The culturable ocular bacterial microbiota of beef cattle and its commensal members that can inhibit pinkeye-associated pathogens

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The objective of this study was to characterize the culturable fraction of the bacterial microbiota residing in the bovine eye and to investigate whether commensal members of this community could inhibit the pinkeye-associated pathogens Moraxella bovis and Moraxella bovoculi. Results indicate that the bovine eye harbors a relatively diverse culturable bacterial community, and some of these commensal species can inhibit pinkeye pathogens, suggesting the possibility to develop bacterial therapeutics based on these commensal isolates to mitigate pinkeye infections in cattle in place of antibiotics.

Summary

In this study, we cultured a wide range of bacterial species using both aerobic and anaerobic culture conditions and five different growth mediums from ocular swabs obtained from beef cattle with (n = 35) and without (n = 29; healthy control) pinkeye infections. We taxonomically identified a subset of these bacterial isolates using near-full length 16s rRNA gene sequencing and tested a subset of these isolates for their ability to directly inhibit the growth of *Moraxella bovis* and *Moraxella bovoculi*, the primary pinkeye pathogens. We identified 6 bacterial isolates that can inhibit the growth of *M. bovis* and *M. bovoculi in vitro*. Using scanning electron microscopy (SEM), we further investigated the morphological and structural damage that occurred to *M. bovis* and *M. bovoculi* cells after incubation with culture supernatants of selected isolates that demonstrated growth inhibition *in vitro*. The SEM imaging provided clear indication of damage to *Moraxella* cell structure. Together, these commensal ocular bacteria that displayed antimicrobial activity against *Moraxella in vitro* are viable candidates for the development of bacterial therapeutics to treat and prevent pinkeye in cattle.

Introduction

Pinkeye, clinically known as infectious bovine keratoconjunctivitis (IBK), is a highly contagious disease of the eye and is one of the most significant health challenges impacting producers in the Midwest (Martin et al., 2019) with an estimated $150 million in annual losses to US beef producers (Bartenslager et al., 2021). The development of pinkeye in cattle is multifactorial, and it is commonly believed that irritation to the eye from face flies, tall grass, and ultraviolet (UV) light predisposes animals to pinkeye development. However, the primary ecological agents that are known to contribute to pinkeye infection are the bacterial pathogens *Moraxella bovis* and *Moraxella bovoculi*. Currently, pinkeye vaccinations against these pathogens are limited in their efficacy, and as such, producers are left with very little protection from outbreaks. Challenges in preventing and treating pinkeye infections may be a result of the knowledge gap surrounding the ocular microbiome (Bartenslager et al., 2021). To date, there are very few studies that have investigated the bacterial community of the bovine eye using culture independent metagenomic sequencing-based techniques (Cullen et al., 2017; Bartenslager et al., 2021). Research suggests that the bovine eye does harbor bacterial communities and that they may be important in ocular health. A relatively rich and site-specific bacterial community has recently been reported in the eyes of healthy newborn calves (Luecke et al., 2023). The genera *Moraxella* was well represented in those samples, and it is currently unclear if early colonization of the eye acts to prime the newborn's immune system against pathogens or if it predisposes them to infection. Given this information, we conducted the present study to 1) characterize the ocular microbiota of healthy and pinkeye-infected beef cattle using sequencing and culturing techniques, and 2) isolate and screen commensal ocular bacterial isolates for their antimicrobial activity against *M. bovis* and *M. bovoculi*.

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Procedures

Ocular swabs from the cornea and conjunctiva of cattle exhibiting IBK symptoms (n = 35) as well as control swabs from healthy animals (n = 29) were collected from multiple herds across North Dakota as well as from the NDSU Beef Cattle Research and Teaching Center and from the NDSU Veterinary Diagnostic Laboratory (Table 1). Ocular swabs were collected using Puritan Opti-Swabs with the Liquid Amies Collection and Transport System (Puritan, Guilford, ME) and were stored on ice for transport to the lab. Once in the lab, samples were aliquoted and spread on up to five types of agar plates (Blood, Columbia Blood, De Man, Rogosa and Sharpe (MRS), Wilkins-Chalgren, and Multi-slice agar), all with various growth mediums to support the growth of a wide range of microorganisms. Plated samples were incubated both aerobically and anaerobically for 24 – 48 h. Bacterial colony growth was quantified, and unique colonies were sub-streaked onto fresh media and incubated for 24 h. Isolated bacteria were then cryopreserved (n = 658). Genomic DNA was extracted from a subset of preserved bacterial isolates (n = 351) and used for taxonomic identification by the near-full length 16S rRNA gene sequencing. Of the 351 identified isolates, 53 were tested for growth inhibitory effects against *M. bovis* and *M. bovoculi* using the agar slab method as described previously (Amat et al., 2019). Following the agar slab experiments, a selection of candidate bacteria that exhibited relatively strong inhibition of *Moraxella* growth were used to evaluate changes to cell morphology of *Moraxella* by inoculating *Moraxella* into the cell free supernatant of the candidate bacteria, incubating for 14 h, and observing changes using scanning electron microscopy (SEM) as described previously (Amat et al., 2019).

Results and Discussion

The 351 bacterial isolates identified using near-full length 16s rRNA gene sequencing were represented by 6 different bacterial phyla and 61 different bacterial genera. Bacterial phyla included Bacillota (44%), Firmicutes (23%), Actinomycetota (13%), Pseudomonadota (13%), Actinobacteria (5%), and Proteobacteria (2% Figure 1A). Of the 61 bacterial

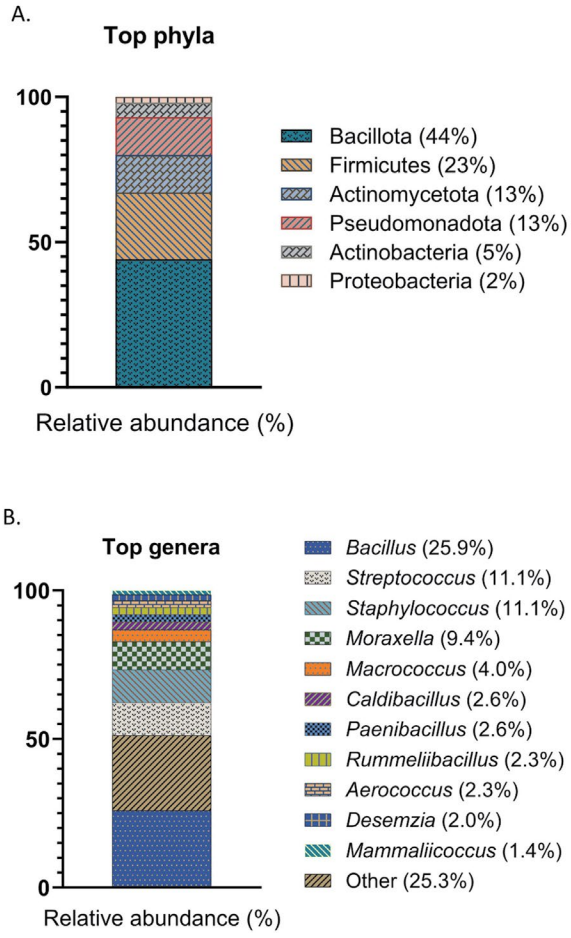

Figure 1: Proportion of bacterial phyla (A) and genera (B) representing a subset of the bacterial isolates isolated from ocular swabs of healthy and pinkeye affected beef cattle (n = 351 isolates).

Table 1: Number of swabs collected and total number of isolated bacteria that were cryopreserved.

Sources	4 ND Veterinary Clinics, NDSU Beef Herd, NDSU VDL					No. of Summer Swabs				43
						No. of Winter Swabs				21
Swab Type	Total Number of Swabs	Aerobic Isolates				Anaerobic Isolates				Total Isolate Number
		MP	CB	Blood	MRS	MP	Blood	WC	CB	
Control	29	30	61	30	43	14	11	74	18	281
Pinkeye	35	41	37	108	67	12	10	80	22	377
Subtotal	64	71	98	138	110	26	21	154	40	658

genera, *Bacillus* (26%), *Streptococcus* (11%), *Staphylococcus* (11%), *Moraxella* (9%), and *Macrococcus* (4%) were the most prevalent (Figure 1B). A total of 33 *Moraxella* isolates were identified, and they consisted of *M. bovis* and *M. bovoculi*. Of the 53 isolates tested for inhibition against Moraxella using the agar slab method (Figure 2), 6 isolates showed zones of inhibition ranging from an average of 13 mm to 25.7 mm (Table 2). *Weizmannia coagulans* (43Y MRS-C), *Lactobacillus fermentum* (ATTC 9338), and *Paenibacillus polymyxa* (42G WC-F) showed relatively strong inhibition against *Moraxella*, while *Alkalihalobacillus rhizosphaerae* (25F CB-B) and *Lentilactobacillus buchneri* (23D MRS-A) showed medium growth inhibition and *Weissella paramesenteroides* (23D MRS-F) displayed weak growth inhibition. *Weizmannia coagulans* (43Y MRS-C), *Lactobacillus fermentum* (ATTC 9338), and *Lentilactobacillus buchneri* (23D MRS-A) cell-free culture supernatants were incubated with *M. bovis* and *M. bovoculi* for 14 h and prepared for SEM imaging. The *W. coagulans* and *L. fermentum* isolates exhibited the greatest amount of cell damage to *M. bovoculi*. Complete cell lysis was observed, indicating that *W. coagulans* and *L. fermentum* effectively inhibit the growth of *M. bovoculi*. *Lentilactobacillus buchneri* exhibited only minor cell damage to *M. bovoculi*, with few structural damages to the *M. bovoculi* cells (Figure 3). When the cell-free culture supernatant of *W. coagulans* and *L. fermentum* was incubated with *M. bovis*, noticeable cell damage occurred, but it was not to the extent of the damage that occurred to *M. bovoculi* (Data not shown). Irregular cell shape and damages to the cell wall of *M. bovis* were observed, which indicates that *W. coagulans* and *L. fermentum* may be viable candidates for the development of bacterial therapeutics against *M. bovis*.

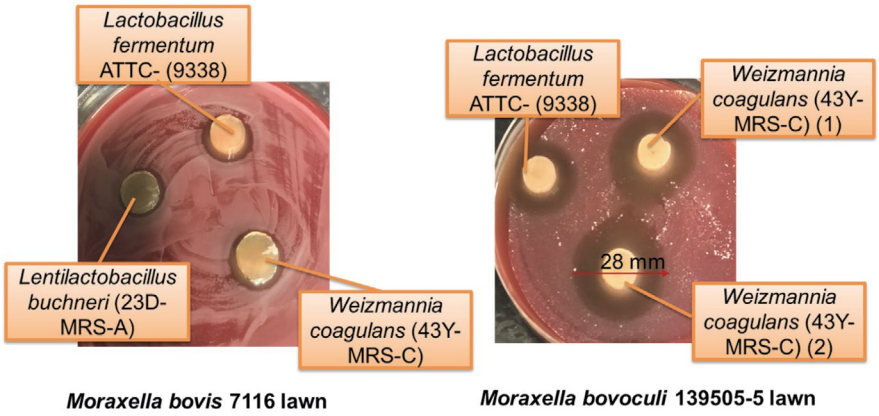

Figure 2: Example of the agar slab method. This method allows for visualization of antimicrobial activity of bacteria by placing a small agar slab (10 mm in diameter) containing a bacterial strain of interest on the surface of a plate containing a fresh inoculation of *M. bovis* or *M. bovoculi*. After co-incubation, growth inhibition of *M. bovis* or *M. bovoculi* can be observed by the formation of a zone of inhibition surrounding the agar slab containing the bacteria of interest.

Table 2: Six bacterial isolates that exhibited antimicrobial activity against *M. bovis* and *M. bovoculi* when tested using the agar slab method.

Isolate ID	Species	Average ZOI (mm)
43Y MRS-C	*Weizmannia coagulans*	25.7
ATTC 9338	*Lactobacillus fermentum*	18.5
42G WC-F	*Paenibacillus polymyxa*	17.2
25F CB-B	*Alkalihalobacillus rhizosphaerae*	16.4
23D MRS-A	*Lentilactobacillus buchneri*	14.1
23D MRS-F	*Weissella paramesenteroides*	13.0

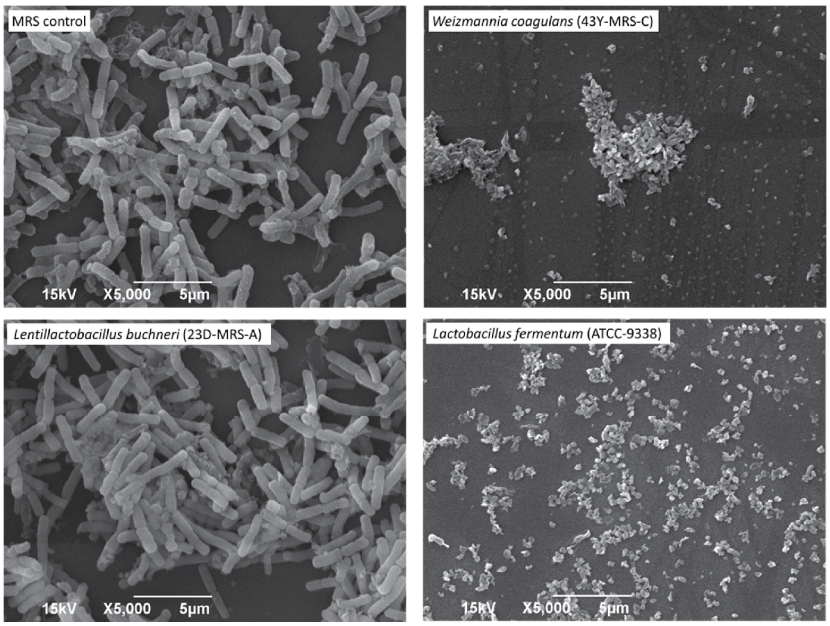

Figure 3: Scanning microscopy imaging (SEM) images of *Moraxella bovoculi* 139505-5 after incubation with cell-free culture supernatants of screened bacterial strains originated from the bovine ocular swab. Bacteria were incubated with cell-free culture supernatants before fixation and microscopy. MRS control represents untreated (incubated with MRS broth only) *Moraxella bovoculi* 139505-5 cells.

These results indicate that the bovine oculus harbors relatively diverse culturable bacteria. In addition, some of the commensal bacterial isolates can inhibit the growth of *M. bovis* and *M. bovoculi*, potentially through the production of antimicrobial agents that can damage the cell structure and cell morphology of the pathogens. This information adds to the current understanding of the bovine ocular microbiota and indicates that commensal bacterial species within the ocular microbiota may be able to be harnessed to combat against pinkeye pathogens and modulate ocular microbiome-mediated eye health in cattle.

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Literature Cited

Amat, S., E. Timsit, D. Baines, J. Yanke, and T. W. Alexander. 2019. Development of Bacterial Therapeutics against the Bovine Respiratory Pathogen Mannheimia haemolytica. Appl. Environ. Microbiol. 85:e01359-01319.

Bartenslager, A. C., N. D. Althuge, J. D. Loy, M. M. Hille, M. L. Spangler, and S. C. Fernando. 2021. Longitudinal assessment of the bovine ocular bacterial community dynamics in calves. Anim. Microbiome 3:16.

Cullen, J. N., A. Lithio, A. S. Seetharam, Y. Zheng, G. Li, D. Nettleton, and A. M. O'Connor. 2017. Microbial community sequencing analysis of the calf eye microbiota and relationship to infectious bovine keratoconjunctivitis. Vet. Microbiol. 207:267-279.

Luecke, S. M., D. B. Holman, K. N. Schmidt, K. E. Gzyl, J. L. Hurlbert, A. C. B. Menezes, K. A. Bochantin, J. D. Kirsch, F. Baumgaertner, K. K. Sedivec, K. C. Swanson, C. R. Dahlen, and S. Amat. 2023. Whole-body microbiota of newborn calves and their response to prenatal vitamin and mineral supplementation. Front. Microbiol. 14: 207601.

Martin, M. S., S. A. Grau, B. W. Rutherford, T. Grandin, and L. N. Edwards-Callaway. 2019. Survey of cow-calf producer perspectives on management strategies and industry challenges. Part 1: handling practices, and health and industry challenges. Transl. Anim. Sci. 3:195-203.