24h at 37^oC – in 5mL of broth similar to the original solid medium that they grew in. Each sample was incubated at 37^oC in the presence of gastric juice (0.85% NaCl, pH 2.0) for 3h and distributed (2% v/v) in three wells in a 96-well ELISA plate containing 0.2mL of pure MRS (*Difco*) broth each. The control of each sample was done with incubation at 37^0C for 3h in the presence of saline (0.85% NaCl) pH 7.0. They were alsodistributed (2% v/v) in three other wells containing 0.2mL of pure MRS broth each. Each plate containing 15 samples was incubated at 37^oC for 18h. Absorbance at 620_{nm} was read every 30min on a Spectramax 340 spectrophotometer (*Molecular Devices*, USA). Using Origin 8.5 (*OriginLab*, USA), differences in growth curve areas for each sample – control and in the presence of gastric juice – were calculated and the percentage of *in vitro* inhibition by gastric juice was obtained. This procedure was adapted from Walker and Gilliland (1993). Two repetitions were made.

Selected microorganisms were activated two times (24h at 37^0C) in 5mL of the broth similar to the original solid medium that they grew in.

Each sample was distributed (2% v/v) in three wells in a 96-well ELISA plate containing 0.2mL of pure MRS (*Difco*) broth each, and in three wells containing 0.2mLof MRS (*Difco*) broth with 0.3% oxgall (*Difco*) each. Each plate containing 15 samples was incubated at 37°C for 18h. Absorbance at 620_{nm} was read every 30min on a Spectramax 340 spectrophotometer (*Molecular Devices*). Using Origin 8.5 (*OriginLab*), differences in growth curve areas for each sample (control and in the presence of 0.3% oxgall – *Difco*) were calculated and the percentage of *in vitro* inhibition by biliary salts was obtained (adapted from Walker and Gilliland, 1993). Two repetitions were made.

Selected microorganisms were activated once – 24h at 37°C – in 5mL of brothand then in agar similar to the original solid medium that they grew in. Then, each microorganism was transferred to 3.5mL of saline (0.85% NaCl) until turbidity equal to 0.5 on McFarland scale was obtained. Using a swab, each microorganism was transferred to a Petri dish containing MRS (*Difco*) agar. Antimicrobial discs (*Oxoid*, UK) were equally distributed on the surface of the agar. The antimicrobials used were: penicillin (PEN, 10U), oxacillin (OX, 1μg), vancomycin (VAN, 30μg), ceftazidime (CAZ, 30μg), streptomycin (S, 30μg), clindamycin (DA, 2μg), erythromycin (E, 5μg), ciprofloxacin (CIP, 5μg), gentamicin (CN, 10μg), and tetracycline (TE, 30μg). Each dish was incubated at 37°C for 48h. Quality control was done using *Escherichia coli* ATCC 25922. The inhibition halos were measured with a Mitutoyo digital paquimeter (*Mitutoyo*, Brazil). This procedure was executed in duplicate with three repetitions and was adapted from Charteris (1998).

Selected microorganisms were activated twice – 24h at 37°C – in 5mL of broth similar to the agar that they originally grew in. A 5μL spot was made from each activated microorganism in the center of a Petri dish containing MRS (*Difco*) agar. Each Petri dish was incubated for 48h at 37°C. Then, 1mL of chloroform was added to the cover of each Petri dish and left to rest for 30min under UV. Another 30min with the cover open were needed to evaporate the chloroform. The revealing microorganisms were then added onto the surface of the former dishes through 7.5μL of their recent culture in 3.5mL of semi-solid BHI (*Difco*) for *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 15313, *Pseudomonas aeruginosa* ATCC 28853, *Salmonella enterica* var. Typhimurium ATTCC 14028 and *Staphylococcus aureus* ATCC 29213; or semi-solid MRS (*Difco*) agar for *Enterococcus durans* sample 23 isolated from sheep milk in the present study. Each dish was incubated for 48h at 37°C. The inhibition halos of each microorganism against the revealing microorganisms were measured with a Mitutoyo digital paquimeter (*Mitutoyo*). This procedure was executed in duplicate with three repetitions and was adapted from Tagg *et al*. (1976). The data obtained was analyzed using the Kruskal-Wallis test, since results from this kind of test usually present an abnormal behavior, and the level of significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Using Sanger's method of sequencing the 16srDNA gene from microorganisms isolated from sheep milk, the following species were identified: nine (56.25%) out of 16 identified species were *Enterococcus faecium*, five (31.25%) were *Enterococcus durans* and two (12.5%) were *Enterococcus casseliflavus* (Table 1).

Medina *et al*. (2001), when identifying microorganisms isolated from sheep milk, also found a high percentage (48%) of bacteria from the *Enterococcus* genus. In a similar study, a lower (33%), but also significant percentage of bacteria from the *Enterococcus* genus was found in sheep milk (Oksuztepe *et al*., 2005).

According to Gilliland *et al*. (1984), when a 0.3 absorbance is achieved after at least 2h of incubation at 37°C in presence of gastric pH between 1.5 and 4.0, a microorganism can be considered tolerant or resistant to gastric pH. Considering this, 13 (81.25%) out of 16 samples tested can be considered tolerant to gastric pH (Table 2).

Table 1. *Enterococcus* spp. isolated from sheep milk identified by the sequencing of 16S rDNA gene using Sanger's method

Sheep (Breed)	Sample	Species
1433 (half-blood)	3.1	*E. faecium*
L04 (Lacaune)	4.2	*E. faecium*
1516 (half-blood)	5	*E. durans*
L09 (Lacaune)	6.2	*E. faecium*
L09 (Lacaune)	6.4	*E. faecium*
194 (half-blood)	8.2	*E. durans*
1448 (half-blood)	10.3	*E. casseliflavus*
81 (Santa Inês)	12.1	*E. faecium*
81 (Santa Inês)	12.2	*E. faecium*
102 (half-blood)	14.1	*E. faecium*
102 (half-blood)	14.3	*E. casseliflavus*
102 (half-blood)	14.4	*E. faecium*
1469 (half-blood)	16	*E. durans*
300 (half-blood)	20.4	*E. durans*
300 (half-blood)	20.5	*E. faecium*
1508 (half-blood)	23	*E. durans*

Table 2. Maximum absorbance achieved by enterococci samples isolated from sheep milk after 3h incubation at 37^0C in the presence of gastric pH (2.0) and subsequent 18h incubation at 37^0C in plain MRS broth

Sheep (Breed)	Sample (Species)	Maximum Absorbance
81 (Santa Inês)	12.1 (*E. faecium*)	0.987
L09 (Lacaune)	6.2 (*E. faecium*)	0.986
102 (half-blood)	14.1 (*E.faecium*)	0.966
300 (half-blood)	20.5 (*E.faecium*)	0.945
81 (Santa Inês)	12.2 (*E.faecium*)	0.944
300 (half-blood)	20.4 (*E. durans*)	0.933
1469 (half-blood)	16 (*E. durans*)	0.891
1516 (half-blood)	5 (*E. durans*)	0.699
102 (half-blood)	14.3 (*E. casseliflavus*)	0.493
L09 (Lacaune)	6.4 (*E. faecium*)	0.366
1433 (half-blood)	3.1 (*E. faecium*)	0.938
102 (half-blood)	14.4 (*E. faecium*)	0.324
L04 (Lacaune)	4.2 (*E. faecium*)	0.320
194 (half-blood)	8.2 (*E. durans*)	0.261
1448 (half-blood)	10.3 (*E. casseliflavus*)	0.223
1508 (half-blood)	23 (*E. durans*)	0.166

Morandi *et al.* (2005) found tolerance to acid pH from *E. faecium* samples such as what was found in the present work.

Tolerance to gastric juice was also considered for samples that achieved less than 40% of inhibition, therefore, 12 (75%) out of 16 samples were considered tolerant (Tab. 3). Sample 10.3 was not considered tolerant because it did not achieve a 0.3 absorbance (Table2).

In a similar study, a 38.5% inhibition by gastric juice from *E. durans* and 56.8% from *E. faecium* samples were observed, confirming the resistance to gastric pH by enterococci samples observed in the present study (Cueto-Vigil *et al*., 2010).

Table 3. Inhibition percentage by gastric pH (2.0) of 16 enterococcisamples isolated from sheep milk

Sheep (Breed)	Sample (Species)	Inhibition (%)
81 (Santa Inês)	12.1 (*E. faecium*)	0.00
102 (half-blood)	14.1 (*E. faecium*)	0.00
300 (half-blood)	20.4 (*E. durans*)	0.73
300 (half-blood)	20.5 (*E. faecium*)	2.08
81 (Santa Inês)	12.2 (*E. faecium*)	5.66
1469 (half-blood)	16 (*E. durans*)	7.26
L09 (Lacaune)	6.4 (*E.faecium*)	16.01
L04 (Lacaune)	4.2 (*E. faecium*)	19.74
1433 (half-blood)	3.1 (*E. faecium*)	26.29
1448 (half-blood)	10.3 (*E. casseliflavus*)	26.75
L09 (Lacaune)	6.2 (*E. faecium*)	31.97
1516 (half-blood)	5 (*E. durans*)	36.00
102 (half-blood)	14.3 (*E. casseliflavus*)	39.60
194 (half-blood)	8.2 (*E. durans*)	42.42
102 (half-blood)	14.4 (*E. faecium*)	57.62
1508 (half-blood)	23 (*E. durans*)	75.28

According to Gilliland *et al*. (1984), when 0.3 of absorbance is achieved after at least 8h of incubation at 37^0C in presence of 0.3% oxgall, a microorganism can be considered tolerant or resistant to biliary salts. Considering this, 11 (68.75%) out of 16 samples tested can be considered tolerant to biliary salts (Table 4).

Table 4. Maximum absorbance achieved by enterococci samples isolated from sheep milk after 18h incubation at 37^0C in presence of 0.3% of oxgall

Sheep (Breed)	Sample (Species)	Maximum Absorbance
1433 (half-blood)	3.1 (*E. faecium*)	0.938
1516 (half-blood)	5 (*E. durans*)	0.883
194 (half-blood)	8.2 (*E. durans*)	0.843
L09 (Lacaune)	6.4 (*E. faecium*)	0.841
1469 (half-blood)	16 (*E. durans*)	0.841
L04 (Lacaune)	4.2 (*E. faecium*)	0.834
L09 (Lacaune)	6.2 (*E. faecium*)	0.827
102 (half-blood)	14.4 (*E. faecium*)	0.824
1508 (half-blood)	23 (*E. durans*)	0.799
102 (half-blood)	14.3 (*E. casseliflavus*)	0.768
300 (half-blood)	20.5 (*E.faecium*)	0.576
102 (half-blood)	14.1 (*E.faecium*)	0.245
81 (Santa Inês)	12.2 (*E.faecium*)	0.225
81 (Santa Inês)	12.1 (*E. faecium*)	0.213
300 (half-blood)	20.4 (*E. durans*)	0.132
1448 (half-blood)	10.3 (*E. casseliflavus*)	0.039

Tolerance to 1% oxgall from *E. durans* and *E. faecium* samples isolated from Feta cheese was observed in another study (Ambadoyiannis *et al*., 2005). Pereira and Gibson (2002) found tolerance to 0.4% oxgall from an *E. durans* sample.

Tolerance to biliary salt was also considered for samples that achieved less than 40% of inhibition, therefore, nine (56.25%) out of 16 samples were considered tolerant to biliary salts (Table 5). Sample 14.1 was not considered tolerant because it did not achieve a 0.3 absorbance (Table 4).

Table 5. Inhibition percentage by biliary salts (oxgall 0.3%) of 16 enterococci samples isolated from sheep milk

Sheep (Breed)	Sample (Species)	Inhibition (%)
1433 (half-blood)	3.1 (*E. faecium*)	23.22
102 (half-blood)	14.3 (*E. casseliflavus*)	26.68
1516 (half-blood)	5 (*E. durans*)	26.69
194 (half-blood)	8.2 (*E. durans*)	26.89
102 (half-blood)	14.4 (*E. faecium*)	27.51
L04 (Lacaune)	4.2 (*E. faecium*)	30.14
1508 (half-blood)	23 (*E. durans*)	31.76
L09 (Lacaune)	6.2 (*E. faecium*)	32.55
L09 (Lacaune)	6.4 (*E.faecium*)	32.75
102 (half-blood)	14.1 (*E. faecium*)	34.14
1469 (half-blood)	16 (*E. durans*)	41.64
300 (half-blood)	20.5 (*E. faecium*)	44.27
81 (Santa Inês)	12.2 (*E. faecium*)	58.76
81 (Santa Inês)	12.1 (*E. faecium*)	58.76
300 (half-blood)	20.4 (*E. durans*)	63.01
1448 (half-blood)	10.3 (*E. casseliflavus*)	85.88

All enterococci samples were resistant to ceftazidime, oxacillin and streptomycin; and sensitive to clindamycin (sample 12.1 was only moderately sensitive), erythromycin, penicillin and tetracycline (sample 20.4 was only moderately sensitive). Samples 12.1 and 14.1 were the only ones moderately sensible to ciprofloxacin and sensitive to vancomycin, and the other samples were resistant. These two samples were also the only ones resistant to tetracycline, and the others were sensitive. Samples 14.4 and 20.5 were the only ones sensitive to gentamicin, and the other samples were resistant (Table 6).

Table 6. Enterococci antimicrobial susceptibility[a]

Sample	Antimicrobial									
	CAZ	CIP	DA	E	GN	OX	P	S	TE	VA
3.1	R	R	S	S	R	R	S	R	S	R
4.2	R	R	S	S	R	R	S	R	S	R
5	R	R	S	S	R	R	S	R	S	R
6.2	R	R	S	S	R	R	S	R	S	R
6.4	R	R	S	S	R	R	S	R	S	R
8.2	R	R	S	S	R	R	S	R	S	R
10.3	R	R	S	S	R	R	S	R	S	R
12.1	R	MS	MS	S	R	R	S	R	R	S
12.2	R	R	S	S	R	R	S	R	S	R
14.1	R	MS	S	S	R	R	S	R	R	S
14.3	R	R	S	S	R	R	S	R	S	R
14.4	R	R	S	S	S	R	S	R	S	R
16	R	R	S	S	R	R	S	R	S	R
20.4	R	R	S	S	R	R	S	R	MS	R
20.5	R	R	S	S	S	R	S	R	S	R
23	R	R	S	S	R	R	S	R	S	R

[a]CAZ: ceftazidime (30 μg), CIP: ciprofloxacin (5 μg), DA: clindamycin (2 μg), E: erythromycin (5 μg), GN: gentamicin (10 μg), OX: oxacillin (1 μg), PEN: penicillin (10 U), S: streptomycin (30 μg), tetracycline (30 μg), and vancomycin (VAN, 30 μg). R: resistant, MS: moderately sensible, S: sensible.

Resistance to ciprofloxacin and vancomycin was found by most of the enterococci samples tested in a similar study (Coque *et al*., 1996), corroborating the findings of the present study. Cueto-Vigil *et al*. (2010) found sensitivity to clindamycin, erythromycin, penicillin and tetracycline by most of enterococci samples isolated from cheese, such as the enterococci isolated from sheep milk in this work.

Mannu *et al*. (2003), when comparing susceptibility to antimicrobials of enterococci from different origins, found that enterococci from raw sheep milk were sensitive to penicillin, tetracycline and vancomycin, and the ones isolated from sheep feces were sensitive to vancomycin, moderately sensitive to tetracycline and resistant to penicillin. This work leads to the supposition that the enterococci isolated from sheep milk in this study are from milk and not from feces - or any other contamination.

The inhibition of tested enterococci were considered significant ($P<0.05$) against *E. coli* and *L. monocytogenes* when compared to the other revealing microorganisms tested. The inhibition was less intense, but still significant ($P<0.05$), against *E. faecalis* and *S. aureus* when compared to the poor inhibition against *S.* Typhimurium, and the non-existent inhibition against *P. aerugionosa* and *E. durans* - sample 23 isolated from sheep milk (Table 7). It is interesting to notice here that an *E. faecalis* pathogenic sample was significantly ($P<0.05$) inhibited while an *E. durans* sample from sheep milk was not. None of the enterococci samples showed statistical difference in their behavior against all pathogens, indicating similar inhibition profiles according to Kruskal-Wallis test ($P>0.05$).

A diversity of bacteriocins produced by enterococci is known as good inhibitors to *L. monocytogenes* and *S. aureus* growth, according to Giraffa (1995). In Ennahar *et al*. (2001) and Sarantinopoulos *et al*. (2002) studies in different cheeses, different *E. faecium* samples inhibited reference *L. monocytogenes* and *S. aureus* samples. These results confirm the results observed in the present study.

A study developed with enterococci from goat cheese against reference pathogens found, as in this work, inhibition against *E. coli*, *L. monocytogenes* and *S. aureus* (Psoni *et al*., 2006).

In Strompfová *et al.* (2006) and Taras *et al*. (2006) works, *in vivo* inhibition of different *E. faecium* probiotic samples against *E. coli* in piglets were proven. These results confirm what was demonstrated *in vitro* in the present work.

Table 7. Means (mm) of inhibition halos of enterococci samples against reference microorganisms[a]

Reference Microorganism	Mean (mm) halo Inhibition
Escherichia coli	65.60a
Listeria monocytogenes	57.87a
Enterococcus faecalis	24.11b
Staphylococcus aureus	19.84b
Salmonella Typhimurium	3.85c
Pseudomonas aeruginosa	0.00d
Enterococcus durans(sample 23)	0.00d

[a]Means followed by distinct letters are different by Kruskal-Wallis test ($p<0.05$).

CONCLUSION

E. durans, *E. faecium* and *E. casseliflavus* samples isolated from sheep milk (from Lacaune, Santa Inês and their crossbreeds) can present *in vitro* probiotic properties such as resistance to gastric juice, biliary salts and antagonism against reference pathogens such as *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*. Therefore, their use as probiotics in dairy products is promising, although more *in vitro* and *in vivo* studies are needed to prove their full probiotic potential and their inability to transfer antimicrobial resistance genes.

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