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Lactic Acid Bacteria as Biological Control of *Staphylococcus aureus* in Coalho Goat Cheese

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SUMMARY

The aim of this study is to investigate the bacterial population in coalho goat cheese produced in the semi-arid northeast region of Brazil, to analyse the antibiotic resistance profiles of the identified pathogenic bacteria, to detect the staphylococcal enterotoxin genes and to evaluate the addition of autochthonous lactic acid bacteria (LAB) with technofunctional properties for the control of *Staphylococcus aureus* growth. In the analysed samples, strains of *Escherichia coli* (*N*=11), *Salmonella* spp. (*N*=18), *Listeria* spp. (*N*=6) and *S. aureus* (*N*=9) were classified as multidrug resistant (MDR). The most commonly isolated pathogen from the studied coalho goat cheese was *S. aureus*. Its isolates were positive for the genes encoding enterotoxins A (*sea*), B (*seb*), C (*sec*) and D (*sed*). The autochthonous LAB with the potential to inhibit *S. aureus* were identified as *Enterococcus faecium*. These strains were selected for *in vitro* tests of protective, safety, technological and functional properties. In the coalho goat cheese food matrix, these selected autochthonous LAB were able to reduce the enterotoxigenic MDR *S. aureus* load by approx. 3 log units.

Key words*:* coalho goat cheese, pathogens, multidrug resistance, staphylococcal enterotoxins, microbiological safety, technofunctional properties of *Enterococcus faecium*

INTRODUCTION

Brazil is the largest producer of goat's milk in South America, and 93 % of the goat population is concentrated in the Northeast region. Goat raising is as a promising activity due to changes in the food supply chain and market diversification. Among the outstanding products of goat's milk, the coalho cheese is a traditional product of Northeast Brazil, usually produced from raw milk and is greatly valued by consumers (*1*,*2*). Artisanal cheese is considered part of the cultural context and identity of a region (*3*).

Although artisanal goat cheese is one of the main products used to generate income for smallholders, it is still primarily handmade, often without suitable facilities. Thus, there is no standardization of the process, and the use of raw milk is common, endangering the health of consumers (*2*). The absence of quality control standards for goat's milk and dairy products in Brazil is a major hindrance to agribusiness specializing in dairy goats. The market access to these products depends strongly on the application of appropriate technology to obtain the quality standards required by legislation (*4*).

Regarding the microbiological risks of goat cheese, in general, artisanal cheese produced from goat's milk has poor microbial quality (*5*). *Staphylococcus aureus* is one of the most important foodborne pathogens in cheese due the manual contact of the handlers during the manufacture of the product (*6*,*7*). Tools to increase the microbiological safety of artisanal goat cheese would include the addition of specific antibacterial starter cultures, such as selected lactic acid bacteria (LAB) with anti-*Staphylococcus* activity. The incorporation of these cultures in the technological procedures for product preparation is crucial, not only to increase microbiological safety by inhibiting pathogens but also to improve the technological and functional properties and enhancement of the cheese flavour, important attributes for dairy derivatives of goat origin (*8*).

For the semi-arid northeastern region of Brazil, it is a fundamental necessity to characterize the microbiology of handmade goat cheese to increase the microbiological and technological quality of the product. This is because of the possibility of required registration

for the geographical indication (IG) by the Ministry of Agriculture, Livestock and Supply (MAPA, Brazil), which is regulated through Normative Instruction (NI) no. 30 of 7 August 2013 (*9*). This NI allows that the artisan cheese traditionally produced from raw milk can be matured for less than sixty days when technical and scientific studies prove that a reduction of the maturation period does not affect the safety of the product. Additionally, the smallholders will be able to cater to the food acquisition program (Programa de Aquisição de Alimentos, PAA, Brazil), a programme in which the Brazilian government acquires food products from goat breeding to supply day-care centres, hospitals and other institutions with government assistance. Thus, this study aims to investigate bacterial microorganisms in coalho goat cheese produced in the Brazilian semi-arid northeast region by analysing the resistance profile of the pathogens, by detecting the enterotoxin genes in the multidrug-resistant (MDR) *S. aureus* isolates and by evaluating the addition of autochthonous LAB for controlling *S. aureus* isolate of the goat cheese.

MATERIALS AND METHODS

Sample collection

A total of 40 small-scale commercially produced coalho goat cheese samples were acquired from goat farmer associations in eight municipalities in the semi-arid northeast region of Brazil in the state of Pernambuco and Bahia, comprising: Petrolina, Santa Maria da Boa Vista, Lagoa Grande, Cabrobó, Dormentes, Afrânio, Casa Nova and Sento Sé (**Fig. 1**). Five samples were analysed from each city, and samples were transported to the laboratory in isothermal boxes under refrigeration and analysed immediately.

Microbiological analysis and determination of pH

A total of 25 g of each goat cheese sample was removed by means of radial cuts and placed in sterile plastic bags containing 225 mL of a sterile 2 % (*m/V*) sodium citrate (Synth, Diadema, Brazil) solution and homogenized for 2 min in a Stomacher® (Mayo Homogenius HG 400; São Paulo, Brazil). Tenfold

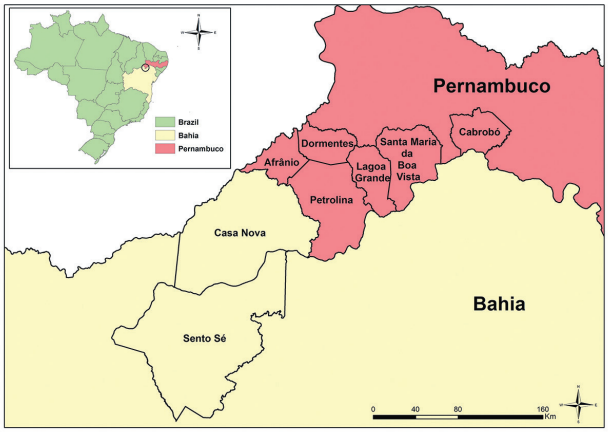

Fig. 1. Map of the municipalities of Bahia and Pernambuco (northeast region, Brazil) where the coalho goat cheese samples were collected

dilutions (10^{-1} to 10^{-10}) of cheese homogenate were prepared in 0.1 % sterile peptone water (HiMedia, Bombay, India) and plated on specific medium to detect mesophilic aerobic bacteria (MAB), lactic acid bacteria (LAB), thermotolerant coliforms and *Escherichia coli, Staphylococcus aureus*, *Salmonella* spp. and *Listeria monocytogenes*.

The MAB and LAB were enumerated on plate count agar (PCA, HiMedia) and de Man, Rogosa and Sharpe agar (MRS, HiMedia), respectively. Plates were incubated at 37 °C for 48 h for MAB and 72–96 h for LAB. The characterization of the LAB isolates included Gram staining (Gram Staining Kit, Laborclin, São Paulo, Brazil), morphology, catalase (Anidrol, Diadema, Brazil), motility (HiMedia) and cytochrome oxidase activities (Probac, São Paulo, Brazil). One isolate was stored from each goat cheese sample.

For the enumeration of thermotolerant coliforms and detection of *E. coli,* the Fluorocult® broth (Merck, Darmstadt, Germany) was used (*10*). The tubes were incubated at 37 °C for 24 h. Positive tubes, after the addition of Kovac's reagent (Probac), served for calculation of the most probable number (MPN). For confirmation of *E. coli*, Gram-negative (Gram Staining Kit, Laborclin), oxidase-negative (Probac) and catalase-positive (Anidrol) colonies were streaked on PCA and incubated at 37 °C for 24 h to perform the indole (tryptone broth, HiMedia), methyl red and Voges-Proskauer (MR-VP medium, HiMedia) and citrate (Simmons citrate agar, HiMedia) (IMViC) biochemical tests.

S. aureus colonies were isolated, identified and confirmed biochemically according to Normative Instruction no. 62 (*11*). Aliquots of 0.1 mL of each cheese sample were plated onto the surface of Baird-Parker agar (HiMedia) in duplicate and incubated at 37 °C for 24–48 h. For the identification, the following tests were conducted for typical colonies selected from the medium: Gram staining, catalase, coagulase (Laborclin), oxidation and fermentation of glucose and mannitol (Synth), as well as the detection of DNAse and thermonuclease (DNase test agar with Toluidine Blue, HiMedia) activities.

Salmonella spp. were detected according to the method of Pignato *et al.* (*12*). Tetrathionate broth (HiMedia) supplemented with iodine and Brilliant Green solutions and Rambach agar (Merck) was used for selective enrichment (18 h at 37 °C) and isolation (24 h at 37 °C), respectively. For biochemical identification (*11*), typical colonies were added into tubes containing triple sugar iron (TSI) agar (HiMedia) or lysine iron agar (LIA; HiMedia) and incubated at 37 °C for 24 h. Presumptive *Salmonella* strains were checked for differential Gram staining, catalase and oxidase activity, motility, and sulphide and indole production (HiMedia). In addition, serological tests were performed using somatic and flagellar polyvalent antiserum (Probac) following the manufacturer's instructions.

Detection of *L. monocytogenes* was detected as described by Capita *et al.* (*13*). Fraser broth (HiMedia) and Palcam agar (HiMedia) were used for selective enrichment (24–48 h at 35 °C) and isolation (24–48 h at 35 °C), respectively. Suspected *Listeria* spp. colonies were streaked onto tryptone soy agar (TSA) plates

supplemented with 0.6 % yeast extract (TSA-YE; HiMedia) and incubated at 37 °C for 24–48 h. Next, appropriate biochemical tests were conducted on Gram-positive isolates to verify the production of catalase, fermentation of carbohydrates, haemolysis on sheep blood agar (HiMedia) and motility at 25 °C.

For quantitative analyses, plates with 30–300 colonies were counted. The microbiological counts were expressed in logarithms of the number of colony forming units per gram (CFU/g). In qualitative analyses (detection of *Salmonella* spp. and *L. monocytogenes*), microorganisms were detected as being present or absent.

For the determination of pH, cheese (10 g) was homogenized in 10 mL of distilled water according to de Almeida Júnior *et al*. (*14*). The analyses were performed in triplicate with a pH meter (PHS-3E-BI; Ion, Araucária, Brazil).

Antimicrobial resistance of pathogens

The antibiotic resistance of isolated pathogens was determined using disc diffusion method on Mueller–Hinton agar with Multidiscos Gram Negativo 12® and Multidiscos Gram Positivo 12® (Laborclin) (**Table 1**). After incubation at 37 °C for 24 h, inhibition zone diameters were measured following the recommendations of the Clinical and Laboratory Standards Institute (*15*). The multidrug resistance profiles were calculated by the index of multiple antibiotic resistance (MAR). Strains with an index equal to or above 0.5 and resistant to three or more tested antimicrobials were classified as having a high MAR index (*16*).

Molecular identification of pathogens and detection of genes for staphylococcal enterotoxins

The DNA of pathogens (two of each genus) with the highest MAR index and of those resistant to three or more tested antimicrobials was extracted using a PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. The polymerase chain reaction (PCR) was performed according to the protocol proposed by de Ávila *et al*. (*17*).

For the *S. aureus* strains with the highest MAR index, genes coding for the classical staphylococcal enterotoxins (SEs: *sea, seb, sec, sed* and *see*) were detected as described by Schneid Kroning *et al*. (*18*). The positive controls used in the reactions for the classic enterotoxin genes were donations of Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, Brazil), strains were from the National Institute for Quality Control in Health (INCQS, Rio de Janeiro, Brazil): *S. aureus* INCQS 00285 (*sea* and *sed*), *S. aureus* INCQS 00005 (*seb*), *S. aureus* INCQS 00080 (*sec*) and *S. aureus* INCQS 00093 (*see*).

Antimicrobial activity of autochthonous LAB against S. aureus strain from coalho goat cheese

The inhibitory effect of autochthonous LAB against *S. aureus* strain QCSA24 (enterotoxigenic and MDR isolate with the greatest MAR index) from coalho goat cheese was tested using the agar disc diffusion method according to Ferrari *et al*. (*19*) with modifications. The suspension of the *S. aureus*

Table 1. Antimicrobial agent discs* used to evaluate the resistance of pathogens and lactic acid bacteria isolated from coalho goat cheese

Microorganism	Antimicrobial class	Antimicrobial agent	(*m*(antimicrobial agent)/μg)/disc
Gram-negative	penicillins	ampicillin	10
	fluoroquinolones	ciprofloxacin	5
	folate pathway inhibitors	sulfazotrim	25
	aminoglycosides	gentamycin	10
		amikacin	20
	cephems	cephalothin	30
		ceftazidime	30
		cefepime	30
		cefoxitin	30
		cefuroxime	30
	β-lactamase inhibitor combinations	amoxicillin+clavulanate	30
	carbapenems	meropenem	10
Gram-positive	penicillins	penicillin	10
		oxacillin	1
	fluoroquinolones	ciprofloxacin	5
	folate pathway inhibitors	sulfazotrim	25
	aminoglycosides	gentamycin	10
	cephems	cefepime	30
	tetracyclines	tetracycline	30
	glycopeptides	vancomycin	30
	phenicols	chloramphenicol	30
	lincosamides	clindamycin	2
	macrolides	erythromycin	15
	ansamycins	rifampin	5

*Multidiscos Gram Negativo 12® and Multidiscos Gram Positivo 12® (Laborclin)

strain containing 10^8 CFU/mL was uniformly spread with sterile swab over the plate with brain heart infusion (BHI; HiMedia) agar. Whatman no. 1 filter paper discs (Sigma-Adrich, Merck, St. Louis, MO, USA) of 6 mm impregnated with 20 µL cell-free supernatant obtained by centrifugation (centrifuge K14-1215; Kasvi, São José dos Pinhais, Brazil) at 2500×*g* for 10 min from each isolate of autochthonous LAB were placed onto the surface of the agar. The pH of the cell-free supernatant was adjusted to 6.5 (1 mmol/L NaOH). After incubation at 37 °C for 24 h the radius (mm) of inhibition zones around the discs was measured. Inhibition zones smaller than 1 mm radius were considered negative. The experiment was performed in triplicate. Six autochthonous LAB with the highest antimicrobial activity against *S. aureus* strain were selected for additional tests described below.

Antimicrobial activity among and molecular identification of autochthonous LAB

A test was performed to observe the antimicrobial activity among autochthonous LAB (*20*). In this assay, LAB strains were grown on MRS broth at 37 °C for 24 h. An aliquot of 1 mL of the LAB isolate (approx. 10^6 CFU/mL, final concentration) and 15 mL of MRS broth (HiMedia) were inoculated onto the plate using the pour plate method. An aliquot of 10 µL of another LAB isolate was spotted onto MRS agar and incubated for 24 h at 37 °C. The detection of a zone of inhibition in the lawn indicated that there was an antagonistic action among the LAB isolates. The experiment was performed in triplicate.

The molecular identification of the six LAB strains was done in the same way as for pathogens.

Safety, technological and functional properties of autochthonous LAB

Phenotypical tests to identify virulence activity of autochthonous LAB were performed, including haemolytic activity (*14*), production of DNase and ability to coagulate rabbit plasma (*19*). The resistance of LAB strains to antimicrobial agents (**Table 1**) was tested by the agar disc diffusion test using Multidiscos Gram Positivo 12® (Laborclin) (*15*).

As probiotic candidates, the six autochthonous LAB strains were tested in solutions that chemically simulate physiological conditions. The survival rate of LAB at pH=2 and in bile salt (2 %) conditions was calculated by determining the total viable counts at time 0 and 3 h on MRS agar according to de Almeida Júnior *et al.* (*14*). In addition, the proteolytic (*14*) and β-galactosidase (*19*) activities of autochthonous LAB were checked. To verify proteolytic activity, the strains were plated on skimmed milk agar (HiMedia) and incubated at 7 °C for 10 days and 37 °C for 48 h. A clear halo around the colonies indicated a positive result. For the activity of the β-galactosidase enzyme, one colony of each isolate was emulsified in the tube containing an ONPG (*o*-nitrophenyl-β-D-galactopyranose) disc (Fluka, Buchs, Switzerland) and 1 mL sterile saline. The tubes were incubated at 37 °C, and the yellow staining (positive reaction) was observed within 6 h. All tests were performed in triplicate.

Control of S. aureus strain by autochthonous LAB in coalho goat cheese

The artisanal goat cheese (coalho cheese) was produced to evaluate the antibacterial activity of LAB against *S. aureus*. The LAB were inoculated using a mix of the six autochthonous LAB strains isolated from goat cheese (UNIVASF CAP QC1, QC4, QC13, QC14, QC18 and QC19) with highest antimicrobial activity against *S. aureus* strain. The experiment was conducted as proposed by Ferrari *et al.* (*19*) with modifications in the use of pathogen and inoculum concentration. This study used a pathogen *S. aureus* isolated from goat cheese (QCSA24, an enterotoxigenic MDR *S. aureus* isolate). The first cheese sample (SA, positive control) was inoculated with *S. aureus*, suspended in 1 % milk in the concentration of 10^8 CFU/mL. The second cheese sample (SA+LAB) was inoculated with the same concentration of 10^8 CFU/mL of *S. aureus* and the mix of six selected *E. faecium* strains. The third cheese sample (NC) served as negative control, without the addition of microorganisms. The artisanal goat cheese was drained and placed in perforated moulds (250 g). Three replicate determinations for each cheese were carried out. The cheese samples were packed in sterile plastic bags and stored at 10 °C for 20 days. The bacteriological analyses (*S. aureus* and LAB) and pH measurements were conducted in five five-day intervals (days 0, 5, 10, 15 and 20) in triplicate.

Statistical analysis

SISVAR® software v. 5.6 (*21*) helped to statistically analyse the antimicrobial activities of autochthonous LAB against *S. aureus* isolate in goat cheese. The results were evaluated using ANOVA and the mean values were compared by a Scott-Knott test (*22*). Quantitative data were analysed by regression. A probability value at $p<0.05$ was statistically significant.

RESULTS AND DISCUSSION

Microbiological quality and pH value of coalho goat cheese

According to the Brazilian Technical Regulation of Identity and Quality, coalho cheese is obtained by the coagulation of milk by rennet, complemented or not by the action of selected LAB, stored under an average temperature of 10–12 °C and normally marketed up to ten days after manufacture (*23*). In addition, according to the Brazilian legislation, it is a product of medium to high humidity, of semi-cooked curd or cooked or otherwise prepared from raw curd (unheated) (*23*). The goat cheese samples collected in this study were prepared from raw curd (unheated) and had high humidity.

Total counts of MAB and LAB in the cheese remained at 6.7 and 6.6 log CFU/g, respectively (**Table 2**). In general, our results showed a high cell number count compared with those found in other goat cheese varieties that were previously observed

Table 2. Log enumerations of microbial groups and determination of pH (mean value±standard deviation) of coalho goat cheese commercialized in municipalities of the semi-arid northeast region of Brazil

Municipality	*N*(microorganism)/(log CFU/g)			*N*(TC)/(log MPN/g)	pH
	MAB	LAB	*Staphylococcus aureus*		
Petrolina	6.7±1.2	6.5±1.3	6.9±0.3	6.7±1.0	5.4±0.1
Santa Maria da Boa Vista	7.6±0.1	7.0±0.5	6.7±0.7	5.7±3.7	5.7±0.2
Lagoa Grande	6.9±0.7	6.0±0.6	5.9±0.6	3.2±2.5	5.7±0.3
Cabrobó	6.0±0.6	6.6±0.6	6.3±0.7	1.17±0.02	5.4±0.1
Casa Nova	7.0±0.7	7.0±0.5	5.82±0.09	1.17±0.02	5.2±0.2
Sento Sé	6.6±0.5	7.0±0.4	5.6±0.3	1.17±0.02	5.24±0.09
Dormentes	6.1±0.7	6.2±0.4	5.7±0.2	1.17±0.02	5.19±0.06
Afrânio	7.1±0.8	6.8±1.0	6.8±0.8	1.17±0.02	5.3±0.2
Average	6.7	6.6	6.2	2.7	5.4

MAB=mesophilic aerobic bacteria, LAB=lactic acid bacteria, TC=thermotolerant coliforms, CFU=colony forming unit, MPN=most probable number

in Morocco, Spain and Greece (*24,25*). Mesophilic lactobacilli are the most important components of the non-starter LAB (NSLAB) microbiota of cheese. These microorganisms can contribute to sensory characteristics and the inhibition of potential pathogens in the product (*25,26*).

As for the current legislation in Brazil for microbiological criteria of cheese with high moisture content, both the Ministry of Health and the Ministry of Agriculture, through the law RDC 12 (*27*) and Ordinance 146 (*28*), respectively, agree that 2 out of 5 samples may contain between 3 and 3.7 log CFU/g thermotolerant coliforms, 2 out of 5 samples may contain between 2 and 3 log CFU/g coagulase-positive staphylococci, while *Salmonella* spp. and *L. monocytogenes* must be absent from the product.

In this study, five, three and two samples from the municipalities of Petrolina, Santa Maria da Boa Vista and Lagoa Grande (Pernambuco State, Brazil), respectively, did not conform to the law for thermotolerant coliforms. On average, the population of thermotolerant coliforms was 2.7 log CFU/g (**Table 2**). The presence of coliforms seems to be usual in raw goat's milk cheese as previously reported (*24*). Among the 40 samples positive for thermotolerant coliforms, in 11 samples there was phenotypic confirmation of *E. coli* (**Table 3**).

Regarding the presumptive enumeration of *S. aureus*, the overall average in the municipalities was 6.2 log CFU/g (**Table 2**). It was noticeable that the population of LAB and *Staphylococcus* was similar in the goat cheese samples, both in the range of 6 log CFU/g. The counts of *Staphylococcus* were higher than recommended by law (**Table 2**) in all the samples. In the biochemical identification (**Table 3**), 13 isolates were confirmed as coagulase-negative *Staphylococcus*, 18 as coagulase-positive *Staphylococcus* and 9 as *S. aureus*.

S. aureus is one of the most common pathogens associated with raw milk cheese. It presents a microbiological safety concern only when it is present at a level higher than 4 log CFU/g and when strains can produce enterotoxins. Generally, the amount of staphylococcal enterotoxins for the establishment of typical symptoms of food poisoning ranges from 20 ng to 1 μg (*29*).

Salmonella spp. were detected in 18 samples of goat cheese (45 %; **Table 3**). *Salmonella* can originate from goat's milk, utensils and equipment, and improper handling during the production of cheese. *Salmonella* spp. survive for extensive periods in goat cheese and represent a potential health hazard. Outbreaks of infections with *Salmonella* spp. have been associated with the consumption of cheese (*30*). In Brazil there is little

Table 3. Biochemical confirmation and multiple antibiotic resistance (MAR) index (≥0.5) of pathogens isolated from coalho goat cheese commercialized in municipalities of the semi-arid northeast of Brazil

Municipality	Number of isolates					
	Escherichia coli	CNS	CPS	*Staphylococcus aureus*	*Salmonella* spp.	*Listeria* spp.
Petrolina	1	5	n.d.	n.d.	5	1
Santa Maria da Boa Vista	n.d.	2	1	2	5	n.d.
Lagoa Grande	3	2	2	1	2	2
Cabrobó	3	n.d.	3	2	2	1
Casa Nova	4	n.d.	4	1	n.d.	1
Sento Sé	n.d.	n.d.	4	1	n.d.	1
Dormentes	n.d.	1	2	2	1	n.d.
Afrânio	n.d.	3	2	n.d.	3	n.d.
Total number of isolates	11	13	18	9	18	6
Number of isolates with MAR index≥0.5	8	n.d.	n.d.	9	12	6

CNS=coagulase-negative *Staphylococcus*, CPS=coagulase-positive *Staphylococcus,* n.d.=not detected

information about the prevalence of *Salmonella* spp. in goat's milk and its derivatives. Oliveira *et al.* (*4*) reported the presence of *S. enterica* in samples of milk on dairy goat farms from the Cariri region in the northeastern state of Paraiba, Brazil.

L. monocytogenes was detected in six samples of goat cheese (15 %; **Table 3**), which is a major concern for consumers of this product in the region. Brazilian law does not tolerate the presence of *L. monocytogenes* in food products. El Galiou *et al.* (*24*) reported the presence of this pathogen in samples of goat cheese. According to Melo *et al.* (*31*), *L. monocytogenes* is a challenging issue for the dairy industry. Cheese offers a suitable environment for this pathogen, which is also persistent in dairy processing plants.

The average pH value of cheese collected from different municipalities in this study ranged from 5.2 to 5.7, and the overall average was 5.4 (**Table 2**). In the Brazilian legislation, the pH of goat's milk is slightly less acidic than of cow's milk (*32*). However, different factors, such as autochthonous microbiota, chemical composition, renneting parameters, ripening time and traditional technology can affect the pH of the cheese.

Antimicrobial resistance test and MAR index

The antimicrobial resistance profile calls attention to the high MAR index of pathogens. In our study, there were pathogens that had MAR indices higher than 0.5 and were resistant to three or more antimicrobials. Among the 11 isolates of *E. coli*, eight had MAR indices higher than 0.5 (**Table 3**). Twelve *Salmonella* spp. isolates had MAR indices higher than or equal to 0.50 (**Table 3**), while six isolates of *Listeria* spp. were classified as MDR (**Table 3**). One isolate of *S. aureus* from the municipality of Casa Nova (QCSA24) had the greatest MAR index 0.91, followed by the isolate from the municipality of Dormentes (QCSA34), with MAR index 0.83 (data not shown).

All *S. aureus* isolates proved to be resistant to three or more classes of antimicrobial agents and had a MAR index higher than or equal to 0.50 (**Table 3**). The coagulase-negative *Staphylococcus* and coagulase-positive *Staphylococcus* strains had MAR indices lower than 0.5 (**Table 3**). These results showed that raw milk cheese may act as vehicle for the transmission of MDR strains of pathogens, and thus constitutes a potential risk for public health. Consumers should avoid the consumption of raw milk cheese of poor microbiological quality.

Identification of pathogens and presence of S. aureus enterotoxin genes

Two isolates of each genus with the highest MAR index that were resistant to three or more tested antimicrobials were biochemically identified as *E. coli* (QCE15 and QCE18). *Salmonella* spp. (QCS5 and QCS7), *Listeria* spp. (QCL5 and QCL19) and *S. aureus* (QCSA24 and QCSA34) were also identified molecularly. The identification by 16S rRNA gene sequencing presented 99 % similarity with *E. coli* (GU594316.1/GU811877.1), *Salmonella* Typhi (DQ480723.1), *S. aureus* (NR_037007.1/NR_113956.1) and *L. monocytogenes* (NR_044823.1). These pathogens have been reported in goat cheese (*24,33*).

The enterotoxin genes A (*sea*), B (*seb*), C (*sec*) and D (*sed*) were detected in the two isolates of *S. aureus* (QCSA24 and QCSA34). Yoon *et al.* (*29*) reported that some *S. aureus* strains isolated from cheese are able to form four different serotypes of staphylococcal enterotoxins (SEs), *sea, seb, sec* and *sed*. The coalho goat cheese produced in the semi-arid northeast region of Brazil is a potential carrier of enterotoxigenic *S. aureus*.

Protective activity and identification of autochthonous LAB

There were observable differences in the inhibitory activity of the autochthonous LAB ($p<0.05$) against the enterotoxigenic MDR *S. aureus* isolate (QCSA24, MAR index 0.91). Twenty-two LAB isolates showed an ability to inhibit *S. aureus* (**Fig. 2**). Six isolates of LAB from the municipalities of Petrolina (QC1 and QC4), Lagoa Grande (QC13 and QC14) and Cabrobó (QC18 and QC19) had pronounced inhibitory activity. These isolates were selected for subsequent testing of the ability to

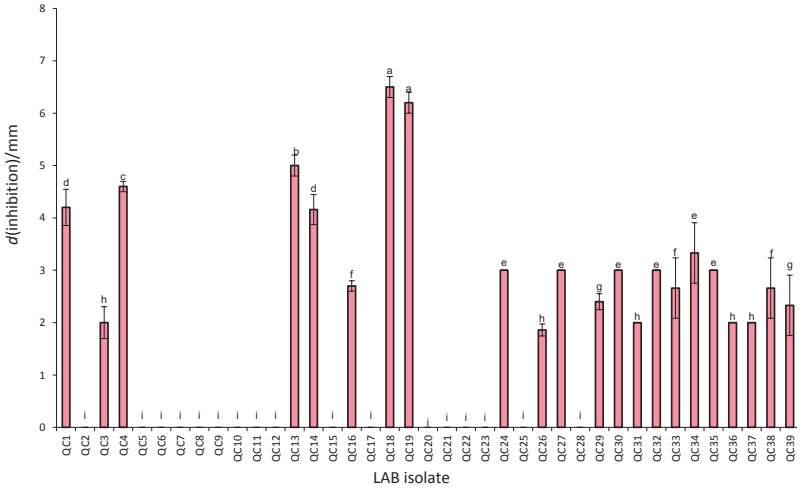

Fig. 2. Antimicrobial activity of the autochthonous lactic acid bacteria (LAB) isolates (UNIVASF CAP) against the enterotoxigenic multidrug resistant *Staphylococcus aureus* isolate (QCSA24) from coalho goat cheese. Values designated by different letters are significantly different ($p<0.05$). Standard error=0.122

confer this inhibitory activity to artisanal cheese. According to Yoon *et al*. (*29*), LAB have been shown to inhibit the growth of *S. aureus* in cheese by the production of diacetyl, organic acids and bacteriocins.

Each autochthonous LAB isolate was tested against another LAB isolate to test the antimicrobial activity among them. None of the six selected LAB isolates exhibited the ability to inhibit another isolate. This finding is interesting for the use of these cultures together as inhibitors against *S. aureus*.

After the selection of autochthonous LAB with antibacterial activity against *S. aureus* and the observation of non-antagonism among them, they were subjected to molecular identification. These LAB isolates were identified based on 16S rRNA gene sequence analysis with 99 % similarity as *Enterococcus faecium* (NR_114742.1), which has been found in Brazilian artisanal cheese products (*34*).

Virulence factors and technofunctional properties of autochthonous E. faecium

For the characterization of virulence factors, the six tested *E. faecium* isolates were negative for haemolytic activity, production of DNase and ability to coagulate rabbit plasma (**Table 4**).

Another important criterion to be investigated is the antibiotic resistance of these strains. Antibiotic-sensitive LAB have no contribution to horizontal transfer of antibiotic resistance genes to pathogenic bacteria. In this study, the strains showed high rates of susceptibility to antimicrobial agents (**Table 4**). Four enterococcal strains showed sensitivity to all investigated antibiotics. The strains *E. faecium* QC1 and QC19 were only resistant to oxacillin. Resistance to this antibiotic in *E. faecium* isolated from cheese was also verified by Amaral *et al*. (*35*). These authors reported that the resistance to oxacillin is an intrinsic feature of *E. faecium,* chromosomally encoded.

These four isolates also demonstrated high tolerance to low pH and bile salts. The survival rates under these conditions ranged from 95.6 to 99.1 % and from 98.5 to 99.2 %, respectively (**Table 4**). The gastric emptying half time and the small bowel transit time in healthy women and men, in general, are estimated to be approx. 3 h (*36*). These criteria are important, since probiotic strains must be able to withstand pH variations and bile in the gastrointestinal tract. In addition, tolerance to low pH is also fundamental for the technological use of these cultures in cheese. Our results corroborate those of İspirli *et al*. (*37*); *E. faecium* isolated from cheese showed the ability to inhibit *S. aureus* and had technofunctional properties for use in cheese production.

The six isolates of *E. faecium* were positive for β-galactosidase and proteolytic activity (**Table 4**). Dos Santos *et al*. (*34*) also reported the isolation of *E. faecium* from Brazilian cheese with β-galactosidase and proteolytic activity. Cultures that have the ability to ferment lactose are very important for the dairy industry. Functionally, the hydrolysis of lactose allows cheese consumption by people intolerant to lactose.

Inhibition of S. aureus growth by E. faecium in coalho goat cheese

The milk used to produce the cheese was in accordance with the standard of identity and quality of product (*32*). After the thermal processing of milk, in the negative control cheese there was no detection of microorganisms. In the positive control cheese (SA), LAB were also not detected. Thus, only our selected *Enterococcus* inoculum was evaluated for the control of pathogenic *S. aureus* in coalho goat cheese.

There was correlation ($p<0.05$) between the storage period and the count of *S. aureus* in all cheese samples (**Table 5**). A lower population of pathogens was present in the cheese inoculated with the mix of *E. faecium* (SA+LAB). Populations of *S. aureus* differed among cheese samples from the 5th day of storage ($p<0.05$).

In the positive control (SA), the regression equation showed that there was a decrease in the *S. aureus* population as a function of the storage period (**Table 5**). According to the regression equation, the lowest count was on day 16, where the pathogen population measured 7.76 log CFU/g. In general, there was a difference of 0.44 log units in the initial and final population of *S. aureus* in the positive control (SA). This small decrease might

Table 4. Safety, technological and functional properties of selected autochthonous Enterococcus faecium from coalho goat cheese

Property	*Enterococcus faecium* (UNIVASF CAP)					
	QC1	QC4	QC13	QC14	QC18	QC19
Haemolytic activity	–	–	–	–	–	–
Production of DNase	–	–	–	–	–	–
Coagulase activity	–	–	–	–	–	–
Inhibition between LAB	–	–	–	–	–	–
Susceptibility rate/%*	91.66	100	100	100	100	91.66
Resistance to antimicrobial agent*	oxacilin	–	–	–	–	oxacilin
Survival rate to pH=2/%	98.3	95.8	99.1	96.8	95.6	97.1
Survival rate to 2 % bile salts/%	98.5	99	99.2	99	98.9	98.7
β-Galactosidase activity	+	+	+	+	+	+
Proteolytic activity	+	+	+	+	+	+

*12 tested antimicrobials, –=negative, +=positive, LAB=lactic acid bacteria

Table 5. The viable count (log CFU/g) of *Staphylococcus aureus* in the cheese samples (SA and SA+LAB) and of *Enterococcus faecium* in cheese sample SA+LAB (with a mix of *E. faecium* UNIVASF CAP) during storage for up to 20 days at 10 °C

Cheese sample	*t*(storage)/day					Log fold decrease day 0–20	Equation
	0	5	10	15	20		
	N(*S. aureus*)/(log CFU/g)[1]						
SA	8.37[a]	7.91[a]	7.94[a]	7.94[a]	7.93[a]	0.44	$y=0.002x^2-0.07x+8.31$ $R^2=0.80$
SA+LAB*	8.39[a]	6.48[b]	6.48[b]	5.68[b]	5.57[b]	2.82	$y=-0.14x+7.81$ $R^2=0.80$
	N(*E. faecium*)/(log CFU/g)[2]					Log fold increase day 0–20	
SA+LAB*	8.50	9.43	9.76	9.93	9.91	1.41	$y=0.07x+8.846$ $R^2=0.77$

For each column, mean values with different letters are significant ($p<0.05$) according to the Scott–Knott test, standard error=[1]0.0384 and [2]0.0245
*Cheese with the inclusion of *E. faecium* mix, SA=*S. aureus*, LAB=lactic acid bacteria

have been influenced by the storage temperature, in accordance with Jakobsen *et al.* (*38*).

There was a difference of 2.82 log units in the initial and final population of *S. aureus* in the cheese sample SA+LAB (**Table 5**). In this cheese, the population of *S. aureus* decreased 0.13 log units per day according to the first degree equation. During the assessment period, there was a reduction of approx. 3 log units of the *S. aureus* population. Sahraoui *et al.* (*5*) also reported an antagonistic effect of autochthonous *Lactococcus* strain from Algerian raw goat's milk against *S. aureus*. Inhibition of *S. aureus* by LAB can be associated with acidification, bacteriocin and H_2O_2 production. Delpech *et al.* (*39*) confirmed that the growth of the foodborne pathogen *S. aureus* can be inhibited in milk and cheese by hydrogen peroxide-producing bacteria.

In relation to the population of selected *Enterococcus* from goat cheese, the inoculum increased linearly over time (0.07 log unit per day), as can be explained by the first degree equation (**Table 5**). The LAB population increased 1.41 log units at the end of storage period (day 0 to day 20). The selected inoculum of *E. faecium* (UNIVASF CAP) and *S. aureus* originated from goat cheese. Our UNIVASF CAP isolates exhibited inhibition of *S. aureus* (isolate with highest MAR index) both *in vitro* and in the goat cheese product. Sahraoui *et al.* (*5*) confirmed that autochthonous LAB, in addition to their bioprotective effect, contribute to the terroir of the product and sustain the Protected Appellations of Origin of the cheese.

It is noteworthy that *Enterococcus* contributes to the development of the flavour of cheese (*26,37*), improving the sensory acceptance of the goat product. In this study we reported an average of 6.2 log CFU/g of *S. aureus* in coalho goat cheese commercialized in the semi-arid region. Thus, the addition of inoculum containing enterococci could be an option so that the product caters to Brazilian legislation by not containing enough pathogen population to produce enterotoxin. As previously discussed, a pathogen population greater than 4 log CFU/g in milk and milk products is necessary for the production of enterotoxin. Thus, the inoculum constitutes an additional hurdle for the pathogen population in the product. Furthermore, the success for the prevention of *S. aureus* growth in cheese requires hygiene measures implemented along the entire production chain.

The pH in positive control (SA) changed following a quadratic equation (**Fig. 3**), with a higher value on the 12th day (6.77), and a minimum reduction at the end of storage. In cheese sample SA+LAB, the pH value decreased linearly over time by 0.05 units per day. Although *S. aureus* have bacterial proteases that induce proteolytic degradation, generating alkaline radicals, the pH decline can be explained by the production of organic acid by the UNIVASF CAP isolates.

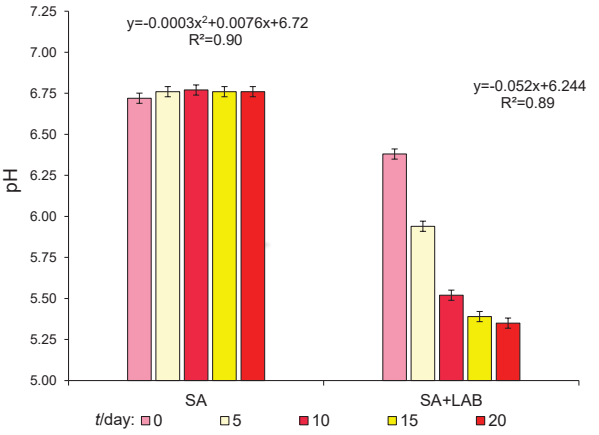

Fig. 3. pH values in cheese samples without (SA) and with (SA+LAB) a mix of *Enterococcus faecium* during storage for up to 20 days at 10 °C. Standard error=0.031, SA=*Staphylococcus aureus*, LAB=lactic acid bacteria

CONCLUSIONS

The cheese commercialized in the semi-arid northeastern region of Brazil is a pathogen vehicle. Multidrug resistant (MDR) strains of *Escherichia coli, Staphylococcus aureus, Listeria* and *Salmonella* are circulating in the product. The pathogen most commonly present in coalho goat cheese was *Staphylococcus*. However, this study demonstrated that selected autochthonous lactic acid bacteria (LAB) from goat cheese (UNIVASF CAP), identified as *Enterococcus faecium*, were able to reduce approx. 3 log units of the enterotoxigenic MDR *S. aureus* isolated from coalho goat cheese. These autochthonous LAB cultures, which are particular biotype of a geographical area, ensure a differentiated coalho goat cheese, exclusive to the region of origin. In addition, these cultures can contribute to increasing the sensory and functional properties of the artisanal goat cheese. Thus, the selected LAB inoculum could be an option for smallholders to produce cheese with a higher microbiological quality by applying good farming and processing practices, such as using milk of good quality and following proper hygiene procedures during cheese-making.

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