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ORIGINAL RESEARCH

Characterisation of *Staphylococcus aureus* strains from milk and goat cheese and evaluation of their inhibition by gallic acid, nisin and velame of the Brazilian caatinga

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Twenty isolates from milk and goat cheese were confirmed as Staphylococcus aureus. *These isolates were characterised for phenotypic properties related to cell adhesion and for the presence of enterotoxin production, intercellular adhesion and β-lactam resistance genes.* Staphylococcus aureus *L47 showed cell adhesion ability and positivity for the* sec, sed, ica*D,* mec*A and* bla*Z genes. Three antimicrobial compounds were tested singly or in pairs for growth control of strain L47: gallic acid (GA), nisin and essential oil (EO) of* Croton heliotropiifolius *(velame). At 24 h, EO and EO + nisin showed higher inhibitory activity against* S. aureus *L47 in goat milk.*

Keywords Biofilm, Enterotoxins genes, β-Lactam resistance, *Staphylococcus aureus* control, Antimicrobial compounds, Essential oil.

INTRODUCTION

Goat milk and cheese are at risk of contamination by enterotoxigenic and multidrug-resistant *Staphylococcus aureus* strains (Castro *et al.* 2018). The pathogen is highly prevalent and is the most frequent contaminant in milk and goat milk products (Xing *et al.* 2016; Castro *et al.* 2018; Aragão *et al.* 2019). *Staphylococcus aureus* remains on surfaces and utensils due to its ability to form biofilms. Intercellular interactions with the exchange of genetic material in biofilms result in bacteria with less susceptibility to existing antimicrobials. This could lead to serious damage to the dairy industry and a potential risk to public health (Bogdanovičová *et al.* 2017; Liu *et al.* 2017). In the Brazilian north-eastern semi-arid region, in the milk cooperatives involving small producers, the characterisation of *S. aureus* strains in goat milk and dairy derivatives and development of strategies for the control of these strains are necessary.

Since pathogens have emerged that are resistant to sanitisers, food industries have intensely investigated natural antimicrobial agents that can inhibit the growth, adhesion and biofilm formation of *S. aureus* (Unlu *et al.* 2018). Among these agents, nisin (Zhao *et al.* 2016), gallic acid (GA) (Liu *et al.* 2017) and the essential oil (EO) of 'velame' (*Croton heliotropiifolius*) (Alencar Filho *et al.* 2017) have been highlighted as important natural antimicrobials.

EOs are antimicrobial, aromatic and bioactive volatile compounds extracted from plants (Sadekuzzaman *et al.* 2018). The caatinga biome of the Brazilian north-eastern semi-arid region features several plant species with EOs with potential applications, such as *C. heliotropiifolius*, popularly known as 'velame' (Figure 1). Velame is used by the local population as a herbal remedy (tea) (Alencar Filho *et al.* 2017) and as a preservative in goat meat products (Rigo 2015). Its EO is recognised as a promising alternative source for antimicrobial agents and

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natural antioxidants (Araújo *et al.* 2017; Brito *et al.* 2018). According to Araújo *et al.* (2017), the EO of the aerial part of *C. heliotropiifolius* is effective against multidrug-resistant *S. aureus*. In addition, *C. heliotropiifolius* EO contains bioactive compounds that can contribute to increase the nutritional value of the food (Brito *et al.* 2018).

Thus, in this context, the purposes of this study were (i) to characterise the virulence potential of *S. aureus* strains from goat milk and coalho cheeses on the basis of phenotypic properties related to cell adhesion and the detection of genes related to enterotoxin production, biofilm-related and β-lactam resistance and (ii) to evaluate the inhibitory activity of nisin, GA and *C. heliotropiifolius* EO alone or in synergistic combinations against *S. aureus* strains circulating in goat dairy products produced in the Brazilian semi-arid region.

MATERIALS AND METHODS

Samples

From June 2016 to August 2017, a total of 140 samples, 70 of raw goat milk and 70 of artisanal goat coalho cheese, were collected from 10 rural properties in each of the seven municipalities of the north-eastern semi-arid region: Petrolina, Santa Maria da Boa Vista, Santa Filomena, Dormentes and Afrânio in the state of Pernambuco (PE) and Juazeiro and Uauá in the state of Bahia (BA), Brazil (Figure 2). A commonality of the rural properties was that they all obtained milk for the elaboration of coalho cheese. Coalho cheese is a typical dairy product from the north-east region of Brazil. The artisanal goat coalho cheese samples investigated in this study were obtained by coagulation of raw goat milk by chymosin. The samples were collected under refrigeration and transported immediately to the laboratory in isothermal boxes.

Figure 1 Velame (*Croton heliotropiifolius*) in the caatinga biome. [Colour figure can be viewed at wileyonlinelibrary.com]

Isolation and identification of *Staphylococcus aureus*

A total of 25 mL of milk and 25 g of coalho cheese were analysed. The procedures for obtaining and diluting the goat milk and coalho cheese were performed according to those described by Almeida Júnior *et al.* (2015) and Castro *et al.* (2018), respectively. Isolation and biochemical confirmation of *S. aureus* were conducted using the methodology described by Brasil (2003). Aliquots of 0.1 mL of each sample were plated onto the surface of Baird Parker agar (Sigma-Aldrich, St. Louis, MO, USA), containing 50% egg yolk emulsion (HiMedia, Mumbai, India) and 3.5% potassium tellurite (Sigma-Aldrich) in duplicate and incubated for 24-48 h at 37 °C. For *S. aureus* detection, plates with 20-200 typical presumptive *S. aureus* colonies (colonies with black centres surrounded by clear halos) were counted. The microbiological counts are expressed as the logarithm of the number of colony-forming units per millilitre or per gram (CFU/mL or CFU/g, respectively). According to the methods of Xing *et al.* (2016), two colonies per sample were selected from each plate and subjected to subsequent analyses. For biochemical identification of *S. aureus* from typical colonies, gram staining (Gram Staining Kit, Laborclin, Pinhais, PR, Brazil), catalase assays (Vetec, Duque de Caxias, RJ, Brazil), coagulase assays (Laborclin), mannitol and glucose oxidation and fermentation (O/F) assays (Synth, Diadema, SP, Brazil), and thermonuclease and DNase assays (DNase test agar w/toluidine blue, HiMedia) were performed.

The DNA of isolates that were biochemically identified as *S. aureus* was extracted with a PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the instruction manual. PCR was used to detect the expression of the thermonuclease gene (*nuc*) (Xing *et al.* 2016). The 16S rRNA gene was amplified according to Lange *et al.* (2011). PCR was conducted on an Applied Biosystems 2720 Thermo Cycler (Applied Biosystems, Carlsbad, CA, USA). The information on the primers used is shown in Table 1. The PCR products were separated on 0.8% agarose gels and visualised by staining with Safer® dye (Kasvi, São José dos Pinhais, PR, Brazil) using an LED transilluminator (470 nm) (Kasvi). Partial sequencing of the 16S rRNA gene was performed by Helixxa (Paulínia, SP, Brazil) and

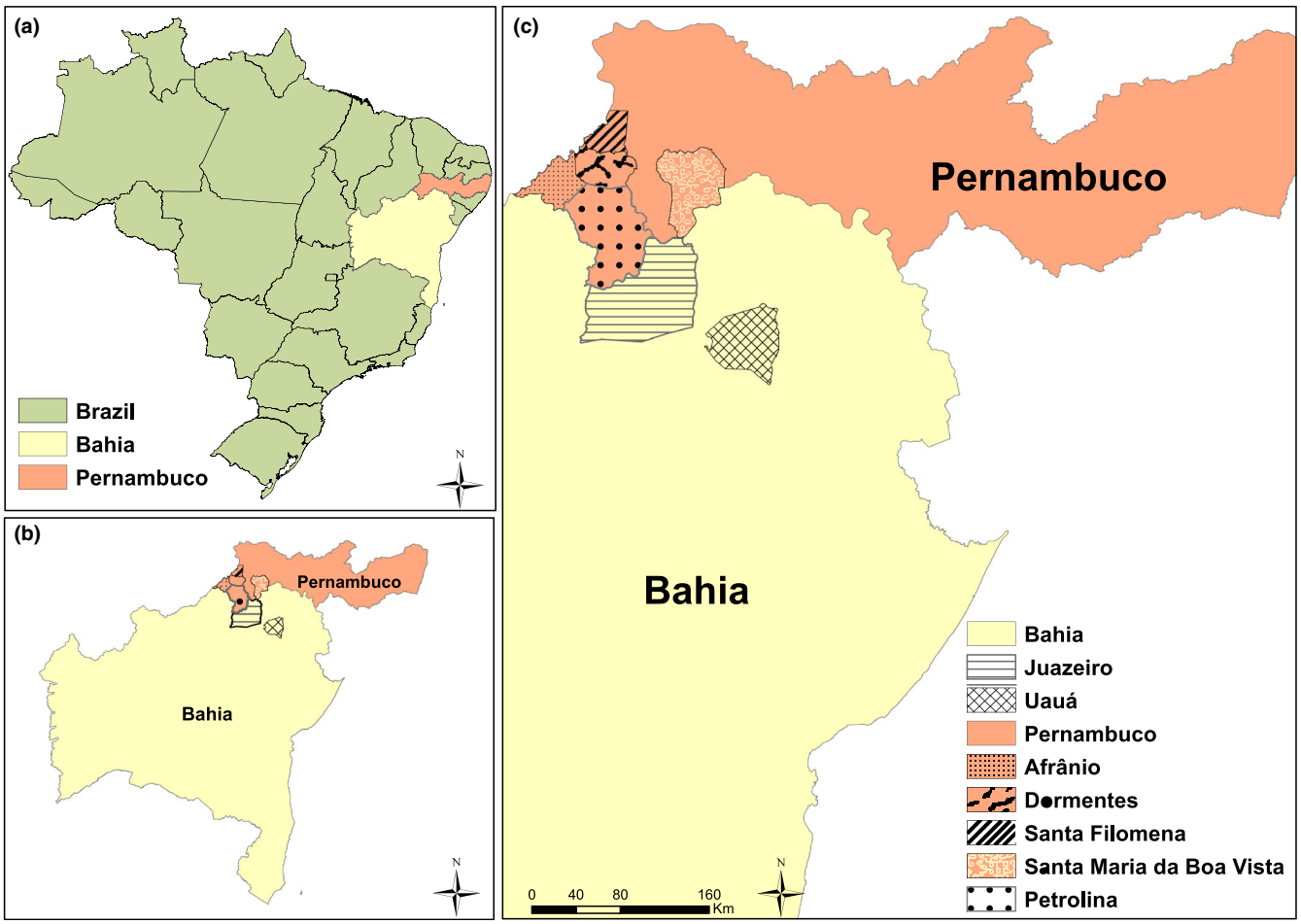

Figure 2 Location map. (a) Brazil. (b) States of Pernambuco and Bahia in the north-east region of Brazil. (c) Cities in which goat milk and cheese samples were obtained.

compared for similarity with deposited sequences in the GenBank public database.

Phenotypic characteristics related to cell adhesion

Hydrophobicity

The isolates were cultured in tubes containing brain heart infusion (BHI) (Sigma-Aldrich) for 4 h and 24 h at 37 °C. The bacterial suspension of each isolate was standardised in phosphate buffered saline (PBS) to an optical density (OD) of 1 at 550 nm. In a tube, 3.6 mL of each bacterial suspension and 0.4 mL of xylol were combined. After homogenisation, the tube was allowed to stand for 20 min. After the removal of the lower layer, the absorbance was measured at 400 nm. The hydrophobicity index (HI) was determined using the following formula: $HI = 100 \times (Vi - Vf)/Vi$, where Vi is the initial absorbance and Vf is the final absorbance. Bacteria with an HI > 70% were considered to be highly hydrophobic, those with an HI < 30% were considered to be highly hydrophilic, and those with an HI 30% < HI < 70% were considered to be moderately hydrophobic. BHI without inoculum was used as a blank (Rodrigues *et al.* 2009).

Exopolysaccharide (EPS)

After each *S. aureus* isolate was cultured in a tube containing 20 mL of BHI for 3 days at 37 °C, the cells were collected by centrifugation (6000 *g*) for 20 min. Ethanol (Vetec) was added at a ratio of 2 parts per 1 part supernatant to precipitate EPS. Vacuum filtration was performed using paper filters (Unifil®, Alvorada, RS, Brazil) that had previously been labelled and weighed. After filtration, the filters were placed in trays and incubated at 60 °C for 3 h, and the weight of each filter was again measured to quantify the EPS produced. EPS production below 10 mg/L was considered to be very low (Van Geel-Schutten *et al.* 1998).

Qualitative determination of biofilm production

The isolates were streaked onto plates containing Congo red agar (CRA, HiMedia), and the plates were incubated for

Table 1 Oligonucleotides used for the identification of *Staphylococcus aureus* isolates and virulence genes.

Gene	*Sequence 5′-3′*	*Reference*
nuc	GCGATTGATGGTGATACGGTTAGCCAAGCCTTGACGAACTAAAGC	Xing *et al.* (2016)
16S-rRNA	AGAGTTTGATCCTGGCTCAGGTATTACCGCGGCTGCTG	Lange *et al.* (2011)
mecA	AAAATCGATGGTAAAGGTTGGCAGTTCTGCAGTACCGGATTTGC	Sawant *et al.* (2009)
blaZ	AAGAGATTTGCCTATGCTTCGCTTGACCACTTTTATCAGC	Sawant *et al.* (2009)
sea	ACGATCAATTTTTACAGCTGCATGTTTTCAGAGTTAATC	Kroning *et al.* (2016)
seb	ATTCTATTAAGGACACTAAGTTAGGGGAATCCCGTTTCATAAGGCGAGT	Kroning *et al.* (2016)
sec	GACATAAAAGCTAGGAATTTAAATCGGATTAACATTATCCA	Kroning *et al.* (2016)
sed	CAAATATATTGATATAATGAAGTAAAAAAGAGTAATGCAA	Kroning *et al.* (2016)
see	CAAAGAAATGCTTTAAGCAATCTTAGGCCACCTTACCGCCAAAGCTG	Kroning *et al.* (2016)
*ica*A	CCTAACTAACGAAAGGTAGAAGATATAGCGATAAGTGC	Kroning *et al.* (2016)
*ica*D	AAACGTAAGAGAGGTGGGGCAATATGATCAAGATAC	Kroning *et al.* (2016)

24 h at 35 °C before being left at room temperature for another 48 h. Colony colour was used to classify each isolate in terms of biofilm production as described by Rodrigues *et al.* (2017).

Quantification of biofilm production

The isolates were inoculated in TSB enriched with glucose and incubated at 37 °C for 24 h. A volume of 200 μL of each culture was then added to each microplate well, and the microplate was incubated at 37 °C for 24 h. The culture medium present in each microplate was discarded, and three washes were performed with 200 μL of autoclaved distilled water. After air drying, 100 μL of 0.25% crystal violet (Laborclin) solution was added, and each microplate was incubated for 3 min at room temperature. Then, three more washes were performed. After drying, 200 μL of alcohol: acetone (80:20) (Vetec) was added, and the OD was measured at 595 nm. *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 and *Staphylococcus epidermidis* ATCC 12228 were used as the positive control and the negative control (NC), respectively. Biofilm production was classified as follows: no biofilm production (sample OD ≤ NC OD), poor biofilm production (NC OD < sample OD ≤ 2× NC OD), moderate biofilm production (NC OD < sample OD ≤ 4× NC OD) and strong biofilm production (sample OD > 4× NC OD (Merino *et al.* 2009).

Virulence and antimicrobial resistance genes

The virulence and antimicrobial resistance genes harboured by the *S. aureus* isolates were characterised by PCR assay. The primers utilised are listed in Table 1. PCR was performed using premixed GoTaq® Green Master Mix 2× solution (Promega Corp., Madison, WI, USA) in a total volume of 25 μL, which included 2 μL of DNA (50 ng/μL), 12.5 μL of Master Mix, 1 μL of each primer (10 pmol/μL) and 8.5 μL of nuclease-free water, for the detection of classic staphylococcal enterotoxin genes (*sea*, *seb*, *sec*, *sed* and *see*), biofilm-related genes (*ica*A and *ica*D) (Kroning *et al.* 2016) and antimicrobial resistance genes against methicillin (*mec*A) and penicillin (*blaZ*) (Sawant *et al.* 2009). *Staphylococcus aureus* ATCC 33591 was used as a positive control

Table 2 Numbers of positive samples and count ranges and averages for *Staphylococcus aureus* in samples of goat milk and goat coalho cheese from 10 rural properties located in seven municipalities of the Brazilian north-eastern semi-arid region.

	Raw goat milk			*Artisanal goat coalho cheese*		
Municipalities	*Positive samples (n = 10)*	*Count range (log_{10} CFU/mL)*	*Counting average (log_{10} CFU/mL)*	*Positive samples (n = 10)*	*Count range (log_{10} CFU/g)*	*Average count (log_{10} CFU/g)*
Afrânio	4	0–3.33	1.07	10	4.59–5.90	5.31
Dormentes	4	0–3.08	1.10	10	4.48–5.69	4.92
Santa Filomena	5	0–3.46	1.47	10	4.56–5.73	4.96
Santa Maria	6	0–3.62	1.77	10	4.49–6.16	5.08
Petrolina	6	0–3.58	1.74	10	4.64–6.91	5.25
Juazeiro	10	2.66–3.46	3.11	10	4.91–6.50	5.81
Uauá	8	0–3.99	2.31	10	4.61–6.48	5.02

Table 3 Origins, virulence and antimicrobial resistance genes and cell adhesion-related phenotypic characteristics of the isolates confirmed as *Staphylococcus aureus*.

			Phenotypic characteristics related to cell adhesion				*Genes detected*								
							Enterotoxins					*Intercellular adhesion*		*β-lactam resistance*	
Sample	*City*	Staphylococcus aureus *isolates*	*Hydrophobicity (%)1*	*EPS (mg)2*	*Qualitative biofilm*	*Quantitative biofilm*	sea	seb	sec	sed	see	ica*A*	ica*D*	mec*A*	bla*Z*
Milk	Petrolina	L2	83.62[a]	16.86[f]	+	Strong	−	−	+	−	−	−	+	+	+
Milk	Santa Filomena	L13	50.05[i]	23.66[c]	+	Moderate	−	−	−	+	−	−	+	+	+
Milk	Santa Filomena	L19	35.56[l]	20[e]	+	Weak	−	−	−	+	−	−	+	+	+
Milk	Santa Maria	L22	82.96[a]	26[b]	+	Strong	−	−	−	+	−	−	+	+	+
Milk	Santa Maria	L26	33.62[m]	10[h]	+	Weak	−	−	+	−	−	−	+	+	+
Milk	Santa Maria	L30	53.23[h]	16.53[f]	+	Moderate	−	−	−	+	−	−	+	+	+
Milk	Dormentes	L34	74.70[c]	10.33[h]	+	Moderate	−	−	−	+	−	−	+	+	+
Milk	Dormentes	L38	62.08[f]	3.73[j]	+	Moderate	−	−	−	+	−	−	+	+	+
Milk	Afrânio	L46	67.60[e]	22.66[d]	+	Moderate	−	−	+	−	−	−	+	+	+
Milk	Afrânio	L47	82.95[a]	36.66[a]	+	Strong	−	−	+	+	−	−	+	+	+
Milk	Juazeiro	L54	79.16[b]	25.33[b]	+	Strong	−	−	+	+	−	−	+	+	+
Milk	Juazeiro	L56	61.36[f]	14[g]	+	Moderate	−	−	+	−	−	−	+	+	+
Milk	Juazeiro	L57	69.32[d]	23.00[d]	+	Moderate	−	−	−	+	−	−	+	+	+
Milk	Uauá	L63	40.48[j]	6.55[i]	+	Weak	−	−	−	+	−	−	+	+	+
Cheese	Petrolina	Q8	55.39[g]	3.44[j]	+	Moderate	−	−	+	−	−	−	+	+	+
Cheese	Petrolina	Q10	83.74[a]	6.44[i]	+	Strong	−	−	+	+	−	−	+	+	+
Cheese	Santa Maria	Q11	62.19[f]	20.20[e]	+	Moderate	−	−	−	−	−	−	+	+	+
Cheese	Santa Maria	Q19	61.20[f]	16.66[f]	+	Moderate	−	−	−	−	−	−	+	+	+
Cheese	Juazeiro	Q22	42.08[k]	10.33[h]	+	Weak	−	−	−	+	−	−	+	+	+
Cheese	Juazeiro	Q23	83.47[a]	23.78[c]	+	Strong	−	−	−	−	−	−	+	+	+

For each column, mean values with different letters are significantly different ($P < 0.05$) according to the Scott–Knott test. +: positive, −: negative.

[1]Standard error (SE) = 0.36.

[2]SE = 0.24.

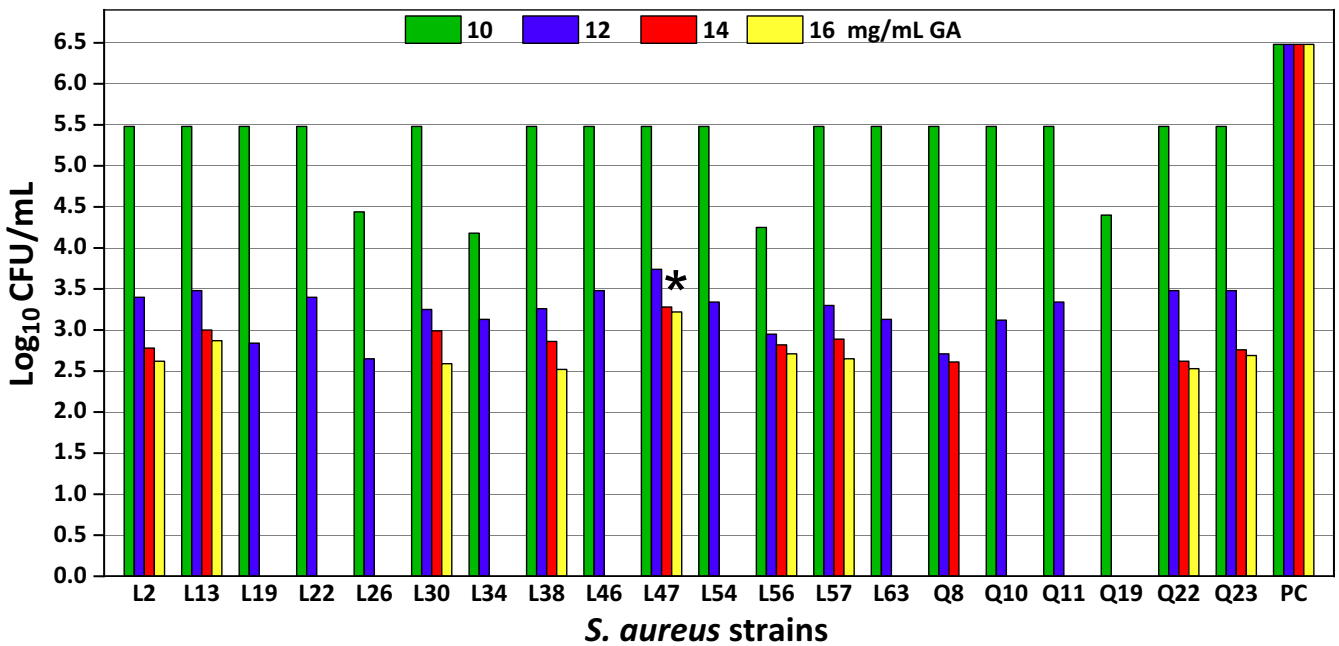

Figure 3 Effects of different concentrations of gallic acid (GA) on the biofilms of *Staphylococcus aureus* strains. *Strain with the highest resistance to 12, 14 and 16 mg/mL GA among the 20 isolated strains analysed according to the Scott–Knott test ($P < 0.05$). PC: positive control. SEs: 10 mg/mL GA, 0.01; 12 mg/mL GA, 0.06; 14 mg/mL GA, 0.003; and 16 mg/mL GA, 0.003.

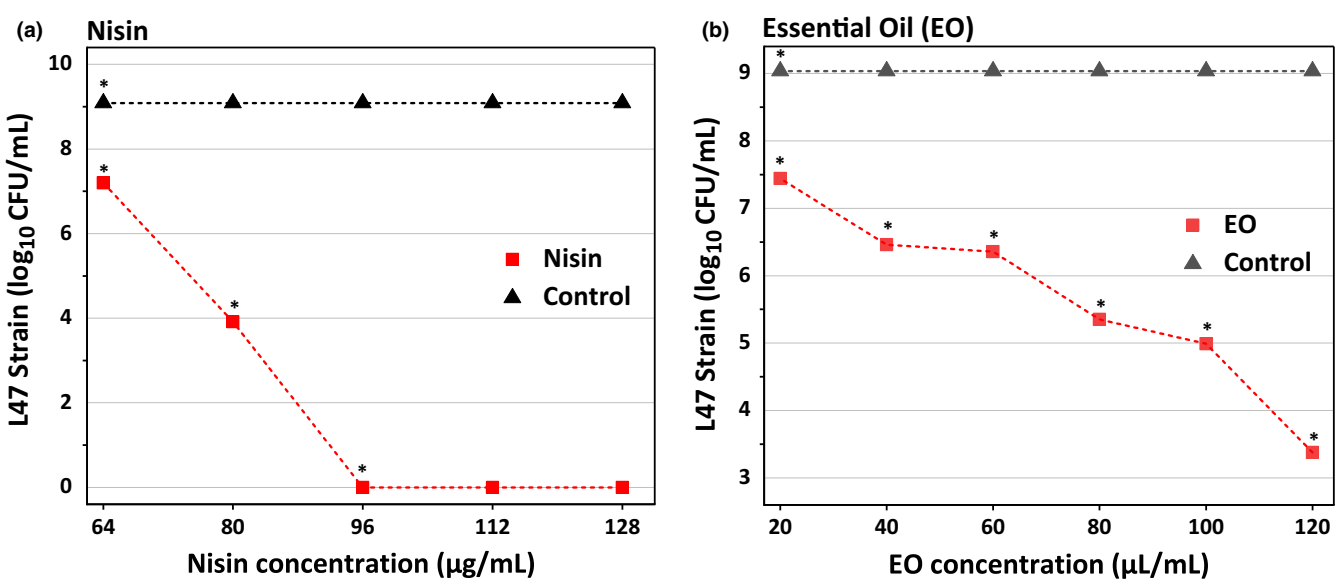

Figure 4 Effects of different concentrations of (A) nisin and (B) essential oil (EO) on *Staphylococcus aureus* L47 strain biofilm. *Mean values differ statistically according to the Scott–Knott test ($P < 0.05$). SEs: nisin, 0.02; EO, 0.007.

in the reactions for *ica*A, *ica*D, *mec*A and *bla*Z. The positive controls for the enterotoxin genes, donated by Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, RJ, Brazil), included *S. aureus* Instituto Nacional de Controle de Qualidade em Saúde (INCQS) strain 00285 (*sea* and *sed*), *S. aureus* INCQS 00005 (*seb*), *S. aureus* INCQS 00080 (*sec*) and *S. aureus* INCQS 00093 (*see*).

GA antimicrobial activity assay against *S. aureus* biofilms

Staphylococcus aureus isolates were cultured in BHI for 24 h at 37 °C. Volumes of 200 µL of each isolate with standardised populations of 10^6 CFU/mL were transferred to the wells of a microplate, and the plate was incubated at 24 h at 37 °C. After biofilm formation, each well was

washed with PBS, GA (Sigma-Aldrich) was added at concentrations of 10, 12, 14 and 16 mg/mL, and the plate was re-incubated under the same conditions as before. Thereafter, the GA was removed, the biofilms were recovered from the wells by swabbing and washing with sterile saline, and plating was conducted on BHI agar. As a positive control, a sterile saline solution without gallic acid was used. The concentration of GA with the greatest inhibitory activity against *S. aureus* growth on Baird Parker agar (causing a decrease greater than 3 $\log_{10}$ CFU/mL from the value in the positive control) and the isolate with the highest resistance to this agent were selected for the following tests (Liu *et al.* 2017).

Nisin antimicrobial activity assay against *S. aureus*

According to the methods of Shi *et al.* (2017), with minor modifications, the cells of the most resistant *S. aureus* isolate (L47) in the previous test were standardised to 10^9 CFU/mL in Müller Hinton (MH) broth (HiMedia). In a cuvette, 1 mL of the standardised inoculum and 1 mL of nisin at each concentration tested (64, 80, 96, 112 and 128 µg/mL) were mixed. After incubation for 24 h at 37 °C, plating assays were conducted on Baird Parker agar. Inoculum in MH broth without nisin was used as the positive control. The concentration of nisin that reduced the *S. aureus* population in Baird Parker agar by at least 5 $\log_{10}$ CFU/mL (without causing total inhibition) from the positive control value was selected for the following tests.

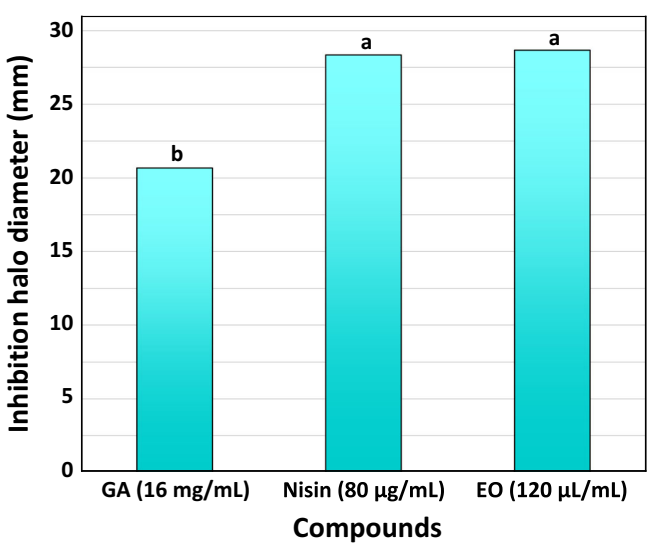

Figure 5 Diameters of the inhibition halos (mm) of gallic acid (GA), nisin and essential oil (EO) against *Staphylococcus aureus* L47. Mean values with different letters are significant ($P < 0.05$) according to the Scott–Knott test. SE: 0.57.

Procurement and assessment of the antimicrobial activity of *Croton heliotropiifolius* (velame) EO against *S. aureus*

Plant material

The aerial part of *C. heliotropiifolius* was collected from the caatinga biome in the city of Petrolina, PE, in December 2018. A voucher specimen was deposited in the San Francisco Valley Herbarium (HVASF) under number 13963, Agrarian Sciences Campus, Federal University of San Francisco Valley (UNIVASF), Petrolina, PE, Brazil.

Distillation of EO

The fresh leaves were weighed, immersed in distilled water (150 g:1500 mL) and hydrodistilled for 2 h to extract the essential oil in a Clevenger apparatus. For removal of the remaining water, anhydrous sodium sulphate (Na_2SO_4; Synth) was added to the obtained organic phase, and then filtration and centrifugation (6000 *g*, 4 °C) were performed. The EO was stored frozen in an amber vial.

Antimicrobial activity of EO

EO solution was obtained according to the methods of Oliveira *et al.* (2006) at a concentration of 120 µL/mL (600 µL of EO and 40 µL of Tween 80 in sterile distilled water to a total volume of 5 mL). Dilutions with concentrations of 100, 80, 60, 40 and 20 µL/mL were obtained and tested for their ability to inhibit *S. aureus* growth. According to the antimicrobial activity test described in Section 2.6, a 1-mL aliquot of the *S. aureus* isolate in MH broth was tested against aliquots of 1 mL of the different EO concentrations. After incubation, the mixtures were plated on Baird Parker agar. In addition, the viability of the isolate under different EO concentrations was tested with 0.5% 2,3,5-triphenyl-tetrazolium (CTT) (Sigma-Aldrich). After incubation, prior to plating, 1 mL of each treatment (isolate + each EO concentration tested) was withdrawn and added to 100 µL of CTT. The isolate only and the EO only were used as the positive and negative controls, respectively. The appearance of red staining indicated a positive result for cell viability and activity. The concentration of EO that reduced the *S. aureus* population on Baird Parker agar by at least 5 $\log_{10}$ CFU/mL (without causing total inhibition) from the positive control value was selected.

Agar disc diffusion: GA, nisin and EO

An agar disc diffusion test was performed using the selected concentrations (determined in the previous tests) of GA, nisin and EO. Volumes of 100 µL of a 10^6 CFU/mL suspension of the *S. aureus* isolate were spread on Petri dishes containing Müller Hinton agar (HiMedia). Paper discs embedded with GA, nisin and OE (5 µL of each selected concentration) were placed onto the agar surface, and the dishes were incubated for 24 h at 37 °C. The diameter of

Table 4 Counts of viable *Staphylococcus aureus* in goat milk (log_{10} CFU/mL) with or without the addition of antimicrobial compounds stored for different hours at 37 °C.

	Log_{10} CFU/mL of Staphylococcus aureus *at different time points (h)*				*Decreases in log units at different time points (h) (from 0 h)*		
Goat milk with antimicrobial compounds	*0*	*3*	*6*	*24*	*3*	*6*	*24*
GA	6.477^{aA}	3.908^{cB}	3.45^{dC}	3.02^{bD}	2.569	3.027	3.457
Nisin	6.477^{aA}	3.153^{eB}	2.829^{gC}	2.771^{eD}	3.324	3.648	3.706
EO	6.477^{aA}	6.477^{bA}	3.202^{eB}	2.690^{fC}	0	3.275	3.787
Nisin + GA	6.477^{aA}	3.656^{dB}	3.060^{fC}	2.895^{cD}	2.821	3.417	3.582
EO + GA	6.477^{aA}	6.477^{bA}	3.684^{cB}	2.843^{dC}	0	2.793	3.634
EO + nisin	6.477^{aA}	6.477^{bA}	3.748^{bB}	2.700^{fC}	0	2.729	3.777
	Log_{10} CFU/mL of Staphylococcus aureus *at different time points (h)*				*Increases in log units at different time points (h) (from 0 h)*		
Goat milk without antimicrobial compounds	*0*	*3*	*6*	*24*	*3*	*6*	*24*
PC	6.477^{aA}	6.826^{aB}	7.178^{aC}	7.880^{aD}	0.349	0.701	1.403

Mean values with different superscript lower-case letters (columns) or different superscript capital letters (rows) are significant ($P < 0.05$) according to the Scott–Knott test. SE: 0.015.

GA = gallic acid, EO = essential oil, PC = positive control.

the inhibition halo formed by each antimicrobial compound (nisin, GA and EO) was measured with a digital calliper, and the intensity of the inhibitory activity was classified according to the system of Shi *et al.* (2017).

Antimicrobial activity of GA, nisin and OE against the *S. aureus* isolate in its environment of origin: goat milk

This test used 8 L of pasteurised goat milk. The *S. aureus* isolate L47 was added to 7 L of pasteurised goat milk to a population of 10^6 CFU/mL. This volume of milk was divided into 7 1-L portions. No antimicrobial was added to one of the portions (positive control), while GA, nisin, EO, nisin + GA, EO + GA and EO + nisin were separately added to each of the other 6 portions. Nothing was added to an additional 1 L of pasteurised goat milk (completing the 8 L used in the experiment); this sample was the negative control. Each 1 L sample was incubated at 37 °C for 24 h. Baird Parker agar was used to count the *S. aureus* isolate L47 colonies at 0, 3, 6 and 24 h. The counts of the colonies were recorded to determine the activity of the antimicrobial compounds alone or in combination on the growth of the isolate in its environment of origin.

Statistical analysis

The tests involving phenotypic characteristics related to cell adhesion, antimicrobial activity and agar disc diffusion were performed in triplicate (exception for the tests: isolation, identification of *S. aureus* and detection of virulence and antimicrobial resistance genes). SISVAR® software (version 5.6, Lavras, MG, Brazil) was used for the statistical analyses. The data were evaluated using ANOVA, and the means were compared by Scott–Knott test. A probability value $P < 0.05$ was considered to indicate statistical significance. The graphics were developed in Origin® (Student version 2018b, Northampton, MA, USA).

RESULTS AND DISCUSSION

Staphylococcus aureus detection, assessment of phenotypic properties for cell adhesion and screening for antimicrobial resistance and virulence genes

In 43 (61.43%) and 70 (100%) milk and cheese samples, respectively, presumptive *S. aureus* was detected (Table 2). For the milk samples, the mean counts in the 7 municipalities ranged from 1.18×10^1 to 1.29×10^3 CFU/mL, while for the cheese samples, the counts ranged from 8.32×10^4 to 6.46×10^5 CFU/g. *Staphylococcus aureus* is the pathogen most commonly isolated from milk, and goat derivatives, milkers and artisan cheesemakers are some of the potential transmitters of this microorganism along the milk chain (Castro *et al.* 2018; Anderson *et al.* 2019; Aragão *et al.* 2019). Our study draws attention to a direct risk to the consumer given the probability of production of enterotoxins due to the quantitative populations of presumptive *S. aureus* in the cheeses from all the properties studied. According to Yoon *et al.* (2016), there is an increased probability of enterotoxin production when *S. aureus* populations are at least 10^4 CFU/g.

After biochemical characterisation and *nuc* gene amplification were performed for the presumptive *S. aureus*

isolates, 20 isolates were confirmed, 14 from milk and 6 from cheese (Table 3). The isolates were identified to have 99% similarity with *S. aureus* (NR_113956.1) by sequencing the 16S rRNA gene.

Among the phenotypic properties related to cell adhesion, high hydrophobicity was observed ($P < 0.05$) in 7 isolates (Table 3): L2, L22, L34, L47, L54, Q10 and Q23. Moderate hydrophobicity was verified in 13 isolates. Regarding the production of EPS, isolate L47 was the major producer ($P < 0.05$), while 4 isolates were weak producers, producing below 10 mg/L EPS (Table 3). In the qualitative evaluation, all isolates presented biofilm-forming ability. Upon quantification, 6, 10 and 4 isolates were classified as strong, moderate and weak biofilm producers, respectively. Isolates L2, L22, L47, L54, Q10 and Q23 were the strong biofilm producers (Table 3). Among the isolates, the L47 isolate most strongly demonstrated phenotypic characteristics related to cell adhesion.

Screening for virulence and antimicrobial resistance genes revealed that the staphylococcal enterotoxin genes *sec* and *sed* were present in 8 (40%) and 12 (60%) isolates, respectively. In addition, 3 isolates had the genes for both enterotoxins, C and D (Table 3). With regard to intercellular adhesion genes, all isolates were positive and negative for *ica*D and *ica*A, respectively (Table 3). All *S. aureus* isolates contained the resistance genes for methicillin (*mec*A) and penicillin (*bla*Z) (Table 3).

The findings of our study are extremely relevant to public health, not only because of the risk of consumer intoxication but also because milk and goat cheese are sources of dissemination of methicillin-resistant *S. aureus* (MRSA). MRSA is resistant to most of the beta-lactam antimicrobials usually used in animal treatments (Papadopoulos *et al.* 2018). However, cell adhesion and biofilm formation abilities amplify the problem, since these traits facilitate antimicrobial resistance gene exchange between bacteria, decrease drug permeability, create barriers and thus contribute to the emergence of new MRSA strains. Coimbra-e-Souza *et al.* (2019) reported that the transmission of antimicrobial resistance among *Staphylococcus* spp. isolated from goat milk is facilitated by the ability of the isolates to form biofilms.

Castro *et al.* (2018) reported that *S. aureus* is the predominant pathogen in artisanal goat coalho cheese from the north-eastern semi-arid region. Because this cheese is a local food product with economic and cultural importance, strategies to increase its safety are needed, such as inclusion of antimicrobial compounds with effective action against *S. aureus*.

Effect of GA on *S. aureus* biofilms

The antimicrobial activity of GA at different concentrations was tested on the 20 isolates of *S. aureus* (Figure 3). All studied concentrations showed inhibitory activity compared to a lack of GA (the positive control). Higher concentrations of GA exhibited greater inhibition of biofilm formation. Total inhibition of biofilm formation for all isolates was achieved at 18 mg/mL GA. At 16 mg/mL GA, there was total inhibition of biofilm formation for 11 *S. aureus* isolates. At concentrations of 12, 14 and 16 mg/mL GA, strain L47 showed the least inhibition of biofilm formation among the 20 isolates ($P < 0.05$), with counts of 3.74, 3.28 and 3.22 $\log_{10}$ CFU/mL, respectively (Figure 3). GA is an antioxidant extracted from plants that is safe for use in the food industry (Li *et al.* 2019). GA inhibits *S. aureus* biofilm formation by regulating *ica* operon expression (Liu *et al.* 2017). GA is a phenolic acid that alters the surface properties of the bacterial membrane as well as the cell permeability. The action of GA on cell membranes is correlated with the phenolic acid concentration. Experiments have shown that GA reduces cell respiratory activity and increases hydrophobicity of the cell surface of *S. aureus* (Borges *et al.* 2013; Luís *et al.* 2013).

To compare the action of GA with that of the other antimicrobial compounds (nisin and EO), we selected the GA concentration that most strongly inhibited biofilm formation by all the isolates tested without causing total inhibition and selected the most resistant strain among the 20 isolates from goat cheese and milk. In addition, bacteria in biofilms were used because of their greater resistance to chemical agents than planktonic bacteria (Liu *et al.* 2017). When testing GA concentrations, a reduction of 3 log units was used as the cut-off, so that the product must be framed with the current Brazilian legislation (Brasil 2001. Given the relatively low inhibition of biofilm formation at the concentration of 16 mg/mL GA for isolate L47 (3.22 $\log_{10}$ CFU/mL), this isolate was subjected to further testing with the other antimicrobial compounds. The concentration of 16 mg/mL GA was used as the standard in subsequent tests.

Effects of nisin and EO on *S. aureus* L47

The effects of nisin and EO were tested on planktonic cells of isolate L47. Based on the average *S. aureus* counts in cheeses produced in the region, the concentrations that decreased the population of isolate L47 by at least 5 log units (without causing total inhibition) from the positive control value were selected for both nisin and EO. The 80 μg/mL nisin (Figure 4a) and 120 μL/mL EO (Figure 4b) concentrations achieved such inhibition. These concentrations were subsequently tested for the inhibition of *S. aureus* growth in goat milk.

Nisin is widely used in the food industry, and it has even been recommended as a preservative for the technical regulation of cheese identity and quality in Brazil (Brasil 1996). Nisin interacts with the *S. aureus* cytoplasmic membrane through lipid II, causing permeabilisation (depolarisation), loss of cellular integrity and inhibition of cell wall biosynthesis (Breukink and Kruij 1999).

Croton heliotropiifolius has medicinal properties. Velame is widely used by local communities in Brazil, especially for the treatment of gastrointestinal disorders and general infections involving fever (Brito *et al.* 2018; Silva *et al.* 2018). Consistent with our results, Araújo *et al.* (2017) reported the antimicrobial activity of *C. heliotropiifolius* against *S. aureus* and stated that EO has potential applications against multidrug-resistant strains of *Staphylococcus* spp. The EO of *C. heliotropiifolius* has high percentages of sesquiterpenes, particularly (E)-β-caryophyllene, demonstrating strong antimicrobial and antioxidant activity (Alencar filho *et al.* 2017; Araújo *et al.* 2017; Brito *et al.* 2018). The antibacterial activity of sesquiterpenes is related to rupture of cell microtubules, decreases in fatty acids and disturbances in the cell membrane that lead to membrane potential alteration, ion loss, proton pump collapse and ATP depletion (Huang *et al.* 2012). In particular, oxygen species present in the environment react with (E)-β-caryophyllene. Photo-oxidised (E)-β-caryophyllene generates oxidation products with extremely strong antibacterial activity against *S. aureus* (Atkinson and Arey 2003; Kim *et al.* 2008).

Agar disc diffusion: GA, nisin and EO

To re-confirm the suitability of the concentrations of GA, nisin and EO selected through the tests described above for the inhibition of *S. aureus*, an agar disc diffusion test was performed. According to Shi *et al.* (2017), in addition to being a simple, inexpensive and widely used method to test the susceptibility of microorganisms, the agar disc diffusion test also presents excellent reliability. In this study, inhibition halos 20.67, 28.33 and 28.67 mm in diameter were observed when isolate L47 was exposed to GA (16 mg/mL), nisin (80 μg/mL) and EO (120 μL/mL), respectively (Figure 5). There was no difference ($P < 0.05$) in inhibitory activity between nisin and EO against isolate L47. This result reflects the antimicrobial potential of EO compared with the other tested compounds.

Antimicrobial activity of GA, nisin and OE against the *S. aureus* isolate in its environment of origin: goat milk

The 3 antimicrobial compounds tested inhibited the growth of *S. aureus* L47 in goat milk. There was a significant interaction between count and time of antimicrobial action ($P < 0.05$). In goat milk without the addition of antimicrobial compounds (positive control), *S. aureus* L47 presented growth of 0.349, 0.701 and 1.403 log units at 3, 6 and 24 h, respectively (Table 4).

At 3 and 6 h, the antimicrobial with the highest inhibitory activity against the isolate L47 was nisin ($P < 0.05$), reducing the microbial counts by 3.324 and 3.648 log units, respectively. The combination of nisin + GA at 3 and 6 h caused reductions of 2.821 and 3.417 log units of pathogen in goat milk, respectively (Table 4). At 3 h, GA was the third most effective antimicrobial compound against *S. aureus* L47. However, GA should be used in combination because of its thermal instability and rapid decomposition (Li *et al.* 2019). The synergism between nisin and GA can be explained by GA-mediated alteration of the surface tension of the *S. aureus* membrane, causing it to become more hydrophobic. Hydrophobic interactions allow insertion of the N-terminal region of nisin into the pathogen's lipid membrane (Breukink and Kruij 1999; Borges *et al.* 2013).

EO, both alone and in combinations with GA and nisin, inhibited the growth of isolate L47 in goat milk beginning at 6 h (Table 4). At 24 h, EO conferred better inhibition than the other treatments, with no significant difference between EO and EO + nisin. *Staphylococcus aureus* L47 populations were reduced by 3.787 and 3.777 log units for EO and EO + nisin, respectively. The EO + GA combination was more effective than the nisin + EO combination at 6 h ($P < 0.05$) (Table 4).

The most effective response of the EO against the growth of *S. aureus* L47 corroborates the findings of a study by Kim *et al.* (2008). As previously reported, this greatest inhibitory action in 24 h can be explained by the time necessary for reactions to take place that lead to photooxidation of β-caryophyllene by oxygen present in the environment, generating new products with strongly enhanced inhibitory action against *S. aureus*. With regard to EO, its synergism with nisin can potentiate collapse of the cytoplasmic membranes of microorganisms. EO alters the permeability of cell membranes and causes the release of cell content. This effect is most evident in combination treatment with nisin (Moosavy *et al.* 2008; Zhang *et al.* 2014; Shi *et al.* 2017). GA can also have a synergistic effect with EO, since the action of EO is improved under hydrophobic and low-pH conditions (Burt 2004).

Thus, in general, on the basis of the results of this study, it is possible to conclude that at 3 and 6 h, nisin was the most effective antimicrobial compound. At 24 h, EO and/or EO + nisin showed higher inhibitory activity against *S. aureus* L47 in goat milk than the other treatments. As artisanal goat coalho cheese is usually marketed the day after its manufacture, EO can be used to control MRSA growth in such products and to prevent staphylococcal poisoning in consumers.

CONCLUSION

Artisanal goat coalho cheese is part of the cultural identity of the population of the north-eastern semi-arid region, and the results of this study draw attention to the quality of this product. In the regional goat dairy chain, MRSA with virulence and colonisation abilities is circulating. Inclusion of natural anti-staphylococcal compounds in cheese could be a solution to this problem. However, for such compounds to be used, acquisition facilities and acceptance by local producers and consumers are needed. Velame is abundantly

found in the caatinga and is a traditional part of everyday life, as it is popularly used as a medicinal herb and for the conservation of goat meat. Therefore, the inclusion of its essential oil will not affect the regional culture or geographical origin of the cheese. Thus, *C. heliotropiifolius* essential oil is an alternative for the control of MRSA in artisanal goat cheese in this Brazilian region.

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REFERENCES

Alencar Filho J M T, Araújo L C, Oliveira A P, Guimarães A L, Pacheco A G M, Silva F S, Cavalcante L S, Lucchese A M, Almeida J R G and Araújo E C C (2017) Chemical composition and antibacterial activity of essential oil from leaves of *Croton heliotropiifolius* in different seasons of the year. *Revista Brasileira de Farmacognosia* **27** 440–444.

Almeida Júnior W L G, Ferrari I D S, Souza J V, Silva C D A, Costa M M and Dias F S (2015) Characterization and evaluation of lactic acid bacteria isolated from goat milk. *Food Control* **53** 96–103.

Anderson K L, Kearns R, Lyman R and Correa M T (2019) Staphylococci in dairy goats and human milkers, and the relationship with herd management practices. *Small Ruminant Research* **171** 13–22.

Aragão B B, Trjani S C, Silva J G, Silva B P, Oliveira R P, Pinheiro Junior J W, Peixoto R M and Mota R A (2019) High frequency of β-lactam-resistant *Staphylococcus aureus* in artisanal coalho cheese made from goat milk produced in northeastern Brazil. *Journal of Dairy Science* **102** 6923–6927.

Araújo F M, Dantas M C S M, Silva L S, Aona L Y S, Tavares I F and Souza-Neta L C (2017) Antibacterial activity and chemical composition of the essential oil of *Croton heliotropiifolius Kunth* from Amargosa, Bahia, Brazil. *Industrial Crops and Products* **105** 203–206.

Atkinson R and Arey J (2003) Atmospheric degradation of volatile organic compounds. *Chemical Reviews* **103** 4605–4635.

Bogdanovičová K, Necidová L, Haruštiaková D and Janštová B (2017) Milk powder risk assessment with *Staphylococcus aureus* toxigenic strains. *Food Control* **73** 2–7.

Borges A, Ferreira C, Saavedra M J and Simões M (2013) Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microbial Drug Resistance* **19** 256–265.

Brasil (1996) Ministério da Agricultura, Pecuária e Abastecimento. Regulamento técnico para identidade e qualidade do queijo. Anexo II - Regulamento técnico geral para o estabelecimento dos requisitos microbiológicos do queijo. Portaria no 146, 07 de março de 1996. Diário Oficial da República Federativa do Brasil 1, pp. 39–77 [Internet document] URL http://extranet.agricultura.gov.br/sislegis-consulta/consultarLegislacao.do?operacao=visualizar&xml:id=1218. Accessed 06/07/2019.

Brasil (2001) Ministério da Saúde. Agência Nacional de Vigilância Sanitária. Regulamento técnico sobre os padrões microbiológicos para alimentos.Resolução RDC n° 12, de 02 de janeiro de 2001. Diário Oficial da República Federativa do Brasil 1, pp. 45–53 [Internet document] URL http://portal.anvisa.gov.br/documents/33880/2568070/RDC_12_2001.pdf/15ffddf6-3767-4527-bfac-740a0400829b. Accessed 06/07/2019.

Brasil (2003) Ministério da Agricultura, Pecuária e Abastecimento. Métodos analíticos oficiais para controle de produtos de origem animal e água.Instrução Normativa n° 62, 26 de agosto de 2003. Diário Oficial da República Federativa do Brasil 1, p. 14 [Internet document] URL http://extranet.agricultura.gov.br/sislegis-consulta/consultarLegislacao.do?operacao=visualizar&xml:id=2851. Accessed 06/07/2019.

Breukink E and Kruij B (1999) The lantibiotic nisin, a special case or not? *Biochimica et Biophysica Acta* **1462** 223–234.

Brito S S S, Silva F, Malheiro R, Baptista P and Pereira J A (2018) *Croton argyrophyllus* Kunth and *Croton heliotropiifolius* Kunth: phytochemical characterization and bioactive properties. *Industrial Crops & Products* **113** 308–315.

Burt S (2004) Essential oils: their antibacterial properties and potential applications in foods - a review. *International Journal of Food Microbiology* **94** 223–253.

Castro R C S, Oliveira A P D, Souza E A R, Correia T M A, Souza V S and Dias F S (2018) Lactic acid bacteria as biological control of *Staphylococcus aureus* in coalho goat cheese. *Food Technology and Biotechnology* **56** 431–440.

Coimbra-e-Souza V, Rossi C C, Freita L J J, Brita M A V P, Laport M S and Marval M G (2019) Diversity of species and transmission of antimicrobial resistance among *Staphylococcus* spp. isolated from goat milk. *Journal of Dairy Science* **102** 1–7.

Huang M, Sanchez-Moreiras A M, Abel C, Sohrabi R, Lee S, Gershenzon J and Tholl D (2012) The major volatile organic compound emitted from *Arabidopsis thaliana* flowers, the sesquiterpene (E)-β-caryophyllene, is a defense against a bacterial pathogen. *New Phytologist* **193** 997–1008.

Kim Y S, Park S J, Lee E J, Cerbo R M, Lee S M, Ryu C H R, Kim G S, Kim J O and Ha Y l (2008) Antibacterial compounds from rose Bengal-sensitized photooxidation of β-caryophyllene. *Journal of Food Science* **73** 540–545.

Kroning I S, Iglesias M A, Sehn C P, Valente Gandra T K, Mata M M and Silva W P (2016) *Staphylococcus aureus* isolated from handmade sweets: biofilm formation, enterotoxigenicity and antimicrobial resistance. *Food Microbiology* **58** 105–111.

Lange C C, Brito M A V P, Brito J R F, Arcuri E F, Guilherme N, Souza M A, Machado R D and Salimena A P S (2011) Uso de PCR e sequenciamento do rDNA 16S para identificação de bactérias do gênero *Staphylococcus* isoladas de mastite bovina. *Pesquisa Veterinária Brasileira* **31** 36–40.

Li K, Guana G, Zhub J, Wu H and Sun Q (2019) Antibacterial activity and mechanism of a laccase-catalyzed chitosan–gallic acid derivative against *Escherichia coli* and *Staphylococcus aureus*. *Food Control* **96** 234–243.

Liu M, Wu X, Li J, Liu L, Zhang R, Shao D and Du X (2017) The specific anti-biofilm effect of gallic acid on *Staphylococcus aureus* by regulating the expression of the *ica* operon. *Food Control* **73** 613–618.

Luís A, Silva F, Sousa S, Duarte A P and Domingues F (2013) Antistaphylococcal and biofilm inhibitory activities of gallic, caffeic, and chlorogenic acids. *Biofouling* **14** 69–79.

Merino N, Toledo-Arana A, Vergara-irigaray M *et al*. (2009) Protein A-mediated multicellular behavior in *Staphylococcus aureus*. *Journal of Bacteriology* **191** 832–843.

Moosavy M H, Basti A A, Misaghi A, Salehi T Z, Abbsifar R, Mousavi H A E, Alipour M, Razavi N E, Gandomi H and Noori N (2008) Effect of *Zataria multiflora* Boiss. essential oil and nisin on *Salmonella typhimurium* and *Staphylococcus aureus* in a food model system and on the bacterial cell membranes. *Food Research International* **41** 1050–1057.

Oliveira F P, Lima E O, Siqueira Júnior J P, Souza E L, Santos B H Ž and Barreto H M (2006) Effectiveness of *Lippia sidoides* Cham. (Verbenaceae) essential oil in inhibiting the growth of *Staphylococcus aureus* strains isolated from clinical material. *Brazilian Journal of Pharmacognosy* **16** 510–516.

Papadopoulos P, Papadopoulos T, Angelidis A S, Boukouvala E, Zdragas A, Papa A, Hadjichristodoulou C and Sergelis D (2018) Prevalence of *Staphylococcus aureus* and of methicillin-resistant *S. aureus* (MRSA) along the production chain of dairy products in north-western Greece Panagiotis. *Food Microbiology* **69** 43–50.

Rigo N (2015) Aroma e sabores da caatinga [Internet document] URL https://come-se.blogspot.com/2015/03/aromas-e-sabores-da-caatinga.html. Accessed 05/04/2019.

Rodrigues L B, Santos L R, Rizzo N N, Tagliari V Z, Oliveira A P, Trenhago G, Rodegheri S C, Taglieti R M, Dickel E L and Nascimento V P (2009) Avaliação da hidrofobicidade e da formação de biofilme em poliestireno por *Salmonella* Heidelberg isoladas de abatedouro avícola. *Acta Scientiae Veterinariae* **37** 225–230.

Rodrigues J B S, Carvalho R J, Souza N E T, Oliveira K S, Franco O A L, Schaffner D, Souza E L and Magnani M (2017) Effects of oregano essential oil and carvacrol on biofilms of *Staphylococcus aureus* from food-contact surfaces. *Food Control* **73** 1237–1246.

Sadekuzzaman M, Mizan M F R, Kim H S, Yang S and Ha S D (2018) Activity of thyme and tea tree essential oils against selected foodborne pathogens in biofilms on abiotic surfaces. *LWT - Food Science and Technology* **89** 134–139.

Sawant A A, Gillespie B E and Oliver S P (2009) Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. *Veterinary Microbiology* **134** 73–81.

Shi C, Zhang X, Zhao X, Meng R, Liu Z, Chen X and Guo N (2017) Synergistic interactions of nisin in combination with cinnamaldehyde against *Staphylococcus aureus* in pasteurized milk. *Food Control* **71** 10–16.

Silva J A, Silva G C, Silva T M S, Costa M A S and Leite S P (2018) Atividade tóxica in vitro de *Croton heliotropiifolius* Kunth (Euphorbiaceae). *Revista Verde de Agroecologia e Desenvolvimento Sustentável* **13** 112–115.

Unlu A, Sar T, Seker G, Erman A G, Kalpar E and Akbas M Y I (2018) Biofilm formation by *Staphylococcus aureus* strains and their control by selected phytochemicals. *International Journal of Dairy Technology* **70** 1–10.

Van Geel-Schutten G H, Flesch F, Ten Brink B, Smith M R and Dijkhuizen L (1998) Screening and characterization of *Lactobacillus* strains producing large amounts of exopolysaccharides. *Applied Microbiology and Biotechnology* **50** 697–703.

Xing X, Zhang Y, Wu Q, Wang X, Ge W and Wu C (2016) Prevalence and characterization of *Staphylococcus aureus* isolated from goat milk powder processing plants. *Food Control* **59** 644–650.

Yoon Y, Lee S and Choi K H (2016) Microbial benefits and risks of raw milk cheese. *Food Control* **63** 201–215.

Zhang Z, Vriesekoop F, Yuan Q and Liang H (2014) Effects of nisin on the antimicrobial activity of D-limonene and its nanoemulsion. *Food Chemistry* **150** 307–312.

Zhao X, Meng R, Shi C, Liu Z, Huang Y, Zhao Z, Guo N and Yu L (2016) Analysis of the gene expression profile of *Staphylococcus aureus* treated with nisin. *Food Control* **59** 499–506.