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Critical points of microbiological contamination in buffalo milking and characterization of *Staphylococcus* spp.

[Pontos críticos de contaminação microbiológica na ordenha de búfalas e caracterização de *Staphylococcus* spp.]

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Abstract: Milking is a critical step in dairy production, being decisive for the microbiological quality of milk. Therefore, this study aimed to evaluate hygienic-sanitary aspects of buffalo milking through microbiological analysis, focusing on the study of bacteria of the genus *Staphylococcus*. Two buffalo milk samplings were carried out at the Estação Experimental Agronômica (EEA) of Federal University of Rio Grande do Sul (UFRGS), which were analyzed for microbiological and physicochemical parameters. Samples from milking surfaces were analyzed by Standard Plate Count (SPC) and identified by MALDI-TOF. *Staphylococcus* spp. isolates were characterized for coagulase production, antimicrobial resistance, hemolytic activity, biofilm formation, and exopolysaccharide (EPS) production. Raw milk showed SPC levels above the limits established by legislation in Sampling 2, and fat and protein contents lower than values reported in the literature in both samplings. SPC on milking surfaces ranged from 2.2×10^2 to 1.05×10^5 CFU/mL, with the highest counts found inside teat cup liners. Among the isolated microorganisms, *Staphylococcus* was the most frequent (32.6 %), followed by *Pseudomonas* and *Acinetobacter* (12.2 % each). The *Staphylococcus* species identified were *S. chromogenes, S. hyicus,* and *S. capitis*. Only *S. chromogenes* (6.25 %) showed coagulase activity. *S. capitis* was resistant to six antimicrobials, while the other isolates were sensitive or intermediate. None of the isolates showed hemolysis or EPS production, whereas all isolates were identified as biofilm producers. The findings highlight the pathogenic potential of the isolates and reinforce the need for continuous monitoring and strict control of milking practices to ensure buffalo milk quality and to prevent the dissemination of pathogens and antimicrobial resistance within the production chain.

Keywords: Antibiogram; biofilm formation; Buffalo milk; monitoring; *Staphylococcus* sp.

Resumo: A ordenha é uma etapa crítica na produção leiteira, sendo determinante à qualidade microbiológica do leite. Portanto, objetivou-se avaliar aspectos higiênico-sanitários da ordenha de búfalas através da pesquisa microbiológica, com foco no estudo de bactérias do gênero *Staphylococcus*. Foram realizadas duas coletas de leite de búfala na Estação Experimental Agronômica (EEA) da Universidade Federal do Rio Grande do Sul (UFRGS), analisadas quanto aos parâmetros microbiológicos e físico-químicos. Amostras de superfícies da ordenha foram analisadas por Contagem Padrão em Placa (CPP) e identificadas por MALDI-TOF. Os isolados de *Staphylococcus* spp. foram caracterizados quanto à coagulase, resistência antimicrobiana, atividade hemolítica,

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produção de biofilme e de exopolissacarídeos (EPS). O leite cru apresentou CPP acima do permitido pela legislação na Coleta 2, e teores de gordura e proteína inferiores à literatura em ambas as coletas. A CPP nas superfícies da ordenha variou de 2,2 x 10^2 a 1,05 x 10^5 UFC/mL, predominando no interior das teteiras. Entre os microrganismos isolados, *Staphylococcus* foi o mais frequente (32,6 %), seguido de *Pseudomonas* e *Acinetobacter* (12,2 % cada). As espécies identificadas de *Staphylococcus* foram *S. chromogenes, S. hyicus* e *S. capitis*. Somente *S. chromogenes* (6,25 %) apresentou coagulase positiva. *S. capitis* foi resistente a seis antimicrobianos, enquanto os demais foram sensíveis ou intermediários. Nenhum isolado apresentou hemólise ou produção de EPS, enquanto todos isolados foram identificados como formadores de biofilme. Os achados indicam o potencial patogênico dos isolados e a necessidade de monitoramento contínuo e controle rigoroso da ordenha para assegurar a qualidade do leite bubalino e prevenir a disseminação de patógenos e resistência antimicrobiana na cadeia produtiva.

Palavras-chave: Antibiograma; formação de biofilme; leite bubalino; monitoramento; *Staphylococcus* sp.

1. Introduction

Buffalo milk is the second most produced milk in the world, accounting for 15 % of global production, second only to cow's milk [1]. Despite this, only 7 % of the milk consumed worldwide is of buffalo origin. During the milking process and immediately after its extraction from the udder, milk may be contaminated by various environmental microorganisms [2].

In this context, the hygienic-sanitary conditions adopted during production, especially in animal handling, equipment sanitation, and milking practices, are essential to ensure milk quality. The adoption of Good Agricultural Practices (GAP) in dairy farming consists of the application of technical and operational procedures that cover all stages of production on rural properties, with the aim of ensuring safe, high-quality milk and dairy products suitable for consumption, in accordance with the required standards [3]. In addition to practices related to animal handling, it is essential that the milker adopts strict personal hygiene measures, as they are in direct contact with the teats, equipment, and milk [4].

Among the main microorganisms of interest in dairy farming, bacteria of the genus *Staphylococcus* stand out, as they can affect parameters such as milk composition and Somatic Cell Count (SCC) [5-6]. Among these, *S. aureus* is an opportunistic pathogen, recognized for its potential for human-animal transmission. It is one of the main causes of infection and disease in a wide range of animal hosts, leading to a significant impact on both public health and agriculture [7]. In the context of livestock production, it stands out as one of the main etiological agents of mastitis – a disease characterized by inflammation of the mammary gland [8] that leads to significant economic losses in the dairy chain. For this reason, mastitis is among the most frequently reported *S. aureus* infections in animals [7]. In this regard, species of the genus *Staphylococcus* are relevant at the interface between animal, human, and environmental health and are frequently discussed from a One Health perspective, particularly due to their zoonotic potential and the dissemination of antimicrobial resistance [9].

In addition, *S. chromogenes* stands out, as it has been reported as the most prevalent species of coagulase-negative *Staphylococcus* (CoNS) associated with mastitis in dairy cattle in several studies [10-12], including in buffalo herds. Another important CoNS species is *S. capitis*, which has the ability to form biofilms and exhibit antimicrobial resistance, in addition to acting as an opportunistic

agent in cases of contamination during milking; it may also be associated with cases of mastitis in ruminants [13]. In this context, the present study aimed to evaluate the hygienic and sanitary aspects of the milking process of buffaloes through the microbiological assessment of different points in the process, with a focus on bacteria of the genus *Staphylococcus*.

2. Material and methods

The present study is exploratory and was conducted as a pilot study based on preliminary data from a limited number of samples. Studies of this nature aim to generate initial information, identify trends, and support the design of more robust future investigations [14].

2.1 Study site and milking procedures

The milk used in this study was collected at the dairy farm of Agronomic Experimental Station of the Federal University of Rio Grande do Sul (UFRGS), located at km 146 of BR-290 highway, in the municipality of Eldorado do Sul, Rio Grande do Sul, Brazil (30°05′32″ S, 51°40′37″ W). The study evaluated a herd composed of 42 animals of the Murrah and Mediterranean breeds, including 12 multiparous female buffaloes (*Bubalus bubalis*), with an average body weight of 603 ± 44.3 kg, aged between 4 and 11 years, and with a mean of 173 days in lactation. Milking was performed three times per week using a bucket-type mechanical milking system, with two teat cup clusters. Milking procedures were conducted in accordance with the protocols established by the Guide to Good Practices in Dairy Farming [3] and the Manual of Good Agricultural Practices in Milking: Buffalo Production [4]. For this purpose, prior to milking, pre-dipping was performed using iodopovidone diluted in water at a 1:3 ration (Globo Vac), followed by the strip cup test and the California Mastitis Test (CMT) using bromocresol purple reagent (2 mL of reagent to 2 mL of milk) (Tadabras). After milking, post-dipping was carried out using undiluted iodopovidone (Globo Vac). Cleaning procedures at the dairy farm were performed by the staff and conducted prior to each milking session using sodium hypochlorite (Polibom), and after each session using chlorinated detergent (Quimiprol). The removal of organic matter to ensure the sanitation of all equipment and utensils used during the milking process was consistently performed. As the study involved animal handling, the project was submitted to the Animal Ethics Committee (CEUA) of UFRGS and was approved under protocol number 43289.

2.2 Microbiological analyses and centesimal composition of buffalo milk

Bulk tank milk samples were aseptically collected from the cooling tank immediately after the end of milking for the evaluation of centesimal composition and Standard Plate Count (SPC). The samples were filtered and homogenized, then stored in insulated containers and transported to the Microbiology and One Health Laboratory of the Institute of Basic Health Sciences (ICBS - UFRGS). For microbiological analysis, SPC was performed on Plate Count Agar (PCA) according to the ISO 4833-2:2013 methodology [15], and the results were expressed as colony-forming units per milliliter (CFU/mL). The centesimal composition of the milk samples was determined using a milk analyzer calibrated for buffalo milk (Akso Master Pro Touch).

2.3 Swab sampling of milking control points for microbial enumeration and isolation

Two sampling events (C1 and C2) were carried out on surfaces from different milking points using swabs, which were placed into tubes containing 9 mL of 0.1 % peptone water. The samplings were conducted 30 days apart. Sampling points were selected based on the highest risk of microbial contamination of the milk, from the point of its collection. These included the inner surfaces of teat cups A and B (ITa and ITb), the filter screen (TC), the interior of the cooling tank

(IR), and the teats of four lactating buffaloes during milking (TZ09, TZ03, TB25, and TB31). Some sampling points (IR and TC) were collected using a template with a known delimited area (25 cm² and 100 cm²) to determine the number of colonies per area, with results expressed as colony-forming units per square centimeter (CFU/cm²). Samples were stored in an insulated container with ice and transported to the Microbiology and One Health Laboratory of ICBS - UFRGS. For microbiological analysis, the SPC was performed on Plate Count Agar (PCA), according to ISO 4833:2:2013 methodology [15], and the results were expressed as colony-forming units per square centimeter (CFU/cm²).

2.4 Selection and identification of microorganisms by MALDI-TOF mass spectrometry

Microorganism selection on PCA medium was performed based on colony morphology, considering different phenotypic characteristics. The selected cultures were identified by MALDI-TOF mass spectrometry using the direct transfer technique. This technique consisted of depositing a small amount of sample from a previously isolated culture on a Petri dish onto a spot on the MALDI-TOF target plate, followed by overlaying the sample with α-cyano-4-hydroxycinnamic acid (HCCA) matrix. Analyses were performed using a MALDI Biotyper 4.0 (Bruker Daltonics, Bremen, Germany). After acquisition of the sample spectrum, the MBT RTC (Real Time Classification) software identified the best match by comparing it with spectra stored in the instrument database, generating results with different confidence scores. Scores ≥2.000 indicate species-level identification, scores between 1.700 and 1.999 indicate genus-level identification, and scores <1.700 were interpreted as unreliable identification.

2.5 Selection of *Staphylococcus* spp. and evaluation of virulence factors

After the identification of different genera and species of microorganisms by MALDI-TOF, bacteria belonging to the genus *Staphylococcus* spp. were selected. The identified bacterial cultures were analyzed for their ability to coagulate plasma (Newprov), with incubation at 37 ± 1 °C under aerobic conditions. The test was read at 4, 6, and 24 h, observing the presence or absence of coagulation. To evaluate hemolysin production, the isolates were inoculated onto Blood Agar Base (Himedia®) supplemented with 5 % defibrinated sheep blood. The plates were incubated at 37 ± 1 °C for 24 h, and, after the incubation period, they were examined for β-hemolysis (complete), α-hemolysis (partial), or γ-hemolysis (absence) [16].

2.6 Antimicrobial susceptibility testing

The Kirby–Bauer method [17] was employed using the following antimicrobials: Penicillin G (10 IU), Erythromycin (15 µg), Cefoxitin (30 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Amikacin (30 µg), Gentamicin (10 µg), Tobramycin (10 µg), Norfloxacin (10 µg), Clindamycin (2 µg), and Rifampicin (5 µg). A bacterial suspension with turbidity equivalent to the 0.5 McFarland standard (10^8 CFU/mL) was prepared and inoculated onto Mueller–Hinton agar. Antimicrobial disks were applied, and the plates were incubated at 37 ± 1 °C for 24 h. The diameters of the inhibition zones were measured in millimeters, and isolates were classified as susceptible or resistant according to the interpretive criteria of the Brazilian Committee on Antimicrobial Susceptibility Testing [18].

2.7 Evaluation of biofilm production

For biofilm production, the method described by Stepanovic et al. [19] was used. *Staphylococcus* spp. isolates were previously cultured on plates containing Tryptic Soy Agar (TSA) and incubated at 37 ± 1 ºC under aerobic conditions for 24 h. After incubation, the isolates were

individually resuspended in 0.85 % saline solution and adjusted to the 0.5 McFarland standard. The assay was performed in flat-bottom polystyrene 96-well microtiter plates. Wells were filled with 180 µL of Tryptic Soy Broth (TSB) and 20 µL of each bacterial suspension. For the negative control, the first column of the microplate was filled with TSB only, followed by the positive control, *Staphylococcus epidermidis* ATCC 35984 [20]. Microtiter plates were incubated at 37 ± 1 ºC for 72 h to allow cell adhesion and biofilm formation, as described by Borucki et al. [21]. After incubation, the wells were aspirated and washed three times with 200 µL of sterile 0.85 % saline solution. Bacterial fixation was performed using 200 µL of 99.8 % methanol (PA) for 20 min. The methanol was removed, and the plates were inverted and allowed to dry overnight at room temperature. Staining was performed with 200 µL of 0.1 % crystal violet for 15 min, followed by washing with sterile distilled water. After drying, the fixed and stained bacterial cells at the bottom of the wells were resuspended in 200 µL of 95 % ethanol for 30 min, and biofilm quantification was then carried out. The optical density (OD) of the bacterial biofilm was measured using a microplate spectrophotometer at a wavelength of 570 nm. Isolates were classified as non-biofilm producers (OD ≤ ODc), weak biofilm producers (ODc < OD ≤ 2 × ODc), moderate biofilm producers (2 × ODc < OD ≤ 4 × ODc), and strong biofilm producers (OD > 4 × ODc). All assays were performed in biological duplicate and technical octuplicate.

2.8 Evaluation of exopolysaccharide (EPS) production

For the exopolysaccharide (EPS) detection assay, the method described by Freeman et al. [22] was used. Bacterial isolates were streaked onto plates containing Congo Red Agar (37 g/L Brain Heart Infusion (BHI), 50 g/L sucrose, 10 g/L agar, and 0.8 g/L Congo red) and incubated at 37 ± 1 ºC for 24 h for the observation of dark colonies, indicative of EPS production. The *Staphylococcus aureus* ATCC 25923 strain was used as a positive control.

3. Results and discussion

3.1 Centesimal composition and microbiological analyses of buffalo milk

Buffalo milk was analyzed for its centesimal composition (Table 1), where protein contents were similar between the samples (≈ 3.6 g/100 g) but lower than reported by Bailone et al. [23]. The fat content was higher in C1 (3.60 g/100 g) compared to C2 (2.93 g/100 g), both lower than the values described by other authors [1-2], and at the minimum established in the SAS-03 Resolution [24]. The lactose content was similar in both samples and above the values described by Costa Filho et al. [25].

The densities observed in this study (1035.99 g/mL and 1036.34 g/mL) were higher than the limits established by the current Brazilian legislation for bovine milk [26-27], as well as the parameters set by specific São Paulo state legislation for buffalo milk [28]. Still, the values obtained are consistent with those reported by other researchers [1-2], which attribute this high density to the structure of buffalo milk fat cells, which are larger than those found in cow's milk [28].

Factors such as nutrition, lactation stage, milking quality, and even environmental factors can influence the centesimal composition of buffalo milk, affecting fat, protein, and total solids contents. In addition, a high somatic cell count (SCC), which can be caused by mastitis, can alter milk composition, such as fat, protein, and lactose [28].

In addition, SPC was performed at C1 and C2, where 1.27×10^5 CFU/mL and 1.83×10^6 CFU/mL were obtained, respectively. The value found in C2 is above the maximum limit allowed by legislation (3.00×10^5 CFU/mL) [26-27], as well as being above the result found by Di Domenico

[28], performed on the same dairy farm in the period between June and November 2021. The author obtained a mean SPC of 3.35 × 10^4 CFU/mL, with the highest value obtained in the August collections at 1.39 × 10^5 CFU/mL, but still lower than the result obtained in C2 in this study [28]. The SPC is usually used as a parameter to assess the hygiene of the entire production process, which suggests that the GAPs were not met at the time of milking in sample 2 (C2), since the result is not within what the legislation recommends, as improper cleaning of milking equipment and utensils can affect the number of bacteria in milk.

Thus, the results observed highlight the importance of interpreting them in light of current Brazilian legislation, particularly Decree No. 9.013/2017 (RIISPOA) [29] and Normative Instructions No. 76 and No. 77 of 2018, issued by MAPA [26–27], which establish criteria for obtaining and maintaining the quality of refrigerated raw milk. These regulations reinforce the mandatory adoption of proper hygiene practices during milking, as well as the systematic control of the microbiological quality of milk. In this context, the non-compliance observed in Sample 2 (C2) underscores the need to meet legal requirements, especially regarding equipment sanitation, milking management, and monitoring of SPC, in order to ensure product safety and comply with the standards established for commercialization.

Table 1. Average proximate composition results of buffalo milk compared with the official parameters established by Normative Instruction No. 76/2018 and SAS – 03/2008.

Parameters	Buffalo milk		NI No. 76/2018 (minimum)	SAS - 03/2008 (minimum)
	Sample 1	Sample 2		
pH	6,77	6,99	-	6,40 a 6,90
Protein (g/100 g)	3,66±0,02	3,64±0,00	2,9	-
Fat (g/100 g)	3,60±0,02	2,93±0,06	3,00	4,5
Lactose (g/100 g)	5,49±0,03	5,47±0,00	4,3	-
NFS (g/100 g)	9,98±0,05	9,94±0,01	8,4	8,57
Minerals (g/100 g)	0,82±0,00	0,82±0,00	-	-
Water (g/100 g)	0±0,00	0±0,00	-	-
Density (g/mL)		1036,34±0,09	1028 a 1034	1028 a 1034
CI (ºC)	-0,680±0,00	-0,670±0,00	-0,512 a -0,536	-0,520 a -0,570
Temperature (ºC)	23,17±1,34	23,93±1,72	-	-
Conductivity (mS/cm)	5±0,00	5,17±0,04	-	-

NI: Normative Instruction; SAS: Secretariat of Agriculture and Supply; NFS: Non-fat solids; CI: Cryoscopic index.

3.2 Standard Plate Count of different milking control points

Samples from swabs collected at different points of the milking process were analyzed for SPC, with values ranging from 4.25 × 10^4 to 8.65 × 10^4 CFU/cm^2 found on the teat cups (Table 2). Similar values were reported at these points by Garcia et al. [30] after milking (4.6 × 10^4 CFU/mL). However, lower values were observed in the study by Santos, Biângulo, and Pereira [31], who investigated the microbiological quality of mechanical milking machines. The researchers analyzed four different teat cups, before and after equipment sanitation, and found SPC values ranging from 1.70 × 10^1 to 2.60 × 10^2 CFU/mL before cleaning. After the sanitation procedure, counts were reduced to a range between 0 and 7.00 × 10^1 CFU/mL. The results of the study highlight the importance of proper milking machine cleaning to reduce bacterial contamination of milk, ensuring its quality, safety for consumption, and higher commercial value [31].

For the analysis of SPC on the animals' teats, sampling was performed before the application of pre-dipping. On the teat surface, values ranging from 2.20 × 10^2 to 1.05 × 10^5 CFU/cm^2 were found (Table 2). Similar values were reported in the study by Garcia et al. [30], whose results ranged

from 4.0 × 10^3 CFU/cm^2 to 1.3 × 10^5 CFU/cm^2 when analyzing the microbial diversity on cow teats. In the study by Matsubara et al. [32], counts of 7.9 × 10^4 CFU/cm^2 were observed before pre-dipping, and 1.0 × 10^4 CFU/cm^2 after pre-dipping, representing a reduction of 87.3 %. The practice of pre-dipping significantly contributes to reducing bacterial contamination on teats [32].

After analyzing the SPC of the filter screen, values ranging from 1.51 × 10^4 CFU/cm^2 to 4.00 × 10^4 CFU/cm^2 were found. Similar values were reported by Siebel et al. [33], who found an average of 2.5 × 10^4 CFU/mL in the filter. In the present study, no visible dirt was observed on the filter; however, the results suggest that this utensil may act as a source of recontamination, even when the milk initially presents good quality. This risk increases if the filter is not replaced frequently, not properly cleaned between milkings, or reused without adequate sanitation [5].

When analyzing the SPC inside the cooling tank, values ranging from 7.3 × 10^2 to 5.5 × 10^3 CFU/cm^2 were found. Silva et al. [34] reported higher values, reaching 1.2 × 10^6 CFU/cm^2 on the side of the equipment. The presence of microorganisms inside the cooling tank can increase the SPC of the milk, reduce its shelf life, raise the risk of proliferation of psychrotrophic microorganisms and pathogens, and compromise both the yield and quality of dairy products [34]. Although there is no specific numerical limit for the number of microorganisms on the internal surface of the tank, Normative Instruction No. 77/2018 [25] requires that equipment undergo strict sanitation and hygiene routines precisely to prevent microbial growth in stored milk.

Table 2. SPC results of microorganisms from the different surfaces analyzed.

Sampling points	Sample 1 (C1)	Sample 2 (C2)
Inner surface - teat cup A (UFC/mL)	8,65 × 10^4	6,95 × 10^4
Inner surface - teat cup B (UFC/mL)	6,35 × 10^4	4,25 × 10^4
Filter screen (UFC/cm^2)	1,51 × 10^4	4,00 × 10^4
Interior of the cooling tank (UFC/cm^2)	5,50 × 10^3	7,30 × 10^2
Teat B025 (UFC/cm^2)	1,30 × 10^3	1,70 × 10^3
Teat B031 (UFC/cm^2)	8,40 × 10^3	1,05 × 10^5
Teat Z003 (UFC/cm^2)	5,80 × 10^2	3,00 × 10^3
Teat Z009 (UFC/cm^2)	2,20 × 10^2	1,75 × 10^3

3.3 Identification of bacterial isolates

Of the 49 identified isolates (100 %), the genus *Staphylococcus* was the most frequently detected, representing 32.6 % (n = 16), and was mainly found on the animals' teats (TZ09, TZ03, TB25, TB31) and inside the teat cups (IT) (Table 3). Similar results were reported by Lavor et al. [6], who isolated microorganisms from the milk of cows with mastitis, in which *Staphylococcus* was also the most frequently identified genus, accounting for 31.1 %. Among the most prevalent pathogens associated with mastitis in bovine and buffalo species are bacteria of the genus *Staphylococcus* spp., with emphasis on *Staphylococcus aureus* and CoNS, such as *S. chromogenes* [10]. Despite the presence and identification of *Staphylococcus* isolates, none of the animals showed a positive reaction to the CMT or the strip cup test on the sampling days. However, it is important to note that not all isolated microorganisms are necessarily associated with mastitis, as many are part of the environmental microbiota or commensal flora of the teat skin [35]. In this context, the presence of *Staphylococcus* species, especially CoNS, should be interpreted with caution, as they may act either as opportunistic colonizers or as etiological agents of mastitis, depending on host conditions and management practices.

The species *S. chromogenes* accounted for 87.5 % (n = 14) of the total isolates within the genus, a value higher than that reported by Israel et al. [10], who found 60 % of *S. chromogenes* on the teats of 27 cows. The species *S. hyicus* and *S. capitis* each accounted for 6.25 % (n = 1) of the total isolates within the genus (Table 3). According to Andrade-Becerra et al. [36], the three species identified in this study are among the most frequently isolated CoNS in subclinical mastitis and are sometimes referred to as environmental staphylococci. In addition, Nagase et al. [37] demonstrated that *S. capitis* can be found on human skin, representing a potential source for the colonization of this species in buffalo mammary tissue, especially in situations where the milker handles teats or milking equipment without proper hand hygiene. These results highlight the circulation of microorganisms among different components of the production system, in line with the One Health approach, characterized by the interaction between animals, handlers, and the production environment.

The second most frequently isolated genus was *Pseudomonas* (n = 5), mainly found inside the cooling tank (IR) (Table 3). Similar results were reported by Bogo et al. [38], in which *Pseudomonas* was the most frequently isolated genus among Gram-negative bacteria from buffalo milk samples. The presence of *Pseudomonas* isolates may be explained by temperature fluctuations during milk storage in the cooling tank, either during milking and/or throughout the day, allowing the growth of psychrotrophic microorganisms [38].

Along with *Pseudomonas*, the genus *Acinetobacter* was also the second most frequently identified (n = 5), being detected on the filter screen (TC) and inside the cooling tank (IR) (Table 3). *Acinetobacter* is a common food contaminant and has already been isolated from milk samples [44]. Species of the genus *Acinetobacter* represent a significant public health concern due to their rapid ability to develop resistance to a wide range of antimicrobial agents, as well as their remarkable persistence in the environment [40].

Table 3. Isolates from different sampling points of buffalo milk milking, identified by MALDI-TOF.

Sampling points	Identified microorganisms	Sampling points	Identified microorganisms
C1 TC1	*Yarrowia lipolytica*	C2 TC7	*Staphylococcus chromogenes*
C1 TC2	*Acinetobacter johnsonii*	C2 IR2	*Lactococcus lactis*
C1 TC3	*Pseudomonas synxantha*	C2 IR3	*Pseudomonas fluorescens*
C1 TC6	*Curtobacterium flaccumfaciens*	C2 IR5	*Buttiauxella noackiae*
C1 IR1	*Acinetobacter guillouiae*	C2 IR6	*Buttiauxella noackiae*
C1 IR2	*Pseudomonas koreensis*	C2 IR7	*Acinetobacter guillouiae*
C1 IR3	*Pseudomonas koreensis*	C2 TZ09-1	*Buttiauxella agrestis*
C1 IR5	*Pseudomonas synxantha*	C2 TZ09-3	*Buttiauxella gaviniae*
C1 ITa-1	*Staphylococcus chromogenes*	C2 TZ09-4	*Arthrobacter protophormiae*
C1 ITa-2	*Staphylococcus chromogenes*	C2 TZ09-7	*Staphylococcus chromogenes*
C1 ITa-3	*Staphylococcus chromogenes*	C2 TZ03-2	*Acinetobacter guillouiae*
C1 ITa-4	*Staphylococcus chromogenes*	C2 TZ03-6	*Lactercia adecarboxylata*
C1 ITb-2	*Enterobacter asburiae*	C2 TZ03-8	*Proteus mirabilis*
C1 TB25-3	*Staphylococcus chromogenes*	C2 ITa-1	*Staphylococcus chromogenes*
C1 TB25-5	*Bacillus thuringiensis*	C2 ITa-2	*Staphylococcus chromogenes*
C1 TB25-6	*Bacillus pumilus*	C2 ITa-3	*Staphylococcus chromogenes*
C1 TB31-1	*Staphylococcus capitis*	C2 ITa-4	*Arthrobacter koreensis*
C1 TB31-4	*Staphylococcus chromogenes*	C2 ITa-5	*Proteus mirabilis*
C1 TB31-5	*Staphylococcus chromogenes*	C2 ITa-7	*Staphylococcus chromogenes*
C2 TC5	*Lactococcus lactis*	C2 ITa-8	*Staphylococcus chromogenes*
C2 TC6	*Acinetobacter guillouiae*	C2 ITb-3	*Staphylococcus hyicus*

C1: Sample 1; C2: Sample 2; TC: Filter screen; IR: Interior of the cooling tank; IT: Inner surface of the teat cup; TB25, TB31, TZ03, TZ09: Buffaloes teats.

3.4 Antimicrobial susceptibility of *Staphylococcus* isolates

In the evaluation of antimicrobial susceptibility, only the *S. capitis* isolate showed resistance to penicillin, erythromycin, cefoxitin, norfloxacin, rifampicin, and clindamycin. The remaining isolates were classified as susceptible or intermediate to the tested antimicrobials. Lopes et al. [41] reported a different resistance profile, in which 68.2 % of their *Staphylococcus* isolates were resistant to one to seven different antibiotics tested. Additionally, 47 % of *S. chromogenes* isolates showed resistance to penicillin [41]; however, in the present study, no isolates of this species exhibited resistance to this antibiotic, with resistance to penicillin observed only in *S. capitis*. All *Staphylococcus* isolates in the present study showed an intermediate susceptibility profile to ciprofloxacin (CIP) and levofloxacin. In contrast, Silva [8] reported different results, where, upon isolating *Staphylococcus* species from sheep milk, only two out of fifteen *S. chromogenes* isolates showed intermediate susceptibility to CIP, while the remaining isolates were susceptible to the antibiotic. It is important to highlight that the presence of antimicrobial resistance may be associated with the prior use of antibiotics in animal management, as well as the selective pressure exerted by therapeutic treatments, favoring the survival and dissemination of resistant strains in the production environment. Furthermore, milking surfaces and equipment may act as reservoirs for these microorganisms, contributing to their persistence and spread throughout the production chain.

The detection of antimicrobial resistance in *S. capitis* is a relevant finding, especially considering the growing global challenge of bacterial resistance. The presence of resistance to multiple antibiotics, such as penicillin, erythromycin, and cefoxitin, raises concerns about the effectiveness of conventional treatments and highlights the potential risk of dissemination of these resistance genes in the environment [42]. In this context, from a One Health perspective, resistant microorganisms present in the dairy production environment may circulate among animals, humans, and the environment, increasing the risk of resistance spread. Considering that species of the genus *Staphylococcus* can act as opportunistic pathogens in both humans and animals [7], the observed resistance may compromise treatment responses in future infections, representing a warning for public health.

3.5 Evaluation of virulence factors of *Staphylococcus* spp.

Of the 16 *Staphylococcus* isolates tested, only one (6.25 %) showed a positive result in the coagulase test. This isolate was identified as *Staphylococcus chromogenes*, obtained from the filter screen (C2 TC7). The remaining microorganisms showed negative results. Different findings were reported by Israel et al. [11], where 36.7 % of *S. chromogenes* isolates demonstrated the ability to coagulate rabbit plasma in the tube coagulase test. Coagulase production is considered an important virulence factor in certain *Staphylococcus* species, as it promotes fibrin clot formation, creating a protective barrier against host defense mechanisms [44]. Recent studies indicate that *S. chromogenes* may show variation in coagulase production, with rare but specific populations exhibiting coagulating activity [10,12]. In addition, CoNS also require attention, as they are recognized as major etiological agents of subclinical infections, such as mastitis. Several CoNS species frequently inhabit the teat canal and teat skin of domestic ruminants and may be transferred to the mammary gland during milking or suckling, without necessarily causing infection in the mammary parenchyma [40].

When evaluating hemolytic activity, all 16 *Staphylococcus* isolates (100 %) showed no hemolysis (Table 4). Similar results were reported by Silva [8], who identified 15 *Staphylococcus chromogenes* isolates from sheep milk samples, all exhibiting a γ-hemolysis profile. This finding supports the characterization of *Staphylococcus chromogenes* as a non-hemolytic coagulase-negative species [10].

Furthermore, in the same study by Silva [8], when evaluating the two *S. hyicus* isolates, one exhibited γ-hemolysis and the other α-hemolysis, a result similar to the present study, in which the *S. hyicus* isolate also showed γ-hemolysis. Although *S. capitis* is generally associated with β-hemolysis, the absence of hemolysis in the isolate identified as *S. capitis* may be related to the possibility that it belongs to the subspecies *ureolyticus*, which is known to lack hemolytic activity [13]. However, in this study, it was not possible to confirm whether the isolate belongs to the subspecies *ureolyticus*, and further studies are needed to investigate identification at the subspecies level.

Table 4. Hemolytic activity, biofilm production, and EPS production by *Staphylococcus* isolates from different sampling points during buffalo milking.

Sampling points	Isolate	Hemolysis	Biofilm	EPS
C1 ITa-1	*S. chromogenes*	γ-hemolysis	Moderate	Negative
C1 ITa-2	*S. chromogenes*	γ-hemolysis	Strong	Negative
C1 ITa-3	*S. chromogenes*	γ-hemolysis	Strong	Negative
C1 ITa-4	*S. chromogenes*	γ-hemolysis	Moderate	Negative
C1 TB31-1	*S. capitis*	γ-hemolysis	Moderate	Negative
C1 TB31-4	*S. chromogenes*	γ-hemolysis	Strong	Negative
C1 TB31-5	*S. chromogenes*	γ-hemolysis	Strong	Negative
C1 TB25-3	*S. chromogenes*	γ-hemolysis	Strong	Negative
C2 ITa-1	*S. chromogenes*	γ-hemolysis	Moderate	Negative
C2 ITa-2	*S. chromogenes*	γ-hemolysis	Strong	Negative
C2 ITa-3	*S. chromogenes*	γ-hemolysis	Strong	Negative
C2 ITa-7	*S. chromogenes*	γ-hemolysis	Moderate	Negative
C2 ITa-8	*S. chromogenes*	γ-hemolysis	Strong	Negative
C2 TZ09-7	*S. chromogenes*	γ-hemolysis	Moderate	Negative
C2 TC7	*S. chromogenes*	γ-hemolysis	Moderate	Negative
C2 ITb-3	*S. hyicus*	γ-hemolysis	Strong	Negative

C1: Sample 1; C2: Sample 2; TC: Filter screen; IR: Interior of the cooling tank; IT: Inner surface of the teat cup; TB25, TB31, TZ03, TZ09: Buffaloes teats.

3.6 Evaluation of biofilm production

Among the 16 *Staphylococcus* isolates, all (100 %) were able to form biofilm (Table 4). More than half of the *S. chromogenes* isolates (n = 9, 57.1 %) were classified as strong biofilm producers, while the remaining *S. chromogenes* isolates (n = 7, 42.9 %) were classified as moderate biofilm producers. This result differs from that reported by Israel et al. [11], where 43.3 % of the isolates of the same species were classified as weak biofilm producers. In addition, the *S. hyicus* isolate was classified as a strong biofilm producer. In the study by Israel et al. [11], which investigated biofilm production by *Staphylococcus* spp. isolated from bovine mastitis, 8 *S. hyicus* isolates were identified, of which 2 were classified as strong biofilm producers, 1 as moderate, and 5 as weak producers. According to Heath et al. [13], the first pathogenicity factor identified in *S. capitis* was its ability to form biofilm. The results of the present study are consistent with this finding, as the isolate of this species was classified as a moderate biofilm producer.

Biofilm formation in *Staphylococcus* is regulated by several genes, including the *ica* (intercellular adhesion), *bap* (biofilm-associated protein), *agr* (accessory gene regulator), *sar* (staphylococcal accessory regulator), *spa* (*Staphylococcus aureus* protein A), *fnbA/B* (fibronectin-binding proteins A and B), and *sasG* (*Staphylococcus aureus* surface protein G) loci, as well as by extracellular DNA (eDNA), among other factors related to adhesion and environmental stress response [45]. Variations in the presence, expression, or mutations in these genes may result in distinct biofilm formation profiles even within the same species [46]. Furthermore, regulatory factors

and mobile genetic elements, such as plasmids or pathogenicity islands, may confer specific adaptive advantages to strains, influencing their ability to colonize and persist in the mammary environment [7].

Biofilm formation is recognized as an important virulence factor, as it hinders the action of the host immune system and exhibits increased resistance to antimicrobial treatments, thereby favoring the persistence and chronicity of infections [46]. The presence of *S. chromogenes* and *S. hyicus* isolates with a high capacity for biofilm formation represents a significant challenge in the management of infections in production animals, as well as in sanitary control practices. These results suggest that infections caused by these *Staphylococcus* species may require prolonged or combined therapies, using antimicrobials with better penetration, in addition to the rational use of antibiotics based on susceptibility testing.

Furthermore, the high biofilm-forming capacity observed in the isolates favors the persistence of these microorganisms on milking surfaces, hindering their removal during cleaning procedures and contributing to cross-contamination [47]. This scenario increases the risk of dissemination throughout the milk production chain, highlighting the interrelationship between the environment, animals, and handlers. Therefore, additional measures are required, including strengthening hygiene practices throughout the milking process, as well as reinforcing the implementation of Good Agricultural Practices.

3.7 Evaluation of exopolysaccharide (EPS) production

When evaluating EPS production, all 16 *Staphylococcus* isolates (100 %) did not show the formation of dark colonies, indicating that they were not EPS producers under the experimental conditions studied (Table 4). The positive control used, *S. aureus* ATCC 25923, showed the formation of black colonies, indicating EPS production.

Despite the absence of detectable EPS production by the *Staphylococcus* isolates analyzed in this study, biofilm formation was observed, demonstrating a dissociation between these two phenomena under the adopted experimental conditions. This apparent contradiction can be explained by the complexity of biofilm formation in bacteria, especially in species of the genus *Staphylococcus*. According to Nguyen et al. [48], although the polysaccharide intercellular adhesin (PIA), a specific type of EPS, is widely recognized as a fundamental component of the biofilm matrix, alternative mechanisms of formation exist. In *S. aureus ica*-negative strains (including some MRSA), cell adhesion may be mediated by surface proteins such as Bap, Spa, FnBPA/B, and SasG, as well as by extracellular DNA (eDNA), resulting in PIA-independent biofilms [49]. Thus, it is possible that the analyzed isolates formed structured biofilms without producing polysaccharides detectable by the method used.

Additionally, biofilms are complex and multifactorial structures, which may be composed of different components of the extracellular matrix, such as proteins, teichoic acids, extracellular DNA (eDNA), and even hydrophobic adhesion forces between cells and surfaces [50]. The absence of EPS detection may not necessarily reflect the true absence of these substances, but rather limitations of the phenotypic methods employed, such as the use of Congo red agar, which preferentially detects certain types of polysaccharides, such as polysaccharide intercellular adhesin (PIA) or poly-N-acetylglucosamine (PNAG) [50].

Another relevant factor is that the expression of genes related to EPS production may be highly regulated by environmental conditions, including pH, temperature, nutrient availability, the presence of antimicrobials, and stress conditions [51].

Thus, the absence of black colonies in the Congo red test does not rule out the ability of the isolates to form biofilms. On the contrary, it highlights the need for complementary approaches, such as genotypic assays targeting genes of the *ica* operon (such as *icaA, icaD, icaB,* and *icaC*), as well as additional tests to identify other components of the extracellular matrix, such as proteins and eDNA.

4. Conclusion

The study enabled the evaluation of the hygienic-sanitary aspects of buffalo milking through the microbiological investigation of different points in the process, highlighting the presence of *Staphylococcus* spp. Elevated bacterial counts were identified on surfaces such as teat cups and teats, as well as SPC values above the legal limit in one of the milk sampling events, indicating possible specific failures in hygiene practices. The genus *Staphylococcus* was the most frequently detected, with isolates showing the ability to form biofilms and, in one case, resistance to multiple antimicrobials. The results demonstrate that there are critical points in the milking process that require improved sanitary control, reinforcing the need for continuous microbiological monitoring to ensure the quality of buffalo milk.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Data availability statement

The complete set of data that supports the results of this study is available upon request from the corresponding author.

Author contributions

Conceptualization: Duarte, G. D.; Motta, A. S.; Lermen, A. M. Data curation: Duarte, G. D.; Formal analysis: Duarte, G. D. Investigation: Duarte, G. D. Methodology: Duarte, G. D.; Motta, A. S.; Lermen, A. M. Project administration: Duarte, G. D.; Motta, A. S. Supervision: Motta, A. S. Visualization: Duarte, G. D. Writing (original draft): Duarte, G. D.; Lermen, A. M. Writing (review and editing): Duarte, G. D.; Lermen, A. M.; Motta, A. S.

Generative AI use statement

The authors did not use generative artificial intelligence tools or technologies in creating or editing any part of this manuscript.

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