





Microbiological characterization of raw goat milk from a small municipality in Brazil: identification of Gram-positive cocci by MALDI-TOF MS

Gustavo Luis de Paiva Anciens Ramos ¹, Emily Cristine Candido da Silva ²,
Lavinia Falcão de Araújo ², Marcelo Soares de Moraes ², Janaina dos Santos Nascimento ²

¹ Universidade Federal Fluminense; gustavoanciens@id.uff.br.

² Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro; emily.silva.etm2020.1@gmail.com; lavinia.araujo.etm2021.1@gmail.com, soares.marcelo2020@gmail.com, janaina.nascimento@ifrrj.edu.br.

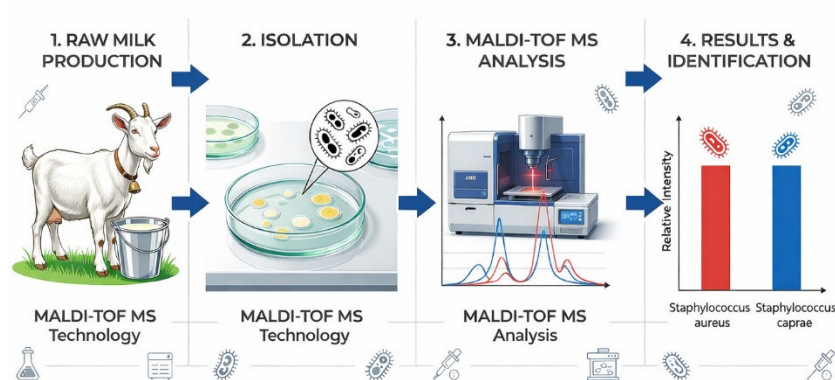
* Correspondence: gustavoanciens@id.uff.br



ABSTRACT

Goat milk is a high-nutritional-value food, but it can also harbor several pathogenic microorganisms. It is not uncommon for this food to be consumed raw, which can pose health risks to consumers. In this study, we sought to verify the diversity of Gram-positive cocci (GPC), especially *Staphylococcus* sp., in raw milk samples obtained in the region of Itaocara, RJ. Microbial identification of the nine samples was performed by mass spectrometry (MALDI-TOF) after isolation in selective culture media. MALDI-TOF analysis of the isolates from raw goat milk samples revealed the predominance of *Staphylococcus* spp., with emphasis on *S. aureus* and *S. caprae*. GPCs of other genera were also detected. Surprisingly, some of these microorganisms were isolated on selective media for Gram-negative bacteria, highlighting the importance of accurate confirmatory identification methods such as MALDI-TOF. The detection of *S. aureus*, a relevant foodborne pathogen, and other species with spoilage or pathogenic potential, such as *S. caprae* and *Mammaliococcus sciuri*, in six of the nine samples highlights potential microbiological risks associated with locally consumed raw goat milk. On the other hand, the presence of lactic acid bacteria (*Lactococcus lactis*, *L. garvieae*, and *Enterococcus* sp.), with technological potential for producing dairy products, was also observed. The recovery of Gram-positive cocci from media selective for Gram-negative bacteria reinforces the limitations of culture-based selectivity and highlights the importance of confirmatory identification methods such as MALDI-TOF MS.

Keywords: *Staphylococcus aureus*, dairy product, mass spectrometry



Graphical Abstract. Comprehensive microbiological workflow for the characterization of raw goat milk. The study integrated: (1) Sampling from small-scale production in Itaocara, RJ; (2) Microbial isolation using selective media; (3) Advanced identification via MALDI-TOF MS technology; and (4) Accurate species-level results highlighting the prevalence of *Staphylococcus aureus* and *S. caprae*. Image generated using AI-assisted tools (GPAI).

INTRODUCTION

Goat farming has been growing, driven by the demand for more natural products and differentiated dairy foods¹. In this context, goat's milk stands out for its unique nutritional profile, including high levels of vitamins, minerals, and medium-chain fatty acids, making it a food appreciated worldwide. Additionally, it is an option for individuals with lactose intolerance or cow's milk allergy².

However, the marketing and consumption of raw goat's milk, a common practice in some regions, raises significant concerns regarding microbiological safety. Raw milk, as it does not undergo heat pasteurization, can harbor a diverse microbiota, including Gram-positive bacteria with pathogenic and deteriorative potential³. Among these bacteria, the *Staphylococcus* genus has been frequently isolated from raw milk of different species, posing a risk to public health due to the production of heat-stable enterotoxins (responsible for food poisoning), biofilm formation, and antimicrobial resistance^{4,5}. The presence of other Gram-positive cocci can also cause disease in humans, goats, and other domestic ruminants (clinical and subclinical mastitis), as well as compromising the sensory quality and shelf life of milk and its derivatives⁶⁻⁸.

On the other hand, other GPC, such as *Lactococcus* and *Enterococcus*, play a crucial role in lactic fermentation, contributing to the development of characteristic flavor, aroma, and texture in cheeses and other fermented products⁹. Therefore, understanding the microbial diversity in raw goat's milk is essential for assessing health risks and identifying the biotechnological potential for producing high-quality artisanal foods.

In this context, the present study aimed to analyze the presence and diversity of potentially pathogenic or spoilage GPC in raw goat's milk samples sold by small producers in the Itaocara region, in the northwest of the state of Rio de Janeiro, and consumed by the local population.

MATERIAL AND METHODS

Sampling

Nine samples of raw goat's milk (Table 1), commonly sold and consumed in natura by the local population, were obtained from small producers in the municipality of Itaocara, in the northwest of the State of Rio de Janeiro, between July and September 2024. The samples come from family farms, which are a major activity in the community of about 23,000 people. The samples were transported under refrigeration to the IFRJ Microbiology Laboratory.

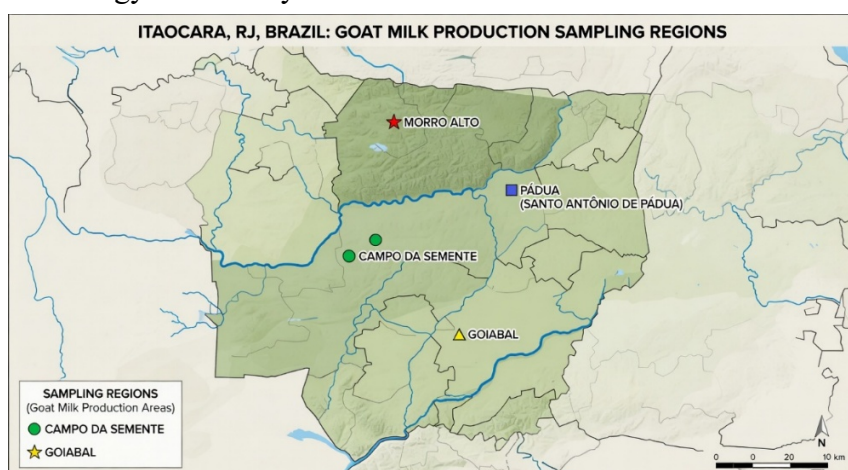


Figure 1. Distribution of microbial species identified in raw goat milk samples from Itaocara, RJ. *Staphylococcus aureus* was the most prevalent species (36.1%), followed by *Staphylococcus caprae* (16.6%).

Regions of Itaocara, RJ	Samples
Morro Alto	A1 e A2
Campo da Semente	CS1 e CS2
Goiabal	G1, G2 e G3
Pádua	P1 e P2

Table 1. Samples of raw goat milk used in this work

Obtaining the isolates

To obtain GPC, primarily to isolate *Staphylococcus* sp., the methodology described in ISO 6888-1: 1999¹⁰ was used. It is known, however, that other GPCs, generally related to *Staphylococcus* sp., can also be isolated using this methodology. Samples were diluted to 10^{-3} , plated on Baird-Parker agar (Himedia, São Paulo, Brazil), supplemented with egg yolk emulsion and potassium tellurite, and incubated at 37°C for 48 h. Typical and atypical colonies (suspected coagulase-positive and coagulase-negative S.) were selected and subcultured on a non-selective medium (Casoy agar, Himedia, São Paulo, Brazil). The plates were incubated at 37°C for 18 to 24h. For a parallel study, the same samples were also inoculated on selective and differential culture media for Gram-negative bacteria (Leeds Agar, Cetrimide Agar, and Tryptone Bile X-glucuronide Agar).

Identification of isolates

The identification of the isolates was performed by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization – Time of Flight) mass spectrometry on a mass spectrometer (Microflex LT, Bruker, United States) at the Medical Microbiology Research Laboratory of the Paulo de Góes Institute of Microbiology, at the Federal University of Rio de Janeiro (UFRJ). Bacterial cultures previously grown on Casoy agar were inoculated in duplicate onto the equipment's metal plate, followed by the addition of 1 µl of 70% formic acid for cell lysis. After the acid dried, the same volume of a matrix of α -cyano-4-hydroxycinnamic acid was applied to crystallize the sample at the end of the process. The plate was then inserted into the equipment, which had been previously calibrated with a control strain of *Escherichia coli*, and the Biotype 3.1 software generated an identification for each isolate. Isolates with species-level accuracy were identified when their spectral similarity score exceeded 2.0.

RESULTS AND DISCUSSION

From the colonies obtained from the microbiological analysis of nine samples of raw goat's milk from different small producers in the region of Itaocara, RJ, some isolates were selected and subjected to MALDI-TOF mass spectrometry analysis. Those that did not show growth in the non-selective medium or did not present precise identification of genus or species were discarded. The isolates were identified as *Staphylococcus aureus* (n=13; 36.1%), *S. caprae* (n=6; 16.6%), *Staphylococcus* sp. (n=4; 11.1%), *Enterococcus faecium* (n=3; 8.3%), *Macroccoccus caseolyticus* (n=3; 8.3%), *Mammaliococcus sciuri* (n=2; 5.6%), *S. kloosii* (n=1; 2.8%), *Enterococcus* sp. (n=1; 2.8%), *Rothia koreensis* (n=1; 2.8%), *Lactococcus lactis* (n=1; 2.8%) and *L. garvieae* (n=1; 2.8%). Percentages are presented as descriptive values of the isolates analyzed in this study. The identification of the isolates from the goat milk sample is presented in Table 2.

Samples	Isolates	Identification	Isolation culture medium
A1	G1L05	<i>Staphylococcus caprae</i>	Leeds
	G1L06	<i>Staphylococcus caprae</i>	Leeds
	G1L07	<i>Staphylococcus caprae</i>	Leeds
	G1B09	<i>Staphylococcus caprae</i>	Baird-Parker
	G1B10	<i>Staphylococcus caprae</i>	Baird-Parker
	G1B12	<i>Staphylococcus caprae</i>	Baird-Parker
	161C	<i>Staphylococcus aureus</i>	Cetrimide
A2	G2B01	<i>Macrococcus caseolyticus</i>	Baird-Parker
	G2B02	<i>Staphylococcus aureus</i>	Baird-Parker
	G2B03	<i>Macrococcus caseolyticus</i>	Baird-Parker
	G2L12	<i>Lactococcus lactis</i>	Leeds
	G2L13	<i>Enterococcus faecium</i>	Leeds
	G2L15	<i>Staphylococcus aureus</i>	Leeds
CS1	G3L03	<i>Staphylococcus aureus</i>	Leeds
	G3L04	<i>Staphylococcus aureus</i>	Leeds
	G3B06	<i>Staphylococcus aureus</i>	Baird-Parker
	G3B07	<i>Macrococcus caseolyticus</i>	Baird-Parker
	G3B08	<i>Staphylococcus aureus</i>	Baird-Parker
	G3T10	<i>Lactococcus garvieae</i>	TBX
	G3T15	<i>Enterococcus faecium</i>	TBX
CS2	111B	<i>Mammaliococcus sciuri</i>	Baird-Parker
	211B	<i>Mammaliococcus sciuri</i>	Baird-Parker
	112B	<i>Staphylococcus kloosii</i>	Baird-Parker
G1	123B	<i>Rothia koreensis</i>	Baird-Parker
G2	106B	<i>Staphylococcus aureus</i>	Baird-Parker
G3	217L	<i>Enterococcus faecium</i>	Leeds
	218L	<i>Enterococcus</i> sp.	Leeds
P1	141B	<i>Staphylococcus aureus</i>	Baird-Parker
P2	152B	<i>Staphylococcus aureus</i>	Baird-Parker
	252B	<i>Staphylococcus aureus</i>	Baird-Parker
	352B	<i>Staphylococcus aureus</i>	Baird-Parker
	452B	<i>Staphylococcus aureus</i>	Baird-Parker
	151B	<i>Staphylococcus</i> sp.	Baird-Parker
	251B	<i>Staphylococcus</i> sp.	Baird-Parker
	351B	<i>Staphylococcus</i> sp.	Baird-Parker
	451B	<i>Staphylococcus</i> sp.	Baird-Parker

Table 2. Gram-positive cocci isolated from the raw goat milk samples. The isolate codes refer to morphological information about the colonies used to organize the study.

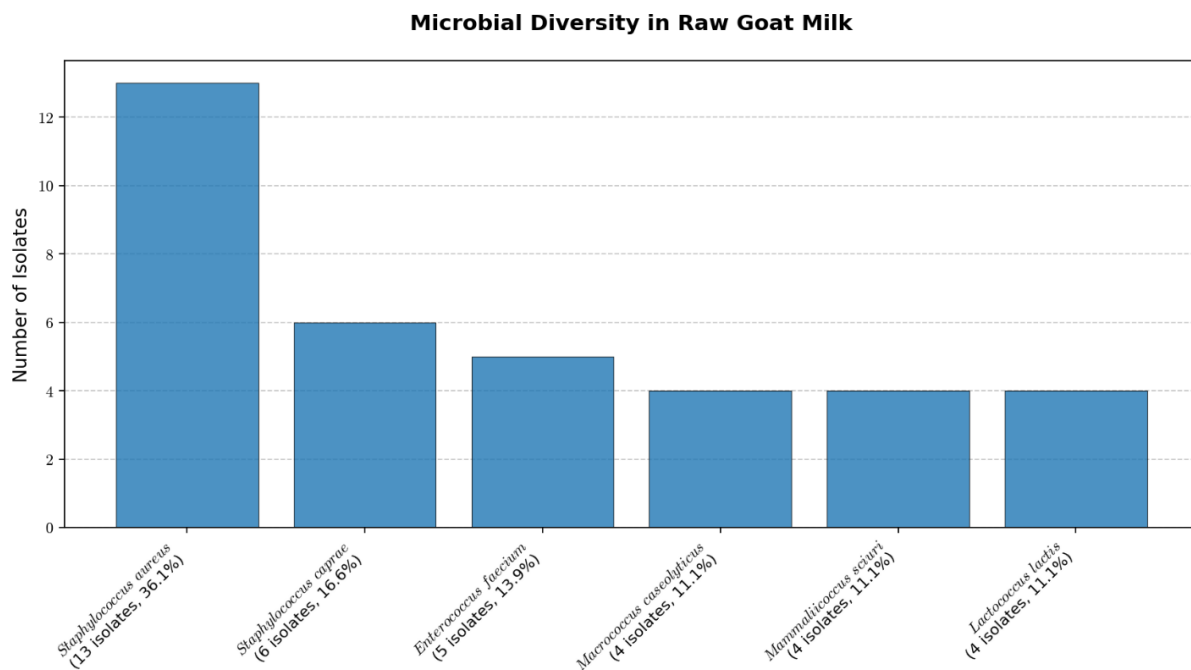


Figure 2. Distribution of microbial species identified in raw goat milk samples from Itaocara, RJ. *Staphylococcus aureus* was the most prevalent species (36.1%), followed by *Staphylococcus caprae* (16.6%).

Some isolates were recovered not only on Baird-Parker agar but also on selective and differential media intended for Gram-negative bacteria (Leeds, Cetrimide, and TBX agars), originally used for a parallel study. The recovery of Gram-positive cocci on these media represents a relevant methodological finding. Although selective media are designed to suppress non-target microorganisms, their specificity is not absolute¹¹. In this study, the growth of *Staphylococcus*, *Enterococcus*, and *Lactococcus* species on media intended for Gram-negative bacteria demonstrates the practical limitations of phenotypic selectivity. These results indicate that relying solely on culture-based characteristics may lead to misinterpretation of microbial identity.

In this context, MALDI-TOF mass spectrometry proved essential for the accurate identification of isolates recovered under these atypical culture conditions, as it supports diagnostic and research microbiology by enabling the reliable identification of microorganisms based on their unique protein profiles. This technique overcomes the limitations of conventional phenotypic methods, which can be time-consuming and, in certain situations, not discriminatory enough to distinguish phylogenetically close species, mainly when growth occurs in atypical culture media¹². For example, MALDI-TOF identification of *Lactococcus garvieae*, *Enterococcus faecium*, and the genera *Micrococcus*, *Mammaliicoccus*, and *Rothia* provides reliable information on the presence of these species in the samples, regardless of the initial isolation medium. It is worth noting that isolates that did not yield conclusive identification are expected and emphasize the importance of continuously updating the MALDI-TOF reference databases¹³, especially for non-clinical samples.

The predominance of *Staphylococcus* isolates (63.8%) was expected; however, the diversity of other isolated species demonstrates the complexity of the microbiota of raw goat milk and underscores the importance of more in-depth studies to characterize these microorganisms functionally. The most prevalent species among the isolates, *S. aureus*, was detected in six of the nine samples analyzed. This finding is consistent with previous studies that reported the frequent occurrence of *S. aureus* in raw milk from different animal species⁸. The presence of this microorganism in raw milk is considered a significant public health risk, since this

bacterium is recognized as an important foodborne pathogen capable of producing heat-stable enterotoxins that can cause food poisoning even after milk heat treatment¹⁴, and the spread of antibiotic-resistant *S. aureus* strains further aggravates this concern¹⁵. However, in the present study, no enterotoxin production or virulence factors were evaluated.

In contrast, *S. caprae* was isolated in only one sample (A1). Although generally considered to have low pathogenic potential, some strains of coagulase-negative staphylococci, such as *S. caprae*, are more associated with subclinical mastitis than with clinical mastitis, which is commonly associated with *S. aureus*¹⁶. Furthermore, *S. caprae* can occasionally cause human infections¹⁷. The presence of other *Staphylococcus* species underscores the importance of monitoring the microbiological quality of raw goat's milk, as some species of this genus may exhibit pathogenic and/or deteriorative characteristics.

Macrococcus caseolyticus, isolated from samples A2 and CS1, is known for its association with changes in the flavor of milk and dairy products that can generate undesirable flavors. However, regarding the safety of using macrococcal strains in food, two important criteria should be evaluated: the presence of antimicrobial resistance determinants and their transfer to other bacteria, since this genus is closely related to *Staphylococcus* sp.^{7, 18}. The presence of *Mammaliococcus sciuri*, previously known as *S. sciuri*, in sample CS2 may indicate potential failures in herd hygiene and health practices during production, since this species is frequently found on animals' skin and may contaminate milk. Its importance lies in its being a bacterium generally resistant to antibiotics, capable of colonizing animals and humans, and considered a potentially opportunistic pathogen^{19, 20}.

Rothia koreensis, found in sample G1, is a bacterium that has not yet been studied; until 2018, it was still classified in the genus *Kocuria*. The few studies that cite the genus *Rothia* associate it with infections in immunocompromised or immunocompetent individuals²¹, but its role in dairy foods is limited to its former nomenclature, *Kocuria koreensis*, with few records of its isolation from raw bovine milk and cheeses^{22, 23}.

Lactic acid bacteria (LAB) were also observed. These microorganisms, commonly found in milk, are mostly non-pathogenic and have great potential for technological application in the production of dairy products. *Lactococcus lactis* and *L. garvieae*, species widely used as starter cultures in cheese production, were isolated in samples A2 and CS1, respectively. These microorganisms play a key role in milk acidification and the development of desirable aromatic compounds²⁴. The detection of *Enterococcus faecium* in three samples (A2, CS1, and G3) is also relevant, as this species can contribute to the ripening and flavor of dairy products such as cheese²⁵. The isolation of these LAB on Gram-negative media (Leeds agar and TBX agar) further highlights the importance of accurate identification techniques such as MALDI-TOF.

This study has some limitations that should be acknowledged. The number of samples analyzed was relatively small, and sampling was restricted to specific small towns, limiting the generalizability of the findings. In addition, this work has a descriptive character, focusing on the isolation and identification of microorganisms. No analyses of virulence genes, enterotoxin production, or antimicrobial resistance profiles were performed. Furthermore, the technological properties of the isolates were not experimentally evaluated. It should also be considered that the culture conditions and the use of selective media may have influenced the spectrum of microorganisms recovered. Therefore, any discussion regarding pathogenic potential, resistance traits, or technological applications is based on previous literature and should not be interpreted as characteristics demonstrated in the isolates analyzed in this study.

Future studies should expand the sampling scope to include a larger number of samples and broader geographic regions, enabling a more comprehensive assessment of the microbiological quality of raw goat milk. Quantitative microbiological analyses would also be valuable for better characterizing contamination

levels. In addition, molecular approaches targeting virulence factors and enterotoxin genes, as well as antimicrobial resistance profiling, would provide further insight into the potential risks associated with the identified microorganisms. Finally, evaluating the technological properties of selected lactic acid bacteria could help explore their potential applications in dairy production.

CONCLUSIONS

This study provides a descriptive overview of Gram-positive cocci present in raw goat milk from small-scale producers in a specific region of Brazil, revealing a diverse microbiota dominated by *Staphylococcus* species. The detection of *S. aureus* in several samples indicates potential microbiological risks at the local level, although broader generalizations cannot be inferred from the limited sample size.

A key finding of this study was the recovery of Gram-positive cocci from culture media intended for Gram-negative bacteria, highlighting the limitations of phenotypic selectivity in routine microbiological analysis. This result reinforces the need for confirmatory identification techniques. In this context, MALDI-TOF MS proved to be a robust and reliable tool for accurate microbial identification, particularly when isolates are obtained under atypical or non-selective growth conditions.

Author Contributions: **Gustavo L. P. A. Ramos:** Data analysis and interpretation, article writing, and critical review of intellectual content. **Emily C. C. Silva, Lavínia F. Araújo, and Marcelo S. Moraes:** Experiment execution, data analysis, and interpretation. **Janaína S. Nascimento:** Concept and design, data analysis and interpretation, article writing, and critical review of intellectual content. **All authors approve the final version to be published and agree to be responsible for all aspects of the work.**

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Informed Consent Statement: Not applicable. This study does not involve human subjects.

Data Availability Statement: The data supporting the findings of this study will be made available by reasonable request to the corresponding author.

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form. The authors have no conflicts of interest to declare.

AI-Assisted Tools Disclosure: The artificial intelligence tool GPAI (<https://gpai.app/>) was used solely to generate the images included in this study. No artificial intelligence system was used to generate, manipulate, or analyze experimental data or statistical results. The authors independently verified all results, analyses, and conclusions, in compliance with the BioNatura Journal policy: <https://bionaturajournal.com/artificial-intelligence--ai-.html>

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Correspondence should be addressed to: gustavoanciens@id.uff.br

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