

Article

Milk microbiota of clinical mastitic cows: an etiological approach

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Simple Summary: Bovine mastitis is a major disease that causes significant losses to dairy farms worldwide. It is caused by a complexity of microorganisms transmitted from cows from contagious or environmental origin, which have showed distinct clinical severity and wide geographical differences among regions and countries. The identification of the microorganism allows dairy farmers to adopt specific control and therapeutic measures. Here, we performed a large-scale study aimed to identify the pathogens involved in 4,273 clinical cases of bovine mastitis in 10 large-dairy farms in Brazilian conditions, with a history of clinical mammary infections, where days in milk, and clinical severity scores of cases were assessed. The main microorganisms identified were of environmental origin (enterobacteria, algae, and environmental streptococci), which also revealed major severity clinical scores, followed by those of contagious origin; enabling recommend specific management and milking measures to prophylaxis/control of the disease, and a rational use of antimicrobials to therapy approaches.

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. In addition, 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 nature, clinical severity scores, and days in milk (DIM) data in 4,273 clinical cases of bovine mastitis among ten large-dairy farms located in the Southeast region of 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, all from the environmental origin. Among 4,273 clinical cases, clinical gravity 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. Most of isolation of pathogens were observed in the first 100 days in milk, and their clinical severity scored as mild (3,612/4,273=84.5%). Our results contribute to the etiological identification, clinical severity scoring, and milking aspects of bovine clinical mastitis in dairy farms with a history of clinical mammary infections.

Keywords: bovine mastitis; milk pathogens; environmental agents; microbiological diagnosis; clinical severity scores; days in milk

1. 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 caused by a great complexity of microorganisms [1].

More than 140 species of microorganism have been identified in milk samples of cows with clinical and subclinical mastitis using conventional microbiological culture, phenotypic tests, [2], and molecular methods [3].

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 its derivatives [4]. 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 globally, being responsible for most cases of clinical and subclinical intramammary infections [5–7].

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 [8].

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 [9]

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) [10].

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 the routine approaches to identify the microorganisms causing the disease [7,11]. 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 varied possible sources of microbial DNA, including from the environment, cows, and milk equipment [3].

Considering the significant 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 10 large-dairy in Brazilian conditions, with a history of mammary infections, where DIM and clinical severity of cases were assessed.

2. Materials and Methods

2.1. 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) 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 [12], 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. 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.

2.2. Milk sampling strategy and clinical diagnosis

The collection of milk samples from cows for diagnosis of clinical mastitis was performed by farmworkers. These employees were submitted to ongoing training to correctly identify clinical cases, severity scores, and collect samples aseptically [13].

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) [10].

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 until sending to the laboratory. Along with the samples, data of clinical severity scores of cases (scores 1, 2 and 3), the main data of milking systems with emphasis in days in lactation, and data regarding the previous treatment were assessed.

2.3. 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. The milk samples were simultaneously plated on MacConkey agar and defibrinated sheep blood agar (5%) under aerobic conditions at 37°C for 72 hours. Microorganisms were classified according to the National Mastitis Council (NMC). Intramammary infection was defined as at least 3 cfu identical colonies. Samples with more than three different colonies types were considered contaminated and discharged [14].

2.4. 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 gravity of mastitis. These parameters were then used for cross-references between: 1) gravity of mastitis and type of pathogen isolated; 2) gravity 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.*, *Streptococcus 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*: *Pseudomonas* sp., *Pseudomonas aeruginosa*; 6) *Bacillus*: *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.

3. 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%), and *Streptococcus uberis* (95/4,273=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.

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

Cultured Pathogens	Frequency	
	n	%
Negative	2084	48.8
Contaminated	649	15.2
Catalase-negative cocci ¹	499	11.7
Coliforms ²	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

¹ Catalase-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);

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

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

⁴ Others: *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).

Among 4,273 cases, clinical gravity 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.

Major clinical severity (scores 2 and 3) was observed among fungi, coliforms, *Trueperella pyogenes*, catalase-negative cocci, and yeasts isolates (Table 2).

Most of coliforms isolates revealed moderate scores (91/196=46.4%) ($p < 0.05$). The mastitis cases caused by *S. aureus* (13/18=72.2%) and *S. agalactiae* (11/12=92.7%), revealed mainly mild clinical severity score.

Table 2. Microbiological identification and severity scores in clinical mammary infections of cows.

Pathogens	Mastitis severity score					
	1 *		2 *		3 *	
	n	%	N	%	n	%
Negative	591	71.7	212	25.7	21	2.6
Catalase-negative cocci ¹	188	67.9	83	30.0	6	2.1
Coliforms ²	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	

* Mild; 2: Moderate; 3: Severe.

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., *Streptococcus dysgalactiae* and *S. uberis*;

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

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

⁴ Others: *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*.

The majority of the mastitis cases that occurred in the first 100 days in milk were scored as mild (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).

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

Pathogens	DIM ¹ ≤ 100 days		DIM ¹ 101 – 200 days		DIM ¹ ≥ 201 days	
	n	%	n	%	n	%
Negative	1780	85.4	124	6.0	180	8.6
Catalase-negative cocci	388	77.8	49	9.8	62	12.4
Coliforms	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>Staphylococcus aureus</i>	23	76.7	4	13.3	3	10.0

<i>Bacillus</i> sp.	24	85.7	1	3.6	3	10.7
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	

¹ DIM: Days in milk.

² NAS: Non-aureus *Staphylococcus*.

4. Discussion

Mastitis is an infectious disease that is characterized as causing the greatest losses to dairy farmers and health problems of herds worldwide. Efforts to control of this disease includes microbiological identification of the causal agents, which enable adopt prophylactic and control measures, as well as rational antimicrobial therapeutic approaches [15]. Nonetheless, a variety of microorganism has been identified in bovine mastitis related-infections [2], with geographical differences among prevalence of the pathogens worldwide. In addition, has recently been proposed the presence of a natural microbial community within the mammary gland (milk microbiota) [16–21], different from organisms-related mastitis [22,23], which may be considered the main motivation of the current study.

Despite the predominance of catalase-negative cocci, coliforms, and NAS groups of the pathogens, a great diversity of microorganisms, particularly bacterial species, followed by a minor isolation of yeast and fungi organisms, were 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 pathogens isolated in milk samples from dairy cattle with mastitis, responsible for the majority of bovine clinical and subclinical intramammary infections [24]. 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 [25].

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 (*Staphylococcus*, *Streptococcus*, *Corynebacterium* and *Mycoplasma* species) usually spread from other mammary cows/quarters, and milk equipment [8]. Thus, the milk microbiome 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 animals [24], and flies from the milking environment [26]. 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 to control or eradicate mastitis of a contagious nature [24,27], including in Brazil [13]. In this regard, 628 mastitic cases occurred in the South [16] and 2,149 cases reported in the Southeast regions from Brazil [17] 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 that catalase-

negative cocci, coliforms, NAS, and *Prototheca* were the prevalent groups of pathogens isolated from cows sampled. The major isolation of the environmental agents among cows sampled is in consonance with other studies that have revealed higher frequency of this group of pathogens in clinical mammary infections [28,29], and could be attributed to a general good management and milking hygiene practices of dairy farms studied, particularly relative to contagious agents [24] 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 [22] farm environment, and milking equipment [30,31]. Among this group, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Enterococcus* spp. are traditionally considered from the environmental origin as causative agents of mammary infections [32]. *S. uberis* has been isolated from bedding stuffs such as silage, and green chop forages, in addition to reproductive tract infections that can contribute to the environmental and teat end contamination [33]. *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 in 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 this pathogen are required to adopt effective prophylactic and control measures [34].

Streptococcus agalactiae was isolated in a minor frequency in the current study, a finding that agree with other authors, due to this pathogen be more frequently involved in subclinical mammary infections [24,35]. Nonetheless, in last decades, an environmental cycle of the microorganism 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, instead exclusively as a contagious agent; a fact that may difficult the control of *Streptococcus agalactiae* in dairy herds [36]. General recommendations target the control of the environmental agents of bovine mastitis includes proper management of stall bedding, lots and pastures, pre-milking teat dipping disinfection, offer food after milking, and milking hygiene and surroundings areas [30].

Coliforms represented other group of bacteria with high frequency in cows sampled. *Escherichia coli* is a well-known species of enterobacteria found in intestinal tract of humans and animals [30] soil, water, organic matter, vegetable, bed of the animals, or contaminating milk equipment [24], as well as flies that inhabit the milking environment of dairy farms [26]. It is characterized by an opportunistic nature, a clonal diversity, and complexity of virulence factors related to enteric and extraenteric infections [37], including in bovine mastitis cases [13]. *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 [28], 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. 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, pre-milking teat dipping antiseptics, effective housing, feeding, milking and drying protocols, along with application of specific vaccine [34,38,39].

Klebsiella species, particularly *K. pneumoniae*, are another pathogenic enterobacteria from environmental origin related to a set of clinical signs in humans [38] and domestic animals [40], including as a causative agent of bovine mammary infections from the environmental origin [28]. 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 agalactiae*), 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 [29].

Prototheca spp. are saprophytic, eucaryotic, unicellular microalgae that infect humans [41] and domestic animals, although bovine clinical mammary infections with chronic evolution, represent the main manifestation of protothecosis in domestic species [42,43]. A recent taxonomic reclassification of the algae in 14 species has been proposed based on mitochondrial *cytB* gene, where *P. bovis* and, less frequently, *P. blaschkeae*, are recognized as the main algae-related bovine mastitis species worldwide [43], including in Brazil [44,45]. Nonetheless, to date, none therapeutic protocol has been considered effectiveness to treat protothecal mammary infections [46], 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 [44,47,48]. 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 [42,43] measures against this alga have been restricted to segregation, chemical dry of quarters, and culling of infected cows [42,43,48].

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 [49], and the infected cows develop probably a relative adaptation into mucous of mammary gland, exhibiting commonly 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 [24,35]. Nonetheless, *S. aureus* possess a variety of virulence mechanisms [50] that allow this pathogen also develop clinical mammary infections as observed in cows sampled, usually refractory to conventional antimicrobial therapy, which require culling of the chronically infected animals [24,35].

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 [49]. 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 [51].

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 restricted to milk abnormalities, followed by moderate gravity scores that includes also signs in mammary glands [10]. Enterobacteria (*E. coli* and *K. pneumoniae*), environmental streptococci, *Prototheca* spp., and *T. pyogenes* represented the main groups/species of microorganisms, a finding that were expected because these pathogens from environmental origin usually induces clinical signs in milk, mammary glands and, occasionally, systemic disorders [10,24,28,40,42,43].

Most of investigations regarding clinical gravity scores of causal agents of bovine mastitis has focused on coliforms [10,13], 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 [52].

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 [53].

A great variety of microorganisms were isolated in the current study among cows with clinical mastitis. This result highlights 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 gradual inclusion of molecular tools to an accurate and rapid diagnosis [31].

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.*, *Bruceella abortus*, *Mycobacterium bovis*, *Listeria monocytogenes*, *Mycoplasma spp.*, which require selective media and special culture conditions to isolation [49], in addition to the activity of antimicrobial components contained in the milk [54].

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.

5. Conclusions

Overall, we observed a wide variety of the microorganisms isolated of cows with clinical mastitis of ten large-dairy farms from Brazil, besides a predominance of pathogens from the environmental origin, *i.e.*, catalase-negative cocci, coliforms, and NAS groups, reinforcing the polymicrobial nature of clinical mammary infections in cattle. In addition, the routine microbiological culture and identification of mastitic pathogens allow adopt specific prophylactic and control measures, as well as rational therapeutic approaches against agents. 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. Our results contribute 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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Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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