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Evaluation of microbiological, cellular and risk factors associated with subclinical mastitis in female buffaloes

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Objective: This study aimed to evaluate the microbiological and cellular milk profile for the diagnosis of subclinical mastitis in female buffaloes and to assess risk factors for predisposition of the disease.
Methods: Analyses were carried out by standard plate count (SPC), identification of species and antibiotic resistance, somatic cell count (SCC), electrical electrical conductivity of milk (ECM), and lactoferrin content in milk. Teat cups were swabbed to evaluate risk factors, observing hyperkeratosis, milking vacuum pressure and cleanliness of the site. Hence, 30 female buffaloes were randomly selected (15 from a group in early lactation and 15 in late lactation).
Results: The most common bacteria in the microbiological examination were *Staphylococcus* spp., *Streptococcus* spp. and *Corynebacterium* sp. In the antibiotic sensitivity test, 10 (58.82%) of the 17 antibiotics tested were sensitive to all isolates, and resistant bacteria were *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus haemolyticus*, and *Escherichia coli*. It was observed that positive samples in the microbiological examination showed total bacterial count between 9.10×10^3 to 6.94×10^6 colony forming units/mL, SCC between 42,000 to 4,320,000 cells/mL and ECM ranging from 1.85 to 7.40 mS/cm. It was also found that the teat cups had high microbial counts indicating poor hygiene, and even faults in the cleanliness of the animals' waiting room were observed. It is concluded that values of SCC above 537,000 cells/mL and ECM above 3.0 mS/mL are indications of mammary gland infection for this herd; however, the association of these values with a microbiological analysis is necessary to more accurately evaluate the health status of mammary glands with subclinical mastitis.
Conclusion: Through phenotypic characterization of bacteria involved in the samples, the genera *Staphylococcus* spp., *Streptococcus* spp., and *Corynebacterimum bovis* were the most prevalent in this study. Faults in environment and equipment hygienization are factors that are directly associated with mastitis.

Keywords: Bubaline; Somatic Cell Count (SCC); Quality; Production

INTRODUCTION

Interest in bubaline species has increased in recent years, being motivated by several factors such as milk production and its derivatives [1]. Buffalo milk has great commercial potential due to high nutritional value in relation to the high levels of fat, protein, solids, and minerals (especially calcium and phosphorus), being used more widely as a raw material for preparation of milk products, since it is increasing the demand for these derived by consumers more demanding and who seek a differentiated product, who values the species in dairy farming [2]. Along with this demand, there is also an increase in the demand for quality products and the need for them to reach the final consumer within the ideally considered standards. Thus, effective knowledge

of the factors that detract from raw materials, and especially regarding the understanding of health-related characteristics of the mammary gland of female buffaloes is necessary so that it is possible to develop and apply the most advanced techniques for prevention and control of mammary infections of these animals, in addition to the need of creating a specific legislation for this species.

One of the factors of greater quantitative and qualitative impact on milk is mastitis, an inflammation of the parenchyma mammary gland due to a predominantly viral process mainly caused by bacteria [3]. Among the main forms of inflammation diagnosis, microbiological analysis of milk is considered to be the most significant when compared to cellular parameters, such as analysis of somatic cell count (SCC) and electrical conductivity of milk (ECM) [4,5].

In-depth study of buffalo milk characteristics and the need for further research for standardizing methods that assist in diagnosing and controlling measures of mastitis guided this study, with the objective to assess the microbiological and cellular profile of milk for the diagnosis of Subclinical Mastitis in buffaloes and to assess risk factors for disease predisposition.

MATERIALS AND METHODS

Animal care

The project for this study was submitted to Ethics Committee in Animal Use (CUSA), receiving opinion number 007/2015, being free and approved for implementation from the legal point of view, according to Law No. 11,794, 2008. "Husbandry practices related to agriculture are not considered research activities" (Art. 1°, § 3°) and "non-experimental interventions related to agricultural practices are not considered experimental" (Art. 3, Paragraph One, III).

Study site

The experiment was conducted at the Tapuio Agropecuaria Ltda., located in the City of Taipu, 50 km from Natal, situated in the Agreste region of the state of Rio Grande do Norte, in Brazil. The climate is characterized as being *As* according to the Köppen classification, being warm with two distinct seasons: summer (rainy) and winter (dry), with the dry season from August to January, and the rainy season from February to July. The average rainfall in 2015 was 603 mm, with an average temperature of 25.3°C and average relative humidity of 79.0%.

Characterization of the property and management of animals

The production unit is considered as being high in milk production, with 550 lactating buffaloes and its own dairy plant. The products are awarded the 100% buffalo purity seal, granted by the Brazilian Association of Buffalo Breeders (ABCB) from the purity control program of cheese produced from buffalo milk.

For this study, 30 lactating buffaloes were randomly selected, of which 15 belonged to the group of higher daily milk yield (Group 1: ≥15 L/animal/d) at the beginning of lactation (19 to 87 days in milk), and 15 to the group of lower daily milk yield (Group 2: ≤8 L/animal/d) at the end of lactation phase (130 to 353 days in milk). The following management practices were conducted for both groups: fully mechanized milking performed by trained staff in an appropriate and calm environment (5 am and 3 pm); pre-dipping and post-dipping; daily cleaning of milking equipment; recording of production rates; individual SCC test performed once a month; weekly performance of total bacterial count test (TBC) from tank milk.

The pre-milking environment consisted of a waiting pen covered to provide shade, a rough cement floor and a drinking fountain. The used milking equipment in circuit closed down double row with 20 milking machines on each side and without the presence of calves.

Buffaloes were managed by rotational grazing in the pastures of *Brachiaria brizantha* and *Panicum maximum* cv. Massai. Lactating buffaloes also received sugarcane (*Saccharum officinarum*) supplemented with 1% urea+ammonium sulphate (9:1) in the dry season, in addition to being provided maize, soybean meal and soybean oil concentrate in mangers located within the pickets.

The buffaloes received a small amount of concentrate during milking, formulated according to the nutritional requirements of each production group. After leaving the milking area the buffaloes were taken to pickets separated by production batches, far from the milking site, thus ensuring that the buffalo remained standing.

Collection of milk samples

General risk factor evaluations for acquisition of mastitis were conducted prior to the milk collections for cellular and microbiological analysis, considering the following items: hygiene conditions of the waiting pen and milking room, cleanliness of milking equipment, monitoring changes of the vacuum pressure in the milking system, and assessing teat injury by the degree of hyperkeratosis. Regarding this, visual evaluations were conducted followed by recording times. Teat cups underwent microbiological analysis by swabbing and the milkers were given disposable gloves at collection time to eliminate the risks of sample contamination.

Sampling methodology consisting of 2/3 morning milk and 1/3 of the evening milk was used for collecting milk intended for SCC analysis and composition. Regarding the other analyzes (standard plate count [SPC], ECM, lactoferrin [Lfe], and microbiological identification tests), the collection was only from morning milkings.

Milk samples were collected directly from the buffalo teats into sterilized jars. Samples were then taken from the milk collecting equipment to analyze SCC, composition, ECM, and Lfe. The samples were collected in triplicate and placed in properly identified standard 40 mL bottles.

Regarding teat cleaning, individual paper towels were used for each teat. Pre-dipping with complete immersion of the teats in specific solution was carried out for disinfecting the teats' surface. Post-dipping was performed for all buffaloes after milking.

Samples for SPC analysis were collected directly from the teats (eliminating the risks of external contamination) into sterile 40 mL flasks containing azidiol preservative, which were then properly identified, packaged under refrigeration and sent to the PROGENE milk quality laboratory at the Federal Rural University of Pernambuco (UFRPE), accredited by the Brazilian Network of Milk Quality (RBQL) which integrates the National Program for Milk Quality Improvement (PNQL).

Electrical conductivity parameters of milk and Lfe content were then analyzed from the same sample. The samples were sent to the milk quality laboratory LABOLEITE (UFRN-Natal) and to the LEA food engineering laboratory (UFRN-Natal) within 24 hours after collection, properly identified and placed in appropriate temperature.

Information regarding physical and chemical qualities (fat, protein, lactose, total solids [TS], non-fat dry extract) and SCC of buffalo milk were provided by the production unit of the study (Tapuio Agropecuaria Ltda, Taipu, Rio Grande do Norte, Brazil).

Hyperkeratosis assessment of buffaloes mammary quarters

All buffaloes used in the experiment were submitted to clinical examination to identify hyperkeratosis of the teats, which were classified according to the intensity observed according to the methodology recommended by the "The Tea Club International" using an adapted model, replacing the teat scores using numbers from I to IV, wherein: I, normal; II, the start of the problem; III, affected animal; IV, serious condition. These observations were made at the very beginning of the biological sample collection required for the study, and the results were recorded in individual files.

Assessment of vacuum pressure

During the milkings, the vacuum level present in each set of milking equipment was observed (dynamic testing) using a clock meter, barometer.

Buffalo milk analysis

For determining the buffalo milk composition, the samples were subjected to analysis by infrared absorption using Bentley 2000 equipment, and SCC was performed by flow cytometry using Somacount 300 equipment at the Milk Quality Analysis laboratory of the Federal Rural University of Pernambuco (PROGENE-UFRPE).

The SPC analysis according to the Official Methods of Analysis [6] was carried out at the same laboratory (PROGENE). Samples were diluted to a ratio of (1:10) in Ringer's solution and then deeply plated in Agar PCA (Acumedia, Lansing, MI, USA) and subsequently incubated at 35°C for 48 h.

Isolating and identifying microbial agents were performed by a laboratory specialized in microbiologically analyzing milk (VidaVet analysis laboratory) in Botucatu (SP), where aliquots of the milk samples were incubated at 37°C for 8 hours for the pre-enrichment stage, and subsequently plated on sheep blood agar, brain heart infusion agar and Sabouraud agar with chloramphenicol, according to [7]. After incubation, morphological characteristics of the isolated colonies and individual innocuous were evaluated, then plated in selective and differential media in order to observe the characteristic phenotypic aspects of the genera [8,9].

Behavior tests on triple sugar-iron agar, tube motility, indole production, acid production from glucose, fermentation of sugars, nitrate reduction, gelatinase production, urease production, citrate and malonate degradation, and other differentials were performed for suspected Enterobacteriaceae, according to the microorganism involved. The *Streptococcus* spp. genus was evaluated through isolation in a selective medium, oxidation potential, and identification by hydrolyzing esculin and hippurate. *Corynebacterium* sp. genus growth was conducted on a selective medium followed by biochemical tests such as the hydrolysis of gelatin, starch, urease, and catalase, according to [7]. After the presumptive identification of *Staphylococcus* spp. colonies, they were subjected to Gram staining, as well as catalase, 3% potassium hydroxide, free coagulase, Voges-Proskauer, urease, and reduced nitrates tests [10].

Antibiotic sensitivity test was also performed by the VidaVet laboratory through the disk diffusion method in agar for the identified samples, according to the methodology recommended by the National Committee for Clinical Laboratory Standards [11], using the bases of commercial products available for clinical treatments of samples from cows with mastitis and different antimicrobials of samples from each buffalo. It is part of the routine of the laboratory to test the drugs most used by veterinarians, in this way the 17 active ingredients used in this sensitivity tests were: PMN = novobiocin (40 μg)+penicillin G procaine (40 μg); CEQ = cefquinome (30 μg); AMO = amoxicillin (10 μg); DUL = danofloxacin (5 μg); CTF = ceftiofur (30 μg); CL = cephalexin (30 μg); CN = gentamicin (10 μg); TE = tetracycline (30 μg); AM = ampicillin (10 μg); ENO = enrofloxacin (5 μg); NEO = neomycin (30 μg); SUT = sulfatrim (25 μg); AMC = ampicillin+colistin (30 μg); CNM = cefalonium (30 μg); ACA = amoxicillin (20 μg) +clavulanic acid (10 μg); OXA = oxacillin (1 μg); and CIP = ciprofloxacin (5 μg).

The samples were then sent to the milk quality lab (LABOLEITE/UFRN) to analyze the electrical ECM and submit them to a test that evaluates the exchange between cations and anions present in milk through an Akso 83 portable electrical conductivity meter. The electrode was immersed in milk samples, allowing the passage of an electrical current that was automatically registered on a digital readout. The readings are expressed in mS/cm.

The concentration of Lfe in milk was determined by enzymatic immunoassay using the ELISA kit blf 5091LFER (R&D Systems,

Minneapolis, MN, USA). Initially, milk was diluted with 100% methanol (1:1 v/v) and homogenized for 30 seconds. Then 50 μL aliquots of the sample were placed in polystyrene microplate with 96 wells, added to 25 μL of the conjugate solution (horseradish peroxidase) and 25 μL of antibody. The microplate was sealed, stirred for 2 seconds and maintained in the absence of light at 25°C. After 60 minutes the wells were washed with Wash solution and then 100 μL of chromogenic substrate (H_2O_2 and tetramethylbenzidine) was added. The reaction was stopped after 30 minutes in the absence of light at 25°C by adding 100 μL sulfuric acid (stop solution). Absorbance was measured immediately after the reaction stop in microplate spectrophotometer (Thermo-Plate Reader Bio-RadLaboratories, Hercules, CA, USA) at 25°C. The calibration curve was constructed from the different concentrations of Lfe standard solution (0.05 to 2.0 μg/mL) and results are expressed as micrograms of Lfe per mL of milk.

Statistical analysis

SAS software [12] was used for statistical analysis, operating by the least square method and generalized linear model procedure. Therefore, a general descriptive analysis of data was conducted grouped by lactation phase. SCC, ECM, and TBC were transformed into natural logarithmic scale in order to linearize the data. The normality (or not) of the milk was verified according to the microbiological results, using the analysis of variance, followed by comparison of means by Tukey test at a 5% significance level. Comparisons of the proportions regarding isolated agents in the microbiological culture were also performed through frequencies.

Finally, correlations between SCC, ECM, TBC were conducted and microorganisms were identified.

RESULTS AND DISCUSSION

Determination of milk constituents of the herd and variations in lactation stages

For evaluating the biochemical tests of buffalo milk, the fat (%), protein (%), lactose (%), TS (%), dry defatted extract (%), and Lfe content (μg/mL) values were determined. The average results obtained in relation to total sample and in each group are detailed in Table 1.

As shown in Table 1, significant changes in milk composition were not observed and the values are close to those found by other studies with buffalo milk in the region [13,14].

Small variations regarding the constituents mentioned in the table are related to the daily production and lactation phase, considering that as buffaloes reach the end of lactation, there is consequently a decrease in production and the colloids are more concentrated (such as fat, for example). With this, the TS content tends to increase, which is called the dilution effect of milk constituents.

Regarding the Lfe content shown in Table 1, it was observed that there was a significant difference between the groups, for which buffaloes from group 1 with high daily production and initial lactating stage had a higher concentration. A drop in milk Lfe concentrations according to the stages of lactation was noticeable. The literature reports that this drop in Lfe content is well established in dairy cows. Newman et al and Pizauro et al [15,16] observed an increase of Lfe content in the period prior to the end of lactation stage in cows. In contrast, Sant'ana et al [17] did not observe any significant variation in Lfe content during lactation. Campanella et al and Giacinti et al [18,19] in analyzing Lfe concentrations in raw buffalo milk found values of 232 μg/mL and 332 μg/mL, respectively. This difference in the average content of Lfe among the studies suggests the influence of the animal's age, the lactation period and the methodology used. The Lfe plays an important role in the protection of the mammary gland, since this protein chelates iron, making it unavailable to micro-organisms and hindering their growth [16]. These results indicate the importance of Lfe in promoting the health of the mammary gland, a bioactive protein with high functional and biological value, considering that higher concentrations were determined during the animal's higher performance phase at the beginning of lactation; a phase in which the animal has a higher susceptibility to contracting infections that can compromise productivity. Thus, it can be hypothesized that Lfe can act in reducing dairy production losses.

Table 1. Means, standard deviations and coefficient of variation of the biochemical constitution of Murrah buffalo milk in relation to total sample and in groups: beginning of lactation (1) and end of lactation (2)

Parameters	Total (n = 30)		Group 1 (n = 15)		Group 2 (n = 15)	
	Mean±SD	CV	Mean±SD	CV	Mean±SD	CV
Fat (%)	6.9±1.6	0.24	6.7±1.7	0.27	7.2±1.56	0.22
Protein (%)	4.2±0.39	0.09	4.1±0.39	0.09	4.2±0.41	0.10
Lactose (%)	4.4±0.35	0.08	4.4±0.43	0.10	4.5±0.25	0.05
TS (%)	16.6±1.6	0.10	16.2±1.7	0.11	16.9±1.4	0.08
DDE (%)	9.6±0.44	0.05	9.5±0.50	0.05	9.6±0.37	0.04
Lfe (μg/mL)	69.5±37.0	0.53	88.8±40.2	0.45	50.3±20.7	0.41

SD, standard deviation; CV, coefficient of variation; TS, total solids; DDE, defatted dry extract; Lfe, lactoferrin.

Determination of the microbiological content of the herd's milk and variations in lactation stages

Of the samples submitted to a microbiological isolation test, 21 (70%) were positive, meaning that bacterial growth was observed. Of these, five samples were considered contaminated, as growth was very intense making it difficult to identify microbial groups. Six samples presented two bacterial types. A singular microbiological evaluation was conducted in order to characterize the etiologic agents present in the milk studied. Nine different species were isolated, with greater bacterial activity in the group at the end of lactation stage (2), as shown in Table 2.

The frequency observed for the positive microbiological examination is similar to those obtained by [20], who isolated

Table 2. Percentages of positive and negative results of microbiological tests from the 30 samples of milk collected in groups with different stages of lactation

Group	Microbiological test		
	Positive (%)	Negative (%)	Total
1. Beginning of lactation	10 (66.7)	5 (33.3)	15
2. End of lactation	11 (73.33)	4 (36.77)	15

microorganisms in 78.6% and 60.5% of bubaline milk samples, respectively.

Considering the microbiological identification of the isolates from the 15 milk samples collected from Group 1 females, there was growth in 10 samples, and the most frequently identified microorganisms were *Streptococcus* (30%). For the 15 milk samples from group 2, growth was observed in 11 samples, and the most frequent microorganisms encountered were *Staphylococcus* (54.55%), as shown in Table 3.

It is worth noting that of the 30 total samples, 5 were classified as contaminated. According to the laboratory, this classification is given when there is a significant growth of different colonies of more than four different types, making it impossible to identify them.

The total microorganisms isolated according to the number of isolation in pure culture revealed that *Staphylococcus* (41%) had the highest frequency, but there was also relevance with the *Staphylococcus epidermidis* (*S. epidermidis*) species, followed by *Streptococcus* (32%), where *Streptococcus uberes* had the highest prevalence, and *Corynebacterium bovis* (*C. bovis*) (14%). *Micrococcus* spp. (9%) and *Escherichia coli* (4%) microorganisms were also identified.

The data found in this study regarding the high frequency of isolated *Staphylococcus* spp. corroborate the findings of [21-23], who reported frequencies of 45%, 50.62%, and 26.6%, respectively, and considered as the most expressive genus among the other genera isolated in these studies. The authors concluded that this bacterium is the main etiological agent involved in cases of bubaline mastitis.

Oliveir et al and Dhakal et al [24,25] isolated the genus *Staphylococcus* spp. from Murrah buffalo milk samples with subclinical mastitis, highlighting it as the main pathogen with a frequency of 33.3% of the analyzed samples. Recently, Medeiros et al [4] observed a 21.8% isolation frequency for *Staphylococcus* and 16.7% for *Corynebacterium* in buffalo milk samples. Chavoshi and Husaini [26] found negative results for the microbiological analysis of *Coagulase-negative staphylococci* (34.5%), *Streptococcus* spp. (21%), and *Bacillus* spp. (13%).

Table 4 shows the absolute and relative values of genera and species identified in samples of buffalo milk of the studied herd.

In the present study, the species *S. epidermidis* was isolated with the highest frequency (18%). Similar results are reported by [25], who collected data from 60 buffaloes with Subclinical Mastitis with SCC>200,000 cel/mL from five farms, and found that the predominant pathogens belonged to the species *S. epidermidis* and *Streptococcus* spp.

Other researchers found different results in terms of frequencies with a prevalence of the same microorganisms highlighted in this study, among which can be mentioned [27], who found a prevalence of 59.25% of *Corynebacterium* spp. and 17.59% of *Staphylococccus* spp.

Langoni et al [28] isolated 31.7% of *C. bovis* as the most frequent microorganism in bubaline milk samples with subclinical mastitis. Bonini et al [29] investigated the bacterial profile in samples of buffalo milk samples, and found positive results of 51.47% for *Corynebacterium* spp. and 17.65% corresponding to the *Staphylococccus* genus. According to Fagiolo and Lal (2002), *Streptococcus* spp. (39%) was the main pathogen associated with subclinical mastitis, followed by *Staphylococcus* spp. and *Corynebacterium* spp. with frequencies of 37% and 24%, respectively.

The difference observed in the studies described above is possibly related to sample size, type of management employed, as well as sanitary measures adopted for milkings, sample collection and site/installations. All these factors may have favored the contamination and proliferation of different infectious agents in the mammary gland. Even with different frequencies from those found in this study, they are still among the main bacteria found in this study.

Some actions in preparing the buffaloes in the milking room favor infection by *Staphylococcus* spp. bacteria penetration, as

Table 3. Number of microorganisms isolated from Murrah buffalo milk at different stages of lactation

Agent	Group 1		Group 2	
	N	(%)	N	(%)
Staphylococcus	2	20.0	6	54.6
Streptococcus	3	30.0	4	36.4
Corynebacterium bovis	2	20.0	1	9.1
Micrococcus spp.	2	20.0	0	0
Escherichia coli	1	10.0	0	0
Total	10	100	11	100

Table 4. Proportion of the 22 isolated microorganisms (genus and species) of milk of 30 buffaloes

Isolated pathogens	N°	Frequency (%)
Staphylococcus epidermidis	4	18.2
Staphylococcus chromogenes	3	13.6
Staphylococcus haemolyticus	1	4.5
Staphylococcus warneri	1	4.5
Streptococcus uberis	3	13.6
Streptococcus agalactiae	2	9.1
Streptococcus dysgalactiae	2	9.1
Corynebacterium bovis	3	13.6
Micrococcus spp.	2	9.1
Escherichia coli	1	4.5
Total	22	100

well as poor hygiene of the milker and the milking equipment, which can be disseminating vehicles of this bacteria into the milk ducts with high contamination and easy transmission.

The isolation frequency of *E. coli* in this study was low, with a percentage of 5%. This result may be compared to those obtained in a study by [30], who isolated *Staphylococcus aureus* (54.0%), *Streptococcus* spp. (12.9%) and *E. coli* in lower frequencies (12.9%). Different proportions were obtained by [31] in assessing 60 samples of buffalo milk with mastitis, obtaining 30% of the samples with *E. coli*.

The presence of subclinical mastitis in the bubaline herd can cause economic losses to producers and is a source of infection for other animals on the property, in addition to also being a public health problem due to the possibility of transmitting mastitis pathogens and enterotoxins through the consumption of contaminated milk and dairy products, thus causing risk to consumers health [16,32].

Profile of antibiotic sensitivity

Nine isolated strains from different genus and species were used to carry out the resistance profile test. From completion of the antibiogram, it was possible to draw a resistance profile for the different antibiotics tested: CEQ, DUL, TE, AM, ENO, CTF, SUT, ACA, CNM, and CIP.

The results of the antibiogram indicated that 10 (58.82%) of the 17 antibiotics tested were sensitive to all isolates with 100% efficiency, being: CEQ, DUL, TE, AM, ENO, CTF, SUT, ACA, CNM, and CIP, followed by OXA, AMO, PMN, AMC with 89% efficiency, and CL, CN, and ENO with 78% efficiency.

The sensitivity profile, resistance and intermediate susceptibility to antibiotics of isolated bacteria from the 30 samples indicate that the highest resistance rates were among the bacteria *Streptococcus uberis*, *Streptococcus dysgalactiae* (resistant to CL, CN, and NEO) and *E. coli* (resistant to OXA, AMO, and PMN); all opportunists as etiologic agents of mastitis, and similar to the results found by [33]. Antibiotic resistance found in this study was also the same as those found by [34]. Strains of *Staphylococcus chromogenes*, *S. epidermidis*, *Staphylococcus warneri*, *Streptococcus agalactiae*, and *C. bovis* were sensitive to all antibiotics tested.

Antimicrobial therapy is one of the main tools for controlling mastitis in herds, allowing treatment to be performed with greater efficiency and safety based on the results of microbiological culture and complemented by the sensitivity test [35]. However, performing it is not always possible in the field, requiring the treatment of acute or chronic clinical cases without prior knowledge of sensitivity to antimicrobial agents.

The present study found that two of the nine isolated bacterial agents (22%) from the 30 buffaloes were resistant to gentamicin. Langoni et al [28] indicated gentamicin as an effective antibiotic for the treatment of animal mastitis from bacterial origin, and one of the most commonly used on farms. Other studies conducted at different moments and conditions have proven the effectiveness of gentamicin against isolated mastitis agents, but also point to the developing resistance to this antibiotic.

Another antibiotic widely used in the treatment of mastitis is TE, which showed 100% efficiency in this study. Similar to these studies, all isolated samples of *Staphylococcus* spp. and *Streptococcus* spp. were sensitive to the same active principle [36].

This test highlights the importance of evaluating antimicrobial sensitivity *in vitro* before the indication of treatment, especially under inadequate conditions of environmental hygiene and milking. Antibiotics such as gentamicin and neomycin, which in different studies showed high efficacy *in vitro* against mastitis agents, but can be ineffective, especially when its use is frequent and inappropriate. Therefore, it can be inferred that this test has outstanding technical importance, especially for professionals working in the field and who lack the infrastructure of laboratory procedures for treatment of mastitis in buffaloes.

Determining cellularity parameters of the milking herd and the variations at different lactation stages

In assessing the evidence of the cellularity of buffalo milk, the electrical ECM (mS) and SCC (thousand cells/mL) values, the average results in relation to total sample and in each group of lactation phase are detailed in Table 5.

It can be observed that mean SCC for the herd was 471,000 cells/mL and the ECM was 3.09 mS/mL. In comparing the cellular content behavior of milk between groups with different lactation phases, group 2 showed higher levels. This increase may be indicative of mastitis, considering that these analyzes (SCC and ECM) are described as routine tests, being well established for the diagnosis of this disease in cattle. Due to the lack of legislation for the buffalo species, there are no reference values.

In determining the ECM, the results were lower in Group 1, being buffaloes in the early lactation phase. These results are similar to those by [37], who found that in the beginning of the

Table 5. Means, standard deviations and coefficient of variation for cellularity parameters of buffalo milk herd and for the groups at the beginning of lactation (1) and end of lactation (2)

Parameters	Total (n = 30)		Group 1 (n = 15)		Group 2 (n = 15)	
	Mean±SD	CV	Mean±SD	CV	Mean±SD	CV
ECM	3.1±1.1	0.36	2.9±0.59	0.20	3.2±1.4	0.45
SCC	470.8±775.5	1.65	256±267.8	1.04	685±37.1	1.51

SD, standard deviation; CV, coefficient of variation; ECM, electrical conductivity of milk; SCC, somatic cell count.

lactation phase, ECM was low, observing a gradual and significant increase in intermediate phases and at the end of lactation ($p< 0.007$). Hussain et al [38] found mean values of 4.9 mS/cm for healthy buffaloes and 6.1 mS/cm for animals with mastitis, being considered as a good mastitis diagnostic test.

Electrical conductivity is based on the principle that animals with mastitis present an alteration in the ionic charge of milk due to an epithelial injury and/or alteration of vascular permeability. This condition causes an increase in the sodium and chlorine concentrations in milk and reduces levels of potassium and lactose, thereby generating increased electrical conductivity so that an electric current will flow more easily through mastitic milk due to its high ionic content [39,40].

Values for the SCC of the buffalo milk showed significant differences between the groups, where an increased SCC was observed throughout lactation (Table 5). These results are similar to those found by [37,41], who observed a significant increase in SCC in buffalo milk throughout lactation, with SCC values ranging from 36,000 cells/mL in the initial phase, to 54,500 and 95,500 cells/mL at the end of lactation stage. Salari et al [42] reported an increase in the SCC in buffaloes that were in an advanced stage of lactation, and Araújo et al [2] observed a rising trend for SCC according to the number of births and the end of lactation; a fact that is caused by a higher natural epithelium desquamation of the mammary gland.

Evaluation of the correlation between cellular and microbiological constituents of buffalo milk at different lactation stages

The mean results of subclinical mastitis diagnostic parameters obtained in both lactation phase groups and mean tests are shown in Table 6, where the values were transformed into logarithmic scale.

It is observed that there was a significant difference in SCC values according to lactation stage where the buffaloes had a tendency to increase in accordance with its higher productivity or being at the end of lactation stage. ECM and TBC parameters showed no significant differences between groups in different stages of lactation, even observing that TBC presented a higher score in the group at the beginning of lactation, which can be explained by collection occurring directly from the mechanical collector of the milking equipment, thus contributing to the increase in the bacterial population.

High levels of bacteria have a negative effect on the milk quality, especially with regard to flavor, shelf life and safety of the food products available to the consumer. According to Murray et al [43], bacterial counts above 1.0×10^3 colony forming units (CFU)/mL is indicative of hygiene deficiencies in milk production; therefore, data obtained from this study reveal that the dairy farm should channel efforts toward improving the hygienic conditions during milking to reduce the levels of microbial contamination, as inadequate hygiene conditions undermine the quality of milk, and dirt and microorganisms present at the milking site can immediately be incorporated into the product.

The animals evaluated in this study were asymptomatic; milk samples were subjected to cellular and microbiological tests for disease detection. By analyzing the data, it was observed that the values varied, especially with SCC between groups, and with significant differences for the Tukey test at 5%. When correlated, a positive significant correlation was observed ($r = 0.42$; $p<0.05$) between TBC and ECM parameters, and a non-significant negative correlation ($r = –0.04$; $p>0.05$) between TBC and SCC.

Correlations of diagnostic mastitis parameters held in different groups revealed that there was a significant negative correlation ($r = –0.51$; $p>0.05$) for group 1 between SCC and microbiological parameters, while there was a significant positive correlation in group 2 between TBC and SCC ($r = 0.58$; $p>0.05$) and between TBC and ECM ($r = 0.56$; $p>0.05$).

In order to have a more accurate reference of mastitis diagnosis in the studied population, a correlation of cellular and microbiological content was performed with the microbial isolates also found in this study, showing that SCC values found in milk samples ranged from 42,000 to 4,320,000 cells/mL, with a median of 257,000 cells/mL, regardless of the isolated microorganism. ECM varied from 1.85 to 7.40 mS/cm with a median of 3.11 mS/mL. On the other hand, TBC varied between 9.10×10^3 to 6.94×10^6 CFU/mL, with a median of 3.21×10^5 CFU/mL, thus indicating the presence of subclinical mastitis in buffaloes which have counts above these medians.

With these ranges of cellularity parameters, it can be suspected that the sample is positive and requires microbiological examination to confirm the infectious agent involved in intramammary infection to implement prevention and control measures. It is known that there are other non-infectious factors which can also elevate SCC in buffalo milk, such as lactation stage and season of the year.

For the negative samples of microbiological examination, the values of the SCC found ranged from 55,000 to 537,000 cells/mL, with an overall average of 232,000 cells/mL and a median of 221,000 cells/mL. This average is higher than those found by [16,37,44], who observed SCC ranging from 24,000 cells/mL,

Table 6. Means and standard deviations in logarithmic scale, coeffcient of variation of cellularity parameters and total bacterial count of buffalo milk in groups at the beginning of lactation (1) and end of lactation (2)

Parameters	Group 1 (n = 15)		Group 2 (n = 15)	
	Mean±SD	CV	Mean±SD	CV
ECM (mS/cm)	$1.0^a \pm 0.20$	0.19	$1.1^a \pm 0.40$	0.37
SCC (thousand cells/mL)	$5.0^a \pm 1.04$	0.20	$6.0^b \pm 0.95$	0.15
TBC (CFU/mL)	$6.1^a \pm 1.8$	0.29	$4.8^a \pm 2.6$	0.53

SD, standard deviation; CV, coefficient of variation (%); ECM, electrical conductivity of milk; SCC, somatic cell count; TBC, total bacterial count; CFU, colony forming units.
[a,b] Means followed by different lowercase letters in the same row differ by Tukey test ($p<0.05$).

30,500 cells/mL and 44,400 cells/mL, respectively, in healthy Murrah buffalo milk. ECM ranged from 1.52 to 3.30 mS/cm with a median of 2.88 mS/mL, and TBC had a variation of 2.0×10^3 to 3.4×10^6 CFU/mL, with a median of 1.1×10^5 CFU/mL.

The results corroborate data evaluated by [45], who observed SCC above 200,000 cells/mL and positive bacterial growth in cultures of buffalo milk samples with the presence of subclinical mastitis. Dhakal et al [25] obtained an average milk SCC of 171,000 cells/mL for animals without mastitis, 799,000 cells/mL for animals with subclinical mastitis, and 6,039,000 cells/mL for animals with clinical mastitis. Medeiros et al [4] found an average of 328,000 cells/mL in bubaline milk samples under microbiological examination, leading to the conclusion that SCC values above 280,000 cells/mL are indicative of mammary gland infection.

Because quality standards for buffalo milk do not exist yet, literature data shows low SCC for buffalo milk when compared to bovine/cow milk; however, the present study indicates that there is need for using routine testing, such as SCC and ECM in combination with microbiological data, considering that the latter is more sensitive for this species.

This information is very important for the culture of buffalo dairy, given that buffalo milk is intended entirely for the production of derivatives and that milk quality directly influences the quality of the final product [46].

Evaluation of risk factors associated to bubaline mastitis

Some risk factors for infection of the disease were analyzed, such as inadequate cleanliness of environments, milking equipment, vacuum pressure of the teat cups and hyperkeratosis condition in the animals.

A critical point observed in the waiting room of the animals was the hygiene of the place, where much waste/feces was encountered. Even if the animals undergo cleaning, they are still susceptible to contracting environmental mastitis, so harboring resistant pathogens increases the risk of milk contamination. In the milking site, the waste was removed with strong water hoses and drained into a gutter. Cleaning of facilities and equipment was carried out immediately after milking.

The results of the swabbing samples from the teat cups showed a high level of microbial contamination with more than four kinds of agents, including: Coliforms, Pseudomonas, and *Bacillus* spp., which made it impossible to identify these separately. Collecting conducted from the teat cups was prior to milking, where the devices were ready for use, indicating poor hygienization of the equipment, inefficacy of the used products, or error in the cleaning procedure. According to the employees of the industry, washing and disinfection of mechanized milking equipment were according to the manufacturer's instructions.

The concern with correct cleaning of milking equipment is valid, because according to Zeni et al [47], this practice directly influences the microbial contamination level of milk; its absence or incompetent performance favors the proliferation of a wide variety of microorganisms which adhere to the inner surface of the equipment and accessories forming a biofilm, and its removal is quite difficult by normal cleaning.

Variable vacuum pressure in the system during the experiment was also monitored from the standard milking equipment meter (barometer); however, it remained constant at 40 kbp. This result is in accordance with the recommendations of researchers and technicians in the field, which indicate that the maximum milking efficiency is obtained with the vacuum level between 37 to 41 kPa in the milking set during the maximum milking flow [39].

Most studies evidence that vacuum pressure fluctuations in the milking system increase the incidence of mastitis. According to Blowye and Edmondson [48], mechanical irregularity in the milking system, such as inadequate vacuum pressure, deprived pulse, worn out teat cups, or improper removal of the assembly can cause swelling and lesions to the tip of the teats, acting as a bridge to getting infections such as mastitis, and with one of the most common injuries being hyperkeratosis.

Table 7 show the results for the clinical examination of hyperkeratosis teats. The literature recommends that the percentage of animals in the herd with a score of III and IV should be less than 20% and 10%, respectively [40,49].

It was observed that the degree of hyperkeratosis reached level III in the group at the end of lactation. This happens due to the fact that this is an alteration of the teats' skin which occurs long-term mainly from factors related to milking, as female buffalo contribute to their productive life.

The results show that the studied dairy farm had a herd with low occurrence of hyperkeratosis on the teats, requiring only that preventive actions be intensified so that the teats with scores II and III did not develop into more severe conditions.

Keeping the ends of the teats in good conditions is extremely important because the sphincter muscle in that region plays a crucial role in teat canal contraction keeping it closed between milkings, thereby preventing the entry of pathogens into the mammary gland. This action is aided by mature keratin cells present in the teat canal, and together they represent the primary resistance barrier to mastitis [40]. This is a fact confirmed by Zecconi et al [50], who found a significant increase in new intramammary infections with an increase of 5% in the thickness of the edges of teats, confirming the importance of this analysis in their study,

Table 7. Absolute and relative results of the degree of hyperkeratosis in teats of Murrah buffaloes for groups in the beginning of the lactation (1) and at the end of lactation (2)

Degree	Group 1		Group 2	
	N	(%)	N	(%)
I	50	83.3	38	63.3
II	10	16.7	16	26.7
III	0	0	6	10.0
IV	0	0	0	0

as well as the observation of general factors of hygiene and equipment being risk factors for the occurrence of subclinical mastitis in buffaloes; and thus obtaining an effective quality control of milk and milk products.

CONCLUSION

We conclude that SCC values above 257,000 cells/mL and ECM above 3.1 mS/mL are indicative of mammary gland infection. However, an association of these analyzes with microbiological factors is necessary for a more precise evaluation of the health status of buffalo with subclinical mastitis. The most prevalent bacteria contained in the milk of the researched buffaloes were *Staphylococcus* spp., *Streptococcus* spp. and *C. bovis*, which may be associated with failures in hygienizing the environment and milking equipment.

This study provides information which if applied to treatment and control programs/policies of mastitis in buffalo farming may allow for an increase in herd productivity and support new studies that could contribute to forming/creating specific legislation for the buffalo species, given the importance of taking preventive measures to ensure its product quality and derivatives.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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