

Occurrence of Multi-Resistant *Staphylococcus aureus* in Artisan Goat Coalho Cheese in Northeastern Brazil

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ABSTRACT

Background: The artisanal goat coalho cheese is one of the products obtained that stand out in the dairy goat farming of the Northeast of Brazil. Despite its importance, goat cheese is often made under inadequate hygienic-sanitary conditions and usually uses raw goat's milk, increasing the risk of product contamination. Among the pathogens carried by goat coalho cheese, *Staphylococcus aureus* stands out, being responsible for cases of food poisoning and persistent infections that are difficult to treat. This study aimed to evaluate the contamination, genotypic and phenotypic resistance of *Staphylococcus aureus* isolated from artisanal coalho cheese made with goat milk produced in the Northeast region of Brazil.

Materials, Methods & Results: This study analyzed only artisanal coalho cheeses made with raw goat's milk and purchased directly from farms. Twelve samples of artisanal coalho cheeses made with raw goat's milk were collected (1 sample per property) in 8 municipalities in the state of Pernambuco, Northeast region of Brazil. For microbiological analysis of enumeration of Colony Forming Units (CFU/g) of *Staphylococcus* spp. the methodology recommended by the International Organization for Standardization (2019) and recognized by the Ministry of Agriculture, Livestock and Supply was used. After enumeration, 5 colonies were selected per enumerated plate, a total of 180 *Staphylococcus* spp. was obtained. These were subjected to thermal extraction of genetic material to search for the *nuc* gene by Polymerase Chain Reaction, the isolates carrying the *nuc* gene were subjected to genotypic and phenotypic evaluation of antimicrobial resistance. After the phenotypic analysis, the Multiple Antimicrobial Resistance Index was evaluated. In all samples, *Staphylococcus* spp. and were considered unfit for consumption, with the lowest count being 9.4×10^3 CFU/g and the highest 6.4×10^6 CFU/g. Of the 180 isolates, 28.34% (51/180) were positive for the detection of the *nuc* gene. All resistance genes except *mecA*, *mecC*, and *norB* were detected. Of the 51 *S. aureus* isolates, 31.37% (16/51) were considered multi-resistant and presented a Multiple Antimicrobial Resistance Index above 0.2.

Discussion: After microbiological analysis it was found that all samples of coalho cheese were out of standards and unfit for human consumption in accordance with Ordinance no 146/1996 of the Ministry of Agriculture, Livestock and Supply of Brazil. Furthermore, the contamination of goat coalho cheeses is a risk to public health. During sample collection were found inadequate hygiene conditions in the environment used for cheese production. The presence of *Staphylococcus aureus* can be attributed to hygienic-sanitary failures in cheese production. From a health point of view, it is even more alarming when it comes to *S. aureus* carrying resistance genes. Although the 51 *S. aureus* isolates did not carry the *mecA*, *mecC*, *norB* genes and did not show phenotypic resistance to cefoxitin and oxacillin, all other genes were detected, indicating the circulation of *S. aureus* carrying the *tet(L)* genes, *tet(M)*, *tet-38*, *msrA*, *norA*, and *norC*, which so far had not been reported in the production chain of goat coalho cheese in Brazil. Furthermore, the evaluation of the Multiple Antimicrobial Resistance Index identified the occurrence of multiple resistance to antimicrobials in 31.37% (16/51) of *S. aureus* at high risk to human health. The results obtained are quite worrying and serve as a warning to the scientific community and the Food Safety and Hygiene Inspection Services.

Keywords: goat cheese, contamination, resistance, one health.

DOI: 10.22456/1679-9216.117447

Received: 25 July 2021

Accepted: 24 September 2021

Published: 3 November 2021

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INTRODUCTION

Dairy goat farming plays a highly important socio-economic role in the Northeast region of Brazil, as goats are adapted to the Cantiga biome which is unique in the world. The main product of goat's milk is the goat coalho cheese, which is a typical example of cheese from the Northeast region of Brazil, produced with raw goat's milk, which is recognized as a culturally and economically important product. Its production is handcrafted and the manufacturing technology comes from knowledge passed from generation to generation, following a family tradition. Despite this, the lack of sanitation and production in inadequate environments increases the risk of contamination [21].

Among the contaminating pathogens of goat milk and artisan coalho cheese made with goat milk, *Staphylococcus aureus* stands out, as it is considered the most frequent bacterial species. In addition, *S. aureus* contaminates utensils used for cheese manufacturing, remaining for several days, favouring intercellular interactions to exchange genetic material horizontally resulting in antimicrobial-resistant bacteria, causing losses to the dairy industry, and a potential risk to public health [12].

Despite the importance of artisanal coalho cheese made with goat milk, little information is available on *Staphylococcus* spp. and the profile of multi-resistant bacteria to antimicrobials. This study aimed to evaluate the contamination, genotypic and phenotypic resistance of *Staphylococcus aureus* isolated from artisanal coalho cheese made with goat milk produced in the Northeast region of Brazil.

MATERIALS AND METHODS

Goat coalho cheese samples

In the period between March and December 2019, 12 samples of artisanal coalho cheese made with goat's milk were collected (1 sample per property) in 8 municipalities in the state of Pernambuco, Northeast region of Brazil. One sample was collected in municipality A; 2 in municipality B; 1 in municipality C; 1 in municipality D; 4 in municipality E; 1 in municipality F; 1 in municipality G, and 1 in municipality H (Figure 1).

The purchased cheese samples weighed approximately 500 g, and after collection, were stored in sterilized bags with zip lock¹, identified and packed in isothermal boxes with recyclable ice at a temperature of (4 to 8°C) and transported to the laboratory for microbiological and molecular analysis.

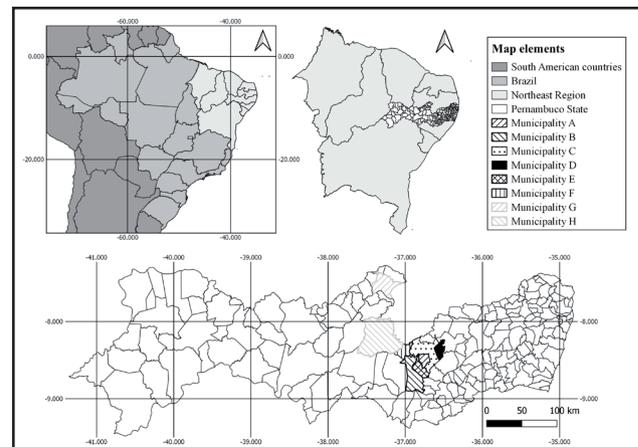


Figure 1. Distribution of samples by sampled municipalities in the state of Pernambuco, Brazil.

Enumeration of *Staphylococcus*

Enumeration of *Staphylococcus* spp. was performed according to the methodology recommended by the International Organization for Standardization [8] with adaptations. Initially, 25 g of different parts of each cheese sample were weighed, then transferred to sterile ziplock[®] bags containing 225 mL of sterile saline solution at 0.85%. Then, they were homogenized for 60 s in Stomacher blender², and from this solution, serial dilutions were made up to 10⁻⁶. From these dilutions, 0.1 mL aliquots were taken and seeded in triplicate on Baird-Parker agar³ enriched with egg yolk emulsion and potassium tellurite⁴. Plates were incubated at 35-37°C for 48 h [8].

After bacterial growth, colonies were counted in plates containing between 20 and 200 colonies and the results were expressed in Colony Forming Unit (CFU/g) [8]. Then, 5 characteristic colonies of *Staphylococcus* spp. were selected from each of the enumerated plates. A total of 180 colonies of *Staphylococcus* spp. were selected from the obtained samples.

Identification of *Staphylococcus aureus*

For the molecular identification of *S. aureus*, DNA extraction was employed in the 180 colonies of *Staphylococcus* spp. For the extraction of genetic material, the methodology proposed by Fan *et al.* [5] was used. The DNA obtained was quantified and analysed for its purity degree in a spectrophotometer with absorbance readings at 260 nm.

For molecular confirmation of the *S. aureus* species, amplification of the specific region of the nuc gene was performed according to the technique described by Brakstad *et al.* [1], with adaptations in

the reagent concentrations. The reactions were adapted to a final volume of 12.5 µL per microtube, containing 100 ng template DNA, primers (10 pmol each) as shown in Table 1, using 6.25 µL of Go-TaqGreen Master Mix⁵ (Table 1).

Then, 10 µL of the reaction were subjected to electrophoresis for 40 min at 100V in a 1.5% agarose gel stained with BlueGreen, visualized and photographed in a photo documenter under ultraviolet light. As a positive control for detection of the *nuc* gene, the strain *Staphylococcus aureus* N315 was used. As a negative control, DNA-Free Water⁶ was used.

Search for resistance genes in S. aureus

For the detection of resistance genes (*blaZ*, *mecA*, *mecC*, *tet(L)*, *tet(M)*, *norA*, *norB* and *norC*, *tet-38*, *msrA*) the conventional polymerase chain reaction (PCR) technique was employed, using the methodology of the references (Table 1), with adaptations in the reagent concentrations. The reactions were formatted to a final volume of 12.5 µL per microtube, containing 100 ng template DNA, primers (10 pmol each), using 6.25 µL of Go-TaqGreen Master Mix⁵. Then, 10 µL of the reaction were subjected to electrophoresis for 40 min at 100V in a 1.5% agarose gel stained with BlueGreen,

visualized and photographed in a photo documenter under ultraviolet light. As a positive control, were used reference strains to detect specific regions. As a negative control, DNA-Free Water⁶ was used.

Analysis of the phenotypic resistance profile

To assess the antimicrobial resistance profile, the disc diffusion technique on Mueller-Hinton agar⁷ was used according to the Clinical and Laboratory Standards Institute methodology [4]. To perform the test were used discs impregnated with Penicillin G (10UI), Cefoxitin (30 µg), Oxacillin (1 µg), Tetracycline (30 µg), Norfloxacin (10 µg), and Erythromycin (15 µg)⁸. The zone of inhibition was interpreted after 24 h of incubation [4].

Calculation of Multiple antibiotic resistance (MAR) index

To calculate the multiple antimicrobial resistance (MAR) index, the Krumperman [10] methodology was used.

Statistical analysis

The results of qualitative diagnostic tests (genotypic and phenotypic resistance) were expressed in their absolute and relative frequencies [6].

Table 1. Genes, oligonucleotide sequences and size of amplified fragments.

Gene	Sequence (5' – 3')	Fragment Size (pb)	References
<i>nuc</i>	F- GCGATTGATGGTGATACGGTI R- AGCCAAGCCTTGACGAAGCTAAAGC	270	[1]
<i>blaZ</i>	F- AAGAGATTGCTATGCTTC R- GCTTGACCACTTTTATCAGC	517	[22]
<i>mecA</i>	2W- TGGTATGTGGAAGTTAGATTGGGAT 2X-CTAATCTCATATGTGTTCCCTGTATTGGC	155	[16]
<i>mecC</i>	1A- CATTAAAATCAGAGCGAGGC 1B- TGGCTGAACCCATTTTGTAT	188	[18]
<i>tet(L)</i>	F- TCGTTAGCGTGCTGTCAATC R- GTATCCCACCAATGTAGCCG	267	[17]
<i>tet(M)</i>	GTG GAC AAA GGT ACA ACG AG CGG TAA AGT TCG TCA CAC AC	406	[17]
<i>norA</i>	F- TGCAATTCATATGATCAATCCC R- AGATTGCAATTCATGCTAAATATT	150	[26]
<i>norB</i>	F- ATAAGGTAAGATAACTAGCA R- ATCTCTATTTGCCCTCCCTATA	150	[25]
<i>norC</i>	F- ATAAATACCTGAAGCAACGCCAAC R- AAATGGTTCTAAGCGACCAA	200	[25]
<i>tet-38</i>	F- TTCAGTTTGGTTATAGACAA R- CGTAGAAATAAATCCACCTG	200	[24]
<i>msrA</i>	F- TCCAATCATTGCACAAAATC R- AATTCCCTCTATTTGGTGGT	890	[14]

RESULTS

In the microbiological analysis of the cheeses, the growth of characteristic colonies of *Staphylococcus* spp. was observed in 100.0% of the analysed samples. Regarding the count of CFU/g of *Staphylococcus* spp., all cheeses were considered unfit for consumption, with the lowest count (9.4×10^3) observed in property 7 in municipality B and the highest (6.4×10^6) in property 12, in municipality H. Of the analysed samples, 91.60% (11/12) had values equal to or greater than 10^5 CFU/g in Baird-Parker agar (Table 2).

Of the 180 *Staphylococcus* spp. submitted to PCR to detect the *nuc* gene, 28.34% (51/180) were positive. Regarding the contamination of cheeses by property, 66.67% (8/12) of the samples were contaminated by *S. aureus* (Table 3).

The genotypic and phenotypic resistance data are shown in Figure 2. There was no detection of the *mecA*, *mecC* and *norB* resistance genes in the 51 *S. aureus* isolates.

Of the 51 *S. aureus* isolates, 31.37% (16/51) were considered multi-resistant and presented a MAR index above 0.2 (Table 4).

Table 2. CFU/g counts in Baird-Parker agar of characteristic colonies of *Staphylococcus* spp. in different municipalities and properties.

Municipalities	Properties	CFU/g
Municipality A	1	1.22×10^5
	2	1.1×10^6
Municipality B	3	1.57×10^5
	4	1.23×10^5
Municipality C	5	1.06×10^5
Municipality D	6	1.03×10^5
	7	9.4×10^3
	8	1.53×10^6
	9	6.2×10^5
Municipality E	10	2.9×10^5
Municipality F	11	6.2×10^5
Municipality G	12	6.4×10^6

Table 3. Absolute and relative frequencies of properties with cheese samples positive for *Staphylococcus aureus*.

Properties	Number of isolates from <i>Staphylococcus</i> spp.	<i>nuc</i> gene	
		Positive	
		F.A.	F.R. (%)
Property 1	15	0	0.0
Property 2	15	1	6.67
Property 3	15	2	13.34
Property 4	15	10	66.67
Property 5	15	10	66.67
Property 6	15	0	0.0
Property 7	15	6	40.0
Property 8	15	9	60.0
Property 9	15	1	6.67
Property 10	15	12	80.0
Property 11	15	0	0.0
Property 12	15	0	0.0
Total	180	51	

Table 4. Distribution of resistance pattern and multiple antimicrobial resistance (MAR) index of 16 *Staphylococcus aureus* isolates isolated in goat cheese coalho.

Antimicrobial resistance profile	Number of resistant isolates	MAR
Pen, Tet, Nor e Eri.	1	0.66
Pen, Tet e Nor	4	0.50
Pen, Nor e Eri	2	0.50
Pen e Tet	3	0.33
Pen e Eri	4	0.33
Nor e Eri	1	0.33
Pen e Nor	1	0.33

Pen= Penicillin G 10 UI; Tet= Tetracycline 30 µg; Nor= Norfloxacin 10 µg; Eri= Erythromycin 15 µg.

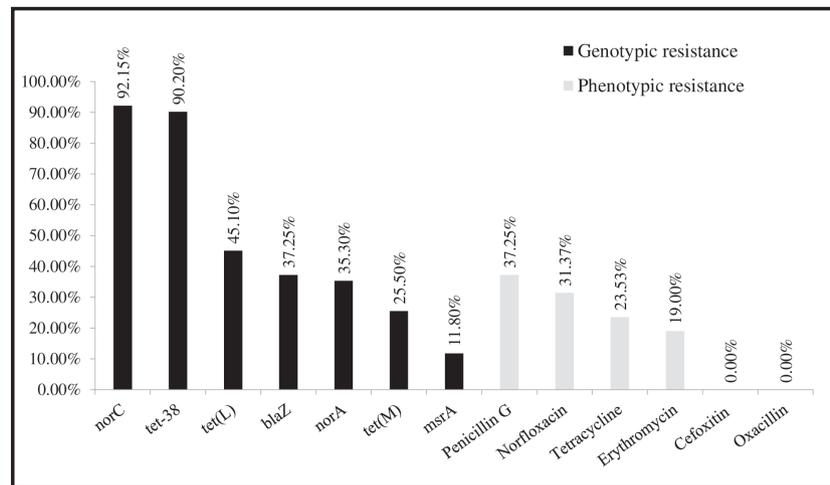


Figure 2. Distribution of genotypic and phenotypic resistance frequencies of *Staphylococcus aureus* isolated in goat cheese.

DISCUSSION

The results obtained in the microbiological analysis revealed that all the evaluated samples of coalho cheese were not up for standards and unfit for human consumption per Ordinance No. 146/1996 of the Ministry of Agriculture, Livestock and Supply (MAPA) of Brazil, which recommends 1×10^3 CFU/g for high moisture cheeses, as goat cheese coalho is classified [2]. In addition to non-compliance with the legislation these cheeses present an imminent risk of poisoning by staphylococcal enterotoxins for their consumers, since they presented values equal to or greater than 10^5 CFU/g for *Staphylococcus* spp. in Baird-Parker agar, being considered at high risk for food poisoning worldwide [9]. Souza *et al.* [23] in a similar study carried out in another region of Brazil has also detected 17.4% of *S. aureus* in 160 analysed samples. Contamination of goat coalho cheese is a public health risk. During sample collection, were found

inadequate hygiene conditions in the environment used for cheese production.

From a sanitary point of view, the contamination of the analysed cheeses is even more alarming when it comes to *S. aureus* carriers of resistance genes. Although the 51 *S. aureus* isolates did not carry the *mecA*, *mecC*, *norB* genes, and did not show phenotypic resistance to cefoxitin and oxacillin, all other genes were detected, indicating the circulation of *S. aureus* carrying the *tet(L)*, *tet(M)*, *tet-38*, *msrA*, *norA* and *norC* genes which, so far, had not been reported in the production chain of goat coalho cheese in Brazil.

The occurrence of genotypically and phenotypically resistant *S. aureus* reveals the spread of genetically evolved strains [3]. The abusive use of antimicrobials in veterinary and human medicine, as well as residual antibiotics that are excreted or discharged in nature, probably contribute to the high frequency of antimicrobial-resistant bacteria [11]. In addition, there is the possibility of acquired resistance,

through mutations in genes (chromosomal mutation), transfer of genes from one microorganism to another by plasmids (conjugation or transformation), transposons (conjugation), integrations, and bacteriophages (transduction) [7].

Selective pressure and the emergence of new resistant strains is a very concerning genetic mechanism and the prevalence detected in this study for the *bla_Z*, *tet(L)*, *tet(M)*, *tet-38*, *norA*, and *norC* genes may become even higher due to the evolvability of *S. aureus* [3]. In addition, the occurrence of 11.8% (6/51) of the *msrA* gene in *S. aureus* found in goat cheese is concerning, as this gene is responsible for conferring resistance to the classes of macrolides, lincosamides, and streptogramins, the main drugs used as alternatives in the treatment of glycopeptide-resistant *Staphylococcus* in humans [20].

Although macrolides, lincosamides, and streptogramins are not used in the treatment of caprine mastitis or other infections in animals [15], 19% (10/51) of *S. aureus* isolates exhibited phenotypic resistance to erythromycin. Thus, it is suggested that *S. aureus* carriers of the *msrA* gene detected in our study are of human origin since, in human medicine, these antimicrobials are of usual prescription as alternative antibiotics in the treatment of persistent staphylococcal infections [27]. In addition to the occurrence of resistance genes, the MAR assessment allowed the identification of multiple resistance to antimicrobials in 31.37% (16/51) of *S. aureus* that presents a high risk to human health [13].

The high rate of genotypic resistance and low phenotypic detection in isolated *Staphylococcus aureus* may be associated with changes in gene expression triggered by mutations, phages, plasmids, and transposons. Therefore, the multiplicity of factors associated with phenotypic resistance requires careful

investigation. The detection of different genetic resistance markers, as well as the study of the regulation of gene expression, allows for a deeper understanding of the real value of their detection in predicting antimicrobial resistance, as recommended by the Clinical and Laboratory Standards Institute [19].

CONCLUSION

The results obtained in microbiological analyses reveal that all evaluated cheeses were unfit for consumption and serve as a warning to the scientific community and the Food Safety and Hygiene Inspection Services. The detection of multi-antimicrobial resistant *S. aureus* in cheese is a risk to the health of consumers, in addition to causing a negative economic impact on the production chain of this type of cheese, which is highly consumed by residents and tourists. The need for regular monitoring of this production chain to control and minimize *S. aureus* contamination of the cheese is emphasized.

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Acknowledgements. We thank the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco for the scholarship (IBPG-0467-5.05/17).

Declaration of interest. The authors declare no conflicts of interest. Only the authors are responsible for the content and writing of the article.

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