Assessment of the Fecal Microbiota in Beef Calves

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Background: There is increasing interest in the fecal microbiota, but study in calves has been limited.

Hypothesis/Objectives: To evaluate the fecal microbiota of beef calves and cows on different farms, and to preliminarily explore the impact of antimicrobial exposure.

Animals: A total of 172 animals, 156 (91%) calves and 16 (9.3%) cows, were enrolled from 5 cow-calf farms.

Methods: The fecal bacterial microbiota was assessed through sequencing of 16S rRNA gene (V4 region) amplicons.

Results: There were significant differences in the relative abundances of numerous phyla between calves on different farms. Farms could be separated into 2 groups: 1 (farms B and C) dominated by Firmicutes and 1 (farms A, D, and E) with predominance of Proteobacteria and Actinobacteria. Richness (median 2,974 versus 1,477, $P = .008$), diversity (51.4 versus 29.1, $P = .0029$), and evenness (0.73 versus 0.68, $P = .006$) were higher in cows. Over-represented operational taxonomic units (OTUs) in cows tended to be from the classes Bacilli and Bacteroidia, whereas Clostridia and Actinobacteria were most prominently over-represented in calves. There were differences in community membership ($P = .028$) and structure ($P = .029$) in calves that had a history of antimicrobial exposure compared those that did not. Eight (89%) over-represented OTUs in the untreated group were Firmicutes (7 from the order Clostridiales), compared to only 3 (38%) (2 Clostridiales) in the untreated group.

Conclusions and Clinical Importance: Interfarm variation should be investigated to determine the causes and potential implications for health and production. Antimicrobial exposure may have an impact on the fecal microbiota at individual and farm levels.

Key words: Antimicrobial; Bacteriology; Bovine; Gastrointestinal.

The body harbors vast microbial populations (micro-biotas) that interact closely with the host. As research advances, it is clear that the intestinal microbiota exerts influences both locally and extra-intestinally, and alterations of the microbiota can be associated with a wide range of infectious, inflammatory, metabolic, and other diseases in many species.[1–5]

While comparative data can provide insight, the unique nature of the ruminant gastrointestinal tract hampers extrapolation of data from nonruminant species. Even within ruminant species, major changes in gastrointestinal tract anatomy and the associated microbiota occur as calves age and progress from milk feeding to the development of a functional rumen.[6,7] Nevertheless, a few recent studies have started to describe the fecal and ruminal microbiota in cattle,[6–12] providing initial insight into this potentially important area.

Abbreviations:

DNA	deoxyribonucleic acid
LEfSe	linear discriminant analysis effective size
OTU	operational taxonomic unit
PCoA	principle coordinate analysis
RDP	ribosomal database project
RNA	ribonucleic acid

Antimicrobial administration has been shown to have an impact on the intestinal microbiota in various species, including humans, pigs, horses, and laboratory animals,[13–16] but there has been limited study in cattle, despite the commonness of antimicrobial exposure in commercial beef and dairy cattle. Penicillin administration was shown to significantly alter the microbiota in calves <6 months of age;[17] however, the methods that were used (automated ribosomal intergenic spacer analysis and terminal restriction fragment length polymorphism analysis) could not provide insight into the nature of those changes. A study by next-generation sequencing reported decreased bacterial richness in calves with pneumonia that were treated with antibiotics, as well as calves with diarrhea that were not treated.[6] Another next-generation sequencing study identified changes in some taxa in dairy calves fed milk with antibiotic residues compared to untreated controls; however, these were relatively limited in number and restricted to the genus level.[7] Further study of factors that influence the microbiota, particularly in farm environments, is required to better understand this important microbial community and how it is related to health and disease.

The objectives of this study were to evaluate the fecal microbiota of beef calves and cows on different farms and the impact of antimicrobial treatment on the fecal microbiota in calves.

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Materials and Methods

Study Population

Fecal samples were collected from calves on 5 western Canadian cow-calf farms between April 17 and May 31, 2013. Farm selection was based on 3 criteria: geographical distribution, willingness of veterinarians, and producers to participate, and that the producers maintained treatment records. The number of cow-calf pairs per farm varied between approximately 50 and 250. The timing of the sampling coincided with when the cattle producers were routinely handling their animals. Producers were instructed to obtain 25–30 calf samples and that the sampling should include an equal number of samples from those that had received antimicrobial treatment ("treated") and those that had never received antimicrobials ("nontreated"); nontreated calves were randomly selected. A graduate student collected samples from farm C, which included samples from calves and cows. Individual data regarding calf birthdates, treatment dates, and sampling dates were not provided by all producers. In general, beef calves in western Canada are typically born between February and April. Therefore, calves would have been <4 months of age. Three farms (A, D, and E) were located in close proximity to each other near Kamloops, British Columbia. The fourth farm (C) was located north of Saskatoon, Saskatchewan, and the last farm (B) was located in close proximity to Brandon, Manitoba. Sampling involved inserting a minimum of 2 swabs 2–3 cm into the rectum. Samples were also collected from cows on 1 farm (farm C).

At the time of sampling, the swabs were placed in a cooler on ice until they could be frozen ($-20°C$), later that day. Frozen samples were transferred to the Western College of Veterinary Medicine, where they were stored at $-82°C$ until being shipped on dry ice to the Ontario Veterinary College. Antimicrobial exposure data were also collected when possible. This study was approved by the University of Saskatchewan Animal Research Ethics Board (protocol #20130032).

Sequencing

DNA was extracted from fecal samples by a commercial kit.[a] The V4 region of the 16S rRNA gene was amplified by PCR,[18] and amplicons were sequenced by Illumina (2×250).[b] Because of the large number of samples, multiple sequence runs were required. Samples were mixed so that individual sequence runs contained samples from multiple farms.

Data Analysis

MOTHUR v1.34[19] was used for sequence processing and analysis. After assembly of paired end reads, sequences that were not consistent with the target amplicon size (240 bp) or contained any ambiguous base calls or long runs (>8 bp) of holopolymers were removed. Sequences were aligned with the Silva 16S rRNA reference database[20] and those that did not align with the V4 region were removed. Archaea were also removed. Chimeras were detected by UCHIME[21] and removed. Taxonomy was assigned by the ribosomal database project (RDP) classifier[22] for database-dependent analysis. Sequences were binned into operational taxonomic units (OTUs) at a 3% dissimilarity level for database-independent (de novo OTU clustering) analysis.

Relative abundances were compared by linear modeling by robust (Huber) estimation to down-weight outliers, with P values that were adjusted for false discovery rate by the Benjamini–Hochberg technique. For subsequent analysis, subsampling was performed to normalize sequence number through random selection of a number of sequences that corresponded to the minimum number for any sample. Alpha diversity indices were calculated, consisting of inverse Simpson's index (diversity), Shannon's evenness index (evenness), and Chao1 index (estimated richness). These were compared by Steel–Dwass or Wilcoxon tests.

Dendrograms were developed based on the Yue and Clayton measure of dissimilarity (a measure of community structure that considers shared OTUs and their relative abundances) and the traditional Jaccard index (a measure of community membership that only considers the number of shared OTUs, not their abundance). Unweighted unifrac[23] was used to compare community membership and structure between groups. Principal coordinate analysis (PCoA) and linear discriminant analysis effective size (LEfSe)[24] were performed. The number of different meta-communities (enterotypes) that the data could be clustered into was determined based on Dirichlet multinomial mixtures method for probabilistic modeling,[25] with the K value that derived the minimum Laplace approximation indicating the number of different meta-communities. P values of <.05 were considered significant for all analyses.

Results

A total of 189 samples were collected from the 5 farms, 16 samples from cows and 172 from calves. However, 16 calf samples were either not processed because of labeling issues or the sample was of insufficient volume. Results were generated from 172 samples, 156 (91%) calves and 16 (9.3%) cows. The number of calves sampled per farm ranged from 25 to 48 (median 27); only cows (n=16) from farm C were sampled. Forty-two (29%) of the calves had prior antibiotic exposure at some point, which are identified herein as "treated" calves. Of the 42 treated calves, 11/25 (44%) were from farm A, 0/25 from farm B, 4/48 (8.3%) from farm C, 13/31 (42%) from farm D, and 14/27 (52%) from farm E ($P < .0001$). Although the farms recorded whether a calf received antimicrobial treatment, only farm D provided detailed data on what treatment was used. In conversations with the other producers, they were reasonably sure what each calf was treated with, but because it was not recorded at the time of treatment, it was equivocal as to what antibiotic was actually administered. Therefore, specific treatment data for those farms were not analyzed. Antimicrobials administered on farm D included sulfamethazine (n=8), oxytetracycline (n=5), florfenicol (n=3), trimethoprim-sulfadoxine (n=2), enrofloxacin (n=1), and penicillin (n=1). Because of the limited antimicrobial use data and the variety of drugs that were used in treated calves on that farm, evaluation of the impact of specific antimicrobials was not performed and antimicrobial treatment was analyzed as a binary (yes/no) variable.

A total of 8,555,682 sequences from 172 samples passed all quality control filters (mean 49,742/sample, median 46,022, range 10,493–195,449). The entire dataset was used for comparison of relative abundances. A random subsample of 10,493 sequences per sample was used to normalize sequence numbers for other analyses.

Comparison of Nonantimicrobial-Treated Calves Between Farms

Twenty-five different phyla were identified, but only 5 accounted for >1% of sequences each (Table 1). There were significant differences in the relative abundances of

Actinobacteria ($P < .001$), Bacteroidetes ($P < .001$), Chloroflexi ($P < .001$), Firmicutes ($P < .001$), Fusobacteria ($P = .015$), Proteobacteria ($P = .0002$), Spirochetes ($P = .016$), and Verrucomicrobia ($P = .010$) between farms. There was apparent grouping of farms B and C and farms A, D, and E. Farms B and C were characterized by a microbiota dominated by Firmicutes, as opposed to a predominance of Proteobacteria and higher relative abundance of Actinobacteria in the other farms. The predominant families and genera are presented in Table 2.

There were significant differences in diversity (Fig 1) and evenness, but not richness on farms A, D, and E compared to farms B and C (Table 3). Similar grouping can be visualized with PCoA (Fig 2). Significant differences in community membership (Jaccard index) and structure (Yue and Clayton index) were identified between all farms (all $P < .001$).

When farms A, D, and E were grouped together and compared by LEfSe to farms B and C, 313 OTUs were identified as differentially enriched between the 2 groups. The 10 OTUs with the highest linear discriminant analysis score from each group are presented in Figure 3. Nine of the 10 most enriched OTUs from farms B and C were Firmicutes (including 8 from the order Clostridiales), whereas 7/10 from farms A, D, and E were Proteobacteria. Similarly, numerous Clostridiales were present at higher relative abundances in farms B and C, including *Faecalibacterium, Ruminococcus, Lachnospira, Blautia,* and *Dorea,* as well as other potentially relevant genera such as *Lactobacillus* (all $P < .001$).

Samples could be assigned to 3 different metacommunities. Community 1 contained 42/43 (98%) samples from farm C and 11/25 (44%) from farm B. Community 2 contained 11/25 (44%) samples from farm B, 1/43 (2.3%) from farm C, and 1/18 (5.6%) from farm D. The third community contained all samples from farms A and E, the 17/18 (94%) samples from farm D and 3/25 (12%) from farm B.

Evaluation of the Impact of Antimicrobial Treatment of Calves

Because of the significant differences in the microbiota between healthy calves on farms B and C versus farms A, D, and E, and the number of calves treated on each farm group (4 versus 38, respectively), analysis of the impact of antimicrobials was performed with only farms A, D, and E. This consisted of 38 treated and 45 untreated calves. There were no differences in

coverage ($P = .94$) or richness ($P = .55$), but antimicrobial-treated calves had lower diversity (median 12.7 versus 15.1, $P = .015$) and evenness (0.59 versus 0.60, $P = .04$) compared to untreated calves. No differences in community membership ($P = .60$) or structure ($P = .59$) were identified by unifrac. The lack of impact of antimicrobial exposure can also be visualized by PCoA (Fig 4). There were no differences in the relative abundances of any phyla, with limited differences at lower taxonomic levels. When the 100 genera with the highest relative abundances were analyzed, there were no significant differences after adjustment for false discovery rate.

To further evaluate the impact of antimicrobials, analysis of farm E alone was performed, as this was the farm with a relatively equal distribution of treated ($n = 14$) and untreated ($n = 13$) calves. No differences in richness ($P = .46$), diversity ($P = .28$), or evenness ($P = .26$) were identified. There was a significant difference in community membership ($P = .028$) and structure ($P = .029$) between treated and untreated calves. When phyla were compared, the only difference was a significantly greater relative abundance of Spirochetes in treated calves (0.0019 versus 0.0007, $P = .013$). Seventeen significantly enriched OTUs were identified by LEfSe (Table 4). Eight (89%) in the untreated groups were Firmicutes (with 7 of those being from the order Clostridiales), compared to only 3 (38%) (2 Clostridiales) in the untreated group.

To highlight the potential impact of pooling data from all farms, basic analysis of the impact of antimicrobials on the entire calf dataset was performed. When all farms were pooled, there were numerous significant differences at the phylum level, with treated calves having lower relative abundances of Actinobacteria, Bacteroidetes, and Firmicutes, and higher Deinococcus–Thermus and Proteobacteria, mimicking the interfarm differences in untreated calves. There were also significant differences in community membership ($P = .002$), with a difference in structure that neared significance ($P = .050$).

### Comparison of Cows and Calves from the Same Farm

Samples were available from 16 cows and 48 calves from the same farm (farm C). The microbiota of adult cows had significantly greater estimated richness (median 2,974 versus 1,477, $P = .008$), diversity (51.4 versus 29.1, $P = .0029$), and evenness (0.73 versus 0.68, $P = .006$) compared to the 44 untreated calves. At the

Table 1. Comparison of the median relative abundances of predominant phyla in the fecal microbiota of calves that did not receive antimicrobials on 5 farms.

Phylum	Farm A (n = 14)	Farm B (n = 25)	Farm C (n = 44)	Farm D (n = 18)	Farm E (n = 13)
Actinobacteria	0.19	0.0059	0.007	0.095	0.12
Bacteroidetes	0.01	0.031	0.023	0.02	0.0068
Proteobacteria	0.58	0.11	0.020	0.57	0.65
Verrucomicrobia	0.00057	0.0067	0.0009	0.002	0.0012
Firmicutes	0.19	0.80	0.92	0.23	0.15

Table 2. Relative abundances (in brackets) of predominant families and genera in the fecal microbiota of 2 groups of calves that did not receive antimicrobials.

Family		Genus	
Farms B/C (n = 69)	Farms A/D/E (n = 45)	Farms B/C (n = 69)	Farms A/D/E (n = 45)
Ruminococcaceae (0.16)	Caulobacteriaceae (0.16)	*Brevundimonas* (0.066)	*Brevundimonas* (0.16)
Lachnospiraceae (0.15)	Xanthomonadaceae (0.10)	Unclassified Lachnopiraceae (0.054)	*Pseudomonas* (0.079)
Peptostreptococcacaeae (0.058)	Pseudomonadaceae (0.081)	*Clostridium* cluster XI (0.051)	*Devosia* (0.077)
Unclassified Clostridiales (0.051)	Peptostreptococcaceae (0.079)	*Faecalibacterium* (0.047)	*Clostridium* cluster XI (0.071)
Lactobacillaceae (0.044)	Hyphomicrobiacea (0.078)	*Lactobacillus* (0.044)	*Stenotrophomonas* (0.057)
Moraxellaceae (0.037)	Alcaligenaceae (0.046)	Unclassified Ruminococcaceae (0.040)	*Pusillimonas* (0.040)
Xanthochromadaceae (0.037)	Paenibacillaceae (0.040)	*Psychrobacter* (0.034)	*Lysobacter* (0.039)
Clostridiaceae (0.030)	Micrococcaceae (0.025)	*Devosia* (0.029)	*Arthrobacter* (0.021)
Hyphomicrobiaceae (0.029)	Moraxellacaeae (0.025)	*Clostridium* cluster XIVa (0.028)	*Paenibacillus* (0.020)
Alcaligenaceae (0.027)	Phyllobacteriaceae (0.024)	*Butyricicoccus* (0.025)	*Sanguibacter* (0.020)
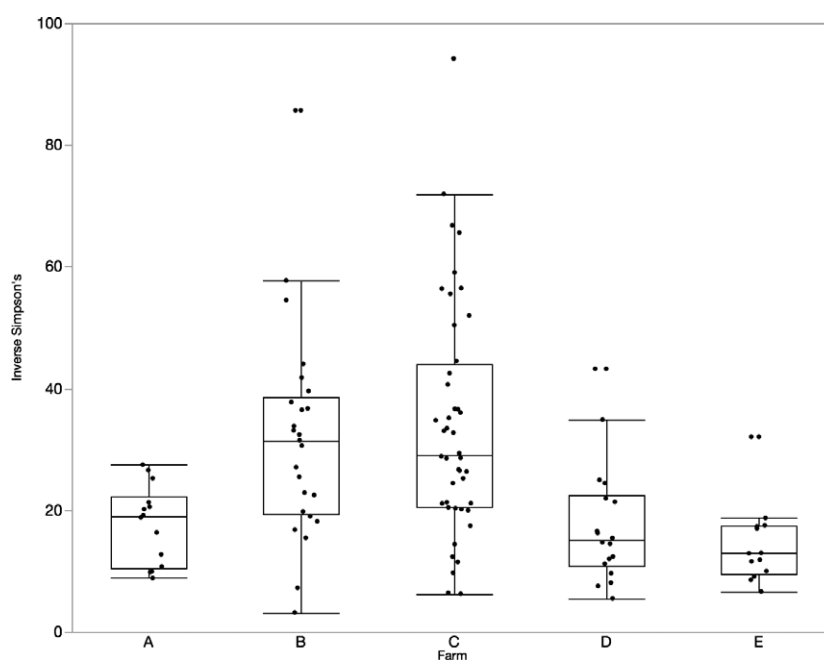
Fig 1. Comparison of bacterial diversity (inverse Simpson's index) in the fecal microbiota of 114 beef calves from 5 farms that had not been treated with antimicrobials.

Table 3. Alpha diversity indices of the fecal microbiota of 114 calves from 5 farms that were not treated with antimicrobials.

	Farm A (n = 14)	Farm B (n = 25)	Farm C (n = 44)	Farm D (n = 18)	Farm E (n = 13)
Richness	1,091[a]	1,566[a]	1,485[a]	1,246[a]	1,057[a]
Evenness	0.57[a]	0.71[b]	0.68[b]	0.59[a]	0.55[a]
Diversity	19[a]	31.5[b]	29.1[b]	15.1[a]	9.43[a]
Coverage	0.97[a]	0.96[a]	0.97[a]	0.97[a]	0.97[a]

Different superscripts indicate significant differences ($P < .05$).

phylum level, there were no differences in major phyla, with Firmicutes accounting for 89% of sequences in untreated calves and 87% in cows. Cows had significantly greater relative abundances of Actinobacteria (0.015 versus 0.007, $P = .002$), Chloroflexi (0.00008 versus 0.00002, $P = .008$), Planctomycetes (0.0001 versus 0.00001, $P = .014$), and TM7 (0.00009 versus 0.00002, $P = .0014$), as well as fewer Bacteroidetes (0.015 versus 0.023, $P = .014$), Chlamydiae (0.000009 versus 0.000086, $P = .008$), and Proteobacteria (0.013 versus 0.020 $P = .012$). Significantly different predominant genera are presented in Table 5.

Significant differences in community membership ($P = .046$) and structure ($P = .040$) (Fig 5) were
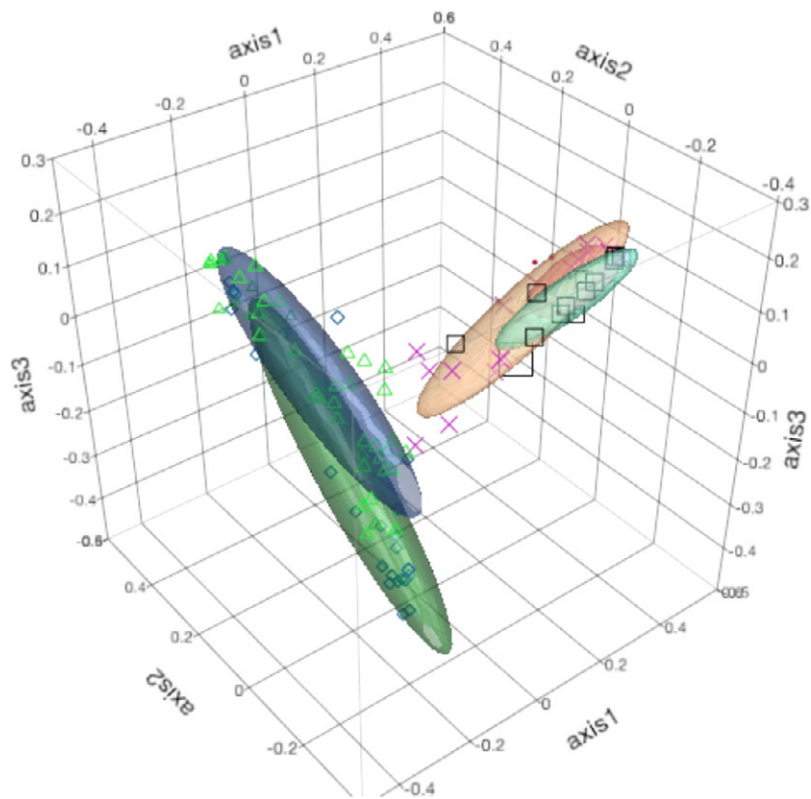
Fig 2. Principle coordinate analysis with 60% ellipsoid coverage of the fecal microbiota of 114 calves from 5 farms that were not treated with antimicrobials. Ellipsoid colors: red: farm A; blue: farm B; dark green: farm C, orange: farm D, light green: farm E.
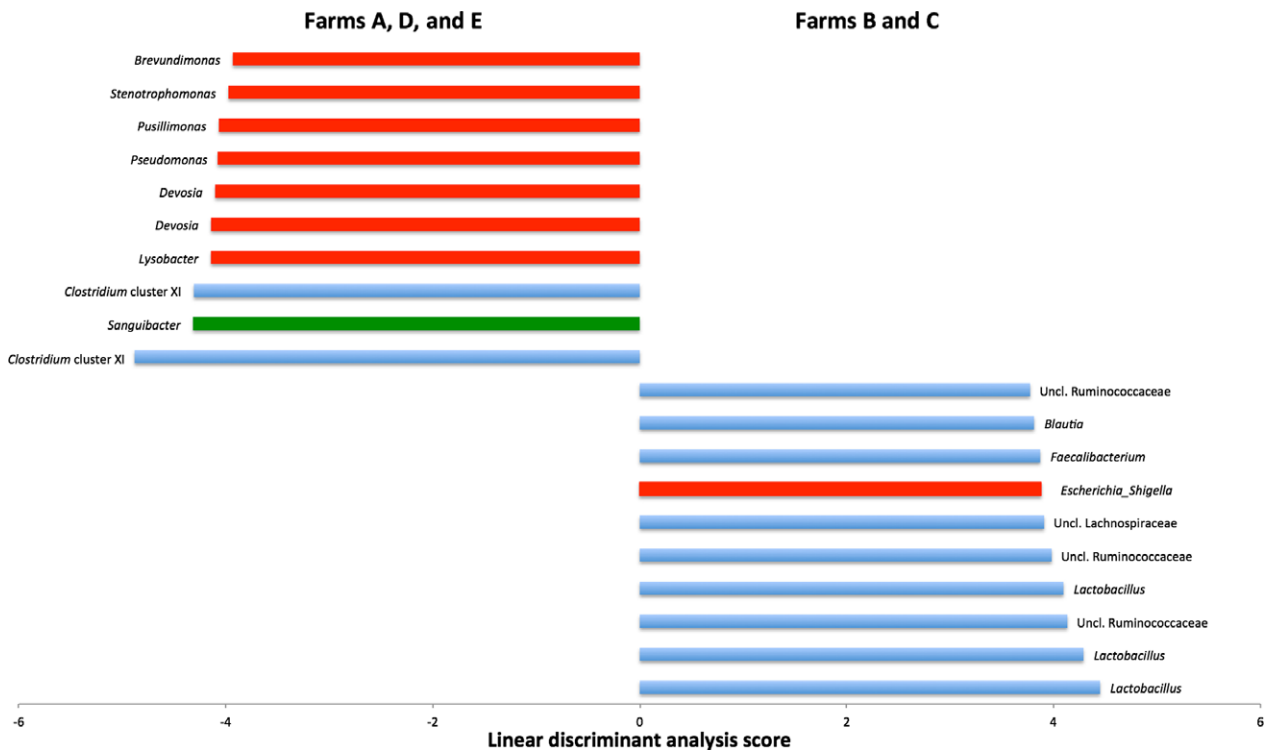
Fig 3. LEfSe results depicting the 10 operational taxonomic units (OTUs) with the highest linear discriminant analysis scores when comparing the fecal microbiota of nonantimicrobial-exposed calves from 2 groups of farms (n = 69 and n = 45). red = Proteobacteria, blue = Firmicutes, green = Actinobacteria.
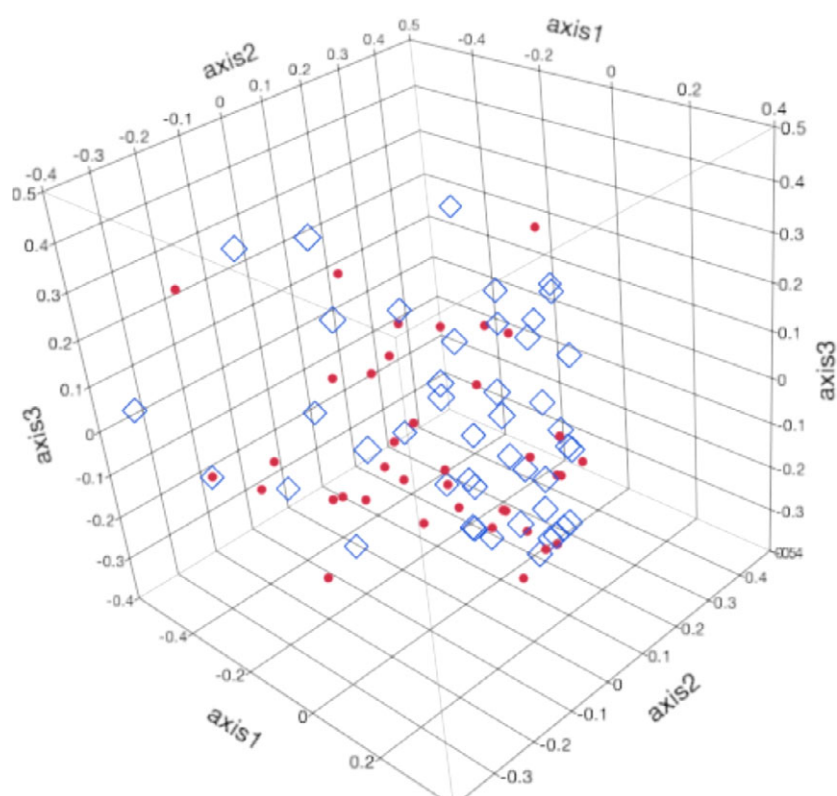
Fig 4. Three-dimensional scatterplot of principle coordinate analysis of the fecal microbiota of 38 antibiotic-treated and 45 untreated calves. blue diamonds = untreated, red dots = treated.

Table 4. Significantly enriched operational taxonomic units (OTUs) from the fecal microbiota of calves from 1 farm (farm E) that were (n = 13) or were not (n = 14) treated with antimicrobials.

Untreated	Treated
Arthrobacter	*Devosia*
Paenibacillus	*Pseudaminobacter*
Unclassified Lachnospiracaeae[a] (2 OTUs)	*Bacteroides*
Blautia[a]	5 genus incertae sedis (Verrucomicrobia)
Unclassified Ruminococcaceae[a]	*Jeotgalibacillus*
Unclassified Clostridiales (3 OTUs)[a]	*Treponema*
	Pseudoflavonifractor
	Clostridium cluster XIV[a]

[a]Order: Clostridiales.

identified between cows and calves. Laplace approximation predicted the presence of 2 separate metacommunities, corresponding to the cow and calf groups.

Five hundred and eighty-one significantly enriched OTUs were identified via LEfSe. Those with the highest linear discriminant analysis scores are presented in Figure 6 and Table 6. Significant OTUs in cows tended to be from the classes Bacilli and Bacteroidia, whereas Clostridia and Actinobacteria were most prominently

over-represented in calves (despite the greater overall relative abundance of the phylum Actinobacteria in cows).

Discussion

The fecal microbiota of calves is rich and diverse, and potentially highly variable between farms. While comparison of the microbiota between farms was not an initial goal of this study, it became apparent that a striking difference was present and that this could profoundly impact analysis. Previous studies of calves have involved single farms or research facilities, precluding an ability to assess interfarm variation. Marked differences between study groups have been apparent, although, with 1 study identifying a Bacteroidetes-dominant microbiota[26] and another a Firmicutes-dominant microbiota,[6] something that could be because of methods, analysis, or true biological differences. Here, farms B and C had a microbiota dominated by Firmicutes, similar to what was reported in 1 study of calves,[6] as well as studies of adult dairy and beef cattle.[8–10] These 2 farms therefore could be interpreted as having the "expected" microbiota, given the consistency of Firmicutes predominance in other studies. However, the other farms had a Proteobacteria-dominant microbiota that was unexpected in a group of healthy individuals. The phylum Proteobacteria consists of a broad group of Gram-negative bacteria, including

Table 5. Genera among the 100 most abundant that were significantly different in the fecal microbiota of cows (n = 16) and calves (n = 48) from 1 farm.

| Genus	Cows	Calves	P Value
Unclassified Clostridiales	0.28	0.18	.003
Lactobacillus	0.035	0.18	.001
Clostridium cluster XI	0.12	0.025	<.0001
Blautia	0.018	0.075	.003
Clostridium cluster XIV	0.004	0.021	.023
Bosea	0.000047	0.000016	.015
Unclassified Peptostreptococcaceae	0.0090	0.0025	<.0001
Unclassified Clostridia	0.013	0.0067	.0002
Mogibacterium	0.020	0.0058	<.0001
Unclassified Paenibaccilaceae	0.00022	0	<.0001
Syntrophomonas	0.0057	0.0022	.0002
Verrucomicrobia genus incertae sedis	0.00085	0.0002	.033
Papillibacter	0.0072	0.0014	.016
Olsenella	0.0051	0.0011	<.0001
Alkalibacter	0.0037	0.00068	.023
Johnsonella	0.0050	0.00083	<.0001

Enterobacteriaceae, and increases in Proteobacteria are often associated with disease such as inflammatory bowel disease or a general state of "dysbiosis."[27–29] Yet, these samples were from healthy calves on well-managed and productive farms, albeit farms where antimicrobial use was common.

There has been limited study of factors that influence the fecal microbiota in cattle. Dietary differences have not been noted to have a substantial impact on phylum-level composition.[9,10] Various other factors could account for major differences in the identified microbiota, including differences in breed, management practices, and study methodology; however, data from this study of similar farms from similar regions by the same methodology suggest that there may be major undetected influences on the fecal microbiota. Farms A, D, and E were from the same region of British Columbia, but had little interaction. There was movement of a small number of bulls from farm D to farm E during the year of sampling, but the bulls were kept in a different area than the cows and calves that were sampled. Farms A and E were approximately 8 km apart and shared the same valley and watershed. Farms B and C were from 2 different Canadian provinces. The potential effect of geography is unknown, and it is difficult to develop a reasonable hypothesis of why the microbiota might be different in different provinces in the same country. Interestingly, a similar difference in microbiotas was noted in the fecal microbiota of wood bison, another ruminant species in western Canada, where 2 different microbiota types were identified within a group of semi-free ranging animals.[30] These were similar to the community types reported here, with 1 group exhibiting a Proteobacteria-dominated microbiota and the other dominated by Firmicutes. Whether ruminants can have such profound differences in the fecal microbiota, particularly a Proteobacteria-dominated microbiota, in the absence of any impact on health and production, requires investigation, given the association of Proteobacteria increases with gut inflammation.

The interfarm differences noted here are important for multiple reasons. All farms are well-managed, productive cow-calf operations, with no identifiable serious health or production problems. The differences between farms suggest that there may be important and currently unidentified management practices that can significantly influence the microbiota. It also highlights a consideration for future observational microbiota studies and limitations in making broad assessments of the "normal" microbiota from limited study populations. If there are potentially major differences between similarly managed farms, these inherent differences might be misinterpreted as an effect of a studied parameter rather than recognized as an inherent interfarm difference. This highlights the need for careful consideration of controls for microbiota studies, something that was apparent when all farms were combined for the analysis of the impact of antimicrobials. When all farms were considered, various significant differences between treated and untreated animals were noted. However, similar differences were also noted when comparing untreated calves on the farms. Thus, the differences were a farm effect, not an effect of antimicrobial exposure of the individual animal, highlighting the potential for erroneous conclusions if appropriate controls are not studied. By similarly managed farms as controls without assessing the microbiota, pre- or intrastudy might lead to erroneous conclusions.

Antimicrobials can impact the microbiota through depletion of some members (and correspondingly loss of their functions), the impacts of overgrowth of resistant members and direct influences of the drug on host tissues (with subsequent modification of the local environment). It is well established that antimicrobials can affect the intestinal microbiota in various species,[13,31,32] although characterizing those changes and determining the relevance can be challenging. The apparent impact of antimicrobial treatment in these calves was limited. While studies in some species have reported major effects of antimicrobials, the impact can be variable depending on drug, dose, and duration. Treated calves had decreased microbial diversity, which is consistent with studies in humans, horses, and pigs,[13,15,32,33] and a recent study of dairy calves fed milk containing antibiotic residues.[7] The clinical relevance of this is unclear as optimal microbial diversity is not well understood but decreases in diversity often accompany disease and lower diversity may lead to a more limited ability of the microbiota to respond to different stressors. Despite the change in diversity, there were limited differences in specific taxa, similar to a study in dairy calves that only identified differences in relative abundances at lower taxonomic levels.[7] However, when calves from only 1 farm were studied to provide the most closely matched cases and controls, differences in membership and structure were noted. Similar to the broader analysis and study in dairy calves, taxonomic differences were still restricted to lower levels (genus), where multiple
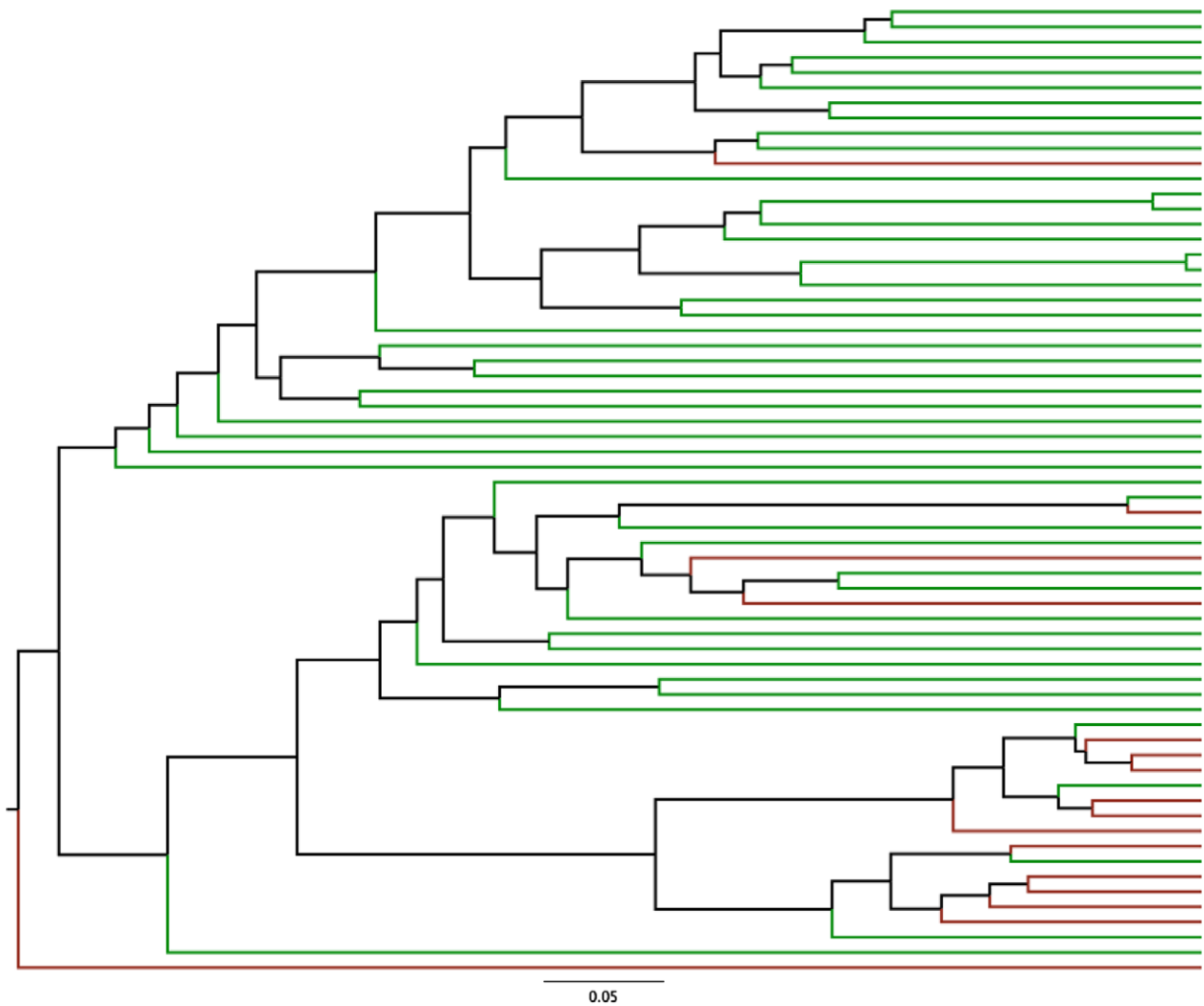
Fig 5. Dendrogram of the community structure of the fecal microbiota of cows (red, n = 16) and calves (green, n = 48) from the same farm, based on the Yue and Clayton index of dissimilarity.

differences were noted. Most of the OTUs identified as enriched in untreated calves by LEfSe were Firmicutes, particularly Clostridiales. Clostridiales, such as the butyrate-producing Lachnospiraceae and Ruminococcaceae families that were enriched in untreated animals, are increasingly associated with "gut health" in various species, including humans, horses, and dogs.[34–36] *Faecalibacterium,* another butyrate-producing member of Clostridiales, was associated with health and growth in a study of calves.[6] While there was no significant difference in this study, the cited study provides more support for the important of this group of Clostridiales.

There are limitations to the antimicrobial component of the study. Specific antimicrobial use data were limited, and not all calves that had been treated were currently undergoing treatment. This is an inherent disadvantage of many field-based studies where sampling and data collection can be a challenge. Greater changes could have been present during treatment, with resolution or attenuation of those changes by the time of sampling. The effect of disease that resulted in

treatment and treatment itself also cannot also be discerned. Longitudinal study of calves before, during, and after antimicrobial administration would provide additional insight. Regardless, the differences that were noted and the similarity to negative changes reported in other species suggest that antimicrobials could incite potentially deleterious effects on the gut microbiota.

It was interesting to note that antimicrobial treatment rates were highest on farms A, D, and E, the farms with the Proteobacteria-enriched microbiota that is quite different from results of other studies of calves and adult cattle.[6,8,9,26] This raises questions about whether antimicrobial use practices could have a broader or cumulative impact on farms, where frequent use results in the development of a different microbiota in animals on the farm, regardless of their individual antimicrobial exposure. While this is highly speculative, antimicrobial administration has also been associated with increases in Proteobacteria in humans.[16,32,37] Significant differences in potentially relevant genera were also identified, including under-representation of potentially important
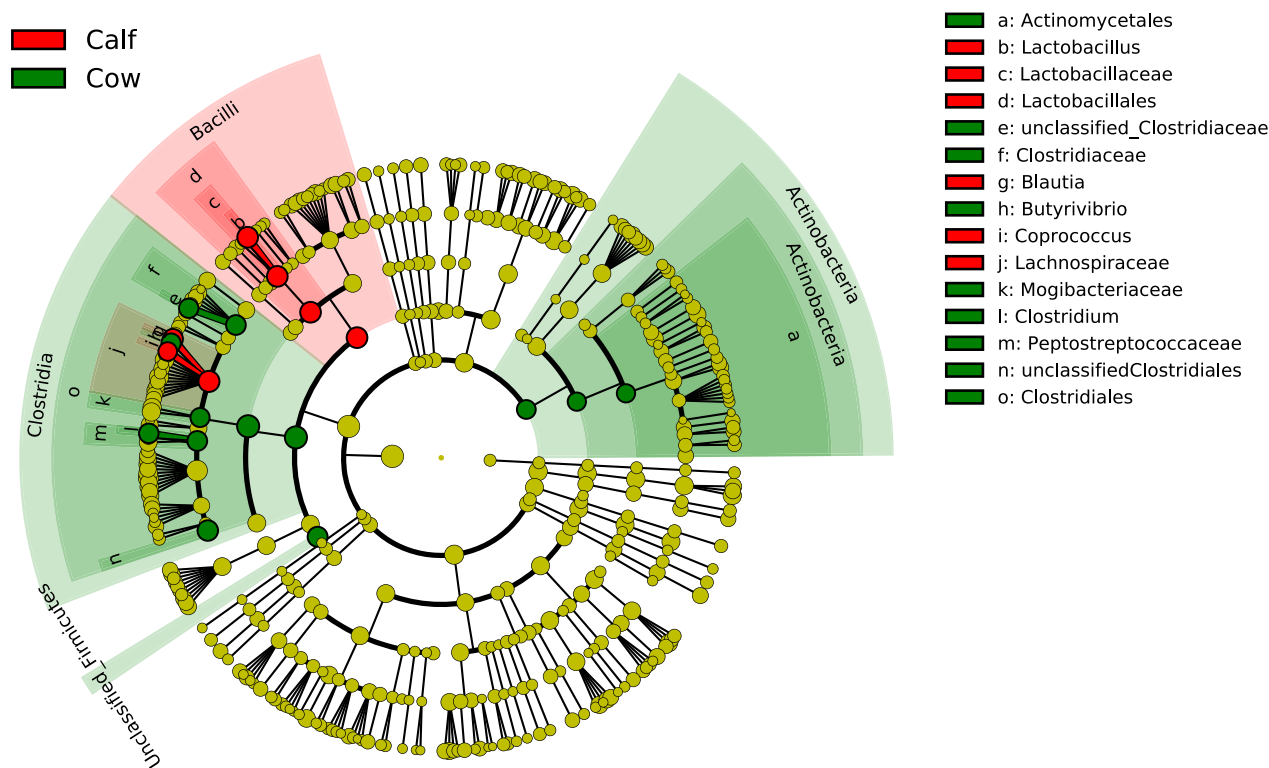
Fig 6. LEfSe comparison of the fecal microbiota of cows (n = 16) and calves (n = 48) from the same farm, depicting operational taxonomic units with a linear discriminant analysis score of ≥4. Red: calf; green: cow.

Table 6. Significantly enriched operational taxonomic units (OTUs) with the highest linear discriminant analysis scores from the fecal microbiota of calves (n = 48) and cows (n = 16) from 1 farm (farm C).

Cows	Calves
Clostridium cluster XI (3 OTUs)	*Lactobacillus* (3 OTUs)
Unclassified Clostridiales (6 OTUs)	*Blautia* (4 OTUs)
Unclassified Lachnospiraceae (2 OTUs)	*Escherichia/Shigella*
Mogibacterium	*Anaerostipes*
	Unclassified Clostridiales (2 OTUs)
	Unclassified Lachnospiraceae
	Faecalibacterium
	Pseudoflavonifractor
	Subdoligranulum

Clostridiales such as *Faecalibacterium, Lachnospira,* and *Ruminococcus,* in the microbiota on these farms. This suggests that further study of the long term or cumulative impact of antimicrobial use on farms is indicated, using populations with more clearly defined on-farm antimicrobial use data.

Unsurprisingly, there were significant differences between the fecal microbiota of cows and calves on the same farm. The greater richness and diversity that were observed in cows is not unexpected because these indices typically increase with age in cattle and other species.[6,7,16,26,38] Some of the taxonomic differences are unsurprising, such as the higher relative abundance of *Lactobacillus* in nursing calves.[6] The potential relevance of many of the other significantly different genera is difficult to determine based on limited understanding of most components of the microbiota.

The fecal microbiota of calves is rich, diverse, and potentially highly variable between farms. Reasons for pronounced interfarm variation need to be elucidated to determine the causes and potential implications for health and production. Antimicrobial exposure can have an impact on the fecal microbiota, and the potential short- and long-term impacts of the microbiota changes associated with antimicrobial exposure require attention.

Footnotes

Acknowledgments

References

1. Abrahamsson TR, Jakobsson HE, Andersson AF, et al. Low diversity of the gut microbiota in infants with atopic eczema. J Allergy Clin Immunol 2012;129:434–440, 440.e431–432.

2. Moreau MM, Eades SC, Reinemeyer CR, et al. Illumina sequencing of the V4 hypervariable region 16S rRNA gene reveals extensive changes in bacterial communities in the cecum following carbohydrate oral infusion and development of early-stage acute laminitis in the horse. Vet Microbiol 2014;168:436–441.

3. Britton RA, Young VB. Role of the intestinal microbiota in resistance to colonization by *Clostridium difficile*. Gastroenterology 2014;146:1547–1553.

4. Minamoto Y, Otoni CC, Steelman SM, et al. Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease. Gut Microbes 2014;6:33–47.

5. Claesson MJ, Jeffery IB, Conde S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nature 2012;488:178–184.

6. Oikonomou G, Teixeira AGV, Foditsch C, et al. Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of Faecalibacterium species with health and growth. PLoS ONE 2013;8:e63157.

7. Van Vleck Pereira R, Lima S, Siler JD, et al. Ingestion of milk containing very low concentration of antimicrobials: Longitudinal effect on fecal microbiota composition in preweaned calves. PLoS ONE 2016;11:e0147525.

8. de Oliveira MNV, Jewell KA, Freitas FS, et al. Characterizing the microbiota across the gastrointestinal tract of a Brazilian Nelore steer. Vet Microbiol 2013;164:307–314.

9. Kim M, Kim J, Kuehn LA, et al. Investigation of bacterial diversity in the feces of cattle fed different diets. J Anim Sci 2013;92:683–694.

10. Rudi K, Moen B, Sekelja M, et al. An eight-year investigation of bovine livestock fecal microbiota. Vet Microbiol 2012;160:369–377.

11. Castro-Carrera T, Toral PG, Frutos P, et al. Rumen bacterial community evaluated by 454 pyrosequencing and terminal restriction fragment length polymorphism analyses in dairy sheep fed marine algae. J Dairy Res 2014;97:1661–1669.

12. Pereira RV, Siler JD, Ng JC, et al. Effect of on-farm use of antimicrobial drugs on resistance in fecal *Escherichia coli* of pre-weaned dairy calves. J Dairy Sci 2014;97:7644–7654.

13. Costa MC, Stämpfli HR, Arroyo LG, et al. Changes in the equine fecal microbiota associated with the use of systemic antimicrobial drugs. BMC Vet Res 2015;11:19.

14. de Sá Del Fiol F, Tardelli Ferreira ACM, Marciano JJ, et al. Obesity and the use of antibiotics and probiotics in rats. Chemotherapy 2015;60:162–167.

15. Schokker D, Zhang J, Vastenhouw SA, et al. Long-lasting effects of early-life antibiotic treatment and routine animal handling on gut microbiota composition and immune system in pigs. PLoS ONE 2015;10:e0116523.

16. Dardas M, Gill SR, Grier A, et al. The impact of postnatal antibiotics on the preterm intestinal microbiome. Pediatr Res 2014;76:150–158.

17. Grønvold A-MR, Mao Y, L'abée-Lund TM, et al. Fecal microbiota of calves in the clinical setting: Effect of penicillin treatment. Vet Microbiol 2011;153:354–360.

18. Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 2013;41:e1.

19. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 2009;75:7537–7541.

20. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res 2013;41:D590–D596.

21. Edgar RC, Haas BJ, Clemente JC, et al. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 2011;27:2194–2200.

22. Cole JR, Wang Q, Cardenas E, et al. The ribosomal database project: Improved alignments and new tools for rRNA analysis. Nucleic Acids Res 2009;37:D141–D145.

23. Lozupone C, Hamady M, Knight R. UniFrac–an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinformatics 2006;7:371.

24. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. Genome Biol 2011;12:R60.

25. Holmes I, Harris K, Quince C. Dirichlet multinomial mixtures: Generative models for microbial metagenomics. PLoS ONE 2012;7:e30126.

26. Klein-Jöbstl D, Schornsteiner E, Mann E, et al. Pyrosequencing reveals diverse fecal microbiota in Simmental calves during early development. Front Microbiol 2014;5:622.

27. Dinh DM, Volpe GE, Duffalo C, et al. Intestinal microbiota, microbial translocation, and systemic inflammation in chronic HIV infection. J Infect Dis 2014;211:19–27.

28. Klase Z, Ortiz A, Deleage C, et al. Dysbiotic bacteria translocate in progressive SIV infection. Mucosal Immunol 2015;8:1009–1020.

29. Wacklin P, Laurikka P, Lindfors K, et al. Altered duodenal microbiota composition in celiac disease patients suffering from persistent symptoms on a long-term gluten-free diet. Am J Gastroenterol 2014;109:1933–1941.

30. Weese JS, Shury T, Jelinski MD. The fecal microbiota of semi-free-ranging wood bison (*Bison bison athabascae*). BMC Vet Res 2014;10:120.

31. Dubourg G, Lagier J-C, Armougom F, et al. High-level colonisation of the human gut by Verrucomicrobia following broad-spectrum antibiotic treatment. Int J Antimicrob Agents 2013;41:149–155.

32. Greenwood C, Morrow AL, Lagomarcino AJ, et al. Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of Enterobacter. J Pediatr 2014;165:23–29.

33. Panda S, El khader I, Casellas F, et al. Short-term effect of antibiotics on human gut microbiota. PLoS ONE 2014;9:e95476.

34. Honneffer JB, Minamoto Y, Suchodolski JS. Microbiota alterations in acute and chronic gastrointestinal inflammation of cats and dogs. World J Gastroenterol 2014;20:16489–16497.

35. Weese JS, Holcombe SJ, Embertson RM, et al. Changes in the faecal microbiota of mares precede the development of postpartum colic. Equine Vet J 2014;47:641–649; doi: 10.1111/evj.12361.

36. Suchodolski JS, Markel ME, Garcia-Mazcorro JF, et al. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. PLoS ONE 2012;7:e51907.

37. Arboleya S, Sánchez B, Milani C, et al. Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. J Pediatr 2015;166:538–544.

38. Slifierz MJ, Friendship RM, Weese JS. Longitudinal study of the early-life fecal and nasal microbiotas of the domestic pig. BMC Microbiol 2015;15:1–12.