



Milk microbiota of clinical mastitic cows: an etiological approach

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ABSTRACT: Bovine mastitis is the most impacting disease of dairy industry, and it is characterized by a complexity of causal agents, which have revealed a geographical variation among regions and countries. The mastitis-related pathogens have been traditionally classified as contagious or environmental, based on habits of the microorganisms and transmission routes. Besides, the severity of mammary infections has been associated with the virulence of the pathogens, and immune and nutritional status of the hosts. Considering this scenario, we investigated the etiological pattern, clinical severity scores, and days in milk (DIM) data in 4,273 clinical cases of bovine mastitis among ten large-dairy farms located in Brazil. *Streptococcus dysgalactiae* (283/4,273 = 6.6%), *Escherichia coli* (190/4,273 = 4.4%), *Prototheca* spp. (112/4,273 = 2.6%), and *Streptococcus uberis* (95/4,273 = 2.2%) were the predominant pathogens isolated. Among 4,273 clinical cases, clinical severity score was available in 43.8% (1,871/4,273) animals. From these, 69.8% (1,306/1,871), 27.3% (510/1,871), and 2.9% (55/1,871) were scored as mild, moderate, and severe, respectively. Pathogens were observed mainly in samples obtained during the first 100 days in milk, and clinical severity scored mainly as mild. Our results contributed to the etiological identification, clinical severity scoring, and milking aspects of mastitic cows in dairy farms with a history of clinical mammary infections.

Key words: bovine mastitis, milk pathogens, environmental agents, microbiological diagnosis, clinical severity scores, days in milk.

Microbiota do leite de vacas com mastite clínica: uma abordagem etiológica

RESUMO: A mastite bovina é a doença mais impactante na indústria de laticínios e é caracterizada por uma complexidade de agentes causais, os quais têm revelado uma variação geográfica entre regiões e países. Os patógenos relacionados à mastite têm sido tradicionalmente classificados como contagiosos ou ambientais, com base na fonte de infecção ou origem e nas vias de transmissão. Além disso, a gravidade das infecções mamárias tem sido associada à virulência dos patógenos, e ao estado imunológico e nutricional dos animais. Considerando esse cenário, investigamos os agentes etiológicos, os escores de gravidade clínica e os dados de dias em lactação (DEL) em 4.273 casos clínicos de mastite bovina em dez fazendas de grande porte, quatro localizadas no sudoeste do estado de São Paulo e seis no sul do Estado de Minas Gerais, Brasil. *Streptococcus dysgalactiae* (283/4.273 = 6,6%), *Escherichia coli* (190/4.273 = 4,4%), *Prototheca* spp. (112/4.273 = 2,6%) e *Streptococcus uberis* (95/4.273 = 2,2%) foram os patógenos predominantes isolados. Entre os 4.273 casos clínicos, a pontuação de gravidade clínica estava disponível em 43,8% (1.871/4.273) dos animais. Desses, 69,8% (1.306/1.871), 27,3% (510/1.871) e 2,9% (55/1.871) foram classificados como leves, moderados e graves, respectivamente. Os patógenos foram observados principalmente em amostras obtidas durante os primeiros 100 dias em lactação, e a gravidade clínica foi principalmente classificada como leve. Nossos resultados contribuem para a identificação etiológica, a pontuação de gravidade clínica e os aspectos de ordenha de vacas com mastite em fazendas leiteiras com histórico de infecções mamárias clínicas.

Palavras-chave: mastite bovina, patógenos do leite, agentes ambientais, diagnóstico microbiológico, scores de severidade clínica, dias em lactação.

INTRODUCTION

Mastitis is the leading disease affecting dairy cows. Its occurrence in herds causes significant economic losses due to lower milk production and quality. The disease is characterized as an inflammation of the mammary gland and is often caused by a great complexity of microorganisms (FREDEBEUL-KREIN et al., 2022). More than 140

species of microorganisms have been identified in milk samples of cows with clinical and subclinical mastitis using conventional microbiological culture, phenotypic tests (WATTS, 1988) and molecular methods (RUEGG, 2022).

Bovine mastitis is considered a polymicrobial disease, and this diversity is responsible for changes in milk price, safety and quality, both of raw milk and dairy products (QUIGLEY et al., 2013).

The etiology of the bovine mammary infections varies widely among regions and countries; although, staphylococci, streptococci, and *Enterobacteriaceae* species are the most common isolated pathogens identified globally, being responsible for most cases of clinical and subclinical intramammary infections (VERBEKE et al., 2014; LEVISON et al., 2016; VAKKAMAKI et al., 2017).

Traditionally, bovine mastitis agents have been classified as environmental and contagious pathogens, based on habitat of the organisms and transmission routes. The environmental mammary infections are represented by agents from the surrounding areas of milking environment, whereas the contagious mastitis infections commonly spread from other cows/mammary quarters (COBIRKA et al., 2020).

The diversity of the agents and different routes of the mammary infections poses a major challenge to the management and control of mastitis in herds (SKEIE et al., 2019). Clinical mammary infections may exhibit a wide variety of clinical signs, which have been scored as mild or score 1 (changes in milk aspect), moderate or score 2 (abnormal appearance in the milk and udder inflammation), and severe or score 3 (additional systemic manifestations) (PINZÓN-SÁNCHEZ & RUEGG, 2011).

The microbiological culture and phenotypical identification methods, permits identifying the pathogen causing the disease, aiming prophylactic and treatment measures to control the mammary infections. Although, the culture method is laborious and slow, it is considered as the routine approaches to identify the microorganisms causing the disease (VAKKAMAKI et al., 2017; ASHRAF & IMRAN, 2018).

In last decades, molecular methods have been introduced in the routine diagnosis of bovine mastitis, including PCR, mass spectrometry, and those based on genomics. Nonetheless, there are challenges to overcome, such as a variety of possible sources of microbial DNA, including from the environment, cows, and milk equipment (RUEGG, 2022).

Considering the significative losses related to bovine mastitis, the influence of mammary infections in the main aspects of milking systems, the geographical variation of etiology of bovine mastitis, the relevance of laboratory identification of causal agents that may influenced the adoption of control measures and therapeutic approaches; we performed a large-scale study aimed to identify the pathogens involved in 4,273 clinical cases of bovine mastitis in ten large-dairy in Brazil, with a history of

clinical mammary infections, where DIM and clinical severity of cases were assessed.

MATERIALS AND METHODS

Farms and herds

A convenience sampling of ten large-dairy farms, six located in the south of the state of Minas Gerais (farms A, B, C, D, E and F) and four in the southwest of the state of Sao Paulo (farms G, H and I), Brazil, were used for the study. The farms and herds were eligible when met the following criteria: bulk tank somatic cell count (SCC) < 400,000 cells/mL (BRAZIL, 2018), presence of mastitis control programs, data recorded in management software, production of Holstein animals >20 L/cow/day, at least 200 lactating cows, mechanical milking system, and a history of clinical bovine mastitis.

Among the ten dairy farms, five had the “free stall” installation, three “compost barn”, and two the “cross ventilation”. Good hygiene of the facilities was present in nine of the ten farms, and good hygiene of the equipment in all farms studied, with the application of a checklist and monitoring of milking and equipment hygiene. All farms had a milking parlor, one double 24 type, four double 8 type, two double-6 type, one double-12 type, one double-20 type and one of the carousel systems. All farms also had an expansion tank. In general, all farms studied showed good equipment hygiene and good quality of workers. All farms had medical-veterinary assistance.

Milk sampling strategy and clinical diagnosis

The collection of milk samples from cows for diagnosis of clinical mastitis was performed by farmworkers. Milk samples were collected from all cows that experienced clinical mastitis from 2017 to 2018. These employees were submitted to ongoing training to correctly identify clinical cases, severity scores, and collect samples aseptically (GUERRA et al., 2020).

The diagnosis of clinical mastitis and clinical severity scores was based on the strip cup test, macroscopic alterations of the milk, and clinical examination of mammary gland and animals. Severity scores were classified as follows: mild (or score 1) cases showed exclusively changes in the milk appearance (e.g., flakes, pus or blood). Moderate cases (score 2) revealed abnormalities in the milk aspect and clinical signs of inflammation of the mammary gland (e.g., edema, redness, pain, abscesses, nodules), whereas cases that revealed additional systemic signs (e.g., inappetence or anorexia, tachycardia, tachypnea,

decubitus, fever, abnormal ruminal movements) were classified as severe (score 3) (PINZÓN-SÁNCHEZ & RUEGG, 2011).

After identifying the cases of mastitis and discarding the first jets of milk, antiseptics of the udder teats was performed with 70% alcohol, the milk collected in sterile bottles, and kept frozen (-20 °C) until sending to the laboratory. Along with the samples, data of clinical severity scores of cases (scores 1, 2 and 3), days in lactation, and the previous treatment history were assessed.

Microbiological culture

The samples were cultured in the Laboratory of the Nucleus of Research on Mastitis (NUPEMAS), at School of Veterinary Medicine and Animal Science, UNESP, Botucatu, SP, Brazil. Approximately 10 µL of each milk sample was streaked onto defibrinated sheep blood agar (5.0%) (Oxoid™, Basingstoke, UK) (an enriched media) and MacConkey agar (Oxoid™, Basingstoke, UK) (a selective media for Gram-negative bacteria). The plates were incubated at 37 °C under aerobic conditions for 72 h. Microorganisms were classified according to the National Mastitis Council (NMC, 1999) based on phenotypic tests. Samples with more than three different colonies types were considered contaminated and discharged (ADKINS et al., 2017).

Statistical analysis

The data were used to produce frequency distributions of the farms and pathogens, along with tabulation of the days in milk (DIM) and severity-of clinical mastitis. These parameters were then used with the Chi-square test for cross-references between: 1) severity of mastitis and type of pathogen isolated; 2) severity of mastitis and DIM; and 3) DIM and type of pathogen isolated.

For analytic purposes, the pathogens were grouped into: 1) Catalase negative cocci: *Enterococcus* (*E.*) *durans*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. mundtii*, *E. saccharolyticus*, *Lactococcus* (*L.*) *garvieae*, *L. lactis*, *Streptococcus* (*S.*) sp., *S. dysgalactiae* and *S. uberis*; 2) Coliforms: *Citrobacter* sp., *Enterobacter* sp., *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*; 3) Non-aureus *Staphylococcus* (NAS): *Staphylococcus* coagulase-negative and *Staphylococcus* coagulase-positive; 4) Others: *Streptomyces* spp., *Actinomyces* spp., *Hafnia* spp., *Micrococcus* sp., *Nocardia* sp., *Pasteurella multocida*, *Serratia* sp.; and 5) *Pseudomonas* (*P.*): *Pseudomonas* sp., *P. aeruginosa*; 6) *Bacillus* (*B.*) sp., *B. cereus*, *B. pumilus*.

The analyses were conducted with the SAS statistical program (SAS Institute, Cary, NC, USA), and P-values < 0.05 were considered significant.

RESULTS

A total of 4,273 milk samples from clinical bovine mastitis cases were cultured to identify mastitis-causing pathogens. *Streptococcus dysgalactiae* was the most common pathogen isolated (283/4,273 = 6.6%), followed by coagulase-negative *Staphylococcus* (238/4,273 = 5.6%), *Escherichia coli* (190/4,273 = 4.4%), *Prototheca* sp. (112/4,273 = 2.6%), *Streptococcus uberis* (95/4,273 = 2.2%), and *K. pneumoniae* (94/4,273 = 2.2%). All the pathogens isolated are grouped and summarized in table 1. The highest isolation was of catalase-negative cocci (499/4,273 = 11.7%) group, followed by coliforms (8.0%). In 48.8% (2,084/4,273) milk samples no growth was observed.

Among all cases, clinical severity was assessed in 43.8% (1,871/4,273) animals. From these 1,871 cases, 69.8% (1,306/1,871), 27.3% (510/1,871), and 2.9% (55/1,871) were clinically scored as mild, moderate, and severe, respectively. Considering specifically for coliforms isolates cases, we had mild (45.4%) and moderate (46.4%) severity clinical scores, while majority of catalase-negative cocci (*S. dysgalactiae* and *S. uberis*) (67.9%) and *Prototheca* sp. (81.8%) revealed mild clinical scores. In addition, mastitis caused specifically by *S. aureus* (72.2%) and *S. agalactiae* (91.7%) were associated mainly with mild clinical severity scores (Table 2).

Most of the mastitis cases that occurred in the first 100 days in milk were scored as mild clinical severity (3,612/4,273 = 84.5%), followed by severe (373/4,273 = 8.7%), and moderate cases (291/4,273 = 6.8%). The distribution of the pathogens recovered in the three different lactation phases revealed that the higher frequency of isolation of pathogens occurred in the 100 initial days in milk and declined thereafter (Table 3).

DISCUSSION

Mastitis is an infectious disease that is characterized as causing the greatest losses to dairy farmers and health problems of herds worldwide. Due to diversity of the causal agents of bovine clinical mastitis, the microbiological identification and antimicrobial susceptibility testing enables adopt prophylactic measures, as well as rational therapeutic approaches to control the mammary

Table 1 - Microorganisms isolated from cases of bovine clinical mastitis in ten large-dairy farms from Brazil.

	-----Frequency-----	
	n	%
Cultured pathogens		
Negative	2084	48.8
Contaminated	649	15.2
Catalase-negative cocci ¹ (e.g., <i>S. dysgalactiae</i> , <i>S. uberis</i>)	499	11.7
Coliforms ² (e.g., <i>E. coli</i> , <i>K. pneumoniae</i>)	341	8.0
NAS ³	245	5.7
<i>Prototheca</i> spp.	112	2.6
Yeast	64	1.5
Others ⁴	58	1.4
<i>Trueperella pyogenes</i>	47	1.1
<i>Pseudomonas</i> spp.	30	0.7
<i>Staphylococcus aureus</i>	30	0.7
<i>Bacillus</i> spp.	28	0.6
Unidentified gram-negative rod	26	0.6
<i>Streptococcus agalactiae</i>	22	0.5
<i>Corynebacterium</i> spp.	20	0.5
Fungi	18	0.4
Total	4273	100

¹Catalase-negative cocci: *Enterococcus* (*E.*) *durans* (n = 1), *E. faecalis* (n = 18), *E. faecium* (n = 13), *E. hirae* (n = 1), *E. mundtii* (n = 1), *E. saccharolyticus* (n = 58), *Lactococcus* (*L.*) *garvieae* (n = 5), *L. lactis* (n = 10), *Streptococcus* (*S.*) sp. (n = 14), *Streptococcus dysgalactiae* (n = 283) and *S. uberis* (n = 95);

²Coliforms: *Citrobacter* sp. (n = 12), *Enterobacter* sp. (n = 32), *Escherichia coli* (n = 190), *Klebsiella oxytoca* (n = 13), and *Klebsiella pneumoniae* (n = 94);

³Non-aureus staphylococci coagulase-negative (n = 238) and *Staphylococcus* coagulase-positive (n = 7);

⁴Others: *Streptomyces* sp. (n = 3), *Actinomyces* sp. (n = 4), *Hafnia* sp. (n = 18), *Micrococcus* sp. (n = 1), *Nocardia* sp. (n = 7), *Pasteurella multocida* (n = 14), and *Serratia* sp. (n = 11).

infections (ANDREWS et al., 2019). Nonetheless, a variety of microorganism has been identified in bovine mastitis related infections (WATTS, 1988), with geographical differences among prevalence of the pathogens worldwide (SWINKELS et al., 2013; RUEGG, 2022). In addition, the presence of a natural microbial community within the healthy mammary gland (milk microbiota) has been proposed (HUNT et al., 2011; CABRERA-RUBIO et al., 2012; OIKONOMOU et al., 2012; OIKONOMOU et al., 2014; ADDIS et al., 2012) relatively different of the main microorganisms-related to mammary infections (GANDA et al., 2016; JAMALI et al., 2018) which may be considered a challenge to mammary glands studies.

Despite the predominance of catalase-negative cocci, coliforms, and NAS groups of the pathogens, a great diversity of bacterial species was identified among cows sampled with bovine clinical mastitis, reinforcing the polymicrobial nature of bovine clinical mammary infections. *Streptococcus dysgalactiae* (6.6%), *Staphylococcus* spp. (5.6%),

Escherichia coli (4.4%), *Prototheca* spp. (2.6%), and *Streptococcus uberis* (2.2%) were the prevalent groups/species of the microorganisms isolated among cows sampled. In fact, enterobacteriaceae, staphylococci, and streptococci have been reported as the most frequent groups of the pathogens isolated in milk samples from dairy cattle with mastitis, responsible for the majority of bovine clinical and subclinical intramammary infections (RUEGG, 2017). On a global level, *S. aureus*, *S. dysgalactiae*, *E. coli* and *S. uberis* were reported as the most relevant etiological agents involving in bovine clinical mastitis in the past 10-15 years (SWINKELS et al., 2013).

Bovine mastitis agents have been traditionally classified as environmental and contagious pathogens based on habitat of the organisms and routes of the infections. The environmental mammary infections are caused by agents from the surrounding areas of milking environment (e.g., enterobacteria, fungi, yeasts, some streptococci, *Prototheca*, and actinomycetes species). In turn, the contagious mastitis agents

Table 2 - Microbiological identification and severity scores in cows' clinical mastitis.

	-----Mastitis severity score-----					
	-----1*-----		-----2*-----		-----3*-----	
Pathogens	n	%	N	%	n	%
Negative	591	71.7	212	25.7	21	2.6
Catalase-negative cocci ¹ (e.g., <i>S. dysgalactiae</i> , <i>S. uberis</i>)	188	67.9	83	30.0	6	2.1
Coliforms ² (e.g., <i>E. coli</i> , <i>K. pneumoniae</i>)	89	45.4	91	46.4	16	8.2
NAS ³	76	79.2	17	17.7	3	3.1
<i>Prototheca</i> sp.	54	81.8	11	16.7	1	1.5
Yeast	22	66.7	8	24.2	3	9.1
Others ⁴	21	77.8	6	22.2	0	0.0
<i>Trueperella pyogenes</i>	23	69.7	10	30.3	0	0.0
<i>Pseudomonas</i> sp. ⁵	22	78.6	6	21.4	0	0.0
<i>Staphylococcus aureus</i>	13	72.2	4	22.2	1	5.6
<i>Bacillus</i> sp. ⁶	12	70.6	4	23.5	1	5.9
Unidentified gram-negative rod	10	76.9	3	23.1	0	0.0
<i>Streptococcus agalactiae</i>	11	91.7	1	8.3	0	0.0
<i>Corynebacterium</i> sp.	3	42.9	3	42.9	1	14.2
Fungi	2	25.0	6	75.0	0	0.0
Total	1137		465		53	

* 1: Mild; 2: Moderate; 3: Severe.

¹Catalase-negative cocci: *Enterococcus* (*E.*) *durans*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. mundtii*, *E. saccharolyticus*, *Lactococcus* (*L.*) *garvieae*, *L. lactis*, *Streptococcus* (*S.*) sp., *Streptococcus dysgalactiae* and *S. uberis*;

²Coliforms: *Citrobacter* sp., *Enterobacter* sp., *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*;

³NAS (Non-aureus staphylococci): *Staphylococcus* coagulase-negative and *Staphylococcus* coagulase-positive;

⁴Others: *Streptomyces* sp., *Actinomyces* sp., *Hafnia* sp., *Micrococcus* sp., *Nocardia* sp., *Pasteurella multocida*, *Serratia* sp.

⁵*Pseudomonas*: *Pseudomonas* sp., *Pseudomonas aeruginosa*;

⁶*Bacillus*: *Bacillus* (*B.*) sp., *B. cereus*, *B. pumilus*.

(*Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium* and *Mycoplasma* species) usually spread from other mammary cows/quarters, and milk equipment (COBIRKA et al., 2020). Thus, the mastitic microbiota may be considered a complex community of microorganisms, which inhabit different sources, since mammary gland, teats of animals, milking equipment, and hands of milking workers, as well as water, feces, organic matter, bed of farm animals (RUEGG, 2017), and flies from the milking environment (BERTOLINI et al., 2022). This complexity of microorganisms poses as a challenge to the management of animals in relation to the health of the mammary gland and the prevention of mammary infections.

The incidence of bovine mastitis caused by pathogens from the environmental origin, such as coliforms and environmental streptococci, has increased in herds that have had success in control or eradicate mastitis of a contagious nature (RUEGG, 2017; RUEGG, 2022), including in Brazil (GUERRA et al., 2020). In this regard, 628 mastitic cases occurred

in the South (JOBIM et al., 2010) and 2,149 cases reported in the Southeast regions from Brazil (PAULA et al., 2015) were subjected to microbiological culture, and revealed that ~50% and 56% of all cases of clinical mastitis were caused by environmental pathogens, respectively, which is in line with the results of our study, since catalase-negative cocci, coliforms, NAS, and *Prototheca* were the prevalent groups/microorganisms isolated from cows sampled. The major isolation of the environmental agents among cows sampled is in consonance with other studies that also have revealed higher frequency of this group of pathogens in clinical mammary infections (SCHUKKEN et al., 2012, KLAAS & ZADOK, 2018). This finding could be attributed to a general good management and milking hygiene practices of dairy farms studied, particularly relative to contagious agents (RUEGG, 2017) despite the high prevalence yet of contagious pathogens in Brazilian dairy herds.

Streptococcus is a heterogeneous genus, and includes several species found in mammary glands of cows (GANDA et al., 2016) farm environment,

Table 3 - Microbiological identification and their distribution by days in milk of cows with clinical mastitis.

	-----DIM ¹ ≤ 100-----		----DIM ¹ 101-200----		-----DIM ¹ ≥ 201-----	
Pathogens	n	%	n	%	n	%
Negative	1780	85.4	124	6.0	180	8.6
Catalase-negative cocci (e.g., <i>S. dysgalactiae</i> , <i>S. uberis</i>)	388	77.8	49	9.8	62	12.4
Coliforms (e.g., <i>E. coli</i> , <i>K. pneumoniae</i>)	262	76.8	32	9.4	47	13.8
NAS ¹	208	84.9	15	6.1	22	9.0
<i>Prototheca</i> sp.	94	83.9	12	10.7	6	5.4
Yeast	50	78.1	6	9.4	8	12.5
Others	46	79.3	4	6.9	8	13.8
<i>Trueperella pyogenes</i>	36	76.6	5	10.6	6	12.8
<i>Pseudomonas</i> sp.	28	93.3	2	6.7	0	0.0
<i>Bacillus</i> sp.	24	85.7	1	3.6	3	10.7
<i>Staphylococcus aureus</i>	23	76.7	4	13.3	3	10.0
Unidentified gram-negative rod	23	88.5	3	11.5	0	0.0
<i>Streptococcus agalactiae</i>	19	86.4	1	4.5	2	9.1
<i>Corynebacterium</i> sp.	19	95.0	0	0.0	1	5.0
Fungi	15	83.3	1	5.6	2	11.1
Total	3015		259		350	

¹DIM: Days in milk.²NAS: Non-aureus staphylococci.

and milking equipment (HOGAN & SMITH, 2012; EL-SAYED et al., 2017). *Streptococcus* (e.g., *Streptococcus dysgalactiae*, *Streptococcus uberis*) and *Enterococcus* species are traditionally considered from the environmental origin as causative agents of mammary infections (PETERSSON-WOLFE et al., 2008); *S. dysgalactiae* has an environmental and contagious transmission pattern. *S. uberis* has been isolated from bedding stuffs such as silage, and green chop forages, which can contribute to the environmental and teat end contamination (HOGAN et al., 1989). *Streptococcus dysgalactiae* (6.6%) represented the major bacterial species isolated among cows sampled in the current study, highlighting the need for consider this species of streptococci as a relevant pathogen among clinical cases of bovine mastitis. Nonetheless, the usual routes of transmission of this pathogen as an environmental cause of bovine mastitis remains unclear, and improved knowledge regarding the transmission mechanisms of the agent are required to adopt effective prophylactic and control measures (EL-SAYED & KAMEL, 2021).

Streptococcus agalactiae was isolated in low frequency among cows sampled. This finding agrees with similar studies, due to this pathogen be more frequent in subclinical mammary infections (RUEGG, 2017; CHENG & HAN, 2020). In last

decades, an environmental cycle of *S. agalactiae* in dairy farms has been proposed, where the farm environment is considered a reservoir of the pathogen to the transmission to mammary glands of cows; a fact that may difficult the control measures of the pathogen in dairy herds (JØRGENSEN et al., 2016).

Coliforms represented 8% of the microorganisms isolated from cows sampled, particularly *E. coli* (190/4,273 = 4.4%) and *K. pneumoniae* (94/4,273 = 2.2%). *Escherichia coli* is a well-known species of enterobacteria found in intestinal tract of humans and animals (HOGAN & SMITH, 2012), soil, water, organic matter, vegetable, bed of the animals, or contaminating milk equipment (RUEGG, 2017), as well as flies that inhabit the milking environment of dairy farms (BERTOLINI et al., 2022). It is characterized by an opportunistic nature, a clonal diversity, and complexity of virulence factors related to enteric and extraenteric infections (DENAMUR et al., 2021), including in bovine mastitis cases (GUERRA et al., 2020). *E. coli*-related mammary infections have been seen mainly causing clinical cases, occurring in a short-time period (2-10 days), mainly in first weeks of lactation (SCHUKKEN et al., 2012), which are data consistent with results of the current study, where *E. coli* was prevalent in cows with clinical infections, mainly in

initial 100 days in milk. In the current study, 4.4% of sampled animals revealed isolation of *E. coli*, which is similar with a study also conducted in Brazil with bovine mastitic cows from 37 dairy farms from two states, where the pathogen was identified among 7.6% (51/670) milk samples (RANGEL & MARIN, 2009). This result reinforces the relevance of *E. coli* as a primary agent of clinical mammary infections in dairy herds and need for attention regarding control measures to environmental agents in farms studied, including proper herd management and milking hygiene practices, pre-milking teat dipping antiseptics, effective housing, feeding, milking and drying protocols, along with application of specific vaccine (EFFAH et al., 2020; EL-SAYED et al., 2021; TOMAZI et al., 2021).

Klebsiella species, particularly *K. pneumoniae*, are another pathogenic enterobacteria from environmental origin related to a set of clinical signs in humans (EFFAH et al., 2020) and domestic animals (RIBEIRO et al., 2022), including as a causative agent of bovine mammary infections (SCHUKKEN et al., 2012). Similar to *E. coli*, mammary infections associated with *Klebsiella* species have been seen as clinical infections in the first weeks of the lactation, a fact that also was observed in cows sampled in the current study, highlighting the impact of this enterobacteria in clinical mammary infections, and the importance to adopt prophylactic, control, and therapeutic measures in dairy herds against *Klebsiella* species. Despite the traditional division of bovine mastitis pathogens as contagious or environmental agents, a binary behavior has been noted mainly in *Klebsiella pneumoniae* and other environmental bacteria (e.g., *Streptococcus uberis* and *Streptococcus dysgalactiae*), which besides an environmental origin, have revealed a contagious behavior (i.e., persistent infections), a fact that may represent a new challenge to control and preventive approaches of bovine mastitis (KLAAS & ZADOKS, 2018).

Prototheca spp. are saprophytic, eucaryotic, unicellular microalgae that infect humans (KANO, 2019) and domestic animals, although bovine clinical mammary infections with chronic infection, represent the main manifestation of protothecosis in domestic species (JAGIELSKI et al., 2019a; JAGIELSKI et al., 2019b). A recent taxonomic reclassification of the algae in 14 species has been proposal based on mitochondrial *cytB* gene, where *P. bovis* and, less frequently, *P. blaschkeae*, are recognized as the main algae-related bovine mastitis species worldwide (JAGIELSKI et al., 2019b), including in Brazil (ALVES et al., 2017; FIDELIS

et al., 2021). Nonetheless, to date, no therapeutic protocol has been considered effectiveness to treat protothecal mammary infections (TASHAKKORI et al., 2022), which has motivated a set of *in vitro* studies evaluating the algacidal effect of different compounds, including antimicrobials, antifungal, sanitizer, natural extracts, essential oils, nanoparticles, and herbicides (MORANDI et al., 2016; ALVES et al., 2017; RIBEIRO, 2021). In the current study, 2.6% of cultured milk samples revealed isolation of the genus *Prototheca*, a fact that is concerning, due to absence of specific treatment approaches and, at this moment, the control (JAGIELSKI et al., 2019a; JAGIELSKI et al., 2019b) measures against this alga have been restrict to segregation, chemical dry of quarters, and culling of infected cows (JAGIELSKI et al., 2019a; JAGIELSKI et al., 2019b; RIBEIRO, 2021). General recommendations target the control of the environmental agents of bovine mastitis (e.g., *E. coli*, *K. pneumoniae*, environmental streptococci) includes proper management of stall bedding, lots and pastures, pre-milking teat dipping disinfection, offer food after milking, and proper milking practices and hygiene of surroundings areas (HOGAN & SMITH, 2012).

Staphylococci (*Staphylococcus aureus* and NAS) also were identified among mastitic milk samples in cows studied. This complex group of the pathogens usually are less frequent in clinical mammary infections because belongs to the normal microbiota of mucous membranes of domestic animals (QUINN et al., 2013), and the infected cows develop probably a relative adaptation into mucous of mammary gland, exhibiting usually subclinical infections. Unlike enterobacteria, *Prototheca* species, and some environmental streptococci, the prophylactic and control measures against *Staphylococcus*-related mammary infections are focused on management and hygiene milking procedures, and post-milking teat dipping antiseptics (RUEGG, 2017; CHENG & HAN, 2020). Nonetheless, *S. aureus* possess a variety of virulence mechanisms (CAMPOS et al., 2022) that allow this pathogen also developed clinical mammary infections as observed in cows sampled, usually refractory to conventional antimicrobial therapy, which require culling of the chronically infected animals (RUEGG, 2017; CHENG & HAN, 2020).

Trueperella pyogenes and corynebacteria belongs a complex group of pleomorphic microorganisms from Actinomycetales order, found in mucous membranes, skin, flies, and the environment of animals, which also are involved in clinical and subclinical mammary infections in cows (QUINN, 2023). Both organisms were isolated in

a minor frequency in the current study. However, *Trueperella pyogenes* usually causes chronic clinical mammary infections in cows that commonly has been refractory to the conventional therapy, probably due to development of pyogranulomatous reactions, requiring chemical dry of teats or culling of infected animals (RIBEIRO et al., 2015).

A miscellaneous of other microorganisms were identified among cows sampled, e.g., fungi, yeast, *Bacillus* spp., *Pseudomonas* spp., reinforcing the polymicrobial nature of clinical mammary infections in cattle.

Most cows sampled revealed mild clinical severity scores, which are restrict to milk abnormalities, followed by moderate scores that includes also signs in mammary glands (PINZÓN-SANCHEZ & RUEGG, 2011), whereas a low number of cows revealed severe clinical signs. Catalase-negative cocci (e.g., *S. dysgalactiae*, *S. uberis*), enterobacteria (e.g., *E. coli* and *K. pneumoniae*), and *Prototheca* spp. represented most frequent groups/species of microorganisms identified among cows sampled, a finding that was expected because these pathogens from environmental origin usually induces clinical signs in milk, mammary glands and, occasionally, systemic disorders (PINZÓN-SANCHEZ & RUEGG, 2011; SCHUKKEN et al., 2012; RUEGG, 2017; JAGIELSKI et al., 2019a; JAGIELSKI et al., 2019b; RIBEIRO et al., 2022).

Most of investigations regarding clinical severity scores of causal agents of bovine mastitis has focused on coliforms (PINZÓN-SANCHEZ & RUEGG, 2011; GUERRA et al., 2020), although, to date, no specific genetic profile has been identified, suggesting that in addition to virulence factors, peri- and post-partum nutritional conditions and immune status could be involved in clinical severity of mammary infections (SUOJALA et al., 2011).

In our study, the majority of isolations occurred at the initial 100 days of lactation. This result was expected, since animals are more likely to be infected by pathogens acquired in the first days in milk, probably related to a decrease of immune response to the pathogens and abnormalities in energy balance (HERTL et al., 2023).

A great variety of microorganisms were isolated in the current study among cows with clinical mastitis. This result highlighted the need for routine of microbiological culture of mastitic milk (laboratories or cultures in farms) to identify the pathogens, enabling adopt specific prophylactic and control measures to the environmental or contagious organisms, as well as a rational use of antimicrobial therapy. Nonetheless, microbiological culture,

biochemical identification of the causative agents, and *in vitro* antimicrobial sensitivity profiles are time-consuming, requiring a gradual inclusion of molecular tools to an accurate and rapid diagnosis (EL-SAYED et al., 2017).

A great number of mastitic milks resulted negative (48.8%) in microbiological culture. This result may be attributed to the culture of samples where the pathogen is not viable anymore (due to a previous immune response of mammary gland), or infections caused by non-conventional microorganisms involved in clinical bovine mastitis, e.g., *Brucella abortus*, *Mycobacterium bovis*, *Listeria monocytogenes*, *Mycoplasma* spp., which require selective media and special culture conditions to isolation (QUINN, 2023), in addition to the activity of antimicrobial components contained in the milk (TSUGAMI et al., 2017), as well as the possibility of intermittent release of some agents, such as *Staphylococcus aureus*, requiring the collection of consecutive samples for microbiological diagnosis (SEARS et al., 1990).

A convenience sampling, no statistical analyses of all data related to milking systems versus the pathogens isolated (except days in milk), and no species identification by molecular methods may be considered limitations of the current study.

CONCLUSION

Overall, we observed a wide variety of the microorganisms isolated from cows with clinical mastitis of ten large-dairy farms from Brazil, reinforcing the polymicrobial nature of clinical mammary infections in cattle; besides a predominance of pathogens from the environmental origin, i.e., catalase-negative cocci, enterobacteria, and *Prototheca* sp. In addition, the routine microbiological culture and identification of mastitic pathogens allow recommend specific prophylactic/control measures against agents from environmental or contagious origin identified in farms studied. Most of animals revealed mild and moderate clinical score of severity, whose infections occurred mainly in the first 100 days in milk, as a probable reflex of the profile of the pathogens isolated in farms studied. This study contributed to the etiological identification, clinical severity scoring, and milking aspects of bovine clinical mastitis in dairy farms with a history of clinical mammary infections.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflicts of interest.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

This study was carried out in accordance with the Comitê de Ética no Uso de Animais (CEUA) guidelines of the Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil (permit N° 136/2017-CEUA). The approval date was June 08, 2017.

AUTHORS' CONTRIBUTIONS

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

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