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MICROBIOLOGY

Identification and antimicrobial resistance profile of Enterobacterales and *Staphylococcus* spp. isolated from subclinical mastitis milk

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Abstract: This study aimed to identify and evaluate the antimicrobial resistance profile of Enterobacterales and *Staphylococcus* spp. isolated from raw milk samples from mammary quarters with subclinical mastitis (SCM), from three small dairy farms suppliers of raw milk for cheese production, in Rio de Janeiro state. Raw milk from each mammary quarter was investigated for signs of SCM using the California Mastitis Test (CMT). Raw milk with signs of SCM (SCMM) was seeded on Compact-Dry EC and Compact Dry X-SA. The strains were identified by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and evaluated for antimicrobial resistance, by disk diffusion test. SCM was observed in 13 (8.02%) mammary quarters from farm A, 24 (25%) from farm B and 7 (7.95%) from farm C. *Staphylococcus aureus* was the most isolated on farms A (55.56%) and B (75.00%). *Enterobacter roggenkampii* was isolated for the first time from SCMM, being the most prevalent specie on farm C (50.00%). Methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-resistant *Staphylococcus chromogenes* (MRS) were isolated from SCMM from farms A and B. The health of the dairy herd requires great attention, mainly reflected in the control of MRSA/MRS.

Key words: carbapenem-resistant Enterobacterales, extended-spectrum β-lactamase, multidrug-resistance, methicillin-resistant *Staphylococcus*, methicillin-resistant *Staphylococcus* aureus.

INTRODUCTION

Mastitis is an inflammation of the mammary gland parenchyma (Byomi et al. 2020), usually associated with an infection (Bobbo et al. 2017). Mastitis is the most common disease of the dairy herd and can be manifested as clinical (CM) or subclinical mastitis (SCM) (Bobbo et al. 2017, Byomi et al. 2020). CM shows visible signs, such as erythema, flushing and edema in the animal, in addition to clots, flakes and change in the viscosity of the milk. The milk from mammary quarters diagnosed with CM should be discarded (Adkins & Middleton 2018). SCM shows no signs in the animal or in the milk. The milk from mammary quarters diagnosed with SCM is used in the dairy chain (Busanello et al. 2017) (Figure 1). The diagnosis of SCM is made by checking the increase in somatic cell in milk (Adkins & Middleton 2018, Bobbo et al. 2017), through specifics test, such as California Mastitis Test (CMT) (Ruegg & Reinemann 2002) (Figure 2). Enterobacterales and *Staphylococcus* spp. are the main agents of mastitis (Klibi et al. 2019).

Furthermore, the excessive use of antimicrobials on dairy farms to prevent and treat mastitis, favors the selective pressure, accelerating the development of antimicrobial resistance (Verraes et al. 2013). Antimicrobial resistance reduces de options of therapy,

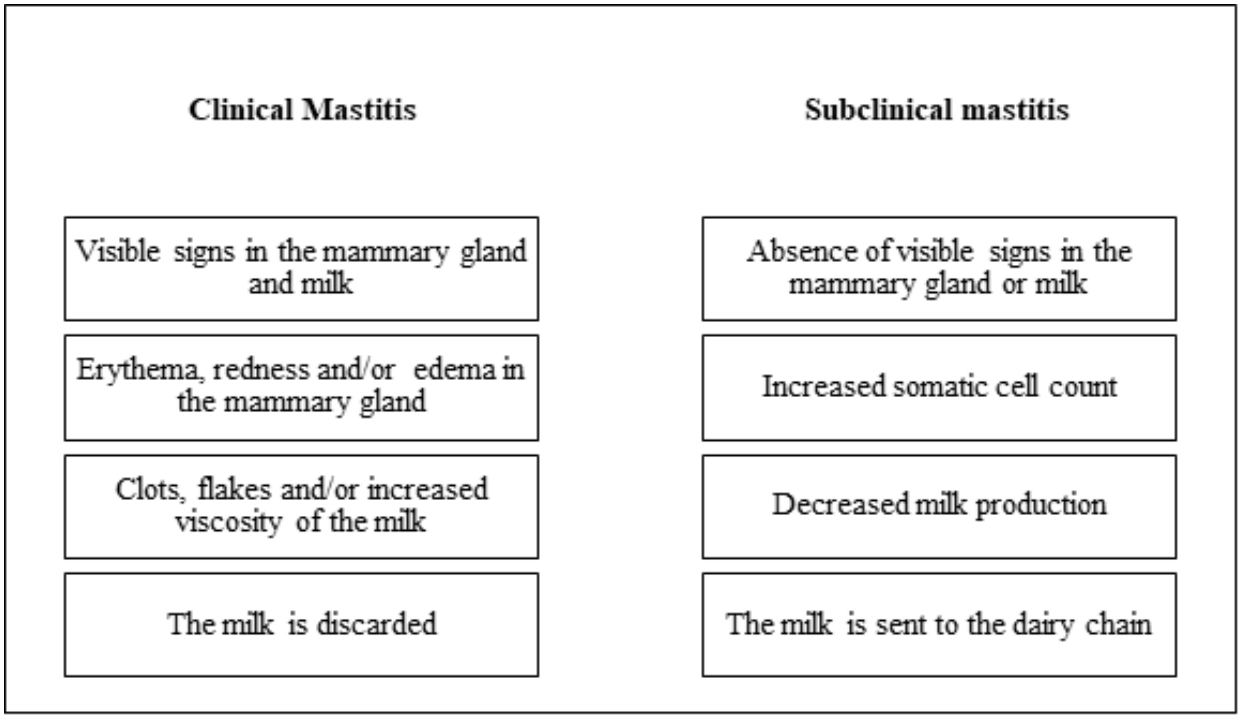

Figure 1. Comparison of clinical and subclinical mastitis. Clinical mastitis is characterized by visible symptoms, requiring milk disposal, whereas subclinical mastitis shows no apparent signs in the animal's mammary gland or milk, but implies in increased somatic cell count and reduced milk production and is sent to the dairy chain.

increasing morbidity and mortality indicators. Antimicrobial resistance is one of the top ten global public health (Walsh et al. 2023). Which the main agents are extended-spectrum beta lactamase (ESBL)-producing Enterobacterales and Methicillin-resistant *Staphylococcus* spp. in addition to carbapenem-resistant Enterobacterales (CRE), which are a serious and urgent threat to health (CDC 2019). Carbapenems are one of the last resources in the treatment of extended-spectrum beta lactamase (ESBL)-producing Enterobacterales infections (Tepeli et al. 2023). The causes and effects of increased antimicrobial resistance are related to several sectors involving human and animal health and the environment, which the main link is food. Therefore, the World Health Organization (WHO) recognized in its Global Action Plan against Antimicrobial Resistance the need for a One Health approach, at global, national and regional levels, involving multidisciplinary areas (WHO 2015).

This study aimed to identify and evaluate the antimicrobial resistance profile of Enterobacterales and *Staphylococcus* spp. isolated from subclinical mastitis milk (SCMM), from mammary quarters of dairy cattle from three small dairy farms suppliers of raw milk for cheese production in two cities of Rio de Janeiro state (Vassouras and Rio Bonito).

MATERIALS AND METHODS

Study area

The study was carried on January to April 2022, in three small dairy farms, suppliers of raw milk for cheese production, located in two distinct geographical areas of the state of Rio de Janeiro, Brazil. Farms A and B, were located in the city of Vassouras (22° 24' 14" S 43° 39'

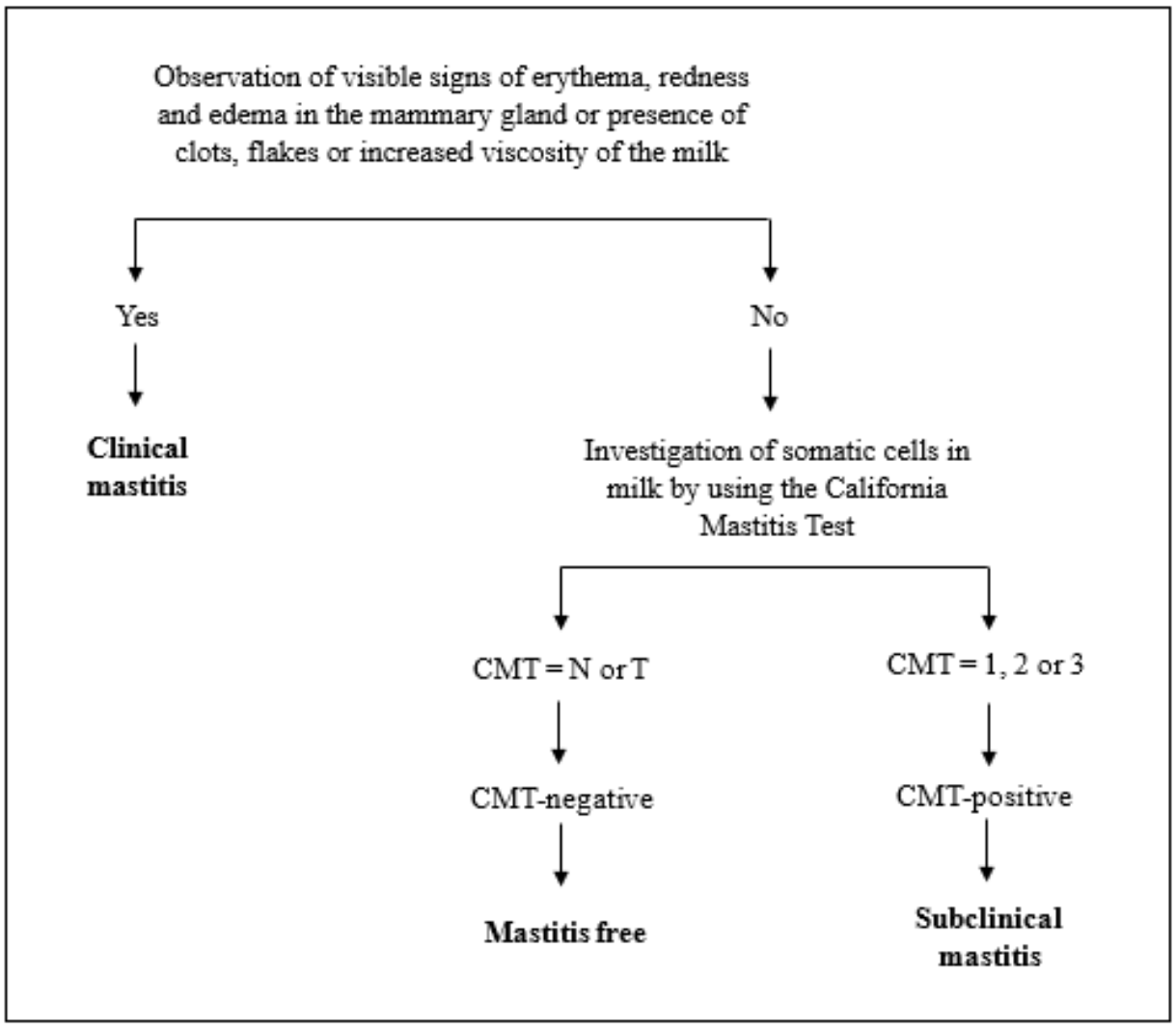

Figure 2. Mastitis diagnosis. CMT: California Mastitis Test; N: negative, no evidence of precipitation; T: traces, possible infection, presence of precipitation that disappeared with movement of the mixture; 1: weak positive infection, precipitation without tendency to gel; 2: positive infection, immediate thickening of the mixture, with slight gel formation that moves towards the center of the paddle; 3: strongly positive infection, formation of a gel.

46" W) and farm C, in the city of Rio Bonito (22° 42' 28" S, 42° 37' 33" W) (Figure 3).

Milk sampling

A total of 346 mammary quarters (farm A=162; farm B=96; farm C=88), from 90 lactating dairy cows (farm A=43; farm B=24; farm C=23) were examined, by experienced veterinarians, for signs of mastitis. CM was investigated by checking for erythema, redness and edema in the mammary gland and the presence of clots or flakes and increased viscosity of the milk. SCM was observed by qualitative investigation of somatic cells in freshly milked raw milk, using the California Mastitis Test (CMT) (TADABRAS). The milk from each mammary quarter was deposited on the CMT-paddle (TADABRAS), mixed and homogenized with the Bromocresol Violet reagent. After 10 seconds, the qualitative assessment of somatic cells was performed,

Figure 3. Map of the State of Rio de Janeiro.◆City of Vassouras (Latitude: 22° 24' 14" S, Longitude: 43° 39' 46" W). ● City of Rio Bonito (Latitude: 22° 42' 328"S, Longitude: 42° 37' 33" W).

and the result was interpreted based on the increase in viscosity, due to the formation of a gel, produced from the release of nuclear material from the leukocytes present in the sample. Score were generated according to the degree of anti-inflammatory response, whether or not accompanied by violet coloring. Scores were identified as: N – negative, no evidence of precipitation; T - traces, possible infection, presence of precipitation that disappeared with movement of the mixture; 1 - weak positive infection, precipitation without tendency to gel; 2 - positive infection, immediate thickening of the mixture, with slight gel formation that moves towards the center of the paddle; 3 - strongly positive infection, formation of a gel (Ruegg & Reinemann 2002). Mammary quarters with CMT score 1, 2 or 3 were diagnosed with SCM. The milk from each mammary quarter with SCM, named SCM milk (SCMM), was collected in an approximate volume of 50 mL, in sterile vials, transported in an isothermal box, with ice packs, maintaining the temperature between 1 and 8 °C (ISO 2007) and analyzed within 48 h.

Bacterial isolation

For the isolation of Enterobacterales and *Staphylococcus* spp., 10 mL of each SCMM sample was homogenized in 90 mL of buffered peptone water (BPW; Himedia), supplemented with 0.15% (v/v) Tween 20 (BPW-T). After, 1 mL was inoculated on Compact Dry® EC and Compact Dry® X-SA plates and incubated at 37 °C for 24 to 48 h, as recommended by the manufacturer (Nissui).

From each culture medium, colonies with different morphologies were selected, cultured in tryptone soy broth (TSB) and incubated at 37

°C for 18 to 24h. The culture in TSB was seeded in tryptone soy agar (TSA) and incubated at 37 °C for 18 to 24 h. A small portion of the cell mass in TSA was transferred to 1.5 mL of TSB with 20% (v/v) glycerol (TSB-G), homogenized in a vortex tube shaker and stored at -20 °C.

Bacterial identification

Bacterial strains at -20 °C were cultured in TSA, incubated at 37 °C for 18 to 20 h and identified by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), using the MALDI Biotyper Microflex LT® equipment (Bruker Daltonics/BD), generating a score between 0 and 3 points (Rodrigues et al. 2017). A score between 0.000 and 1.699 corresponded to unreliable identification; between 1.700 and 1.999 probable genus identification; between 2.000 and 2.299 secure genus identification and probable specie identification; and between 2.300 and 3.000 highly probable specie identification.

Assessment of the phenotypic antimicrobial resistance profile

Enterobacterales and *Staphylococcus* spp., identified with a MALDI TOF MS score ≥2.000 (secure genus and highly probable specie), were tested for antimicrobial resistance by the disk diffusion test (CLSI 2020). Resistant and intermediate strains were reported as non-susceptible. Strains non-susceptible to at least three different classes or subclasses of antibiotics were named as multidrug-resistant (MDR) (Magiorakos et al. 2012). Intrinsic resistance patterns, as described in CLSI (2020), were not included in the MDR profile (CLSI 2020).

Enterobacterales were tested for resistance to Ampicillin (AMP, 10 µg), Amoxicillin-Clavulanate (AMC, 20/10 µg), Piperacillin-Tazobactam (PPT, 100/10 µg), Cefazolin (CFZ, 30 µg), Cefotaxime (CTX, 30 µg), Cefuroxime (CRX, 30 µg), Ceftazidime (CAZ, 30 µg), Aztreonam (ATM, 30 µg), Imipenem (IPM, 10 µg), Meropenem (MER, 10 µg), Ertapenem (ERT, 10 µg), Gentamicin (GEN, 10 µg), Amikacin (AMI, 30 µg), Tetracycline (TET, 30 µg), Minocycline (MIN, 30 µg), Levofloxacin (LVX, 5 µg), Ciprofloxacin (CIP, 5 µg), Nalidixic Acid (NAL, 30 µg), Sulfamethoxazole-Trimethoprim (SUT, 1.25/23.75 µg), Trimethoprim (TRI, 5 µg), Chloramphenicol (CLO, 30 µg), Nitrofurantoin (NIT, 300 µg) and Fosfomycin (FOS, 200 µg). *Escherichia coli* ATCC 25922 was used as quality control (CLSI, 2020). Suspected ESBL strains (non-susceptibility to CAZ, CTX and/or ATM) were subjected to the combined disk diffusion confirmatory test, as described in CLSI (2020). An increase of ≥ 5 mm in the zone of inhibition between the CAZ or CTX disk combined with clavulanic acid, relative to the disc without the inhibitor, confirmed ESBL production. *Escherichia coli* ATCC 25922 was used as ESBL-negative control and *Klebsiella pneumoniae* ATCC 700603 was used as ESBL-positive control. Strains non-susceptible to IPM, ERT or MER were reported as CRE (CLSI 2020).

Staphylococcus spp. were tested for antimicrobial resistance to Penicillin G (PEN, 10 U), Cefoxitin (CFO, 30 µg), Gentamicin (GEN, 10 µg), Erythromycin (ERI, 15 µg), Tetracycline (TET, 30 µg), Levofloxacin (LVX, 5 µg), Ciprofloxacin (CIP, 5 µg), Nitrofurantoin (NIT, 300 µg), Clindamycin (CLI, 2 µg), Sulfamethoxazole-Trimethoprim (SUT, 1.25/23.75 µg), Trimethoprim (TRI, 5µg), Chloramphenicol (CLO, 30 µg), Rifampicin (RIF, 5 µg) and Linezolid (LNZ, 30 µg). *S. aureus* ATCC 25923 was used as quality control (CLSI 2020). *S. aureus* resistant to CFO were reported as MRSA (methicillin-resistant *S. aureus*) and non-aureus *Staphylococcus* resistant to CFO were reported as MRS (methicillin-resistant *Staphylococcus*) (CLSI 2020). MRSA and MRS were reported as MDR, since methicillin-resistance indicates

resistance to all β-lactams, with the exception of ceftaroline (CLSI 2020, Magiorakos et al. 2012).

Statistical analysis

Descriptive statistics were used to describe absolute and relative differences (%) of occurrence of milk with SCM and mastitis-free, the presence of Enterobacterales and *Staphylococcus* spp., and the occurrence of strains resistant to antimicrobials. Furthermore, the difference between the number of mammary quarters with SCM and MF in each farm, the difference in the number of mammary quarters with SCM, the difference in the number of strains of Enterobacterales and *Staphylococcus* spp isolated, the difference in the number of non-susceptible strains and the difference in the number of MDR strains between the three farms, were evaluated by the Chi-square test or Fisher's Exact test, using the Prism 5® program, version 5.01. A value of $p < 0.05$ was considered as the level of significance.

RESULTS AND DISCUSSION

Mastitis diagnosis, bacterial identification and its antimicrobial resistance profile provide valuable insights into dairy herd health and microbial diversity in subclinical mastitis (SCM). The absence of clinical mastitis suggests effective disease management, but the high prevalence of SCM on farm B indicates the need for improved control measures. In addition, bacterial identification revealed patterns of bacterial distribution and resistance, showing the role of geographic location and farm management practices in shaping pathogen profiles.

Diagnosis of mastitis

The majority of mammary quarters evaluated (302; 87.28%) were mastitis-free (CMT-negative test). CM was not observed in any of the 346 mammary quarters evaluated, showing that the herd's health management is preventing serious cases of the disease in the three farms. However, 44 (12.72%) mammary quarters were diagnosed with SCM (CMT-positive test). A significant association ($p<0.0001$) was observed between SCM and farms, with a considerably higher number of mammary quarters diagnosed with SCM on farm B (24; 25%) than on farms A (13; 8.02%) and C (7; 7.95%) (Table I). It shows that on farms A and C subclinical mastitis seems to be under control, but farm B needs to improve the health management of its herd to produce better quality and safety milk.

While clinical mastitis milk (CMM) is discarded, SCMM is used for human consumption or for production of dairy products (Busanello et al. 2017). Even though SCM does not present visible sings in the animal or in the milk, it can have a harmful impact on the dairy chain, due to the increase in the number of somatic cells, lower price per litre of milk, low yield of the dairy herd, increased enzymatic activities in milk, which can generate defective products, reduced milk quality and due to possible contamination

Table I. Health of the mammary quarters of the dairy herd from three farms in the state of Rio de Janeiro (January to April 2022), investigated by checking for erythema, redness and edema in the mammary gland and the presence of clots or flakes and increased viscosity of the milk for diagnosis of clinical mastitis, and qualitative investigation of somatic cells in freshly milked raw milk, using the California Mastitis Test for diagnosis of subclinical mastitis.

Farm	SCM (%)	MF (%)	Total
A	13 (8.02)	149 (91.98)	162
B	24 (25.00)	72 (75.00)	96
C	7 (7.95)	81 (92.05)	88
Total	44 (12.72)	302 (87.28)	346

MF: mastitis-free; SCM: subclinical mastitis. p<0.0001 (p-value of the difference between the number of mammary quarters with SCM and MF among the farms).

with pathogenic and spoilage bacteria (Murphy et al. 2016). To assure the quality of raw milk, SCM should not exceed 15% in the herd (Ruegg & Pantoja 2013). In view of this, the results observed indicate that the high percentage of mammary quarters with SCM (25%) (Table I) may compromise the quality of milk from farm B.

Bacterial identification

The 44 SCMM samples were subjected to isolation of Enterobacterales and *Staphylococcus* spp. A total of 60 bacterial colonies, of different morphotypes, with a score ≥2,000, were identified by MALDI TOF MS. Of these, 14 (23.33%) were identified as Enterobacterales and 46 (76.67%) were identified as *Staphylococcus* spp. The number of Enterobacterales and *Staphylococcus* spp. isolates was significantly different ($p<0.0001$) between the three farms, with a discrepancy occurring on farm C (Table II). This is maybe due to the fact that most of the isolates were obtained from farms A and B, where bacteria of the genus *Staphylococcus* prevailed. *Staphylococcus* spp., mainly *Staphylococcus aureus* are usually contagious agents of mastitis and spread throughout the dairy herd. Which lead us to deduce that mastitis in these farms is of contagious origin and strains of the same origin are possibly circulating in the herds of the region, since farms A and B are located in the same city in the south central region of the state, while farm C is located in a municipality in the metropolitan region of the state (Figure 3). The data also suggests that geographic location may influence Enterobacterales and *Staphylococcus* spp. diversity in SCMM.

Farm C presented the lowest number of bacterial isolates, with a higher prevalence of Enterobacterales (3; 75%), while on farms A (8; 22.22%) and B (3; 15%) this group was the

Table II. Abundance of Enterobacterales and *Staphylococcus* spp. in subclinical mastitis milk in three dairy farms, in the state of Rio de Janeiro (January to April 2022).

Microorganisms	No. of strains (%)						Total	
	Farm A		Farm B		Farm C			
Enterobacterales	8	(22.22)	3	(15.00)	3	(75.00)	14	(23.33)
Citrobacter braakii	1	(2.78)	0	(0.00)	0	(0.00)	1	(1.67)
Citrobacter freundii	1	(2.78)	0	(0.00)	1	(25.00)	2	(3.33)
Enterobacter asburiae	2	(5.56)	0	(0.00)	0	(0.00)	2	(3.33)
Enterobacter bugandensis	2	(5.56)	1	(5.00)	0	(0.00)	3	(5.00)
Enterobacter cloacae	1	(2.78)	0	(0.00)	0	(0.00)	1	(1.67)
Enterobacter roggenkampii	0	(0.00)	1	(5.00)	2	(50.00)	3	(5.00)
Lelliottia amnigena	1	(2.78)	0	(0.00)	0	(0.00)	1	(1.67)
Serratia marcescens	0	(0.00)	1	(5.00)	0	(0.00)	1	(1.67)
Staphylococcus spp.	28	(77.78)	17	(85.00)	1	(25.00)	46	(76.67)
Staphylococcus aureus	20	(55.56)	15	(75.00)	0	(0.00)	35	(58.33)
Staphylococcus chromogenes	7	(19.44)	2	(10.00)	0	(0.00)	9	(15.00)
Staphylococcus xylosus	1	(2.78)	0	(0.00)	0	(0.00)	1	(1.67)
Staphylococcus saprophyticus	0	(0.00)	0	(0.00)	1	(25.00)	1	(1.67)
Total	36	(60.00)	20	(33.33)	4	(6.67)	60	(100.00)

$p<0.0001$ (p-value of the difference between the number of Enterobacterales and *Staphylococcus* spp. among the farms).

least prevalent (Table II). Enterobacterales can contaminate raw milk from animal faeces or contaminated equipment and environment (Sobeih et al. 2020) and are frequently isolated from SCMM (Klibi et al. 2019). Eight different species of Enterobacterales, belonging to the genera *Citrobacter, Enterobacter, Lelliottia* and *Serratia*, were isolated from the SCMM samples (Table II). *Enterobacter* stood out among the Enterobacterales isolated, with *Enterobacter roggenkampii* as the most prevalent specie (2; 50%) in the SCMM of farm C (Table II). *Enterobacter roggenkampii* has been reported in sewage waste from a hospital in China, associated with colistin-resistance (Xu et al. 2021). To our knowledge, this is the first report of the isolation of *Enterobacter roggenkampii* from SCMM and the data suggests that mastitis in this farm is of environmental origin.

Staphylococcus spp. occurred predominantly on farms A (28; 77.78%) and B (17; 85%) while, on farm C only one (25%) coagulase-negative strain, identified as *Staphylococcus saprophyticus*, was described (Table II). *Staphylococcus saprophyticus* has been described less frequently in SCM, appearing to be less virulent than other coagulase-negative staphylococci (Waller et al. 2011). *Staphylococcus aureus* was the most prevalent specie on SCMM samples from farms A (20; 55.56%) and B (15; 75.00%). *Staphylococcus aureus* has been reported as one of the main agents of SCM (Klibi et al. 2019). Coagulase-negative staphylococci were identified with lower prevalence, with emphasis on *Staphylococcus chromogenes* from farms A (7; 19.44%) and B (2; 10.00%) (Table II). *Staphylococcus chromogenes* is one of the main coagulase-negative staphylococci involved in mastitis in cows (Waller et al. 2011), being described as the non-aureus staphylococci most isolated from SCMM in Brazil (de Oliveira et al. 2022) and in the world (De Buck et al. 2021). One strain (2.78%) of *Staphylococcus xylosus* was identified on farm A. *Staphylococcus xylosus* is among the coagulase-negative staphylococci described in CM, also being described in SCM (Waller et al. 2011, Cheung & Otto 2023).

Antimicrobial resistance profile

Of the 60 strains isolated, 50 (83.33%) were non-susceptible to at least one antimicrobial, 13 (92.86%) Enterobacterales and 37 (80.43%) *Staphylococcus* spp. A significant association (p=0.0107) was observed between non-susceptible strains and the three farms, with the majority of strains isolated on farm A (33; 55%) and farm B (14; 23.33%) (Table III). *Staphylococcus aureus* represented the majority of non-susceptible strains on farm A (18; 54.55%) and B (10; 71.43%). While, on farm C, only three (5%) non-susceptible strains, one *Citrobacter freundii* and two *Enterobacter roggenkampii*, were identified (Table III).

A total of 20 (33.33%) strains were identified as multidrug-resistant (MDR), five (35.71%) Enterobacterales and 15 (32.61%) *Staphylococcus* spp. MDR strains were isolated on farms A (18; 30.00%) and B (2; 3.33%) (Table III).

The high rate of antimicrobial non-susceptible strains in SCMM samples is concerning. The identification of strains that are non-susceptible to antimicrobials, especially those used in the treatment of human infections, in non-hospital environments raises awareness regarding the control of resistance to antimicrobials. The treatment of human and animal infections is impaired when the infectious agent is non-susceptible to antimicrobials, which makes antimicrobial resistance a major public health problem, affecting morbidity and mortality rates. Antimicrobial non-susceptible bacteria in raw milk indicates that food may be an important disseminator of these strains. Processing, such as pasteurization, can reduce

Table III. Non-susceptibility and multidrug-resistance in Enterobacterales and *Staphylococcus* spp. isolated from subclinical mastitis milk in three dairy farms, in the state of Rio de Janeiro (January to April, 2022).

Microorganisms	No. of non-susceptible strains (%)						Total		No. of MDR strains (%)						Total	
	Farm A		Farm B		Farm C				Farm A		Farm B		Farm C			
Enterobacterales (n=14)	7	(21.21)	3	(21.43)	3	(100.00)	13	(92.86)	5	(27.78)	0	(0.00)	0	(0.00)	5	(35.71)
Citrobacter braakii (n= 1)	1	(3.03)	0	(0.00)	0	(0.00)	1	(100.00)	1	(5.56)	0	(0.00)	0	(0.00)	1	(100.00)
Citrobacter freundii (n=2)	1	(3.03)	0	(0.00)	1	(33.33)	2	(100.00)	1	(5.56)	0	(0.00)	0	(0.00)	1	(50.00)
Enterobacter asburiae (n=2)	2	(6.06)	0	(0.00)	0	(0.00)	2	(100.00)	1	(5.56)	0	(0.00)	0	(0.00)	1	(50.00)
Enterobacter bugandensis (n=3)	2	(6.06)	1	(7.14)	0	(0.00)	3	(100.00)	2	(11.11)	0	(0.00)	0	(0.00)	2	(66.67)
Enterobacter cloacae (n=1)	1	(3.03)	0	(0.00)	0	(0.00)	1	(100.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)
Enterobacter roggenkampii (n=3)	0	(0.00)	1	(7.14)	2	(66.67)	3	(100.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)
Lelliottia amnigena (n=1)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)
Serratia marcescens (n=1)	0	(0.00)	1	(7.14)	0	(0.00)	1	(100.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)
Staphylococcus spp. (n=46)	26	(78.79)	11	(78.57)	0	(0.00)	37	(80.43)	13	(72.22)	2	(100.00)	0	(0.00)	15	(32.61)
Staphylococcus aureus (n=35)	18	(54.55)	10	(71.43)	0	(0.00)	28	(80.00)	9	(50.00)	1	(50.00)	0	(0.00)	10	(28.57)
Staphylococcus chromogenes (n=9)	7	(21.21)	1	(7.14)	0	(0.00)	8	(88.89)	3	(16.67)	1	(50.00)	0	(0.00)	4	(44.44)
Staphylococcus xylosus (n=1)	1	(3.03)	0	(0.00)	0	(0.00)	1	(100.00)	1	(5.56)	0	(0.00)	0	(0.00)	1	(100.00)
Staphylococcus saprophyticus (n=1)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)
Total (n=60)	33	(55.00)	14	(23.33)	3	(5.00)	50	(83.33)	18	(30.00)	2	(3.33)	0	(0.00)	20	(33.33)

MDR: multidrug-resistant = non-susceptibility to at least one antimicrobial from three different antimicrobial classes; Non-susceptible: resistant or intermediate resistant to at least one antimicrobial. Intrinsic resistance was not considered.

the risk of contamination of dairy products by non-susceptible bacteria, including MDR (Verraes et al. 2013).

The antimicrobial susceptibility profile of Enterobacterales from SCMM on farms A and B was similar. Non-susceptibility to AMC and CFZ was prevalent on farms A and B; and AMP on farm A (5; 62.50%, for each antimicrobial). Non-susceptibility to AMP was also observed on farm B (1; 33.33%). It was expected, since some Enterobacterales are intrinsically resistant to these antimicrobials, although some genes may be low expressed (CLSI 2020). Additionally, non-susceptibility to CRX (12.50% and 33.33%), ATM (25% and 33.33%), NAL (12.50% and 33.33%), TRI (12.50% and 33.33%) and SUT (12.50% and 33.33%) was observed on farms A and B, respectively (Table IV). Non-susceptibility to GEN (12.50%) and FOS (25%) was observed only on farm A, while non-susceptibility to NIT (33.33%) was observed only on farm B (Table IV). The susceptibility profile of Enterobacterales from SCMM on farm C was different from the profile observed on farms A and B, where only strains non-susceptible to ATM and NAL were observed (Table III). Non-susceptibility to ATM can occur

Table IV. Prevalence of phenotypic antimicrobial resistance of Enterobacterales isolated from subclinical mastitis milk, on dairy farms in the state of Rio de Janeiro from January 2022 to April 2022.

Antimicrobial	Farm (%)					
	A		B		C	
AMP	5	(62.50)	1	(33.33)	0	(0.00)
AMC	5	(62.50)	2	(66.67)	0	(0.00)
CFZ	5	(62.50)	2	(66.67)	0	(0.00)
CRX	1	(12.50)	1	(33.33)	0	(0.00)
CAZ	0	(0.00)	0	(0.00)	0	(0.00)
CTX	0	(0.00)	0	(0.00)	0	(0.00)
ATM	2	(25.00)	1	(33.33)	3	(100.00)
PPT	0	(0.00)	0	(0.00)	0	(0.00)
TET	0	(0.00)	0	(0.00)	0	(0.00)
MIN	0	(0.00)	0	(0.00)	0	(0.00)
GEN	1	(12.50)	0	(0.00)	0	(0.00)
AMI	0	(0.00)	0	(0.00)	0	(0.00)
NIT	0	(0.00)	1	(33.33)	0	(0.00)
NAL	1	(12.50)	1	(33.33)	3	(100.00)
CIP	0	(0.00)	0	(0.00)	0	(0.00)
LVX	0	(0.00)	0	(0.00)	0	(0.00)
CLO	0	(0.00)	0	(0.00)	0	(0.00)
TRI	1	(12.50)	1	(33.33)	0	(0.00)
SUT	1	(12.50)	1	(33.33)	0	(0.00)
FOS	2	(25.00)	0	(0.00)	0	(0.00)
IPM	0	(0.00)	0	(0.00)	0	(0.00)
MER	0	(0.00)	0	(0.00)	0	(0.00)
ERT	0	(0.00)	0	(0.00)	0	(0.00)

AMC: Amoxicillin-Clavulanate; AMI: Amikacin; AMP: Ampicillin; ATM: Aztreonam; CAZ: Ceftazidime; CFZ: Cefazolin; CRX: Cefuroxime; CTX: Cefotaxime; CIP: Ciprofloxacin; CLO: Chloramphenicol; ERT: Ertapenem; FOS: Phosphomycin; GEN: Gentamicin; IPM: Imipenem; LVX: Levofloxacin; MER: Meropenem; MIN: Minocycline; NAL: Nalidixic Acid; NIT: Nitrofurantoin; PPT: Piperacillin-Tazobactam; SUT: Trimethoprim-sulfamethoxazole; TET: Tetracycline; TRI: Trimethoprim.

through the ESBL-production (CLSI 2020). ESBL can hydrolyse monobactams (ATM) and third-generation cephalosporins and are encoded on plasmids, which can be transferred to other bacteria (Tepeli & Zorba 2018). The disk diffusion test with clavulanic acid, an ESBL inhibitor, was negative for the six Enterobacterales non-susceptible to ATM, indicating that non-susceptibility was conferred by a mechanism other than ESBL-production. Susceptibility to carbapenems (IPM, ERT and/or MER) and third-generation cephalosporins (CTX and/or CAZ) was observed in all Enterobacterales isolated from SCMM from farms A, B and C (Table IV). The isolation of carbapenem-resistant Enterobacterales from foods is not common. On the other hand, ESBL-producing Enterobacterales are more frequently described (Sierra et al. 2023).

Staphylococcus spp. from farms A and B were mostly non-susceptible to PEN (26; 92.86% and 11; 64.71%. respectively). Resistance to PEN has been widely reported in *Staphylococcus* isolated from bovine mastitis in Brazil, ranging from 30.4% to 100% (Rabello et al. 2020). PEN inhibits bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs), which are essential for peptidoglycan formation. Resistance to PEN occurs through enzymatic inactivation due to the production of β-lactamases. Non-susceptibility to CFO was observed on farms A (9/28; 32.14%) and B (2/17; 11.76%) (Table V). CFO is a surrogate for phenotypic detection of methicillin-resistance. Methicillin is a prototype of the penicillinase-stable penicillin, semisynthetic drugs that were developed to treat infections caused by beta-lactamase-producing *S. aureus*. MRSA are resistant to all beta-lactam agents except the new fifth-generation cephalosporins and has been reported from mastitis cases in Brazilian herds, but with low prevalence (Rabello et al. 2020). Susceptibility to GEN and LVX was observed in all *Staphylococcus* spp. from farms A and B and to CIP and CLO in all *Staphylococcus* spp. from farms A and to TET in all *Staphylococcus* spp. from farms B. *Staphylococcus* spp. from farm C were susceptible to all antimicrobials tested (Table V).

A total of 11 *Staphylococcus* spp. were Methicillin-resistant. From these, nine (81; 82%) were isolated SCMM from in farm A and 2 (18.18%) in farm B. No MRS was observed in farm C. MRSA was the major (8; 72.72%) and MRS *Staphylococcus chromogenes* (3; 27.27%) was also detected. On farm A, 7 (77.78%) MRSA and 2 (22.22%) MRS (*Staphylococcus chromogenes*) and on farm B, 1 (50%) MRSA and 1 (50%) MRS

Table V. Prevalence of phenotypic antimicrobial resistance of *Staphylococcus* spp., isolated from subclinical mastitis milk on dairy farms in the state of Rio de Janeiro, from January 2022 to April 2022.

Antimicrobial	Farm (%)					
	A		B		C	
PEN	26	(92.86)	11	(64.71)	0	(0.00)
CFO	9	(32.14)	2	(11.76)	0	(0.00)
GEN	0	(0.00)	0	(0.00)	0	(0.00)
ERI	12	(42.86)	2	(11.76)	0	(0.00)
TET	1	(3.57)	0	(0.00)	0	(0.00)
LVX	0	(0.00)	0	(0.00)	0	(0.00)
CIP	0	(0.00)	1	(5.88)	0	(0.00)
NIT	1	(3.57)	1	(5.88)	0	(0.00)
CLI	10	(35.71)	3	(17.65)	0	(0.00)
SUT	4	(14.29)	1	(5.88)	0	(0.00)
TRI	3	(10.71)	2	(11.76)	0	(0.00)
CLO	0	(0.00)	1	(5.88)	0	(0.00)
RIF	7	(25.00)	2	(11.76)	0	(0.00)
LNZ	12	(42.86)	3	(17.65)	0	(0.00)

CFO: Cefoxitin; CIP: Ciprofloxacin; CLI: Clindamycin; CLO: Chloramphenicol; ERI: Erythromycin; GEN: Gentamicin; LNZ: Linezolid; LVX: Levofloxacin; NIT: Nitrofurantoin; PEN: Penicillin; RIF: Rifampicin; SUT: Trimethoprim-sulfamethoxazole; TET: Tetracycline; TRI: Trimethoprim.

(*Staphylococcus chromogenes*) were identified (Table VI).

Resistance to methicillin in *Staphylococcus* spp. occurs through the mechanism of alteration of the antibiotic's target site. This is due to the presence of the mecA and/or mecC genes, which encodes an altered penicillin-binding protein, called 2a (PBP2a). Antimicrobials have an affinity for specific molecules, where they bind to exert their action, called the target site. When a change occurs at the target site, the antimicrobial does not recognize the molecule and loses its affinity. Therefore, it cannot bind to the altered site and does not exert its action (Verraes et al. 2013). The production of PBP2a, which has lower affinity to β-lactams, makes Staphylococci able to escape from almost all beta-lactams (Schnitt & Tenhagen 2020). MRSA strains has been reported in dairy farms around the world. MRSA/MRS are classified as MDR because they show resistance not only to CFO, but to several β-lactams (CLSI 2020). MDR strains are worrying because, in addition to reducing treatment options, they can transfer a greater variety of resistance genes to other sensitive *Staphylococcus* spp. (Schnitt & Tenhagen 2020). Alves et al. (2020) identified 26% of *S. aureus*, isolated from SCMM, presenting the MRSA genotype, but none were resistant to CFO. Although methicillin resistance is not related to foodborne illness (Schnitt & Tenhagen 2020), most MRSA strains are staphylococcal enterotoxin producers, increasing the risk of food poisoning (Pinchuk et al. 2010).

CONCLUSIONS

To the best of our knowledge, *Enterobacter roggenkampii* was first reported in SCMM, suggesting the need for future investigations about its relevance in the milk production chain. In addition, *Staphylococcus aureus* has been identified as an important contaminant of SCMM. A high prevalence of non-susceptible *Staphylococcus* spp. was observed on farms with a high prevalence of SCM and on those where the disease seemed to be controlled, but were geographically close, suggesting that environmental factors may influence the spread of this pathogen. ESBL and CRE were not a concern in the SCMM of the farms evaluated. However, MRS and MRSA stood out as a potential risk to public health. The study concluded that the diversity of Enterobacterales and *Staphylococcus* spp., as well as their resistance profile in the SCMM, may be influenced by the geographic location of the farms. These findings reinforce the need for a One Health perspective to monitor and control many agents of the spread of antimicrobial resistance in dairy production.

Table VI. Methicillin-resistance in *Staphylococcus* spp. isolated from subclinical mastitis milk, on dairy farms in the state of Rio de Janeiro, from January 2022 to April 2022.

Microorganisms	Farm (%)						TOTAL	
	A		B		C			
Staphylococcus spp.	9	(81.82)	2	(18.18)	0	(0.00)	11	(100)
Staphylococcus aureus	7	(77.78)	1	(50.00)	0	(0.00)	8	(72.72)
Staphylococcus chromogenes	2	(22.22)	1	(50.00)	0	(0.00)	3	(27.27)

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