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Seasonality of the conjunctival microbiota in cattle from a microregion in midwestern Brazil

Sazonalidade da microbiota conjuntival do gado bovino de uma microrregião do Centro-Oeste do Brasil

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Highlights

To describe the conjunctival microbiota in cattle in a microregion of the *Cerrado.*
Bacillus cereus was the most prevalent agent in both seasons and cattle categories.
M. bovoculi was found only during the rainy season in both cattle categories.
The prevalence of the fungal microbiota (11.8%) was lower than previously reported.

Abstract

This study aimed to identify the bacterial and fungal organisms in the conjunctival fornix of healthy cattle in a microregion located in midwestern Brazil. Additionally, we evaluated whether seasonality and cattle category (dairy *vs.* beef) play a role in our findings. The conjunctival fornix of 74 Holstein x Gyr and 70 Nelore cattle were sampled from February to March (rainy season) and August to September (dry season) of 2017. Bacterial and fungal strains were isolated by classical bacteriological and mycological methods. Some bacterial species were also identified by polymerase chain reaction. From the 144 animals included in the study, a total of 12 bacterial genera were identified, with *Bacillus cereus* being the most prevalent agent in both seasons and cattle categories. The total number of bacteria was not influenced by seasonality or cattle category (P = 0.25). The number of *Moraxella bovoculi* identified in dairy (5/74) and beef cattle (7/70) did not differ statistically (P = 0.76). However, seasonality played a role in *M. bovoculi* identification, being found only during the rainy season in the dairy cattle (P = 0.01), and only during the dry season in the beef cattle (P = 0.04). During the dry season, the number of gram-positive bacteria was significantly higher than the number of gram-negative bacteria in beef cattle (P = 0.009). Fungi were isolated in 20/144 samples. The number of fungi isolated in dairy cattle (16/144) was significantly higher than that isolated in beef cattle (4/144) (P = 0.007). Although a higher number

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of fungi were isolated in both cattle categories during the rainy season, this finding was not significant (P = 0.53). In this microregion located in midwestern Brazil, *Bacillus cereus* was the most prevalent and *Corynebacterium* spp. and Streptococcus spp. the least prevalent bacteria found in the conjunctival fornix of healthy cattle. The prevalence of the conjunctival fungal microbiota (11.8%) was lower than previously reported, with a higher number of isolates found in dairy cattle. *Trichosporon* spp. (41.2%) and *Aspergillus* spp. (35.3%) were the most frequent genera, being most commonly isolated in the rainy season.
Key words: Bovine infections keratoconjunctivitis. Dairy cattle. Beef cattle. Season. Eye.

Resumo

Objetivou-se identificar organismos bacterianos e fúngicos no fórnice conjuntival de bovinos saudáveis em uma microrregião localizada no Centro-Oeste do Brasil. Ademais, foi avaliado se a sazonalidade e a categoria do gado (leite vs. corte) influenciariam nos resultados. Colheram-se amostras do fórnice conjuntival de 74 Holandês x Gir e de 70 Nelores de fevereiro a março (estação chuvosa) e de agosto a setembro (estação seca) do ano de 2017. Cepas bacterianas e fúngicas foram isoladas e identificadas por métodos bacteriológicos e micológicos convencionais. Algumas espécies de bactérias foram identificadas pela reação em cadeia da polimerase. Dos 144 animais incluídos no estudo, um total de 12 gêneros bacterianos foram identificados, sendo *Bacillus cereus* o agente mais prevalente em ambas as estações e categorias de gado. O número total de bactérias não foi influenciado pela sazonalidade e pela categoria do gado (P = 0.25). O número de *Moraxella bovoculi* identificadas no gado de leite (5/74) e de corte (7/70) não diferiram (P = 0.76). Porém, a sazonalidade teve impacto na identificação da *M. bovoculli*, sendo encontrada apenas na estação chuvosa no gado de leite (P = 0.01) e seca no gado de corte (P = 0.04). Durante a estação seca, o número de bactérias gram-positivas foi maior que o de gram-negativas no gado de corte (P = 0.009). Fungos foram isolados em 20/144 amostras. O número de fungos isolados no gado de leite (16/144) foi significativamente maior que os isolados no gado de corte (4/144) (P = 0.007). Embora maior quantidade de fungos tenha sido isolada durante a estação chuvosa em ambas as categorias de gado, esse achado não foi significativo (P = 0.53). Nessa microrregião localizada no Centro-Oeste do Brasil, o *Bacillus cereus* foi a bactéria mais prevalente encontrada no fórnice conjuntival de bovinos saudáveis, enquanto que *Corynebacterium* spp. e *Streptococcus* spp. foram as menos prevalentes. *Trichosporon* spp. (41.2%) e *Aspergillus* spp. (35.3%) foram os gêneros mais frequentes, sendo mais comumente isolados na estação chuvosa.
Palavras-chave: Ceratoconjuntivite infecciosa bovina. Gado de leite. Gado de corte. Estação. Olho.

Introduction

The normal ocular surface is home to a wide range of both commensal and transient bacterial and fungal populations. Individual species identified vary depending on the host species, age, geography, climate, season, local environment, and sampling/culture techniques (Bonelli et al., 2014). These noninvasive organisms are thought to play an important homeostatic role by competing with pathogenic species for space and nutrients to limit their ability to colonize the ocular surface (Gould et al., 2021).

In certain circumstances, nonpathogenic bacteria or fungi may become pathogenic, for instance, in an immunocompromised host, after breach of an epithelial surface, or via coinfection with another agent. In the latter instance, organisms that are individually incapable of inducing disease can in combination exert a cumulative or synergistic effect that leads to clinical disease (Bonelli et al., 2014; Gould et al., 2021). Bacteria can be cultured from the conjunctival sac of approximately 87% of healthy cows. In this species, the predominant gram-positive isolates are *Corynebacterium* sp., *Streptococcus* sp., *Staphylococcus* sp., and *Bacillus* sp., whereas the predominant gram-negative isolates are nonhemolytic *Moraxella* sp., *Mycoplasma* sp., *Branhamella* sp., *Neisseria* sp., *Acinetobacter* sp., coliforms, and *Actinobacillus* sp. (Kojouri et al., 2007; Gould et al., 2021). Fungal cultures may be isolated from the conjunctival sac of healthy cows in 55 to 100% of cases, most commonly being found *Cladosporium* sp., *Penicillium* sp., and *Aspergillus* sp. (Samuelson et al., 1984; Sgorbini et al., 2010).

From the commensal bacteria isolated from the conjunctival microbiota, *Moraxella* spp. are the most important agent, once it can cause Infectious Bovine Keratoconjunctivits (IBK) with a considerable economic impact (Angelos, 2015; Martins, 2021). However, other pathogens from the conjunctival microbiota and the environment can be responsible for contaminating the corneal ulcerate site leading to corneal melting, corneal perforation, severe uveitis, vision loss, and ultimately loss of the affected eye (Angelos, 2015; Martins, 2021). Most previous studies that evaluated the conjunctival bacterial microbiota of cattle have only focused on isolating *Moraxella* spp. in samples from field outbreaks. In addition, those studies have been conducted in North America, Europe, Asia, Uruguay, and southern Brazil where the climate is temperate or cold (Libardoni et al., 2012; O'Connor et al., 2012; Sosa & Zunino, 2013; Loy & Brodersen, 2014; Schnee et al., 2015; Maboni et al., 2015; Ely et al., 2019; Comin et al., 2020; Angelos et al., 2021; Ivanov et al., 2021). Likewise, the aforementioned considerations are valid with regard to the fungal microbiota (Samuelson et al., 1984; Sgorbini et al., 2010). In the midwestern Brazil there are no studies of the conjunctival microbiota of cattle with healthy eyes. Therefore, one of the aims of the present study was to survey and identify the bacterial and fungal organisms in the conjunctival fornix of healthy cattle eyes in a microregion of the *Cerrado* of Mato Grosso, Brazil, where the climate is considered to be of the savanna type. Considering that in cattle, changes in the ocular microbiota have been reported over time (Bartenslager et al., 2021), this study also aimed to identify whether seasonality and cattle category (dairy vs. beef) play a role in our findings.

Material and Methods

Animals and sample collection

The animals used in this study belonged to two different farms located at Santo Antônio do Leverger, in Mato Grosso state, Brazil. One hundred and forty-four animals were selected from two different farms. In one farm, 74 mixed Holstein x Gyr cows (dairy cattle) were selected. In another one, 70 animals of the Nelore breed (beef cattle) were available. The ages of the animals ranged from 1 to 6 years on both farms. To be enrolled in the study all the animals should be considered healthy based on a general and anterior segment ophthalmic examination. For this purpose, the adnexa and anterior segment of both eyes were examined with a binocular magnifying loupe and a transilluminator 3.5 V (Heine, Germany). The evaluation was conducted indoors commencing with distant examination of globe position, size, motility, and the presence of blepharospasm or ocular discharge. Menace response, palpebral reflex, dazzle reflex, and direct and consensual pupillary light reflexes were assessed in both eyes. Fluorescein dye test was also performed (Fluorescein strips; Ophthalmos Ltda, São Paulo, SP, Brazil). Individuals who presented any ophthalmic or general abnormality during the clinical triage listed above were excluded. Cows with visible milk alterations or positive results in the California Mastitis Test were excluded. Additionally, a washout period of 15 days was used as an inclusion criterion for animals treated with antimicrobials for any reason.

Samples were taken during the months of February to March (rainy season), and August to September (dry season) of 2017. On both farms, the animals selected in each season were the same. Conjunctival specimens were obtained randomly from one of the eyes without topical anesthesia, always by the same operator. During the collection, the selected eye was retropulsed through the closed upper eyelid and a sterile swab was run along the surface of the ventral conjunctival fornix. Special care was taken to avoid touching the vibrissae, eyelids, or eyelashes.

Bacteriological culture

Cultures were always obtained by the same operator. Conjunctival samples were plated in Columbia agar with 5% sheep blood and MacConkey agar. The plates were incubated aerobically at 37±1°C for 24 to 72 h. The samples were considered positive when one or more colonies were observed (≥100 CFU/mL). Bacteria were identified using morphology, gram stain, and standard veterinary microbiological techniques and identified to the genus, and, whenever was possible, to the species level by using classical methods or polymerase chain reaction (PCR) (Lopes et al., 2022).

For DNA amplification of *Moraxella* by PCR, the primers employed were ISRup (5'-ACCGACGCTTATCGCAGGTCACTA-3') and ISRdown (5'-GTG TCGAAGCAAAATCAGGGTCGT-3') for amplification of the 16S-23S intergenic region, with fragments of 650 bp for *M. bovis* and 600 bp for *M. bovoculi* and *M. ovis,* and enzymatic restriction with 1U of the enzyme RsaI, for the distinction of Moraxella species (Libardoni et al., 2012; Maboni et al., 2015; Ely et al., 2019).

Fungal culture

Cultures were always obtained by the same operator. Samples were plated onto Sabouraud Dextrose Agar and malt extract agar. The plates were incubated at 25 oC and examined daily from day 4 post incubation over a 21-day period. Only fungal genera were identified.

Statistical analysis

Prism version 7.04 (GraphPad Software, Inc., La Jolla, California, USA) was used for all the analyses. The number of colonies and the most prevalent type of cell wall structure found during both seasons and cattle categories were compared. For all the analyses, Fisher´s exact or Chi-Square tests were used, and statistical significance was set at $P < 0.05$.

Results and Discussion

Bacterial genera isolated from the conjunctival fornix

This was the first survey that described the conjunctival microbiota in cattle in a microregion of the *Cerrado*, Brazil. From the 144 animals included in the study, a total of 12 bacterial genera were identified, with *Bacillus cereus* being the most prevalent agent in both seasons and cattle categories (Table 1). As reported previously, *Bacillus cereus* was the most prevalent agent found in the conjunctival fornix of dairy cattle and in American Bisons (Davidson et al., 1999; Kojouri et al., 2007). In contrast, *Corynebacterium* spp. showed a lower prevalence in the present study (1.14%) when compared to another study conducted in Holstein cows (38.9%) (Kojouri et al., 2007). Additionally, in both cattle categories evaluated in the present study, *Corynebacterium* spp. was isolated only in the dry season (Tables 1, 2). Although in both cattle categories a higher number of bacteria was identified during the dry season (184) than in the rainy season (124), the total number of bacteria was not influenced by seasonality and category ($P = 0.25$) (Tables 1, 2). Regarding gram staining, during the dry season, the number of gram-positive agents was significantly higher than the number of gram-negative agents only in beef cattle ($P = 0.009$) (Tables 1, 2). In the current study, we did not consider age as a factor for statistical analysis. In fact, such an observation might not have a statistical impact on our findings, as two studies using high-throughput sequencing technology demonstrated that the ocular microbiota of cattle of different ages are similar (Cullen et al., 2017; Bartenslager et al., 2021). Although IBK is the main corneal disease that affects cattle, the results of the present study are still important because cases of traumatic corneal ulcer contaminated with pathogens other than *Moraxella* spp. may occur in individuals with high zootechnical value, who are usually isolated and treated.

Table 1
Bacteria identified in the conjunctival fornix of dairy cattle during the rainy and dry seasons

Bacteria	Rainy season Number (%)	Dry season Number (%)	Total
Bacillus cereus	24 (34.79)	35 (36.08)	59
Citrobacter sp.	9 (13.04)	11 (11.34)	20
Corynebacterium sp.	-	1 (1.03)	1
Enterobacter sp.	18 (26.10)	7 (7.21)	25
Escherichia coli	4 (5.79)	6 (6.19)	10
Klebsiella sp.	-	5 (5.15)	5
Micrococcus sp.	-	1 (1.03)	1
Moraxella bovoculi	5 (7.24)	-	5
Proteus mirabillis			-
Proteus vulgaris			-
Pseudomonas sp.	9 (13.04)	11 (11.34)	20
Staphylococcus sp.	-	19 (19.60)	19
Streptococcus sp.	-	1 (1.03)	1
Total (%)	69 (100)	97 (100)	166

Table 2
Bacteria identified in the conjunctival fornix of beef cattle during the rainy and dry seasons

Bacteria	Rainy season Number (%)	Dry season Number (%)	Total
Bacillus cereus	12 (21.80)	38 (43.67)	50
Citrobacter sp.	3 (5.46)	5 (5.74)	8
Corynebacterium sp.	-	1 (1.14)	1
Enterobacter sp.	3 (5.46)	1 (1.14)	4
Escherichia coli	18 (32.72)	9 (10.34)	27
Klebsiella sp.	-	-	-
Micrococcus sp.	-	-	-
Moraxella bovoculi	-	7 (8.04)	7
Proteus mirabillis	4 (7.28)	-	4
Proteus vulgaris	3 (5.46)	-	3
Pseudomonas sp.	4 (7.28)	14 (16.09)	18
Staphylococcus sp.	8 (14.54)	11 (12,64)	19
Streptococcus sp.	-	1 (1.14)	1
Total (%)	55 (100)	87 (100)	142

Moraxella bovoculi isolated from the conjunctival fornix

The number of *M. bovoculi* identified between the cattle categories did not differ statistically (P = 0.76) (Tables 1, 2). However, seasonality had a statistical impact, with a higher number of *M. bovoculi* identified during the rainy season in the dairy cattle (P = 0.01), and during the dry season in the beef cattle (P = 0.04) (Tables 1, 2). Two studies using high-throughput sequencing technology showed that *Moraxella* spp. as one of the top ten genera identified in the conjunctival fornix of healthy cattle (Cullen et al., 2017; Bartenslager et al., 2021). Several virulence factors homologous to those found in *M. bovis* have been found within *M. bovoculi* (Angelos, 2015; Loy et al., 2021). In fact, it has been reported that M. bovoculi was the only bacterium isolated from most individual animal cases of IBK over a four-year period (Angelos, 2015; Loy et al., 2021). Similar results have been reported in samples collected from IBK outbreaks that occurred in the southern region of Brazil (Libardoni et al., 2012; Maboni et al., 2015; Comin et al., 2020). In the present study, this genus of bacteria was not isolated by culture, which may have occurred because samples were not immediately plated on blood agar. Even in samples collected from outbreaks of IBK, *Moraxella* spp. is isolated by culture from approximately 11% of cases (Comin et al., 2020).

In one study that monitored 77 healthy eyes of Angus herd during a four-month period, *M. bovoculli* was identified in only 3.89% of the samples (O'Connor et al., 2012). This was supported by data from another study that showed that the genus *Moraxella* spp. was identified by high-throughput sequencing technology in only 3.29% of the samples collected from the conjunctiva of healthy calves (Cullen et al., 2017). The results reported herein detected a higher incidence of *M. bovoculli* (8.33%) in the general population of cattle during the seasons evaluated. Despite O'Connor et al. (2012) reported identifing *M. bovis* and *M. ovis* in the lacrimal fluid of healthy calves by PCR, in the current study, we were not able to detect those species in the conjunctival fornix throughout the study. Although we have not monitored the occurrence of IBK in any herd included in this study, the staff responsible for managing the cattle did not report the occurrence of any ocular abnormality during the frame between of our collections. In this regard, one study showed that the hazard of IBK incidence in calves was not associated with the detection of *M. bovoculi, M. bovis* and *M. ovis* in lacrimal fluid samples in healthy Angus cattle (O'Connor et al., 2012). Similar results were reported in another study that also found that *Moraxella* spp. were not significantly more abundant in calf eyes that later developed IBK (Cullen et al., 2017). One limitation of the present study was not investigating the prevalence of *Mycoplasma* spp., another opportunist agent also considered one of the top ten genera identified in the conjunctival fornix of healthy cattle (Cullen et al., 2017; Bartenslager et al., 2021). In contrast to *Moraxella* spp., the number of *Mycoplasma* spp. is significantly higher in the conjunctiva of animals with IBK (Cullen et al., 2017; Bartenslager et al., 2021).

Fungi isolated from the conjunctival fornix

In this study, fungi could be isolated in only 20 out of the 144 conjunctival fornixes sampled. From these 20 isolates, the fungal genera most frequently identified were *Trichosporon* spp. (35%) and *Aspergillus* spp. (30%) (Tables 3, 4). This contrasts with the results of previous studies conducted in the USA (Samuelson et al., 1984) and Italy (Sgorbini et al., 2010), where *Cladosporium* spp. and *Penicillium* spp. were the genera most frequently isolated in the conjunctival fornix of healthy cattle. This may be explained by the fact that although *Trichosporon* spp. are broadly spread in nature, this fungus is mostly found in areas where warm and tropical temperatures prevail (Mehta et al., 2021). *Trichosporon* spp. is considered to be an opportunistic agent, with most infections seen in immunocompromised individuals (Mehta et al., 2021). Although no cases of keratomycosis have been reported in the cattle in Brazil, *Trichosporon* spp. was already isolated from herds with mastitis in the southern and southeastern regions of Brazil (Costa et al., 1983; Spanamberg et al., 2008). The prevalence of conjunctival fungal flora in this study (11.8%) was much lower than the prevalence reported for moufflons (45%), cows (95-100%), and sheep (86%) (Samuelson et al., 1984; Sgorbini et al., 2010; Bonelli et al., 2014). Differences in fungal prevalence may be due to different methods of managing herds, breeds available on the farm, and geographical regions (Samuelson et al., 1984; Sgorbini et al., 2010; Bonelli et al., 2014). However, the results from our and other studies showed that cows and other ruminants with conjunctival fungal flora colonization did not present corneal abnormalities, supporting the low incidence of keratomycosis in cattle (Samuelson et al., 1984; Sgorbini et al., 2010; Bonelli et al., 2014). This might be related to better ocular surface defense mechanisms in ruminants compared to horses, where the prevalence of keratomycosis is reportedly higher (Martins, 2021).

Table 3
Fungal isolated from the conjunctival fornix of dairy cattle during the rainy and dry seasons

Fungi	Rainy season Number (%)	Dry season Number (%)
Aspergillus spp.	1	1
Candida spp.	4	-
Fusarium spp.	-	1
Microsporum spp.	-	2
Trichosporon spp.	7	
Total (%)	12 (60%)	4 (20%)

Table 4
Fungal isolated from the conjunctival fornix of beef cattle during the rainy and dry seasons

Fungi	Rainy season Number (%)	Dry season Number (%)
Aspergillus spp.	4	-
Candida spp.	-	-
Fusarium spp.	-	-
Microsporum spp.	-	-
Trichosporon spp.	-	-
Total (%)	4 (20%)	0

Although the number of fungi isolated in dairy cattle (16/144) was significantly higher than that isolated in beef cattle (4/144) (P = 0.007), seasonality did not play a role in the number of fungal isolates in the present study (P = 0.53) (Tables 3, 4). In part, our findings are in accordance with a previous study conducted in Italy, where although the amplitudes recorded for humidity (55 to 80%) and temperature (and 12 to 24 °C) were lower than those recorded here (12 to 95%, 22 to 41 °C), seasonality also did not influence the results (Sgorbini et al., 2010). Differences in the number of fungi isolated between cattle categories may be due to breed characteristics enrolled in each farm (Nelore vs. Holstein x Gyr), as Nelore cattle have natural resistance to various diseases. In the present study, dairy cattle were housed indoors for some periods, while beef cattle were strictly managed outdoors. Although in the present study "housing" was assumed to be "cattle category", a previous study did not report differences between the number of fungi isolated from the conjunctiva in cattle housed indoors vs. outdoors (Sgorbini et al., 2010). However, both breeds evaluated in that study were European (Friesian and Limousin) (Sgorbini et al., 2010).

Microsporum spp. are zoophilic dermatophytes commonly isolated in domestic and wild animals with the potential to infect humans (Chermette et al., 2008). In previous studies conducted in the USA and Italy, this genus of fungi was not isolated from the conjunctival fornix of healthy cattle and sheep (Samuelson et al., 1984; Sgorbini et al., 2010; Bonelli et al., 2014). In the present study, two samples of *Microsporum* spp. were isolated from the conjunctival fornix of dairy cattle during the rainy season (Table 3). However, during the selection of these animals, ringworm-like lesions were not identified in any individual. In these two animals, the skin could be the source of conjunctival contamination. This could be explained by one study conducted with cattle of the southeastern region of Brazi, where *M. canis* and *M. gypseum* could be isolated from the tegument in 13.3 and 23.3% of the individuals, respectively (Surpilli et al., 2018). Therefore, the fungal organisms isolated in the present study may represent a random seeding from the environment where they are ubiquitous.

Conclusions

In this microregion located in midwestern Brazil, *Bacillus cereus* was the most prevalent and *Corynebacterium* spp. and *Streptococcus* spp. the least prevalent bacteria found in the conjunctival fornix of healthy cattle. The total number of bacteria identified was not influenced by seasonality or cattle category. The prevalence of *Moraxella bovoculi* was 8.33% and was influenced by seasonality and cattle category. The prevalence of the conjunctival fungal microbiota (11.8%) was lower than previously reported, with a higher number of isolates found in dairy cattle. *Trichosporon* spp. (41.2%) and *Aspergillus* spp. (35.3%) were the most frequent genera, being most commonly isolated in the rainy season.

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Declarations

The authors have no competing interests.

Ethics approval

All procedures were approved by the Ethics Committee for the Use of Animals in Research of the Federal University of Mato Grosso (protocol number 23.108.171954/2016-79).

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