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Profiling bacterial communities in feedlot cattle affected with bovine foot rot and bovine digital dermatitis lesions using 16S rRNA gene sequencing and quantitative real-time PCR

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Abstract

Background The primary infectious foot diseases in cattle, bovine foot rot (BFR) and bovine digital dermatitis (BDD), commonly associated with *Fusobacterium necrophorum* and *Treponema* spp., respectively, are considered polybacterial in etiology with several additional bacteria involved such as *Porphyromonas levii*, *Bacteroides pyogenes*, and *Fusobacterium mortiferum*. BDD is further classified into several M-stages (M2: active and ulcerative; M4: chronic proliferative). Using quantitative real-time PCR and 16S rRNA gene (V3-V4 region) sequencing, we quantified several specific bacteria and analyzed bacterial communities present in biopsies of visually diagnosed cases of BFR ($n=32$), M2 ($n=17$), and M4 ($n=12$) stages of BDD in feedlot cattle in contrast to inconclusive ($n=14$) clinical cases and healthy ($n=25$) cattle.

Results Bacterial composition of healthy skin differed significantly from that of skin lesions, and between BFR and both lesion stages of BDD, which also differed from each other. All animal groups had generally the same bacterial species, albeit in distinct ratios. Differential abundance analysis relative to the healthy group identified a higher abundance of *Fusobacterium* spp. in BFR and *Treponema* spp. in both BDD-M2 and BDD-M4. *P. levii* had the highest absolute abundance in all animal groups. A significantly higher abundance of *F. necrophorum* was observed in BFR compared to BDD-M2, and *F. mortiferum* in both stages of BDD compared to the inconclusive group. Both BDD M-stages had a significantly higher abundance of *Treponema phagedenis* and *Treponema pedis*. *Treponema medium* was significantly more abundant in BDD-M4 compared to BDD-M2.

Conclusion These results further the evidence of the involvement of *Treponema* spp., in BDD in feedlot cattle. However, it suggests further exploration of the role of *Fusobacterium* spp. in BFR and BDD. Importantly, a

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discriminating polybacterial involvement in these infections was evident demonstrated by changes in the population of multiple bacteria when compared to healthy animals.

Keywords qPCR, Amplicon, NGS, Bacterial composition, Microbiome, Microbiota, Beef, Cow, Canada

Background

Canadian beef production system, largely based on finishing cattle in a feedlot [1], incurs substantial economic losses due to lameness-causing diseases such as bovine foot rot (BFR) and bovine digital dermatitis (BDD) [2]. BFR is an infectious disease of cattle that mainly affects the interdigital skin and subcutaneous tissue of the feet and manifests as an interdigital swelling [3, 4]. BFR was described in cattle as early as 1922 [5]. Despite BFR being widespread and of substantial concern, research studies on etiology are scarce and mostly centered around prevalence, treatment, and vaccination, rather than potential causes [6, 7]. Similar to BFR, BDD is also an important infectious foot disease of beef cattle resulting in lameness. BDD begins in the dermis with clinical signs of acute inflammation including pain and swelling. From the dermis, the infection may extend to the interdigital cleft or under the bulb of the heel affecting several skin layers [8]. Despite advances in understanding both BFR and BDD, there remains a lack of consensus regarding etiology and pathogenesis of these diseases. In addition to the role of the infectious agents in the etiology and pathogenesis, both diseases are currently described as multifactorial including involvement of environmental factors such as season, weather, and climate as well as management-related factors affecting foot hygiene such as housing systems, and the use of footbaths [3, 9, 10].

From a microbiological perspective, several bacteria are believed to be involved in development and progression of BFR. They primarily include anaerobic bacteria such as *Fusobacterium necrophorum*, *Bacteroides melaninogenicus*, *Prevotella melaninogenica*, *Porphyromonas levii*, *Trueperella pyogenes*, *and Dichelobacter nodosus* with *F. necrophorum* suggested as the primary infectious agent in several studies [4, 8, 11–13]. The earliest account of BFR induction in cattle using bacterial isolates involves lesion induction using *F. necrophorum* and *B. melaninogenicus* [14]. Furthermore, recent investigations in dairy cattle hypothesize that BFR is a polybacterial disease [13, 15]. These findings from dairy cattle, however, may not reflect BFR in beef cattle but could explain the limited effectiveness of the current BFR vaccines in this type of cattle [7].

Similarly, the etiology of BDD is not fully understood [16]. BDD has been further classified into various M-stages, namely M1, M2, M3, M4, and M4.1, based on lesion characteristics. The M1 and M2 stages are acute stages with the M2 stage involving lesions greater than 2 cm. The M3 stage involves a healing stage of BDD typically following treatment. The M4 stage is a chronic healed lesion while M4.1 has an acute lesion developing within an area with evidence of a chronic lesion. Healthy feet, with no BDD lesions, have been designated M0 [17, 18]. Noticeable differences in microbial composition have been reported throughout disease progression in BDD [19, 20], although in most studies on BDD, *Treponema* spp. were highlighted as the primary etiological agent because of their higher abundance in lesion biopsies [21–23]. It is noteworthy that varying pathogenicity was reported among species and strains of *Treponema* in the murine subcutaneous abscess model [24, 25]. Recent studies employing metagenomic approaches implied a polybacterial nature of BDD with a continued emphasis on *Treponema* spp. as the primary pathogen [19, 20, 26–29]. Furthermore, a study on BDD in beef cattle isolated a high number of *F. mortiferum* as well as *B. pyogenes*, motivating them to develop a multiplex qPCR assay targeting this bacteria as well [19]. Like BFR, most of these studies have been performed on dairy cattle. Hence, further similar studies in beef cattle may provide stronger evidence regarding the etiology of BDD in beef cattle.

Historically, studies on the etiology of BFR and BDD have used culture-dependent methods. However, culture-independent metagenomic techniques such as deep amplicon sequencing can address the limitations of culture-based methods gaining new insights into etiology of these diseases [30, 31]. With incorporation of 16S rRNA gene sequencing, a deep amplicon sequencing technique, and quantitative real-time PCR (qPCR) to profile qualitative and quantitative aspects of bacteria involved in BFR and BDD, we explored further opportunities for describing the microbiota of these diseases.

The objectives of this study were to (i) describe and compare microbial communities using 16S rRNA gene sequencing in BFR and BDD lesion samples, along with analogous specimens from animals with lesions deemed inconclusive to either disease and specimens collected from healthy cattle feet and (ii) use existing qPCR assays to determine the quantities of each of 7 distinct anaerobic bacteria previously suggested to be important in the etiology and pathogenesis of BFR and BDD. We hypothesized that relative proportions of *Fusobacterium* spp. and *Treponema* spp. were unique to BFR and BDD lesions compared to inconclusive cases and healthy skin. We also hypothesized that higher absolute abundance of *Fusobacterium* spp. within microbial communities of lesions are characteristic of BFR and similarly, an elevated microbial community abundance of *Treponema* spp. is present in

BDD lesions, potentially enabling differentiation of the two diseases based on microbial population characteristics within lesions.

Methods

Sampling strategy and sample collection

Between May 2022 and August 2023, samples were collected from beef cattle in 7 feedlots in Southern Alberta each of which had a history of both BFR and BDD. The beef breeds in this study primarily consisted of pure and mixed breeds of Angus, Charolais, and Hereford. However, due to challenges in accurately identifying breeds of several cattle, all samples were collectively classified as beef breeds. Pen riders identified lame cattle by assessing gait. Only a subset of cattle identified as lame during the study period were sampled depending on convenience and availability of feedlot staff to help with sampling. Cattle were sampled only once throughout the study period. Cattle were allocated into 5 groups, namely, healthy, BFR, BDD-M2 (M2 stage of BDD), BDD-M4 (M4 stage of BDD), and Inc (clinically inconclusive cases). Briefly, BDD-M2 lesions were active, large (>2 cm), ulcerative, mottled, red-grey, and painful. The BDD-M4 lesions were chronic with dark-colored, irregular, and proliferative hyperkeratotic growths that varied from papilliform to mass-like projections [17, 18]. The BFR cases were characterized by painful lesions in the interdigital space with necrotic edges, and swollen feet with the swelling symmetric to the axial midline of the foot [3]. The inconclusive group was cattle that could not be conclusively diagnosed as BFR or BDD based on the visual inspection of the gross lesion. Inconclusive cases were cattle with lesion characteristics of both BFR and BDD in the same foot or had only some lesion characteristics of either BFR or BDD which were not enough to discriminate them visually as either disease. The healthy (control) group had no visible lesions and were identified by the pen riders as healthy pen mates.

Once feedlot pen riders identified lame cattle, they were removed from the pens, moved to feedlot hospital facilities, and restrained in a headgate chute squeeze. Only 1 leg was sampled per animal with a priority for the leg with the highest degree of lameness during the pen walk. In the chute, the affected leg was lifted with a rope, and lesions were inspected and classified. This was followed by superficial cleaning of the foot with clean water and drying with a clean paper towel. Local anesthesia was induced with a subcutaneous injection of lidocaine (3 ml) (Lidocaine HCl 2%, Teligent OÜ, Tallinn, Estonia) without bactericidal preservatives, and the foot left for 3 min before biopsy sampling. A 4 mm skin biopsy punch (Integra™ Miltex™; Integra Life Sciences Corporation, York, PA, USA) was used to obtain skin biopsy specimens from the edge of the foot lesions, as more central lesion locations can be friable, disintegrating, and can lack definitive structure due to necrosis. Skin biopsy samples were immediately stored in a solid transport medium (Anaerobe Tissue Transport media (ATTM); Anaerobe Systems, Morgan Hill, California, USA) and transported back to the University of Calgary at room temperature on the same day.

Skin biopsy processing

Skin biopsy samples were processed in an anaerobic chamber (Bactron 3000; Sheldon Manufacturing Inc., Cornelius, OR, USA) with a static gas concentration (5% CO_2, 5% H_2, and 90% N_2). Tissue extracted using punch biopsy was removed from the transport medium and placed on a sterile Petri dish within the anaerobic chamber. Using a sterile surgical scalpel blade, the epidermis was removed, and the tissue was longitudinally sectioned into 4 approximately equal sections of which 1 was used for DNA extraction. For DNA extraction as well as the potential use of this tissue section for bacterial isolation, ≤ 25 milligrams of tissue were weighed for each sample and stored at -80^{0}C in 450 µl of 50% glycerol and 1050 µl brain-heart infusion (BHI) broth until DNA extraction.

DNA extraction from skin biopsies

DNA extraction was conducted in 2 distinct batches for samples collected in each of the 2 respective sampling years. DNA extraction was performed with Qiagen DNeasy blood and tissue kit (Spin-Colum protocol for tissues; Qiagen, Hilden, Germany) [32] following the manufacturer's recommendation for tissue samples. Briefly, weighed glycerol/BHI frozen biopsy samples were thawed at room temperature and incubated overnight at 56^{0}C in a solution containing 20 µL of proteinase K and 180 µL of ATL buffer. This was followed by an addition of 100% ethanol and subsequent wash buffers. Finally, DNA was eluted using 100 µL of DNAse/RNAse-free molecular-grade water. DNA extraction controls and non-template controls were included to ensure a contamination-free DNA extraction in each of the 2 batches. After confirming the quality of the DNA using the A260/280 ratio, a measure of purity that reflects the presence of potential protein contaminants, the extracted DNA samples were stored at -20^{0}C until further use.

16S rRNA gene sequencing and analysis

Sequencing was done in 2 different batches for samples collected in each of the 2 different years (49 and 52 samples, respectively). Amplification, library preparation, and sequencing of DNA samples were performed by the Center for Health Genomics and Informatics (CHGI) at the University of Calgary, Canada. The V3-V4 hypervariable region of the 16S rRNA gene was amplified using a forward primer

(TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and a reverse primer (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) [33]. Overhang nucleotide adapter sequences compatible with the Illumina platform were added to these sequences. A low-concentration protocol was used for microbiome analysis using the Illumina MiSeq platform (600 cycles; V3 flow cell; 2*300 nucleotides) for DNA samples and controls. The sequencing facility also used its own control for quality control of the sequencing process.

DADA2 v.1.16.0 was used to process and analyze demultiplexed reads separately for the 2 batches [34]. For both batches, the truncation lengths were 280 and 220 for forward and reverse reads, respectively, based on the cutoff average Phred score of 30. Following this, paired-end reads were merged with a minimum overlap of 20 nucleotides, and amplicon sequence variants (ASVs) were inferred. This was followed by removal of chimeric sequences and subsequent taxonomic classification of ASVs using SILVA v.138 [35].

Phyloseq, an R package, was used for further analyses of sequences [36]. The DADA2-processed reads for both batches were combined as a single Phyloseq object. Following this, sequences belonging to bacteria were only retained after mitochondria and chloroplasts were removed. Based on visual inspection of the rarefaction curve, samples were rarefied at 6000 reads, the level at which the new species detected plateaued, leading to 28 samples being removed due to low sequencing depth.

A univariable linear regression model was run for each unique alpha-diversity metric as the outcome variable and animal groups as the predictor variable using emmeans 1.10.6 [37]. Granularity in microbiome variation between the 2 batches was assessed by permutational analysis of variance (PERMANOVA) with 9999 permutations using vegan 2.6–6.1 [38]. For subsequent alpha-and beta diversity analyses, $p < 0.05$ was considered significant. PERMANOVA, based on Bray-Curtis distances, was conducted in a pairwise fashion between the cattle groups to assess any significant differences. Principal coordinate analysis derived from the Bray-Curtis distances [39] was used to visualize differences and similarities in microbial composition between samples. Descriptive relative abundance was plotted as the mean relative abundance of bacteria in all samples within each animal group. DESeq2 v.1.40.2 [40] was used to normalize sequencing depth of the rarefied sequences and identify bacterial taxa with a significant ($\log_2$-fold) change in differences among normalized sequence counts compared across the animal groups. For this, $p < 0.01$ was considered significant. All analyses for 16S rRNA gene sequencing were performed in R v.4.2.3 [41].

qPCR

Three in-house qPCR assays were used. For *Treponema* spp., a multiplex qPCR assay developed for detection of *Treponema* species was used with modifications to quantify *T. medium*, *T. phagedenis*, and *T. pedis* [42]. The modification involved the removal of *Treponema denticola* species from the multiplex as assay optimization repeatedly showed amplification of the non-template controls with *T. denticola*-related primers and probes in the assay. Next, a singleplex qPCR assay was used for *F. necrophorum* [43] whereas a multiplex qPCR assay was used to quantify *P. levii*, *B. pyogenes*, and *F. mortiferum* [19]. For generation of standard curves, DNA concentrations of positive controls were measured using a Qubit dsDNA BR kit and Qubit 4 Fluorometer (Life Technologies, Carlsbad, CA, USA) before each reaction. Slopes and intercepts of standard curves were calculated by plotting the decadic logarithm of the known concentrations of the standards used in the assay against their respective Cq values using Bio-Rad CFX Manager 3.1. The limit of detection for the qPCR assays was ≥ 10 copy numbers, and we never had Cq values ≤ 15 and ≥ 40. Absolute quantity of bacterial species was normalized by the biopsy specimen weight and presented as median copy numbers per milligram of biopsy tissue. A zero-inflated negative binomial regression model was utilized to study the associations between bacterial species within and between the animal groups. For this analysis, the bacterial copy numbers were rounded off to the nearest positive integer. Correlation matrices were generated by calculating pairwise Spearman's correlations between the copy numbers of each bacterial species. Unsupervised Principal Component Analysis (PCA) and supervised Sparse Partial Least Square-Discriminant Analysis (sPLS-DA) along with permutational analysis (999 permutations) on the $\log_{10}$-transformed copy numbers were done to discriminate animal groups based on the absolute abundance of 7 bacterial species used in qPCR assays [44, 45]. All analyses were conducted in R v.4.2.3 [41] with $p < 0.05$ considered statistically significant.

Results

There were 101 biopsies collected and analyzed (Healthy: $n = 25$; BFR: $n = 32$; BDD-M2: $n = 17$; BDD-M4: $n = 12$; Inc: $n = 14$) from 7 feedlots. One of these healthy specimens was excluded from further analyses as it belonged to a dairy breed.

Bacterial characterization using 16S amplicon sequencing

All samples that were sequenced were successfully amplified. The pass filter (%PF), an internal quality filtering procedure on the Illumina platform to remove the least reliable read clusters for analysis, in the first and second batches provided by the Illumina BaseSpace platform

were 90.33 and 91.45%, respectively. The percentage of bases with a quality score of 30 or higher (Q30) was 80.47 and 78.28%, respectively. Of the 10,956,962 reads generated in the first batch and 8,889,314 reads generated in the second batch, approximately 61 and 60%, respectively, of the reads passed the DADA2 quality filtering steps. After merging the 2 batches as Phyloseq objects, 35,322 ASVs were generated. The range of library size for the samples was 847 to 1,55,581 reads with a median of 18,497 reads. However, after filtering mitochondrial DNA, chloroplast, and non-bacterial DNA sequences, 72 samples with 29,941 taxa remained for the final analysis with 28 samples omitted after rarefaction (Healthy: $n = 9$; BFR: $n = 8$; BDD-M2: $n = 4$; BDD-M4: $n = 1$; Inc: $n = 6$).

Batch differences were observed within BFR and healthy groups. Upon further inspection, the batch difference was attributed to samples taken primarily from 1 feedlot. Furthermore, the batch difference within that feedlot was likely attributable to the season of sampling. However, with a low statistical power to investigate the true batch difference and effects of seasons and feedlots of origin, we combined the 2 batches for further analyses.

Alpha- and beta-diversity analysis

Alpha diversity analyses indicated a higher bacterial diversity in healthy skin compared to BDD-M2 (Observed, $p = 0.00005$; Shannon, $p = 0.0006$), BDD-M4 (Observed, $p = 0.004$, Shannon, $p = 0.02$), and the inconclusive group (Observed, $p = 0.006$; Shannon, $p = 0.007$) (Fig. 1). Compared to the BFR group, cattle in BDD-M2 had lower alpha diversity (Observed, $p = 0.003$; Shannon, $p = 0.009$).

Based on PERMANOVA of bacterial taxa, microbial composition of the healthy group differed from all other animal groups (BFR: $p = 0.0019$; BDD-M2: $p = 0.0001$; BDD-M4: $p = 0.0003$; Inc: $p = 0.0271$) (Fig. 2). Furthermore, the BFR group differed from all other groups except the inconclusive group (BDD-M2: $p = 0.0001$, BDD-M4: $p = 0.0031$). Like the healthy group, the BDD-M2 group was also different from the rest of the animal groups (BDD-M4: $p = 0.0183$; Inc: $p = 0.0004$). Finally, the BDD-M4 group was different from all animal groups except for the inconclusive group.

Bacterial composition of BFR and BDD lesions

Firmicutes (amended *Bacillota*) [46] was the most relatively abundant phyla in BFR and M2 and M4 stages of BDD. The BFR group differed from both M-stages of BDD, with a relatively lower relative abundance of *Spirochaetota* and a higher abundance of *Fusobacteriota*. Compared to both BFR and BDD-M4, the BDD-M2 stage had a relatively lower abundance of *Proteobacteria*. Family-level taxonomy differentiated BFR from both M-stages of BDD with a lower abundance of *Spirochaetaceae* and a higher abundance of *Fusobacteriaceae*. Within BDD, the M2 stage had an approximately 2-fold higher proportion of *Spirochaetaceae*. At the genus level, even though *Fusobacterium* spp. was detected in both M2 and M4 stages of BDD, relative abundance of this bacterium was higher within the BFR group. *Treponema* was present in lower abundance in BFR compared to both M-stages

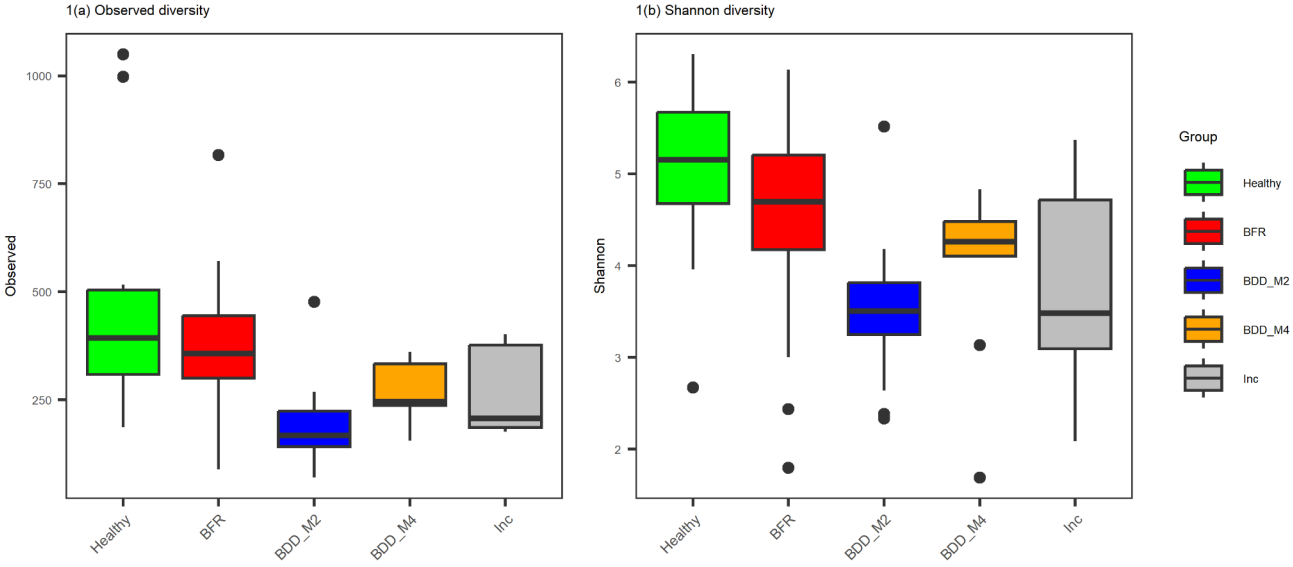

Fig. 1 Alpha-diversity indices for various cattle groups based on the 16S rRNA gene (V3-V4 region). Animal groups (BFR, BDD-M2, BDD-M4, inconclusive, and healthy) were plotted against the X-axis and the Y-axis represents values associated 2 different alpha diversity metrics, namely, **(a)** a unique number of observed bacterial species within each group (richness), and **(b)** Shannon diversity metric based on the unique number of bacterial species and their evenness

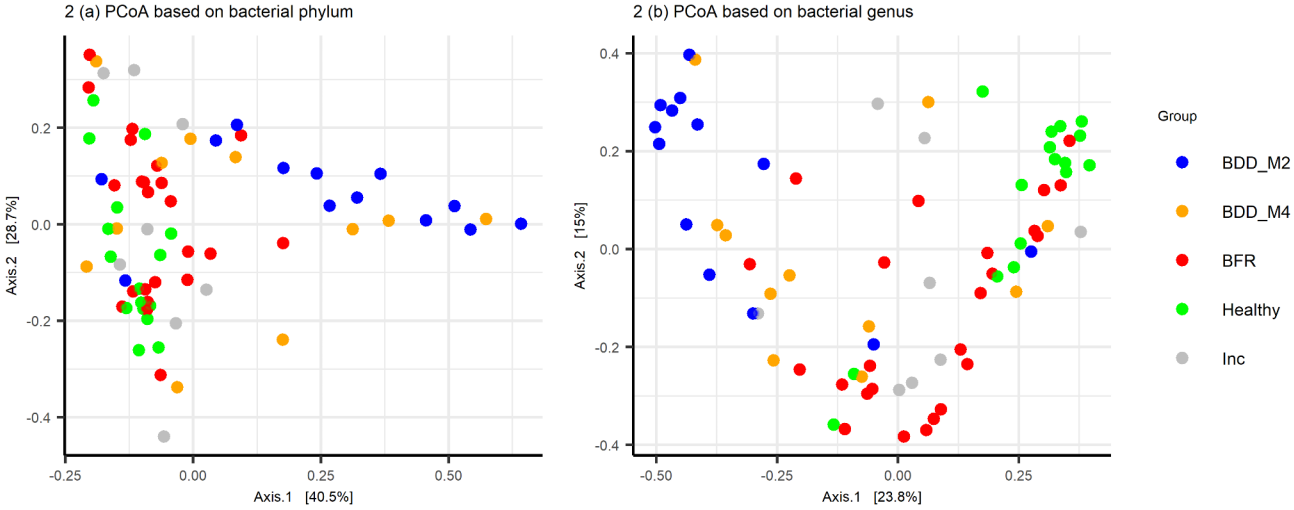

Fig. 2 Principal Coordinate Analysis (PCoA) on various cattle groups based on the 16S rRNA gene (V3-V4 region). Bray-Curtis distances between samples were used to conduct the PCoA analysis. Biopsy samples were colored based on animal groups (BFR, BDD-M2, BDD-M4, inconclusive, and healthy) as indicated by the figure key. Variations between animal groups explained by principal components were associated with bacterial **(a)** phylum and **(b)** genus

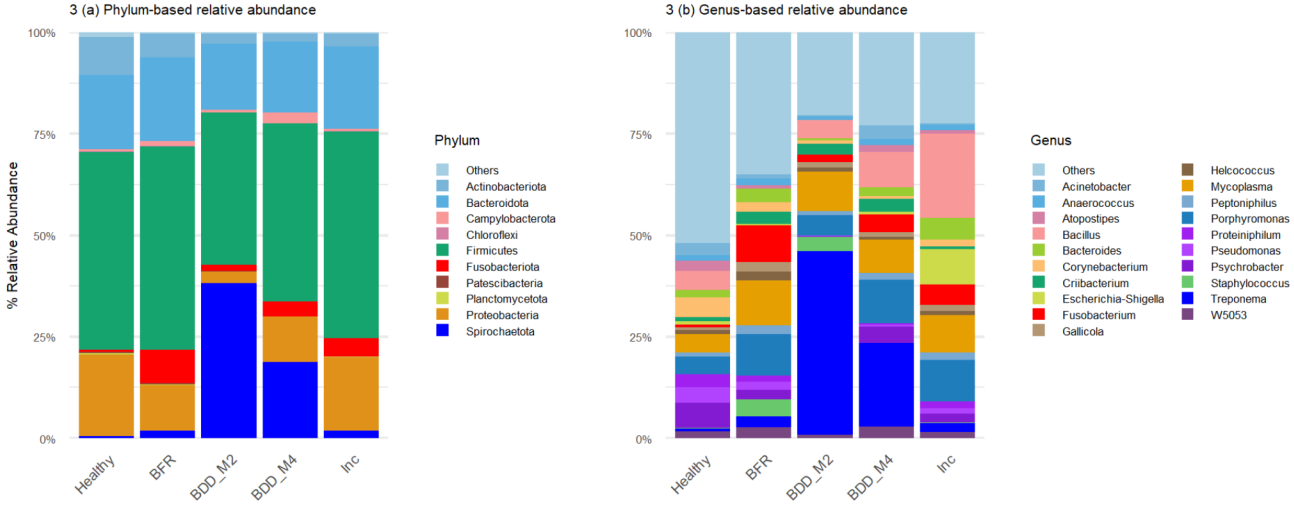

Fig. 3 Relative abundance of bacteria across cattle groups based on the 16S rRNA gene (V3-V4 region). Bars on the X-axis represent various animal groups (BFR, BDD-M2, BDD-M4, Inconclusive, and healthy) and the Y-axis indicates the percentage relative abundance of various bacterial **(a)** phyla and **(b)** genera

of BDD. Within BDD, relative abundance of *Treponema* was approximately 2-fold higher in abundance in the M2 stage compared to the M4 stage. In addition, both *Mycoplasma* and *Porphyromonas* were among the most relatively abundant of the BFR-associated bacteria, and both stages of BDD, with *Porphyromonas* having the highest proportion.

Firmicutes had the highest relative abundance in the inconclusive group, similar to the rest of the groups-Fig. . 3). The proportion of *Proteobacteria* was comparable to that of healthy animals. Family-level analysis indicated a higher proportion of *Bacillaceae* and *Enterobacteriaceae* in the inconclusive group compared to other groups. Relative abundance of Gram-positive families was lower than the Gram-negative phyla in all animal groups including the inconclusive group. At the genus level, a higher relative abundance of *Bacillus* was evident, particularly in select samplesFig. . 4), followed by *Escherichia-Shigella*, *Mycoplasma*, and *Bacteroides*.

For differential abundance analysis of various bacterial species in the diseased animal groups compared to the healthy group, there was a significant $\log_2$-fold increase in relative abundance of several bacterial taxa (Fig. 5). Specifically, *Fusobacterium* had a significantly elevated $\log_2$-fold change (4.18 $\log_2$-fold; $p = 0.0000207$) in abundance in the BFR group. In contrast, *Treponema pedis* had a $\log_2$-fold increase in both BDD-M2 (8.65 $\log_2$-fold; $p = 6.98E^{*}11$) and BDD-M4 (6.38 $\log_2$-fold; $p = 0.0000143$)

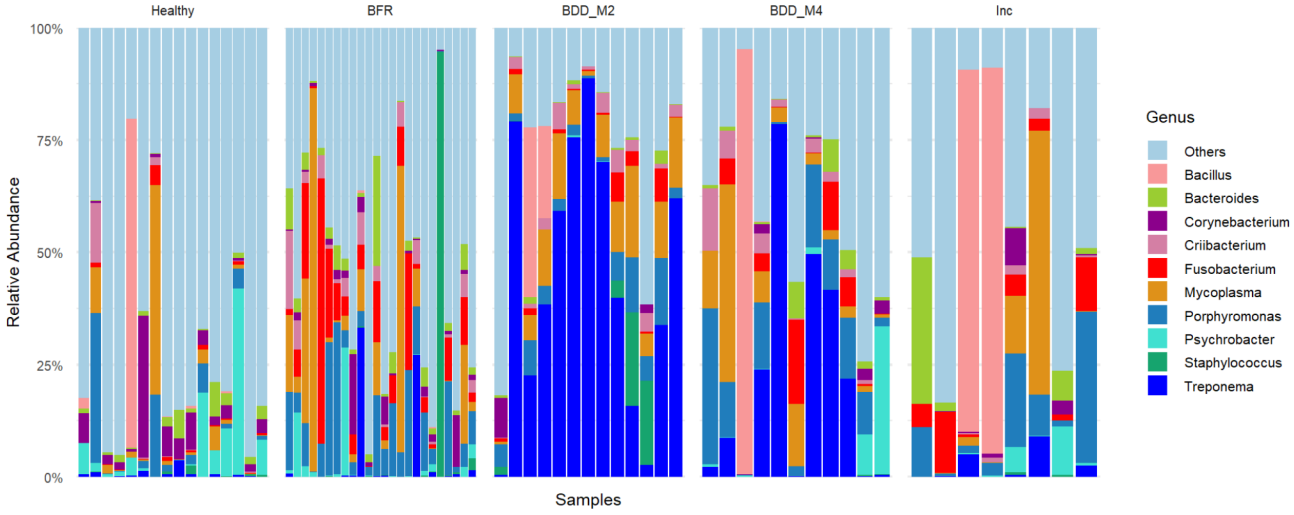

Fig. 4 Relative abundance of bacteria across various samples based on the 16S rRNA gene (V3-V4 region). The X-axis represents various animal groups, with each bar representing a sample, and the Y-axis indicates the percentage relative abundance of different bacterial genera

groups. Of the notable changes in bacteria between various diseased groups, there was a significant decrease of *Treponema pedis* (-5.42 $\log_2$-fold; $p = 0.000024$) in BFR compared to BDD-M2.

Detection and absolute quantification of 7 distinct bacterial species

Quantitative real-time PCR detected the 7 distinct bacterial species in all animal groups, albeit in different percentages (Table 1).

Among each target bacterial species, *P. levii* was the most abundant of all bacteria across all animal groups (Fig. 6). However, a significantly higher absolute abundance of *P. levii* was observed only in the BDD- M2 group compared to healthy ($p = 0.03188$) and inconclusive ($p = 0.00645$) groups (Table 2). *F. necrophorum* was more abundant in all animal groups ($p < 0.0001$) compared to healthy cattle and had a significantly higher abundance in the BFR group ($p = 0.0194$) compared to BDD-M2. *F. mortiferum*, however, was more abundant in both BDD-M2 ($p = 0.02706$) and BDD-M4 ($p = 0.00653$) compared to the inconclusive group. *B. pyogenes* had a significantly higher abundance: BFR and BDD-M2 ($p < 0.0001$), BDD-M4 ($p = 0.0245$), and inconclusive ($p = 0.000153$) compared to the healthy animals. *B. pyogenes* was also more abundant in BDD-M2 compared to BDD-M4 ($p = 0.0233$). *T. medium* was more abundant in BDD-M4 compared to all other groups ($p < 0.0001$). However, the abundance of *T. medium* was not significantly different between BDD-M2 when compared to other groups, except for the inconclusive group where the abundance was higher ($p = 0.04816$). *T. phagedenis* was highly abundant in both BDD-M2 ($p < 0.0001$) and BDD-M4 (BFR and Inc, $p < 0.0001$; healthy, $p = 0.0116$) compared to other groups, except when compared between themselves. *T. pedis* had the highest abundance in the BDD-M2 group with differences observed against all groups (BFR and Inc. $p < 0.0001$; healthy, $p = 0.0416$) except BDD-M4. In BDD-M4, *T. pedis* was more abundant compared to BFR ($p = 0.000262$) and inconclusive ($p = 0.00511$) groups. Additionally, both BFR (for *T. medium*, $p = 0.01$; *T. phagedenis*, $p = 0.00662$; *T. pedis*, $p < 0.0001$) and inconclusive (for *T. medium*, $p = 0.00221$; *T. phagedenis*, $p = 0.000339$; *T. pedis*, $p = 0.000901$) groups had a higher abundance of the *Treponema* spp. compared to healthy cattle. The analysis of within-group differences revealed that *P. levii* was more abundant than the rest of the bacteria across all animal groups and had the highest absolute abundance in BDD-M4 (Table 2). In the BDD-M2 group, *T. medium* had the least abundance compared to all other bacterial species. In BDD-M4, all *Treponema* spp. had different abundances compared to non-Treponemes, except for *P. levii* which only differed significantly from *T. phagedenis*.

PCA and sPLS-DA performed on the absolute abundance of the 7 distinct bacterial species indicated overlaps between animal groups (Fig. 7). Permutation tests based on a combined model with the animal groups as the outcome showed significant differences in absolute abundance of all 7 bacterial species between animal groups ($p = 0.001$). Evaluation of pairwise models indicated significant discrimination between healthy cattle compared to the other groups based on the absolute abundance of the 7 bacterial species. Healthy cattle were distinguishable from the BFR ($p = 0.003$), BDD-M2 ($p = 0.001$), and BDD-M4 ($p = 0.005$) groups, but not from inconclusive group. Additionally, cattle with BFR could be differentiated from those with BDD-M2 ($p = 0.001$) and BDD-M4 ($p = 0.001$). Furthermore, BDD-M2 was also distinguishable from BDD-M4 ($p = 0.004$) and the inconclusive

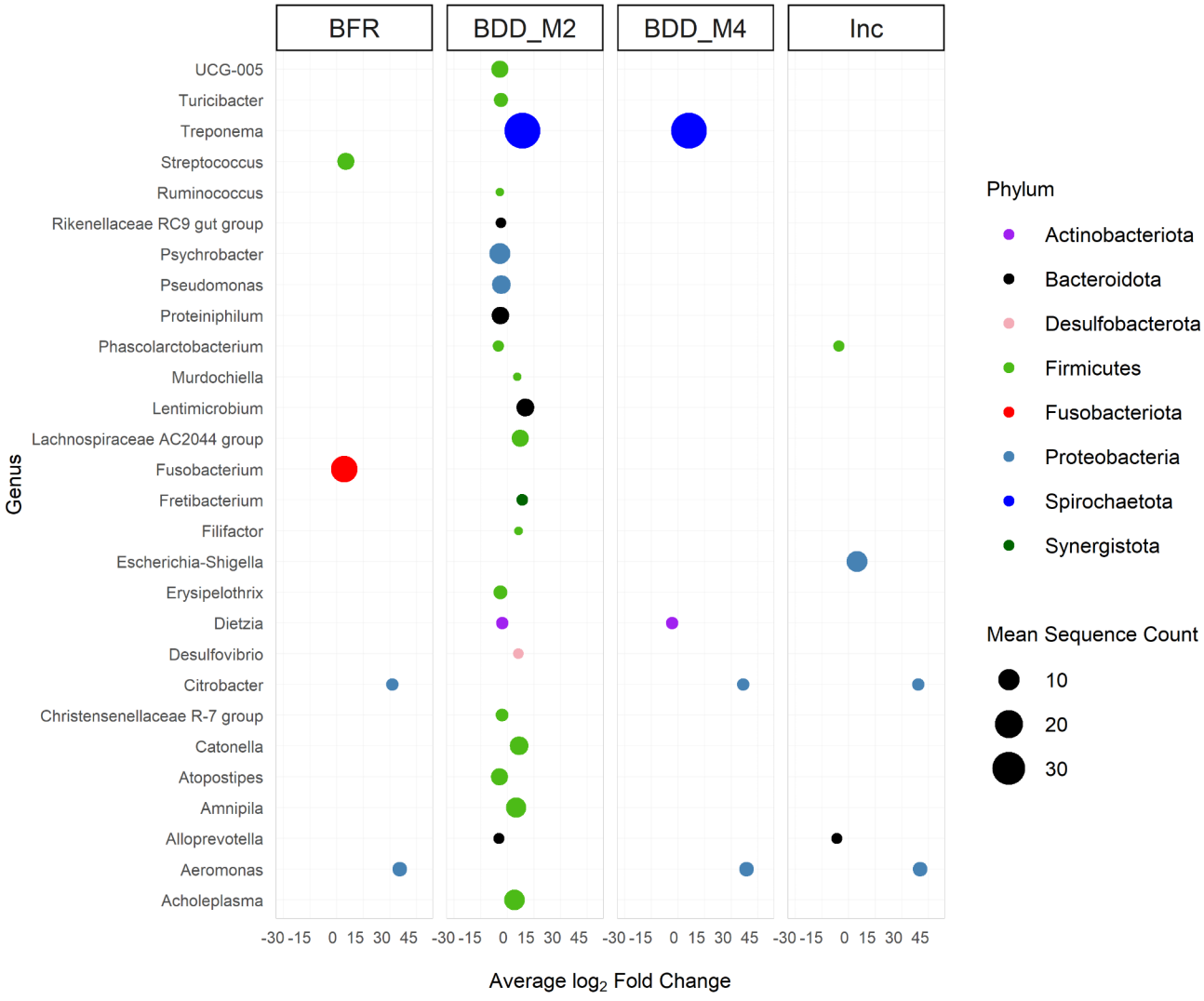

Fig. 5 Differential abundance analysis of bacterial taxa associated with various diseased cattle groups using DESeq2. The X-axis represents log2-fold changes associated with each bacterium in the associated group (BFR, BDD-M2, BDD-M4, or inconclusive) compared to the healthy group. Bacteria are color-coded based on their phylum as indicated on the key, and the associated bacterial genera are indicated by the Y-axis

Table 1 Percentages of 7 distinct bacterial species detected in various cattle groups using qPCR. Percentage values associated with positive samples were rounded to the nearest integers

Category (total samples)	*F. necrophorum*	*F. mortiferum*	*B. pyogenes*	*P. levii*	*T. medium*	*T. phagedenis*	*T. pedis*
	Positive samples (%)						
Healthy $n=25$	15 (60)	9 (33)	8 (33)	19 (75)	4 (17)	16 (63)	11 (45)
BFR $n=32$	28 (88)	26 (81)	21 (66)	31 (97)	9 (28)	23 (72)	14 (44)
BDD_M2 $n=17$	15 (88)	16 (94)	14 (82)	17 (100)	6 (35)	17 (100)	17 (100)
BDD_M4 $n=12$	10 (83)	8 (67)	5 (42)	10 (83)	6 (50)	11 (92)	11 (92)
Inc $n=14$	9 (64)	9 (67)	8 (53)	13 (93)	9 (60)	11 (80)	9 (60)

(0.009) groups. The primary bacteria contributing to the discriminatory ability of X-variate 1 (50% explained variation) namely, *B. pyogenes*, *T. medium*, *T. pedis and Fusobacterium* spp. for BDD-M2 and *T. phagedenis* for BFR, had negative loading values.

Spearman rank correlation analysis performed between absolute abundance of 7 distinct bacteria showed significant positive correlations between these bacterial species within all animal groups including the healthy group (Fig. 8). *P. levii* exhibited a moderate or strong positive correlation with *F. mortiferum* across all cattle groups (range:0.55–0.84), and it also showed an additional strong positive correlation with *F. necrophorum* in BFR (ρ: 0.82). *F. necrophorum* and *F. mortiferum* showed

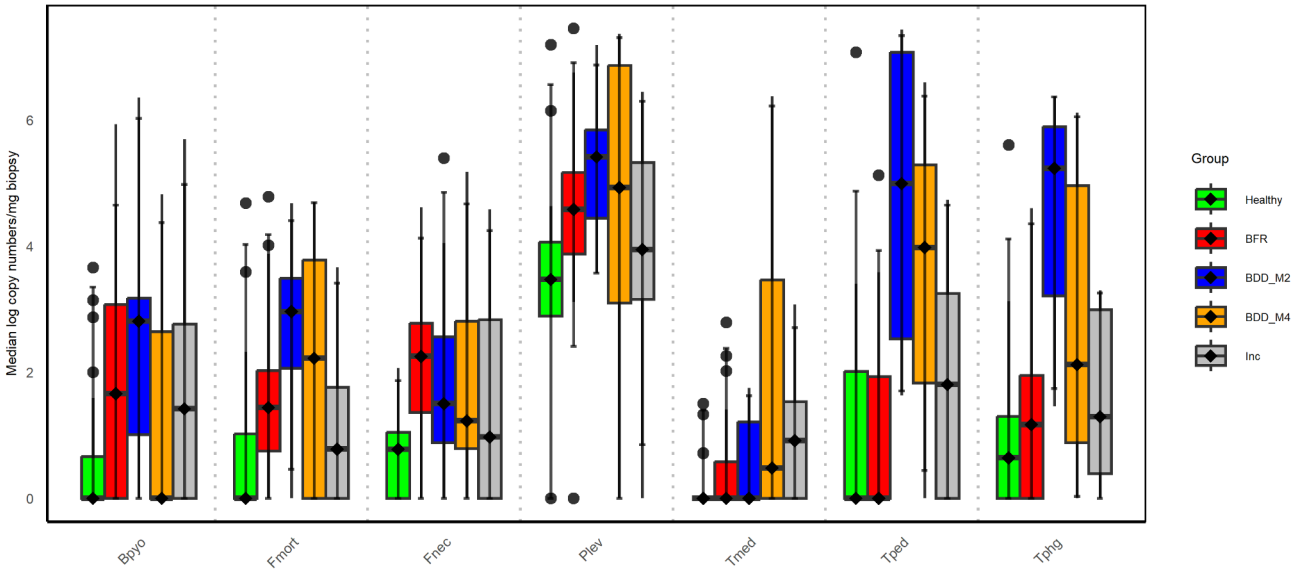

Fig. 6 Absolute abundance of 7 distinct bacterial species between various cattle groups based on qPCR. The Y-axis represents the $\log_{10}$ transformed values of the median bacterial genomic copy number per milligram of tissue in each animal group (BFR, BDD-M2, BDD-M4, or inconclusive). Animal groups are plotted against the X-axis and are distinguished by colors

a significant moderate positive correlation in BFR (ρ: 0.84) and BDD-M4 groups (ρ: 0.62). In BDD-M2 lesions, along with positive correlations between bacterial species; there was a significant negative correlation between *T. medium* compared to *B. pyogenes* (ρ: -0.72) and *P. levii* (ρ: -0.65).

Discussion

Skin biopsies were used to characterize the bacterial compositions of BFR lesions, BDD lesions, and normal healthy skin in feedlot cattle in Alberta. Based on 16S rRNA gene sequencing, microbial composition was significantly different between healthy cattle and those affected by BFR, BDD, or cattle with lesions that were visually inconclusive as either BFR or BDD. We also found that the microbial composition of M2 and M4 stages of BDD were significantly different. BFR and both stages of BDD were differentiated based on 16S rRNA gene sequencing. Although BFR and both stages of BDD were differentiated based on the combined absolute abundances of 7 distinct bacteria, a critical finding of our study is the association of both *F. necrophorum* and *F. mortiferum* with BFR and BDD in feedlot cattle. This association presents a challenge in differentiating BFR and BDD solely based on *Fusobacterium* spp. However, distinctly higher abundances of *Treponema* spp. in both stages of BDD compared to non-BDD groups reinforces the study hypothesis concerning the use of *Treponema* spp. to differentiate BFR and BDD.

Among the 7 bacterial species quantified, the abundance of *B. pyogenes* and *T. medium* were distinctive between BDD-M2 and BDD-M4. This finding aligns with the unique bacterial composition of BDD-M2 and BDD-M4 groups as indicated by 16S rRNA sequencing; however, *B. pyogenes* and *T. medium* were not exclusive to either of the two stages of BDD. Additionally, changes in population of other bacterial species beyond the 7 species quantified were not examined and could explain differences in diseases status. Although *P. levii* was the most abundant bacterium in all animal groups, a significant difference was observed only between BDD-M4 and healthy groups. This may suggest a potential opportunistic nature of *P. levii* with increased bacterial abundance in BDD-M4 lesions. These collective findings, which documented changes in the bacterial abundance within BFR and two stages of BDD, provide substantial evidence of the polybacterial nature of BFR and BDD.

All feedlots from which we sampled our healthy controls also housed cattle with BFR and BDD. The healthy skin of beef cattle was similar to that of healthy dairy cattle in terms of detected phyla [15, 20, 47]. This similarity of detected phyla was also observed for BFR as well as M2 and M4 stages of BDD. *Firmicutes* was the most abundant phylum in BFR and BDD lesions followed by *Bacteroidota*, *Proteobacteriota*, and *Actinobacteriota* (amended *Actinomycetota*) [46] in both dairy and beef cattle [15, 27, 47, 48]. *Mycoplasma* spp. were identified in all animal groups in this study, consistent with a recent study in beef cattle [48]. It would be prudent to explore the involvement of *Mycoplasma* spp. across BFR and BDD because of their unique distribution throughout BDD stages [19]. Bacteria which are often detected from anatomical sites and secretions of cattle and show higher abundance in various stages of BDD in both beef and

Table 2 Bacterial copy number (qPCR) of 7 distinct bacterial species across cattle groups. Significant differences associated with the abundance of a bacterial species across various animal groups (i.e. within-row values) (healthy, BFR, BDD-M2, BDD-M4, and inconclusive) are represented by superscript integers at the end of the associated values. Differences in abundance of various bacterial species within an animal group (i.e. within column values) are represented by superscript lowercase letters preceding the respective associated values. MLCN: Mean log (decadic) copy numbers; MdGCN: Median genomic copy numbers; Min: minimum non-zero genomic copy number; Max: maximum genomic copy number; SD: Standard deviation

Bacteria - Parameters		Healthy (25)	BFR (32)	BDD_M2 (17)	BDD_M4 (12)	Inc (14)
F. necrophorum						
	MLCN ± SD	$^{c}0.695 \pm 0.693^{1}$	$^{b}2.139 \pm 1.2524^{4}$	$^{b}1.859 \pm 1.420^{23}$	$^{c}1.770 \pm 1.574^{24}$	$^{cd}1.529 \pm 1.599^{24}$
	MdGCN	6	178.50	31.4	19.29	12.4
	Min	1.80	6.22	6.79	1.93	2.86
	Max	1.14E + 02	4.07E + 04	2.48E + 05	1.49E + 05	3.79E + 04
F. mortiferum						
	MLCN ± SD	$^{bd}0.713 \pm 1.244$	$^{b}1.588 \pm 1.258$	$^{b}2.756 \pm 1.139^{2}$	$^{c}2.168 \pm 1.932^{2}$	$^{de}1.109 \pm 1.213^{1}$
	MdGCN	0	27.75	907	447.40	6.635
	Min	4.42	4.64	14.30	15.20	2.85
	Max	4.73E + 04	6.04E + 04	4.73E + 04	4.95E + 04	4.61E + 03
B. pyogenes						
	MLCN ± SD	$^{d}0.638 \pm 1.134^{1}$	$^{d}1.705 \pm 1.641^{24}$	$^{c}2.401 \pm 1.796^{23}$	$^{c}1.252 \pm 1.701^{4}$	$^{ab}1.564 \pm 1.752^{24}$
	MdGCN	0	51.20	652	0	26.2
	Min	3.20	2.72	5.79	39.80	24.40
	Max	4.62E + 03	8.53E + 05	2.24E + 06	6.61E + 04	4.88E + 05
P. levii						
	MLCN ± SD	$^{a}3.011 \pm 1.976^{2}$	$^{c}4.540 \pm 1.311$	$^{bd}5.193 \pm 1.005$	$^{b}5.361 \pm 2.627^{1}$	$^{a}4.080 \pm 1.691^{2}$
	MdGCN	2960	38100	254000	130900	9250
	Min	783	1280	3680	340	431
	Max	1.53E + 07	2.79E + 07	1.52E + 07	2.31E + 07	2.75E + 06
T. medium						
	MLCN ± SD	$^{c}0.170 \pm 0.424^{3}$	$^{a}0.379 \pm 0.743^{24}$	$^{a}0.475 \pm 0.674^{23}$	$^{ab}1.976 \pm 2.573^{1}$	$^{e}0.925 \pm 0.980^{4}$
	MdGCN	0	0	0	4.62	12.28
	Min	5.17	3.51	13.70	9.24	1.07
	Max	3.18E + 01	6.15E + 02	5.59E + 01	2.32E + 06	1.16E + 03
T. phagedenis						
	MLCN ± SD	$^{b}1.042 \pm 1.335^{3}$	$^{b}1.190 \pm 1.209^{4}$	$^{bc}4.703 \pm 1.641^{2}$	$^{a}2.775 \pm 2.356^{2}$	$^{de}1.540 \pm 1.312^{14}$
	MdGCN	4.38	14.85	168000	152.65	20.2
	Min	1.66	1.27	29.10	1.31	1.99
	Max	3.94E + 05	3.98E + 03	2.36E + 06	1.28E + 06	1.97E + 03
T. pedis						
	MLCN ± SD	$^{a}1.132 \pm 1.659^{4}$	$^{b}1.009 \pm 1.358^{1}$	$^{d}4.772 \pm 2.241^{23}$	$^{ab}3.687 \pm 2.126^{34}$	$^{bc}1.877 \pm 1.810^{1}$
	MdGCN	0	0	98000	44596	73.30
	Min	32.70	4.09	42.60	40.60	4.91
	Max	1.18E + 07	1.31E + 05	2.66E + 07	3.93E + 06	5.31E + 04

dairy cattle, such as *B. pyogenes*, require further extensive exploration to distinguish their role as primary or opportunistic pathogens [19, 49].

The qPCR analysis revealed changes in the population of *F. necrophorum* and *F. mortiferum* that were otherwise indiscernible from 16S rRNA gene sequencing. Both *Fusobacterium* spp. were associated with BFR as well as BDD. Although *F. necrophorum* was present in higher abundance across all groups compared to healthy cattle, its median copy number was approximately 6-fold higher in BFR compared to both stages of BDD. Also, the significant difference in the abundance of *F. necrophorum* between BFR and BDD-M2 suggests its discriminating potential between BFR and active BDD, further supporting the hypothesis of its primary involvement in BFR. Conversely, despite higher abundance of *F. mortiferum* in both M-stages of BDD, significant differences were observed only when compared to inconclusive group. Notably, median copy number of *F. mortiferum* was approximately 34-fold higher in BDD-M2 and 17-fold higher in BDD-M4 compared to BFR group, indicating its importance in BDD. However, studies on pathogen inoculation in animal models are necessary to establish the potential role of *Fusobacterium* spp. as the

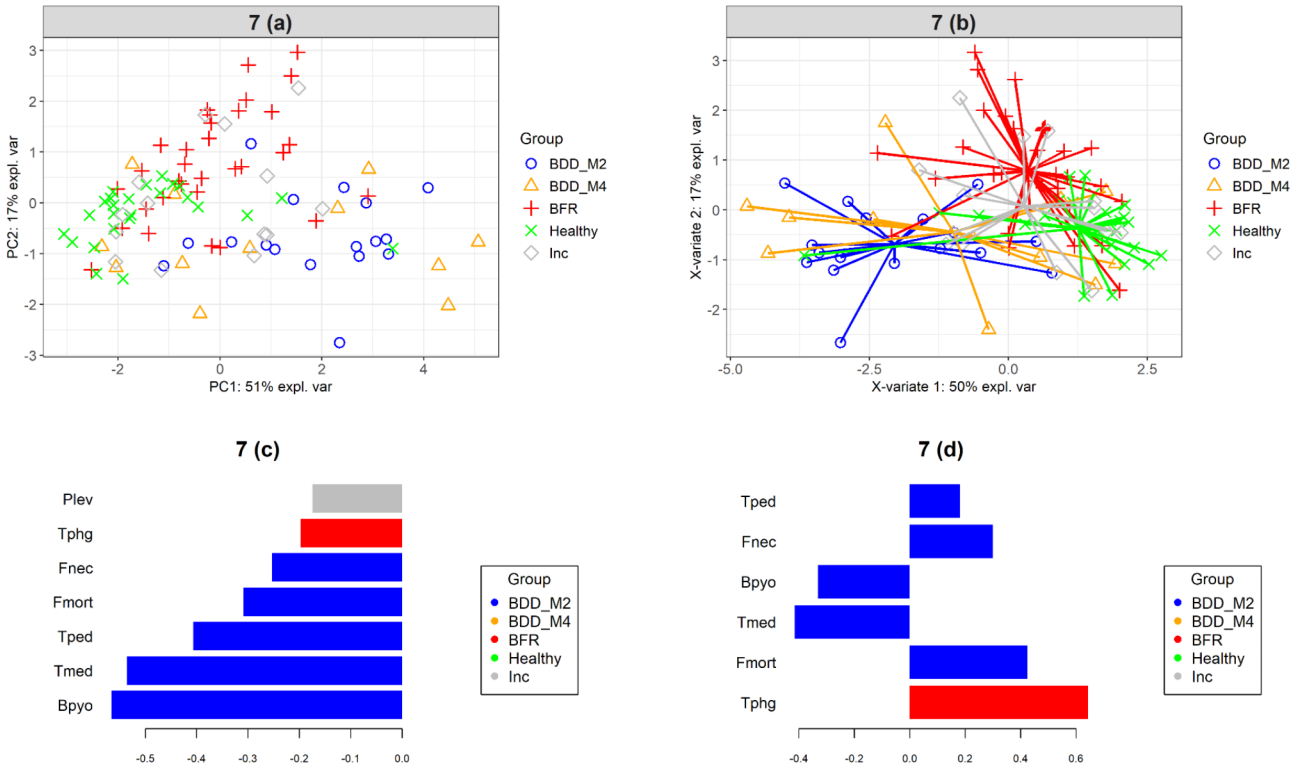

Fig. 7 Discriminant analyses of the cattle groups based on the absolute abundance of seven bacterial species. **(a)** PCA analyses on animal groups (healthy, BFR, BDD-M2, BDD-M4, and inconclusive), **(b)** Plot of sparse PLS-DA, **(c)** Plot loadings of X-variate 1, and **(d)** Plot loadings of X-variate 2, from sPLS-DA method to discriminate various animal groups. Fnec: *F. necrophorum*; Fmort: *F. mortiferum*; Plev: *P. levii*; Bpyo: *B. pyogenes*; Tmed: *T. medium*; Tphg: *T. phagedenis*; Tped: *T. pedis*

primary pathogen in either BFR or BDD. Importantly, the potential role of different strains of *Fusobacterium necrophorum* in BFR and BDD pathogenesis can not be overlooked. Also, the observed differences in the affinity of *Fusobacterium* spp. to different diseases suggest molecular differences between these 2 species. However, it should be noted that a higher quantity of *F. mortiferum* in BDD lesions was observed only in beef cattle but not in dairy cattle [19, 49]. Further cross-sectional and longitudinal studies on the quantification of these *Fusobacterium* spp. in both beef and dairy settings, including their population in healthy cattle feet and their environment, can provide deeper insights into their involvement in BFR and BDD. Additionally, while the categorization of *F. necrophorum* as a passive invader solidifies its role in BFR following the loss of epithelial integrity, a deeper understanding of the virulence factors of *F. mortiferum* is warranted to confirm its pathogenic potential in BFR and BDD [50, 51].

Associations of various *Treponema* spp. with stages of BDD strengthen the hypothesis of their involvement in BDD pathogenesis. However, similar associations were observed in other groups compared to the healthy animals. Despite challenges in differentiating BDD-M2 and BDD-M4 based on *Treponema* spp., these bacteria were approximately 3-fold more abundant in both stages of BDD compared to the BFR group, suggesting their potential as discriminating factors. Specifically, we observed a strong association in the abundance of *T. phagedenis* and *T. pedis* in BDD-M2 compared to other non-BDD groups. In contrast, the population of *T. medium* was two to ten-fold higher in BDD-M4 compared to other groups indicating a strong association of *T. medium* with BDD-M4. This finding contrasts with recent studies where the population of *T. medium* did not differ significantly between the stages of BDD in beef cattle and was associated with BDD-M2 in dairy cattle [19, 42]. Furthermore, contrary evidence from a metagenomic study in dairy cattle revealed a higher relative abundance of *T. medium* and *T. phagedenis* in M2 and M4 lesions, respectively, but not of *T. pedis* [20]. These discrepancies in the association of various *Treponema* spp. with the M-stages of BDD can be explained by potential interchangeability of *Treponema* spp. across these M-stages, indicating that individual *Treponema* spp. might not be restricted to a particular stage. Differences in varying abundance levels between beef and dairy cattle may also indicate potential differences in population changes of *Treponema* spp. in BDD stages between beef and dairy breeds. Furthermore, these variations between studies highlight the limitations

Fig. 8 Heatmap of Spearman's correlation of bacterial abundance across diseased cattle groups based on qPCR. Significant correlations are denoted by an asterisk (*) mark and negative correlations are denoted by a dash (-) before the respective values

of cross-sectional sampling where overgeneralization of a particular stage of BDD might not reflect bacterial composition evident across the beginning and the end of that stage. Hence, longitudinal studies are critical in studying accurate changes in bacterial populations.

The inconclusive group exhibited a considerable visual overlap in microbial composition compared to other animal groups. Relative abundance of several important bacteria such as *Fusobacterium*, *Porphyromonas*, *Mycoplasma*, *Bacteroides*, and *Treponema* in this group was similar to that in either BFR or the M4 stage of BDD. Moreover, no significant changes in the absolute abundance of bacteria targeted for qPCR were evident in the inconclusive group. This suggests that the cases classified as inconclusive may be visually misdiagnosed cases of either the BFR or the BDD-M4 group. Notably, the relative abundance plots indicated a higher relative abundance of *Bacillus* spp. and the *Escherichia-Shigella* complex in the inconclusive group. Upon closer inspection, 2 samples collected on a single day from 1 feedlot, and another 2 samples collected on another day from another feedlot, had a higher proportion of *Bacillus* spp. and *Escherichia-Shigella* complex, respectively. The sample-specific relative abundance of these bacteria in the inconclusive group seems to have skewed the results also indicating their potential role as contaminants. Furthermore, the lack of sequences related to *Bacillus* spp. and *Escherichia-Shigella* complex in the negative controls related to DNA extraction and sequencing indicates that these bacteria were not lab-generated contaminants. Additionally, the absolute bacterial abundance within the inconclusive group did not reveal any striking differences, except for a higher population of *P. levii* compared to other bacteria. Consequently, no bacteria could provide a basis for an exclusive classification of the inconclusive category for diagnostic purposes.

Limitations of this study concern a potentially low statistical power, primarily because of a small sample size associated with both stages of BDD, and the inconclusive category compared to the BFR group. Another limitation of our study was the lack of a bead-beating step in DNA extraction which could have resulted in less representative microbiome composition [52]. Furthermore, a deeper exploration of different feedlots and seasonal changes could provide additional context in the microbiome variations of BFR and BDD. Additionally, we stored our tissue sections in glycerol with BHI media which could potentially influence downstream processes. To minimize significant changes in bacterial composition due to the media, the tissue sections were frozen immediately after submersion and promptly used for DNA extraction upon thawing. This approach minimized the time of contact between the media and bacteria, reducing the likelihood of significant population changes. Furthermore, the extracted DNA consistently showed high quality, with A260/280 ratios close to 1.8 for all samples, ensuring the reliability of downstream processes. We also rarefied our samples, a practice that remains a debate in bioinformatics and microbiome studies [53, 54]. However, strong evidence supports the utility of rarefying samples for addressing uneven sequencing depth [55], a consideration relevant to our study. Moreover, rarefaction was applied across samples from all cattle groups, minimizing potential biases for analyses.

The use of both 16S rRNA gene sequencing and a species-specific qPCR assay enabled the exploration of potentially new etiological agents while intricately studying population changes of bacterial species already deemed important in BFR and BDD pathogenesis. Notably, differences between various groups, particularly between BFR and both stages of BDD, were identical between 16S RNA gene sequencing and qPCR. However, a cautious interpretation of these findings is warranted as factors such as sampling depth, sampling platform, primers, bioinformatics pipeline, and varying copy numbers of the 16S rRNA gene between bacterial taxa can affect outcomes [56–61]. Similarly, findings from qPCR are also subject to various biases [62–64]. This demands an understanding of the genomic characteristics of the bacteria of interest before drawing pivotal conclusions. Also, whereas the idea of the generalizability of the findings in dairy cattle seems enticing, comparisons must be made with caution until the effect of varying risk factors between the beef and dairy management systems is understood in the context of the pathogenesis of BFR and BDD [1, 65, 66].

Conclusion

We concluded that the healthy skin in beef cattle had a higher bacterial diversity, and this diversity decreased in BFR and BDD. This reduction in bacterial diversity in foot skin was marked by a higher abundance of *Fusobacterium* spp. and *Treponema* spp. in both BFR and BDD. Additionally, the change in population of various bacterial species in BFR and BDD indicated a polybacterial involvement. Although bacterial quantity was generally higher in diseased animals, notable differences were observed: BFR differed from BDD-M2 due to a higher quantity of *F. necrophorum* and from BDD-M4 due to a lower quantity of *T. medium*. Additionally, quantities of *T. phagedenis* and *T. pedis* effectively distinguished BFR from both stages of BDD. While a broad association of *Fusobacterium* spp. with BFR could not be established, the distinctively higher abundance of *Treponema* spp. in BDD substantiated the potential for targeting these bacteria in treating and preventing BDD. These findings underscored the complex microbial population dynamics in BFR and BDD and emphasized the importance of a

multifaceted approach in identifying potential etiological agents.

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Author contributions
SP: Project coordination, fieldwork, lab work, raw sequence processing, data curation, analysis, and interpretation, manuscript writing/reviewing/editing. BJC: Fieldwork, metagenomic analysis and interpretation, manuscript reviewing. APD: Fieldwork, qPCR assays, manuscript reviewing. JDB: Research supervision, guidance on lab work and data analysis, manuscript reviewing/editing. DWM: Research supervision, guidance on fieldwork and lab work, manuscript reviewing/editing. KO: Project conception and study design, funding acquisition, overall coordination, supervision and guidance on progression, statistical analysis and interpretation of data, manuscript reviewing/editing. The author(s) read and approved the final manuscript.

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Data availability
Raw FASTQ reads generated from deep amplicon sequencing are accessible at the NCBI SRA database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1153132/) under BioProject accession number PRJNA1153132. Anonymized details on animals and feedlots will be provided upon reasonable request.

Declarations

Ethics approval and consent to participate
Approval of animal use and permission for specimen collection in this study was granted by the University of Calgary Veterinary Sciences Animal Care Committee (VSACC) under the animal care protocol AC21-0126. Written informed consent was obtained from the feedlot producers for the participation of their animals and the publication of the study results.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.
Clinical trial number.
Not applicable.

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