








Isolation of *Escherichia coli* and *Staphylococcus aureus* in raw milk from refrigeration tanks: identification and antimicrobial resistance profiles

Isolamento de Escherichia coli e Staphylococcus aureus em leite cru de tanques de refrigeração: identificação e perfis de resistência antimicrobiana

Patrícia Veiga de Almeida¹ ; Adelino da Cunha Neto² ; Tais Ramalho dos Anjos¹ ; Naudia da Silva Dias¹ ; Kethin Rianne Nunes Calixto de Andrade¹ ; Jade Gabriela Oliveira Nascimento¹ ; Eduardo Eustáquio de Souza Figueiredo² ; Ricardo César Tavares Carvalho¹ 

ABSTRACT: *Escherichia coli* and *Staphylococcus aureus* in milk cooling tank reflects a hygienic deficit in animal management, production environment, and milk obtainment. With implications for public health as agents of infection and food poisoning, and the presence of antimicrobial-resistant strains. Therefore, were investigated in cooling tanks with high counts of somatic cells and total bacteria in milk. Microorganisms, in which a profile of resistance to antimicrobials was investigated, and whether there was a similarity in this profile between the strains of the eight dairy properties. Therefore, eighty-eight samples were obtained, and inoculated on Compact Dry[®] plates. Of this total, 27.27% (24/88) samples tested positive for *E. coli* and 56.81% (50/88) for *S. aureus*. Among 24 *E. coli* strains subjected to disk-diffusion antibiograms, 70.83% were resistant to rifampicin, 50% to ampicillin and 41.67% to cefoxitin and erythromycin, while of the 51 *S. aureus* strains, 94.32% expressed resistance to azetroanam, 86.27% to ampicillin and nalidixic acid, 76.47% to rifampicin and 47.06% to erythromycin and cefoxitin. A criterion of resistance to over three antibiotics was observed for 8.33% (2/24) of the isolated *E. coli* strains and 17.65% (9/51) of the *S. aureus* strains, characterizing them as multidrug resistant (MDR) strains. Resistance phenotypes displayed high similarity between properties F5 and F6 for *S. aureus*, and properties F6 and F8 for *E. coli* when applying the Jaccard index. The presence of these antibiotic-resistant pathogenic microorganisms indicate flaws in milk production handling and sanitary conditions, representing risk to milk consumers.

KEYWORDS: Raw milk; etiological mastitis agents; antimicrobial resistance.

RESUMO: *Escherichia coli* e *Staphylococcus aureus* no tanque de resfriamento de leite refletem um déficit higiênico no manejo animal, ambiente de produção e obtenção de leite. Com implicações para a saúde pública como agentes de infecção e intoxicação alimentar e presença de cepas resistentes a antimicrobianos. Portanto, foram investigados em tanques de resfriamento com altas contagens de células somáticas e bactérias totais no leite. Microorganismos, nos quais foi investigado o perfil de resistência aos antimicrobianos e se havia semelhança nesse perfil entre as cepas das oito propriedades leiteira. Para tanto, oitenta e oito amostras foram coletadas inoculadas em placas de Compact Dry[®], destas 27,27% (24/88) amostras revelaram contaminação com *E. coli*, e 56,81% (50/88) com *S. aureus*. Entre 24 cepas de *E. coli* submetidas a antibiograma por disco-difusão 70,83% foram resistentes a rifampicina, 50% ampicilina, 41,67% cefoxitina e eritromicina, enquanto das 51 cepas de *S. aureus* 94,32% expressaram resistência a azetroanam, 86,27% a ampicilina e nalidíxico ácido, 76,47% a rifampicina, e 47,06% a cefoxitina e eritromicina. O critério de resistência a mais de três antibióticos caracterizou 8,33% (2/24) das cepas de *E. coli*, e 17,65% (9/51) de *S. aureus* como multidroga resistente (MDR). O fenótipo resistência mostrou elevada similaridade entre as propriedades (F5 e F6) para *S. aureus*, e das propriedades (F6 e F8) para *E. coli* aplicando-se o índice de Jaccard. Estes microrganismos patogênicos resistentes a antibióticos, indicam falhas no manuseio e obtenção higiênica do leite, e que estes representam risco aos consumidores do leite.

PALAVRAS-CHAVE: Leite cru; agentes etiológicos de mastite; resistência antimicrobiana.

¹Universidade de Cuiabá, Mato Grosso

²Universidade Federal de Mato Grosso, Cuiabá, Mato Grosso

*Corresponding author: ricardo_carvalho88@hotmail.com

Received: 09/06/2021. Accepted: 11/03/2021

INTRODUCTION

The world's milk production was of 851.8 million tons in 2019 in milk equivalents, with an average consumption of 111.6 kg of milk equivalents per inhabitant (FAO, 2020). In Brazil, a total of 24.46 billion liters were produced in the same year, with an average of 734 million liters/year was obtained in the state of Mato Grosso alone, ranking 11th among the most important milk producing states in the country (IBGE, 2019; BRASIL, 2019).

The quality and/or quantity of produced milk are influenced by several factors, such as those linked to milk obtention, transportation and storage, or associated to the producing animals, such as genetic herd potential, management and feeding, while also being influenced by general animal health and that of their mammary glands (COSTA et al., 2019; ARAÚJO et al., 2017; GUIMARÃES et al., 2017). Clinical and subclinical mastitis are noteworthy among animal health alterations. Microorganisms of contagious and environmental origins, respectively, cause this disease (OSTERAS, 2018). Coagulase negative *Staphylococcus*, *S. aureus*, *Escherichia coli*, *Streptococcus uberis* and *S. dysgalactae* are frequently reported as bacterial clinical mastitis agents, and *S. aureus* and *Corynebacterium bovis* as the agents responsible for subclinical mastitis cases (BETTANIN et al., 2019; OSTERAS, 2018; BI et al., 2016).

Regarding mastitis diagnoses, mastitic milk indicators comprise Somatic Cell (SCC) and Total Bacteria (TBC) counts, with cohort limits established at 500,000 cells/cm³ for SCC and 300,000 CFU/cm³ for TBC, respectively (BRASIL, 2018a). These parameters are, therefore, considered milk hygiene and/or intramammary infection indicators worldwide by official milk control and animal health agencies (EUROPEAN UNION, 2017; BRASIL, 2018a; OSTERAS, 2018).

In addition to conventional microbiological analyses, quick analysis methods like Readycult™ -LMX (BELOTI et al., 2002), Rida Count [Coliforms R 1009] (VASALLO; REYES; CAMBAS, 2013), and 3M™ Petrifilm™ Staph (SOUZA et al., 2015) are routinely applied to microbiological milk analyses. Among these methods, 3M™ Petrifilm™ products are recognized and regulated for foods of animal origin by the Brazilian Ministry of Agriculture, Livestock and Supply-MAPA (BRASIL, 2005). Compact Dry® system products, similar to 3M™ Petrifilm™ products, in these systems, chromogenic substrates are used, are also applied in the microbiological analyses of products of animal origin, such as milk (CASAROTTI et al., 2009).

Regarding the Compact Dry EC® kit, *E. coli* metabolizes the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-Glycuronic acid (X-Gluc), resulting in light blue colonies (MIZUOCHI et al., 2016), while the Compact Dry X-SA® kit indicates *Staphylococcus aureus* as dark blue colonies, resulting from the action of acid phosphatase and β -glucosidase on mannitol and the chromogenic substrate (TERAMURA; MIZOUCHI; KODAKA, 2010).

Several antimicrobials are available for the treatment of mastitis, and failures in this process lead to increased bacterial resistance against usual antibiotics (KRÖMKER; LEIMBACH, 2017; KREWER et al., 2013; CAZOTO et al., 2011). In this regard, studies have been implemented to detect antimicrobial resistance and its mechanisms in several microorganisms, such as *E. coli* and *S. aureus* strains isolated from the milk production chain, classifying them as either resistant or multi-drug resistant (MDR) (MOREIRA et al., 2008; MORITZ; MORITZ, 2016). These strains, when present in milk, can lead to dairy products able to trigger difficult to treat toxoinfections.

The percentages of clinical and subclinical mastitis in dairy cows were reported as 37.5% (30/80), 74.2% (23/31) and 85.2% (92/108) in animals from the cities of Carlinda, Cuiabá and Nossa Senhora de Livramento, in the state of Mato Grosso, Brazil (MARTINS et al., 2006; MARTINS et al., 2010; SILVA; SILVA; BETT, 2017). Concerning *S. aureus*, this microorganism was the causal agent of 44% of clinical mastitis cases, and 21.51% of subclinical mastitis cases in the Cuiabá microregion (MARTINS et al., 2010), and of 17.2% of subclinical mastitis cases in the municipality of Nossa Senhora de Livramento (MARTINS et al., 2006).

In 2013, Cáceres, a municipality located in the Central-South mesoregion of the state of Mato Grosso, was responsible for the production of 9710 liters of cow's milk/year (SOARES et al., 2017). In this context, the present study aimed to investigate cooling tanks in the dairy region of the municipality of Cáceres presenting high Somatic Cell (SCC), and Total Bacteria Counts (TBC) detected over six months concerning the occurrence of *E. coli* and *S. aureus*. The Compact Dry® kit was employed to this end, and the antimicrobial resistance profiles and the similarities between strain resistance profiles, among the eight investigated milk-producing properties were verified for the isolated strains.

MATERIAL AND METHODS

This study was carried out in eight milk production properties located in the municipality of Cáceres, in the central-southern mesoregion of the state of Mato Grosso, Brazil. Raw milk presenting Somatic Cell Counts and Total Bacteria counts (SCC and TBC) higher than the maximum MAPA limit (BRASIL, 2018a) for six months (08/2018 to 01/2019) were analyzed. After 01/2019, weekly samples were collected from the cooling tanks with the aid of a sterile shell for eleven weeks, from 02 to 04/2019. Three hundred-mL aliquots of raw milk were placed in a sterilized polyvinyl chloride bottle, which was then sealed, identified and maintained at 5°C in an isothermal box and sent to the Food Molecular Microbiology Laboratory, belonging to the Faculty of Nutrition, Federal University of Mato Grosso, for bacteriological analyses. At the time of collection, milk temperature in the sampled cooling tanks of the eight assessed properties was determined with the aid

of a calibrated dipstick thermometer (MINIPA, São Paulo, Brazil - [0 to 100° C]).

At the laboratory, following container asepsis, the samples were homogenized by inversion bottle movements, and a 1 mL-aliquot of the milk diluted in 9 mL of 0.1% Peptone Saline Solution (0.1% SSP), at 10^{-1} and 10^{-2} dilutions of the first 1 mL aliquot dilution, were inoculated onto EC-Compact Dry[®] plates (CapLab, Brazil), and 1 mL of the 10^{-2} dilution were inoculated on X-SA Compact Dry[®] plates (CapLab, Brazil), which were then incubated at 35 °C for 24 hours. Reddish colonies were characterized as total coliforms, and light blue colonies, as *E. coli* (MIZUOCHI et al., 2016) on the EC-Compact Dry[®] plates. Characteristic *S. aureus* colonies were dark blue (TERAMURA; MIZOUCHI; KODAKA, 2010) on the X-SA-Compact Dry[®] plates, which were then counted, and the results expressed as Colony Forming Unit per mL (CFU/mL).

The disk diffusion technique was used to analyze *in vitro* antimicrobial susceptibility. *E. coli* and *S. aureus* strains representing property (F) and collection (C) were sown by swarming on Müller-Hinton agar, and disks containing 20 antimicrobial agents (Table 1) were distributed equidistantly on the

plates, which were then incubated at 35° C for 16 to 20 hours, according to Bauer et al. (1966). The inhibition halos were then measured, and the results were compared with standards published by the *Clinical and Laboratory Standards Institute* (CLSI, 2018), classifying the strains as sensitive, intermediate or resistant. Strains were classified as Multidrug Resistant (MDR) when displaying resistance to three or more chemical classes among the tested antibiotics (MAGIORAKOS et al., 2012).

A survey on information adequacy was carried out concerning the requirements of normative instruction n° 77 (BRASIL, 2018a), through observations and *in loco* data records at the eight analyzed properties, with the aid of a structured checklist. A questionnaire containing the following topics: animal hygiene (pre-and-post dipping), milking utensil hygiene (milking buckets, milk gallons teacups), operator hygiene, cooling tank, and tank house hygiene, equipment temperature and resources for investigating mastitis and, finally, positive animal management and treatment (BRASIL, 2018a). The terms compliant, non-compliant, and not applicable were adopted to mark each observation in each assessed property.

To assess microbiological raw milk quality, the quality reference standard of <1 CFU/mL for *Enterobacteriaceae*

Table 1. Antibiotic molecules evaluated in the disk-diffusion assay

Molecule	Code	Class	Disk Content (µg)	Zone diameter Breakpoints (mm) for each serotype					
				<i>Escherichia coli</i>			<i>Staphylococcus aureus</i>		
				S	I	R	S	I	R
Ampicillins	AMP	Penicillin	10	≥17	14 - 16	≤13	≥26	21 - 25	≤22
Aztreonam	ATM	Monobactam	30	≥21	18 - 20	≤17	NR	NR	NR
Cefepime	CPM	Cephem	30	≥25	19 - 24	≤18	≥18	15 -17	≤14
Cefoxitin	FOX	Cephem	30	≥18	15 - 17	≤14	≥22		≤21
Ceftiofur	TIO	Cephem	30	≥21	18 - 20	≤17	≥18	15 -17	≤14
Chloramphenicol	CL	Phenicol	30	≥18	13 - 17	≤12	≥18	13 -17	≤12
Florfenicol	FFN	Phenicol	30	≥19	15 - 18	≤14	≥18	13 -17	≤12
Imipenem	IPM	Carbapenems	10	≥23	20 - 22	≤19	≥16	14 -15	≤13
Gentamicin	GEN	Aminoglycoside	10	≥15	13 - 14	≤12	≥15	13 -14	≤12
Tetracycline	TET	Tetracycline	300	≥15	12 - 14	≤11	≥19	15 -18	≤14
Nalidixic acid	NAL	Quilonone	30	≥19	15 - 18	≤14	NR	NR	NR
Ciprofloxacin	CIP	Fluoroquilonone	5	≥31	21 - 30	≤20	≥21	16 -20	≤15
Enrofloxacin	ENR	Fluoroquilonone	5	≥21	17 - 20	≤16	≥18	15 -17	≤14
Sulfamethoxazole/ Trimethoprim	SXT	Folate Pathway Inhibitor	25	≥16	11 - 15	≤10	≥32	25 -31	≤24
Sulfonamides	SSS	Folate Pathway Inhibitor	300	≥19	15 - 18	≤14	≥17	13 -16	≤12
Trimethoprim	TMP	Folate Pathway Inhibitor	5	≥16	11 - 15	≤10	≥16		≤10
Nitrofurantoin	NIT	Nitrofuran	300	≥17	15 - 16	≤14	≥17	15 -16	≤14
Azithromycin	AZM	Macrolides	15	≥19		≤18	≥18	14 -17	≤13
Erythromycin	E	Macrolides	15	NR	NR	NR	≥23	14 -22	≤13
Rifampicin	RD	Ansamycins	5	≥10		≤8	≥20	17 -19	≤16

Legend: (NR) Naturally resistance; (S) Susceptible; (I) Intermediary; (R) Resistant

(*E. coli* indicators) set in the Brazilian Normative Instruction (IN) N° 76 of 26 November 2018 (BRASIL, 2018b), was applied, while the maximum acceptable limit (LMA) standard recommended by the *Institute of Medicine National Research/Council of the National Academies* (2003), of 500 or $2.70 \text{ Log}_{10} \text{ CFU/mL}$ was applied for *S. aureus*.

Similarities were observed regarding strain resistance to the tested antibiotics (Table 1) among the eight evaluated properties. For this assessment, the antimicrobial susceptibility data were converted into a binary matrix, where 1 indicates resistance and 0 indicates absence of resistance or susceptibility. The data regarding the strains (*E. coli* and *S. aureus*) from each property (F1, ..., F8) were grouped, and the sets were compared ([F1 x F2], [F1 x

F3],...) to verify phenotype similarities applying the Jaccard similarity coefficient, calculated using the PAST software 4.03 (<https://softpedia.com/get/science-CAD/PAST.shtml>). The phenotypic similarities between the properties were evaluated using the mean link between groups method or UPGMA (Unweighted Pair Group Method with Arithmetic Mean). The score for this similarity coefficient ranges from 0 (different) to 1 (similar) according to Sahu; Swain and Kar (2019).

RESULTS AND DISCUSSION

Among the eighty-eight raw milk samples obtained from the cooling tanks of the eight investigated farms with high Somatic Cells and Total Bacteria counts for six months, 67.04% (59/88) were contaminated, 27.27% (24/88) with *Escherichia coli*, and 56.81% (50/88) with *Staphylococcus aureus* (Table 2). Mixed contamination (*E. coli* and *S. aureus*) was observed in 25.42% (15/59) of the positive samples. The exclusive occurrence of only one species was also verified, where 59.32%

(35/59) of the samples were contaminated with *S. aureus* and 15.25% (9/59) with *E. coli* (Table 2).

It was assumed that 100% of the herd of properties with high SCC and TBC counts in the cooling tanks had subclinical mastitis, similar to the percentages historically observed in other Mato Grosso cities, such as 74.2% in Nossa Senhora do Livramento (MARTINS et al., 2006) and 85.2% in Cuiabá (MARTINS et al., 2010). A high incidence of subclinical and clinical mastitis may be due to a number of factors, such as deficient or non-existent mastitis control programs, cattle overcrowding, unhygienic milking practices and variations in farm locations (DEVI; DUTTA, 2018). Concerning the evaluated properties, inaccuracies such as poor environment hygiene, manual milking, failure to perform pre- and post-dipping processes, or incorrect performance, lack of milker and cooling tank hygiene were observed. Failures of this type were also observed in other dairy properties in the state of Mato Grosso, such as in Nossa Senhora de Livramento (MARTINS et al., 2006), Cuiabá (MARTINS et al., 2010) and Carlinda (SILVA; SILVA; BETT, 2017).

Staphylococcus aureus was present in over 50% of the evaluated milk samples (Table 2). Costa et al. (2019) reported a moderate relationship between high somatic cell counts ($\geq 700.000 \text{ SCC/mL}$), and mastitis caused by *S. aureus*. The main identified factors associated with mastitis caused by this microorganism are handling deficiencies during milking, in addition to being linked to teat infection; *S. aureus* may be present on milker hands and in their nostrils (GUIMARÃES et al., 2017; KREWER et al., 2013). Bi et al. (2016) observed that *S. aureus* was significantly more prevalent in small properties compared to large ones in China. The present study was carried out on small farms, which may justify the obtained results (Table 2).

Table 2. Count in $\text{Log}_{10} \text{ UFC/mL}$ of *Staphylococcus aureus* and *Escherichia coli* in the cooling tanks of milk-producing properties in the central-southern mesoregion of the State of Mato Grosso.

Log ₁₀ UFC/mL count of <i>S. aureus</i> (SA) and <i>E.coli</i> (EC), by properties (Farms)																
Farm	FO1		FO2		FO3		FO4		FO5		FO6		FO7		FO8	
Collect	AS	EC	SA	EC	SA	EC	SA	EC	SA	EC	AS	EC	SA	EC	SA	EC
C01	3,91	2,95	2,78	0	0	0	2,76	2,48	3	0	3,94	2,9	3,46	0	3,19	2,67
C02	0	0	3,48	0	0	0	0	0	0	0	0	2,26	3	0	3,09	2,26
C03	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C04	0	0	3	0	0	0	0	0	0	2	0	0	3,78	0	3,2	0
C05	0	0	0	0	3	2,3	0	2,6	0	2,48	3,84	2	0	4	3,7	0
C06	0	0	3,7	2	3,3	2,6	0	0	0	2,48	2,95	2	2,11	2	3,7	0
C07	3,3	0	0	0	3,3	0	0	0	2	2,3	3,04	2	3,7	0	3	0
C08	0	0	0	2,95	3,3	0	0	0	0	3	4	0	3,78	0	2,95	0
C09	0	0	3,3	0	3,3	0	0	0	2	0	3	0	3,7	0	2,78	0
C010	3	0	3	0	3	0	0	0	2,3	0	3,48	2	4,34	0	3,9	2
C011	3,48	0	2,04	2	3	0	0	0	2,3	0	3,3	2	3,95	0	4,04	0

(F) = Farm; (CO) = collect; SA = *Staphylococcus aureus*, and EC = *Escherichia coli*.

The occurrence percentages of *E. coli* (29.54%) in the present study are similar to those observed in dairy farms in China (28.6%) (BI et al., 2016), indicating unsatisfactory hygienic conditions (MARTINS et al., 2016). As a pathogen acquired from the environment the main sources of *E. coli* are contact with humidity, mud and animal feces (BETTANIN et al., 2019). A lack of hygiene at the assessed properties may result in contact with fecal material, the main water and raw milk contamination source by this microorganism. Therefore, water quality and effective hygienic practices during milking are crucial in order to avoid raw milk contamination (RIBEIRO et al., 2019).

Property F4 was displayed the lowest number of contaminated samples (Table 2), two, one by *E. coli* and *S. aureus* (CO1), and the other by *E. coli* alone (CO5). This property presented the best hygiene and general state of conservation. The rest of the properties were hygienically deficient concerning milking, milking utensils and cooling tanks, with predominant *S. aureus* detection (Table 2). At F5, in addition to hygienic deficits, one mastitis case was reported, and at F6, milk during the collection period presented strange characteristics, containing odor and dirt. A high detection of both *S. aureus* and *E. coli* was noted at these properties, in five and seven samples, respectively; differing from the other investigated properties, and explained by the scenario described earlier in this paragraph (Table 1).

E. coli counts ranged from 2 to 4 Log₁₀ CFU/mL, and from 2 to 4.34 Log₁₀ CFU/mL for *S. aureus* (Table 2). *E. coli* counts were above the Brazilian Normative Instruction (IN) N° 76 of 26 November 2018 (BRASIL, 2018b) recommendation, with 26 raw milk samples unfit for consumption or for the preparation of milk-based derivatives (BRASIL, 2018b). Regarding *S. aureus*, 88% (44/50) of the positive samples (Table 2) exhibited counts above the maximum established by the *Institute of Medicine National/Research Council of the National Academies* (2003). Where two samples exhibited counts of 4.04 and 4.34 Log₁₀ CFU/mL. This is a cause for concern, since populations close to 5 Log₁₀ CFU/mL of *S. aureus* could potentially produce heat resistant Staphylococcal Enterotoxins (STAMFORD et al., 2006).

Twenty-four *E. coli* and 51 *S. aureus* isolates were subjected to a susceptibility test to 20 antimicrobials belonging to 13 different chemical classes (Table 1). Resistance commonly expressed by some *E. coli* and *S. aureus* strains was observed against 15 antibiotics from 10 different classes. Namely the β-lactam family in the penicillin (ampicillin), monobactam (aztreonam), 2nd generation (cefoxitin), and 4th generation (cefepime) cephalosporins, phenicol (chloramphenicol and florphenicol), aminoglycoside (gentamicin), tetracyclines (tetracycline), quinolones (nalidixic acid), folate inhibitors (sulfamethoxazole+ trimethoprim), nitrofurans (nitrofurantoin), macrolides (azithromycin and erythromycin) and ansamycin (rifampicin). According to Cuong

et al. (2018), antimicrobials of critical use in mastitis treatment comprise broad-spectrum β-lactams and cephalosporins. However, according to Krömker and Leimbach (2017) low-spectrum β-lactam antibiotics are preferred for topical mastitis treatment.

The *E. coli* isolated from raw milk were resistant to most antibiotics, with the exception of ceftiofur and those belonging to the Fluoroquinolone group (Figure 1). The isolated *E. coli* displayed high resistance to rifampicin (70.83%), ampicillin (50%), cefoxitin and erythromycin (41.67%), similar to strains isolated from milk and dairy products in Iran, which displayed high resistance to ampicillin, of 100% in Kashan and 43.4% in Mashhad (FALLAH et al., 2019; CHALESHTORI et al., 2017). This differs from strains from other regions where lower resistance percentages to ampicillin were detected, for example, of 10% in Bahia, Brazil (BARRETO et al., 2012) and 6.7% in India (RASHEED et al., 2014). Strains isolated from milk and dairy products in the present study displayed resistance to different antibiotic groups (Figure 1), similar to strains found in Iran (FALLAH et al., 2019; CHALESHTORI et al., 2017). However, *E. coli* strains reported in other studies conducted with milk and dairy products in Brazil displayed resistance only to nalidixic acid, cefoxitin and tetracycline (RIBEIRO et al., 2019). Resistance to several antibiotics may be attributed to the presence of certain genes, such as *mcr*-mediated colistin resistance and/or plasmids from the *incF* and *incX* groups, among other factors (SAIDENBERG et al., 2020; ZAMPARETTE et al., 2020; CASTRO et al., 2021). These genes and plasmids have been previously detected in *E. coli* isolated from clinical and food samples in Brazil (SAIDENBERG et al., 2020; ZAMPARETTE et al., 2020) as well as in bovine faeces in Canada (CASTRO et al., 2021).

The *S. aureus* strains isolated herein (51) were sensitive to imipenem, ciprofloxacin and sulfonamide, as well as resistant to 17 other antibiotics, displaying high percentages of resistance to aztreonam (94.12%), ampicillin and nalidixic acid (86.27%), rifampicin (76.47%) cefoxitin (58.85%) and erythromycin (47.06%) (Figure 1). Among the antibiotics employed herein, enrofloxacin, tetracycline, sulfamethoxazole/trimethoprim, ampicillin, gentamicin, ceftiofur, chloramphenicol, erythromycin and sulfonamide are the most reported concerning resistance expressed by *S. aureus* isolated from bovine milk in Brazil (GIRARDINI et al., 2016; BEURON et al., 2014; SILVEIRA FILHO et al., 2014; COSTA et al., 2013; KREWER et al., 2013). Genes encoding different resistance mechanisms (*ermA*, *ermB*, *ermC*, *msrA*, *mecA*, *icaA*, *icaB*, *blaZ*, *tetL*, *tetK*, *tetM*, *aac*, *mepA*, *grrA*, and *gyrA*) have also been reported for *S. aureus* isolated from milk in Brazil (PÉREZ et al., 2020; ARAÚJO et al. 2017; GIRARDINI et al., 2016). The presence and circulation of these genes among *S. aureus* isolated from milk samples justify the results presented in Figure 1.

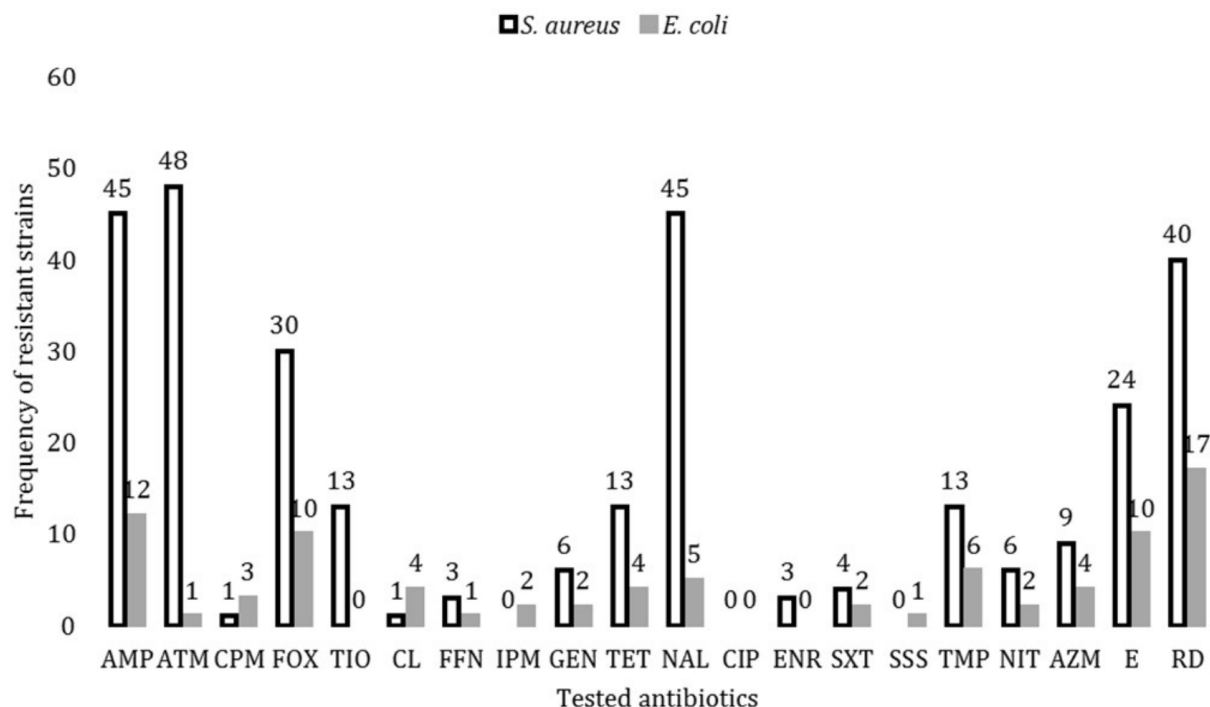


Figure 1. Distribution of the frequency of resistance to antibiotics, such as: Ampicillins (AMP), Aztreonam (ATM), Cefepime (CPM), Cefoxitin (FOX), Ceftiofur (TIO), Chloramphenicol (CL), Florfenicol (FFN), Imipenem (IPM), Gentamicin (GEN), Tetracycline (TET), Nalidixic Acid (NAL), Ciprofloxacin (CIP), Enrofloxacin (ENR), Sulfamethoxazole/Trimethoprim (SXT), Sulfonamides (SSS), Trimethoprim (TMP), Nitrofurantoin (NIT), Azithromycin (AZM), Erythromycin (E), and Rifampicin (RD). In 24 strains of *E. coli* and 51 of *S. aureus* isolated from milk of cows with subclinical mastitis.

Multidrug-resistant (MDR) strains were also observed, at 8.33% (2/24) among the 24 *E. coli* isolates, displaying resistance to 11 antibiotics belonging to 10 different chemical classes. Concerning *S. aureus*, 17.65% (9/51) were MDR to 13 antibiotics belonging to 12 different chemical classes. The appearance of MDR strains in milk is attributed to the indiscriminate use of antibiotics in the treatment of mastitis (KRÖMKER; LEIMBACH, 2017). The presence of these isolates reinforces the importance of choosing the most adequate antimicrobial agents in order to successfully treat mastitis (KREWER et al., 2013).

The detected MDR *E. coli* and *S. aureus* strains displayed common resistance to rifampicin, cefoxitin, tetracycline, chloramphenicol, ampicillin, azithromycin and nitrofurantoin. Resistances to imipenem, cefepime, and gentamicin were detected exclusively in *E. coli*, and resistances to erythromycin, trimethoprim, ceftiofur, sulfamethoxazole/trimethoprim, enrofloxacin and florfenicol were detected only in *S. aureus*. Resistance to erythromycin in *E. coli* and to aztreonam and nalidixic acid in *S. aureus* were not categorized as MDR, which could lead to bias concerning MDR classification, as these species are naturally resistant to these antibiotics.

The Jaccard similarity coefficient was employed to compare similarities or differences in *E. coli*, and *S. aureus* strain behavior regarding their phenotypic resistance to the tested antibiotics among the eight investigated properties.

The findings indicate that properties F5 and F6 displayed the greatest similarity (0.91) concerning *S. aureus* antimicrobial resistance (Figure 2, A), differentiated only by resistance to sulfamethoxazole/trimethoprim in strains isolated from property F5. On the other hand, *E. coli* displayed low similarity among the eight properties, with a maximum of 0.60 among strains isolated from properties F6 and F8 (Figure 2, B). The low similarity among these strains was due to resistance to only chloramphenicol and sulfamethoxazole/trimethoprim, and to azithromycin and tetracycline, expressed respectively, by *E. coli* strains isolated from F6 and F8. Properties F5 and F6 are physically distant, and properties F6 and F8 are physically close.

The differences and similarities observed between the eight milk producing properties regarding the resistance expressed by the isolated *E. coli* and *S. aureus* strains to antibiotics is due to lack of technical assistance from veterinarians and the empirical use of antibiotics by the farm owners. This lack of technical assistance concerning mastitis control also affected the sanitary conditions of the investigated properties, which mostly exhibited hygienic and general conservation deficits, as well as hygienic deficiencies concerning milking, utensils and cooling tanks. Thus, a vicious cycle develops, resulting in reinfections, with high somatic cell and total bacteria counts in raw milk, as well as the presence and high counts of microorganisms like *S. aureus* and *E. coli*.

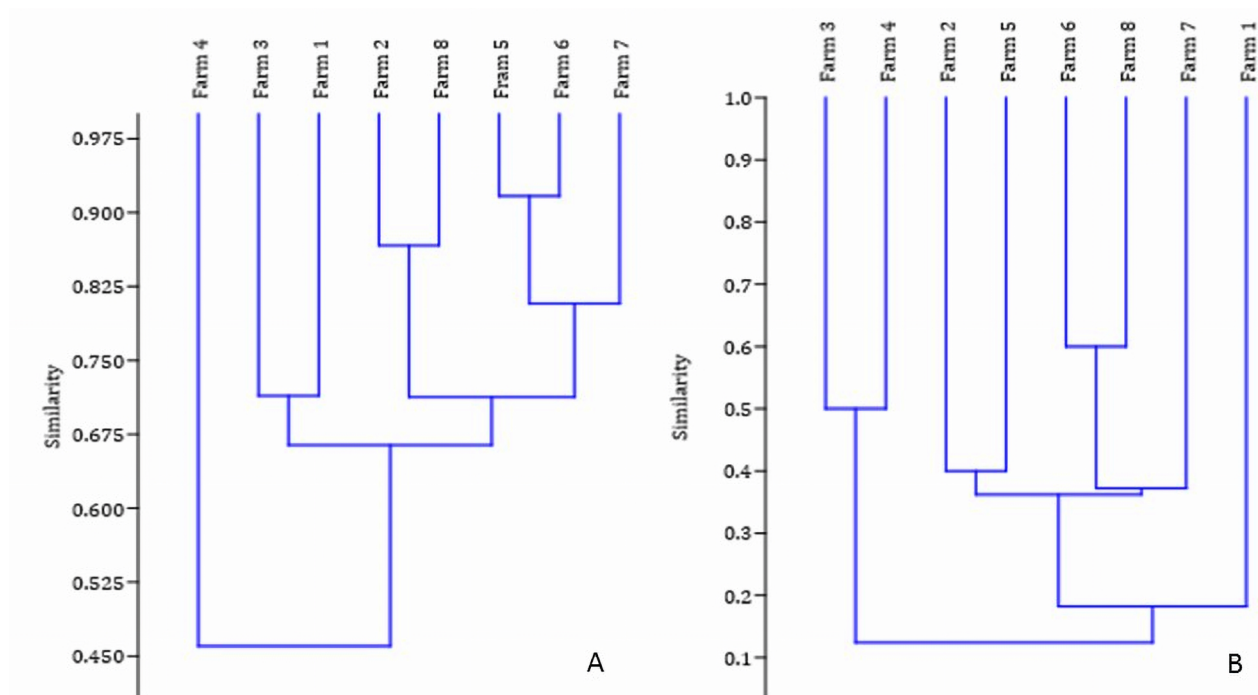


Figure 2. Dendrograms relative to Jaccard Similarity Coefficient, relationships between eight dairy farms, regarding the antibiotic resistance of 51 isolated of *Staphylococcus aureus* (A), and 24 strains of *Escherichia coli* (B) in the region of Cáceres, State of Mato Grosso, Brazil.

CONCLUSION

The findings reported herein confirm a hygienic deficit in the production of the evaluated dairy basin and potentially mastitic milk. This was indicated by high Somatic Cell (SCC), and Total Bacteria (TBC) counts and confirmed by the presence of *E. coli* and *S. aureus* detected by the Compact Dry® kit. The susceptibility profiles of the isolates indicate incorrect handling of animals presenting mastitis, the indiscriminate use of antibiotics, as the isolates were resistant to 18 antibiotics belonging to 13 different chemical classes, and a high resistance similarity expressed by *S. aureus* strains, representing public health risks.

ACKNOWLEDGEMENTS

The authors would like to thank the property owners who authorized visits and samplings from their milk cooling tanks. Thanks are also due to Dr. EESF for allowing use of the

physical structure of the LABMMA (Laboratory of Molecular Microbiology of Food), Faculty of Nutrition, Federal University of Mato Grosso (FANUT/UFMT), for the development of this research. Also this work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, Process: 310181/2021-6.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the conception and writing of the manuscript. All authors critically revised the manuscript and approved the final version.

REFERENCES

ARAÚJO, R. M. P. et al. Virulence factors in *Staphylococcus aureus* and quality of raw milk from dairy cows in a Semiarid Region of Northeastern Brazil. *Acta Scientiae Veterinariae*, v. 45, p. 1491, 2017.

BARRETO, N. S. E. et al. Qualidade microbiológica e suscetibilidade antimicrobiana do leite *in natura* comercializado em Cruz das Almas, Bahia. *Semina Ciências Agrárias*, v. 33, n. 6, p. 2315-2326, 2012.

- BAUER, A. W. et al. Antibiotic susceptibility testing by a standardized single disk method. **American Journal of Clinical Pathology**, v. 45, n. 4, p. 493-6, 1966.
- BELLOTI, V. et al. Use of ReadyCult™ - LMX for enumeration of total coliforms and *Escherichia coli* in milk. **Brazilian Journal Microbiology**, v. 33, p. 49-52, 2002.
- BETTANIN, J. et al. Frequência de Isolamentos dos Agentes Etiológicos da Mastite Bovina no Sudoeste Paranaense. **Revista Brasileira Higiene Sanidade Animal**, v. 13, n. 4, p. 440 – 451, 2019.
- BEURON, D. C., et al. Risk factors associated with the antimicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis. **Pesquisa Veterinária Brasileira**, v. 34, n. 10, p. 947-952, 2014.
- BI, Y. et al. Prevalence of bovine mastitis pathogens in bulk tank milk in China. **PLoS ONE**, v. 11, n. 5, p. e0155621, 2016.
- BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa n. 40, de 12 de dezembro de 2005. Aprova os Métodos Analíticos, Isolamento e Identificação da *Salmonella* na carne bovina, avicultura e produtos derivados de ovos - MLG - 4.03, Metodologia Alternativa de *Salmonella* A-Bax -MLG 4C.01, Isolamento e Identificação de *Listeria Monocytogenes* em carne vermelha, carne de ave, ovos e amostras ambientais, MLG 8.04 - Metodologia Alternativa de *Listeria* A-BAX MLG-8A.01, *Escherichia coli*, MPN AOAC 966.24, Método Petrifilm AOAC 998.08, que passam a constituir Padrões Oficiais para Análise de Microbiologia de Produtos de Origem Animal. **Diário Oficial da União, Brasília**, Seção 1, p. 70, 16 dez. 2005.
- BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa n. 76, de 26 de novembro de 2018. **Diário Oficial da União**, Seção 1, Edição 230, p. 9, 30 de nov. de 2018a.
- BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa n. 77, de 26 de novembro de 2018. **Diário Oficial da União**, Seção 1, Edição 230, p. 10, 30 de nov. de 2018b.
- BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Empresa Brasileira de Pesquisa Agropecuária. Anuário do leite. **Edição digital Embrapa-gado-leite**, p.104, 2019.
- CASAROTTI, S. N. et al. Enumeração de bactérias psicrotólicas em leite cru bovino com a utilização da metodologia tradicional e do sistema Compact Dry®. **Revista do Instituto de Laticínios Candido Tostes**, v. 64, n. 369, p. 64:19-25, 2009.
- CASTRO, V. S. et al. Inconsistent PCR detection of Shiga toxin-producing *Escherichia coli*: Insights from whole genome sequence analyses. **PLoS ONE**, v. 16, n. 9, p. e0257168, 2021.
- CAZOTO, L. L. et al. Antibacterial activity of violacein against *Staphylococcus aureus* isolated from Bovine Mastitis. **Journal Antibiotics**, v. 64, p. 395–397, 2011.
- CHALESHTORI, F. S. et al. Molecular characterization of *Escherichia coli* recovered from traditional milk products in Kashan, Iran. **Veterinary World**, v. 10, n. 10, p. 1264-1268, 2017.
- CLSI. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Disk Susceptibility Tests. 13th ed. Standard M02 (ISBN 1-56238-835-5 [Electronic]). **Clinical and Laboratory Standards Institute**, 950 Wayne, Pennsylvania, 19087 USA, 2018.
- COSTA, G. M. et al. Risk factors for high bulk milk somatic cell counts in dairy herds from Campos das Vertentes region, Minas Gerais State, Brazil: a case-control study. **Pesquisa Veterinária Brasileira**, v. 39, n. 8, p. 606-613, 2019.
- COSTA, G. M. et al. Resistência a antimicrobianos em *Staphylococcus aureus* isolados de mastite em bovinos leiteiros de Minas Gerais, Brasil. **Arquivos Instituto Biológico**, v. 80, n. 3, p. 297-302, 2013.
- CUONG, N. V. et al. Antimicrobial Usage in Animal Production: A Review of the Literature with a Focus on Low- and Middle-Income Countries. **Antibiotics**, v. 7, p. 75, 2018.
- DEVI M.; DUTTA, J. B. Incidence of bovine subclinical mastitis in organized and unorganized farms based on somatic cell count. **International Journal Chemistry**, v. 6, n. 4, p. 1399-1403, 2018.
- EUROPEAN UNION. Overview report: Welfare of Cattle on Dairy Farms. Health and Food Safety. Luxembourg, **Publications Office of the European Union**, ISBN 978-92-79-52981-8, 2017.
- FALLAH, N. et al. Occurrence, pathotypes, and antimicrobial resistance profiles of diarrheagenic *Escherichia coli* strains in animal source food products from public markets in Mashhad, Iran. **Food Control**, v. 121, p. e107640, 2019.
- FAO. Food Agriculture Organization of United Nations. Food Outlook - Biannual Report on Global Food Markets: June 2020. **Food Outlook**, 1. Rome, Italy, p. 169, 2020. <https://doi.org/10.4060/ca9509en>
- GIRARDINI, L. K. et al. Antimicrobial resistance profiles of *Staphylococcus aureus* clusters on small dairy farms in southern Brazil. **Pesquisa Veterinária Brasileira**, v. 36, n. 10, p. 951-956, 2016.
- GUIMARÃES, F. F. et al. Short communication: Outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA)-associated mastitis in a closed dairy herd. **Journal Dairy Science**, v. 100, p. 726–730, 2017.
- IBGE. Instituto Brasileiro de Geografia Estatística. Estatística da Produção Pecuária Jul./Set. Publicado em 12 de dez. de 2019. IBGE, 2019. 50 p.
- Institute of Medicine National Research/Council of the National Academies. Committee on the Review of the Use of Scientific Criteria and Performance Standards for Safe Food. **Scientific Criteria to Ensure Safe Food**. Washington, ISBN: 0-309-50920-3, 2003. 424 p.
- KREWER, C. C. et al. Etiology, antimicrobial susceptibility profile of *Staphylococcus* spp. and risk factors associated with bovine mastitis in the states of Bahia and Pernambuco. **Pesquisa Veterinária Brasileira**, v. 33, n. 5, p. 601-606, 2013.
- KRÖMKER, V.; LEIMBACH, S. 2017. Mastitis treatment - reduction in antibiotic usage in dairy cows. **Reproduction in Domestic Animals**, v. 52, Suppl. 3, p. 21-29, 2017.
- MAGIORAKOS, A. P. et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. **Clinical Microbiology and Infection**, v. 18, p. 268-281, 2012.
- MARTIN, N. H. et al. The evolving role of Coliforms as indicators of unhygienic processing conditions in dairy foods. **Frontiers Microbiology**, v. 7, p. 1549, 2016.
- MARTINS, R. P.; CUNHA NETO, A.; MARQUES, M. R. H. Etiologia da mastite subclínica em vacas do rebanho de uma queijaria em Nossa Senhora do Livramento, MT. **Higiene Alimentar**, v. 20, n. 139, p.104-409, 2006.

- MARTINS, R. P. et al. Prevalência e etiologia infecciosa da mastite bovina na Microrregião de Cuiabá, MT. **Ciência Animal Brasileira**, v. 11, n. 1, p. 181-187, 2010.
- MIZUOCHI, S. et al. Matrix extension study: validation of the compact dry EC method for enumeration of *Escherichia coli* and non-*E. coli* coliform bacteria in selected foods. **Journal of AOAC International**, v. 99, n. 2, p. 451-460, 2016.
- MOREIRA, M. A. S. et al. Resistência a antimicrobianos dependente do sistema de efluxo multidrogas em *Escherichia coli* isoladas de leite mastítico. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 60, n. 6, p. 1307-1314, 2008.
- MORITZ F; MORITZ C. M. F. Resistência aos antimicrobianos em *Staphylococcus* spp. associados à mastite bovina. **Revista Ciência Veterinária Saúde Pública**, v. 3, n. 2, p. 132-136, 2016.
- PÉREZ, V. K. C. et al. Virulence factors and antimicrobial resistance in *Staphylococcus aureus* isolated from bovine mastitis in Brazil. **Brazilian Journal Microbiology**, v. 51, n. 4, p. 2111-2122, 2020.
- OSTERAS, O. Animal health and welfare: Results of the IDF questionnaire data obtained between 2014 and 2016. **IDF Animal Health Report**, v. 12 p. 18-23, 2018.
- RASHEED, M. et al. Antimicrobial drug resistance in strains of *Escherichia coli* isolated from food sources. **Revista do Instituto de Medicina Tropical de São Paulo**, v. 56, n. 4, p. 341-346, 2014.
- RIBEIRO, L. F. et al. Diarrheagenic *Escherichia coli* in raw milk, water, and cattle feces in non-technified dairy farms. **Ciência Animal Brasileira**, v. 20, n. 1-9, p. e-47449, 2019.
- SAHU, M. G.; SWAIN, S. K.; KAR, S. K. Genetically Diversity of *Pseudomonas aeruginosa* Isolated from Chronic Suppurative Otitis Media with Respect to Their Antibiotic Sensitivity Pattern. **International Journal of Otorhinolaryngology and Head and Neck Surgery**, v. 71, Suppl 2, p. S1300-S1308, 2019.
- SAIDENBERG, L. B. P. et al. *mcr*-Positive *Escherichia coli* ST131-H22 from Poultry in Brazil. **Emerging Infectious Diseases**, v. 26, n. 8, p. 1951-1954, 2020.
- SILVA, A. C.; SILVA, F. F.; BETT, V. A. A prevalência de mastites em vacas leiteiras do município de Carlinda (MT), no ano de 2016. **PUBVET - Medicina Veterinária e Zootecnia**, v. 11, n. 8, p. 761-766, 2017.
- SILVEIRA-FILHO, V. M. et al. Antibiotic resistance and molecular analysis of *Staphylococcus aureus* isolated from cow's milk and dairy products in Northeast Brazil. **Journal Food Protection**, v. 77, n. 4, p. 583-591, 2014.
- SOARES, J. C. O. SANTOS L.; CALDAS J. A. P. A pecuária bovina no município de Cáceres-MT: reflexos no contexto socioeconômico. **Ciência Geográfica**, Bauru, v. 21, n. 1, p. 184-197, 2017.
- SOUZA, L. M. J. et al. Evaluation of Petrifilm™ system compared with traditional methodology in count of indicators of sanitary-hygienic quality and pathogenic microorganisms in sheep milk. **Food Science Technology**, v. 35, n. 2, p. 375-379, 2015.
- STAMFORD, T. L. M. et al. Enterotoxigenicidade de *Staphylococcus* spp. isolados de leite in natura. **Ciência e Tecnologia de Alimentos**, v. 26, n. 1, p. 41-45, 2006.
- TERAMURA, H.; MIZOUCI, S.; KODAKA, H. Evaluation of the compact Dry X-SA method for enumerating *Staphylococcus aureus* in artificially contaminated food samples. **Biocontrol Science**, v. 15, n. 4, p. 149-154, 2010.
- VASALLO, A. M.; REYES, D. G.; CAMBAS, A. V. Evaluación del desempeño del método RIDACOUNT para la enumeración de bacterias en leche. **Revista de Salud Animal**, v. 35, n. 1, p. 64-68, 2013.
- ZAMPARETTE, C. P. et al. *IncX4* Plasmid-Mediated *mcr*-1.1 in Polymyxin-Resistant *Escherichia coli* from Outpatients in Santa Catarina, Southern Brazil. **Microbial Drug Resistance**, v. 26, n. 11, p. 1326-1333, 2020.