Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tract of pre-weaned calves

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Running title: Taxonomic identification of GIT microbiome in calves
Abstract

Bacterial colonization in the gastrointestinal tract (GIT) of pre-weaned calves is very important, since it can influence early development and post-weaning production and health. This study investigated the composition of the bacteria along the GIT (rumen, jejunum, ileum, cecum and colon) of pre-weaned bull calves (3-week-old) using pyrosequencing to understand the segregation of bacteria between the mucosal surface and digesta. Phylogenetic analysis revealed that a total of 83 genera belonging to 13 phyla were distributed throughout the GIT of pre-weaned calves with *Firmicutes*, *Bacteroidetes* and *Proteobacteria* predominating. The qPCR analysis of selected abundant bacterial genera (*Prevotella*, *Bacteroides*, *Lactobacillus*, *Faecalibacterium*) revealed that their prevalence was significantly different when comparing among the GIT regions and between mucosa- and digesta-associated communities. Rumen contained the most diverse bacterial population consisting of 47 genera including 16 rumen-specific genera, followed by the large intestine and then small intestine. Bacterial species richness was higher at the mucosal surface than in the local digesta, with the exception of rumen. The majority of bacteria found on the rumen epithelial surface and within the small intestine could not be identified due to a lack of known genus-level information. Thus, future studies will be required to fully characterize the microbiome during the development of the rumen and the mucosal immune system of newborn calves. This is the first study to analyze in depth the bacterial composition of the GIT microbiome in pre-weaned calves, which extends previous findings regarding early rumen colonization and bacterial segregation between mucosa- and digesta- associated microbial communities.
Introduction

The gastrointestinal tract (GIT) of newborns contains a less diverse microbiome than that of adults and the progressive colonization over the time increases this diversity (1). Based on the fecal microbiome of infants, the pioneer gut bacteria are comprised of facultative anaerobes such as *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Enterobacteriaceae* spp. during the first few days of life (2). These species create the reduced-environment that is required for obligate anaerobic gut microbes (3). Besides the facultative anaerobes, anaerobic *Bifidobacterium* species are also present at high levels in the gut of 1-week-old neonates (3). An increasing number of studies are investigating microbial establishment and the factors influencing this process in newborn livestock, such as dairy calves. A recent study reported a link between the prevalence of *Faecalibacterium* during the first week of life and body weight gain as well as diarrhea incidences in 4-week-old calves (4). This suggests a potential role for gut bacteria in both animal health and production. Evidence are also emerging that the initial acquisition and continuous exposure to microbes results in a host-specific gut microbiome, which plays a vital role in the maturation of the mucosal immune system (5-7). Hence, knowledge regarding the pioneer bacterial community of calves provides an opportunity to understand, how perturbations in microbial composition and diversifications may alter health and production in cattle.

The fecal bacterial composition of dairy calves undergoes dynamic changes during the first 12 weeks of life (8). These changes include the appearance of new species such as *Ruminococcus flavefaciens* and *Fibrobacter* species and the disappearance of *Bifidobacterium*, *Enterobacteriaceae*, *Streptococcus*, and *Lactobacillus* species (8), suggesting both diet and gut development may drive changes in the bacterial composition during early life. A study comparing mucosa- and digesta-associated bacterial phylotypes in pre-weaned calves revealed a
much greater richness in mucosa-associated bacterial phylotypes throughout the GIT when compared to the regional digesta (9). Moreover, mucosa-associated bacteria in the murine distal colon not only differed significantly from fecal bacteria but also correlated with the TLR2 and TLR4 gene expression in colon epithelial cells (10). These observations indicate the importance of studying bacterial segregation between mucosal surfaces and digesta throughout the GIT, to better understand host-microbial interactions. Currently, our understanding of the gut microbiome and its segregation between mucosal surfaces and digesta in the neonatal calves is very limited. Therefore, the present study used pyrosequencing of 16S rRNA gene to provide a more complete analysis of the taxonomic segregation of bacteria throughout the GIT, including the rumen, of pre-weaned calves.

**Materials and Methods**

**Sample collection**

This is a companion study conducted using samples collected from 3-week-old Holstein bull calves used in our previous study (9). One-week-old bull calves (n = 8) were purchased from a commercial dairy farm and housed at the Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan. All experimental protocols were approved by the University of Saskatchewan Committee on Animal Care (Animal Protocol: 20020105) and all the procedures were performed following the Canadian Council on Animal Care guidelines.

Calves were fed fresh, non-pasteurised whole milk and Blue Medallion calf supplement (20% CP, 3% C Fat, 5.7% C Fibre, 1% Ca, 0.6% P; Ridley Inc., Fed-Rite, Manitoba, Canada). When calves were 3 weeks old, mucosal tissue and digesta samples were collected from the rumen, jejunum, ileum, cecum and colon within 20 minutes after euthanization. Mucosal tissue samples
were rinsed 3 times with sterile phosphate buffered sulphate (PBS) (pH 7.0) to remove digesta, cut into 4-5 mm² and immersed in RNAlater (Life Technologies, Carlsbad, CA, USA). Digesta was collected from each mucosal tissue collection site and 200 µl of digesta was mixed with 1 ml RNAlater. Samples were stored at -80°C until further analysis.

**DNA extraction and amplicon preparation for pyrosequencing**

Total DNA was extracted from all the samples using the beads beating method as described by Li et al., 2009 (11). DNA quality and quantity was measured using NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Total DNA was then diluted to a final concentration of 25 ng/µl and 50 ng/µl for mucosal tissue and digesta, respectively, and used in amplicon preparation for pyrosequencing.

Conventional PCR was performed for all the samples to amplify V1-V3 region of 16S rRNA gene using primer-containing Titanium A and B adaptors (27F (primer B) – 5’-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-AGAGGTTTGATCCTGGCTCAG-3’; 338R (primer A) – 5’-CCATCTCATCCCTGCGTGTCTCCGACTCAG-RL- TGCTGCTCCCCTGCTTAGGAT-3’) (12). The reverse primer contained 10 different multiplex identifiers (RL1-8, 10, 11; 454 Life Sciences, Branford, CT, USA) and was used to amplify samples from 5 different GIT regions for mucosal tissue and digesta separately using the following program; 94°C for 5 min, 35 cycles of 94°C for 30s, 58°C for 30s and 72°C for 30s followed by 72°C for 7 min. The PCR was first performed for individual animal separately; using primer A with the same assigned RL based on the gut region and sample type (eg: rumen tissue samples with primer A containing RL1; rumen content samples with primer A containing RL2 and so on) and then the PCR products were pooled by the RL index. DNA fragments
(~400bp) were extracted from 1% agarose gel using QIAEX II gel extraction kit (QIAGEN science, MD, USA) following the manufacturer’s instructions. The purified PCR products were then quantified using NanoDrop 1000 and amplicons from each sample were pooled in equal amount to a final concentration of 40 ng/µl and subjected to pyrosequencing.

Pyrosequencing and data analysis

The pooled PCR amplicons were subjected to pyrosequencing using Roche GS-FLX System with Titanium chemistry at Genome Quebec (Montreal, QC, Canada). Data were analysed using Quantitative Insight into Microbial Ecology (QIIME) tool kit (13). The raw sequences obtained from pyrosequencing were filtered through a quality control pipeline and bases of more than 25 quality scores were retained for further analysis. The high quality reads were then assigned to operational taxonomic units (OTUs) at 97% identity threshold using Uclust algorithm (14) and taxonomy was assigned using the latest Greengenes database (Greengenes May 2013 release). Taxonomy assignment was done separately for mucosal tissue and digesta from each gut region (rumen, jejunum, ileum, cecum and colon). The taxonomic identification and comparisons were performed at the genus level. Alpha diversity of mucosa- and digesta-associated bacterial communities of each gut region was obtained using various diversity indices (Chao1, Shannon index and observed species) using the alpha rarefaction pipeline. The hierarchical clustering of microbial communities (Jackknifed beta diversity) was performed using weighted UniFrac distance and Unweighted pair group method with arithmetic mean (UPGMA) clustering. The sequences were deposited in NCBI sequence read archive (SRA) under the accession numbers SRR1036467-SRR1036476.
Quantification of abundant bacterial genera along the GIT

The copy number of 16S rRNA genes of 5 selected bacterial genera (*Prevotella*, *Bacteroides*, *Lactobacillus*, *Clostridium* xiv cluster, *Faecalibacterium*) was estimated using genus specific primers (Supplementary Table 1) and SYBR green chemistry (Fast SYBR® Green Master Mix, Applied Biosystems) with StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The standard curves for each bacterial genus were prepared using purified PCR products amplified with each primer pair. The copy number of 16S rRNA gene/g of fresh tissue or digesta was then calculated using the equation described in Li et al., 2009 (11). The prevalence of bacterial genera was calculated by dividing the 16S rRNA genes copy number of each genus by the 16S rRNA genes copy number of total bacteria.

Statistical analysis

The data obtained from the qPCR were analysed using MIXED procedure in SAS (Version 9.2, SAS Institute Inc., Cary, NC). The gut region and the sample type (mucosal tissue vs. digesta) effects on the bacterial prevalence were determined using a two-way ANOVA and the statistical significances were declared at \( P < 0.05 \).

\[
Y_{ijk} = \mu + G_i + S_j + GS_{(ij)} + e_{ijk}
\]

Where; \( Y \) = bacterial prevalence, \( \mu \) = mean, \( G \) = gut region effect, \( S \) = sample type effect, \( GS \) = gut region by sample type effect, \( e \) = residual error

Results

Bacterial diversity along the GIT of pre-weaned calves
Pyrosequencing analysis of the GIT samples of pre-weaned calves generated a total of 565,854 sequences and 15,771 operational taxonomic units (OTUs) with the Good’s coverage ranging from 0.77 – 0.96. However, the distribution of these OTUs varied among individual GIT regions. The highest number of OTUs was observed in the rumen, followed by the large intestine (cecum and colon) and then the small intestine (jejunum and ileum) (Table 1). The alpha diversity indices (Chao1 and Shannon index) were higher in rumen bacterial community (mucosal tissue and digesta together) compared to those of large and small intestinal communities (Table 1). When the alpha diversity indices were compared between mucosal tissue and digesta of each gut region, the higher values were observed in digesta than mucosal tissue only in the rumen. In contrast, higher Chao1 and Shannon index values were observed in the mucosa-associated communities of jejunum, ileum and cecum when compared to their respective digesta (Table 1). The Chao1 index was higher for colonic digesta than colonic mucosal tissue, whereas the Shannon index was higher for colonic mucosal tissue than colonic digesta (Table 1).

**Gut bacterial composition of pre-weaned calves: Distribution of the predominant bacterial phyla and genera along the GIT**

In total, 13 bacterial phyla were identified within the pre-weaned calf gut microbiota, and these were dominated by **Firmicutes** (42.7%), **Bacteroidetes** (36.3%) and **Proteobacteria** (11.9%). However, the relative abundance of these major phyla varied within the mucosa- and digesta-associated communities. **Firmicutes** (57.6%) were predominant in the digesta, while **Bacteroidetes** (42.3%) were predominant in the mucosa-associated community, when these two communities were compared collectively without considering regional variations. However, the relative abundance of these predominant phyla varied markedly among GIT regions as well as...
between mucosal tissue and digesta (Figure 1). When the bacterial composition was compared regionally, *Bacteroidetes* dominated all digesta-associated communities along the GIT except for the small intestine, where *Firmicutes* (~ 97%) was predominant. *Bacteroidetes* also dominated all the mucosa-associated communities with the exception of the jejunal tissue, where there was a mix of *Firmicutes* and *Proteobacteria* than *Bacteroidetes*.

A total of 83 genera belonging to the 13 phyla were observed throughout the GIT of pre-weaned calves; however, in the present study 32.5% of all sequences were not identified at the genus level. The distribution of predominant bacterial genera (relative abundance ≥ 10% in at least one sample) was also different between mucosa- and digesta-associated communities throughout the GIT (Figure 2). *Bacteroides*, *Prevotella*, *Faecalibacterium*, and *Burkholderia* were predominant in the mucosa-associated community, while *Bacteroides*, *Prevotella*, *Lactobacillus*, *Clostridium* and *Sharpea* were predominant in the digesta-associated community (Figure 2). Among the 83 bacterial genera identified, only 7 genera (*Prevotella*, *Fibrobacter*, *Lactobacillus*, *Clostridium*, *Ruminococcus*, *Sharpea*, *Corynebacterium* – least abundant genus) were detected in both the mucosa- and digesta-associated communities in all GIT regions.

When the bacterial composition among the different GIT regions was compared using Unifrac distance and UPGMA clustering analysis for all OTUs as well as the abundant bacterial genera (20 genera, relative abundance >1%), the rumen and large intestinal bacterial communities clustered separate from the small intestine (Figure 3a & 3b). Among the small intestinal communities, jejunal and ileal mucosa-associated bacterial communities clustered more closely than the digesta-associated communities (Figure 3a & 3b). The rumen digesta- and mucosa-associated communities clustered more closely to each other than other members of the combined rumen and large intestinal communities (Figure 3a & 3b). The distribution of bacterial
genera further revealed 10 shared genera throughout all GIT regions, 10 shared genera between rumen and large intestine, 8 shared genera between small intestine and large intestine, and finally only 4 shared genera between rumen and small intestine (Figure 3c).

Regional segregation of gut bacteria between mucosa- and digesta-associated communities

The phylum *Bacteroidetes* dominated the rumen digesta- and tissue-associated communities of pre-weaned calves (Figure 1). The majority of digesta-associated *Bacteroidetes* (54.8%) were comprised of *Bacteroides* (15.8%), *Prevotella* (15.1%) and *Paludibacter* (4.1%), while *Prevotella* (15.8%) and *Bacteroides* (10.5%) dominated the rumen tissue-associated community (Supplementary Figure S1). *Firmicutes*, the 2nd most abundant phylum in the rumen consisted of 18 different bacterial genera (Supplementary Figure S1). Among these 18 genera, *Megamonas* and p-75-a5 were not present in the rumen digesta-associated community, while *Streptococcus* and *Sarcinia* were absent in the rumen tissue-associated community. *Proteobacteria* was the 2nd most abundant phylum associated with the rumen tissue (30.2%), which was 10 times greater than that in the rumen digesta. However, 27.8% of *Proteobacteria* sequences were not assigned to any genus. Besides, the majority of sequences from ruminal tissue-associated community (58.3%) were not assigned to any genus. In contrast, only 33.1% of sequences from rumen digesta-associated community were not assigned to any genus. The present study revealed 16 rumen specific bacterial genera (*Actinomyces*, *Porphyromonas*, *Butyricimonas*, *Elusimicrobium*, P-75-a5, *Megamonas*, *Desulfobulbus*, *Comamonas*, *Hylemonella*, *Sphaerochaeta*, *Anaeroplasma*, *Synergistes*, *Roseburia*, BF311, TG5 and RFN20) in the pre-weaned calves. Genera *Desulfobulbus*, *Comamonas*, *Hylemonella*, *Megamonas*, TG5 and P-75-a5 were only observed in
the ruminal tissue-associated community, while *Elusimicrobium* and *Anaeroplasma* were present only in the rumen digesta-associated community.

The large intestinal digesta- and mucosa-associated bacterial communities were primarily comprised of *Bacteroidetes* (cecal tissue – 56.8%; cecal digesta – 47.6%; colonic tissue – 52.8%; colonic digesta – 49.8%) (Figure 1). The prevalence of *Bacteroides* was higher in cecal digesta (15.9%) compared to that of cecal tissue (5.9%) (Supplementary Figure S1). In contrast, *Prevotella* accounted for the majority of cecal tissue-associated *Bacteroidetes* (21.7%) compared to the digesta (9.1%). The prevalence of *Prevotella* was higher in colon digesta- and mucosa-associated communities (19.3%) when compared to that of *Bacteroides* (colonic digesta – 15.4%, colon tissue 11%). *Firmicutes* was the 2nd most abundant bacterial phylum in the large intestinal communities (cecal tissue – 21.1%; cecal digesta – 38.2%; colonic tissue – 32.1%; colonic digesta – 25.4%). A higher prevalence of *Faecalibacterium* was observed in all large intestinal communities compared to other *Firmicutes* genera (cecal tissue - 9%, cecal digesta – 9.3%, colonic tissue – 10.8%, colonic digesta – 13.5%) (Supplementary Figure S1). Besides *Faecalibacterium, Clostridium* was also abundant in cecum (12%) and colon (5.8%) digesta-associated communities. A higher prevalence of *Lactobacillus* was only observed in the cecal digesta (6.4%), when compared to other large intestinal communities. The bacterial genera *Escherichia, Succinivibrio, Flavobacterium, Blautia* and L7AE11 were only observed in the large intestine. The present study also revealed 10 bacterial genera (*Paludibacter, Parabacteroides, Oscillopira, Shuttleworthia, Acidaminococcus, Anaerovibrio, Fusobacterium, Ruminobacter, Gallibacterium, and Treponema*) that were shared by rumen and the large intestinal communities but not present in the small intestine.
The small intestinal (jejunum and ileum) bacterial communities displayed a unique segregation between mucosa- and digesta-associated communities. The ileal digesta of pre-weaned calves contained primarily *Firmicutes* (97.7%), consisting of *Lactobacillus* (44.5%), *Clostridium* (16.7%) and *Sharpea* (8.9%) (Supplementary Figure S2). The ileal tissue was comprised of *Bacteroidetes* (33.7%), followed by *Firmicutes* (31.8%) and *Proteobacteria* (24.4%). *Prevotella* (8.2%) dominated the ileal mucosa-associated *Bacteroidetes*, while majority (24.9%) was not identified at genus level. Besides, 49.9% of ileal tissue sequences could not be assigned to any genus. The jejunal digesta-associated community was also dominated by *Firmicutes* (97.4%), which consisted of genera *Sharpea* (31.8%), *Butyrivibrio* (4.2%), *Ruminococcus* (3.2%) and *Lactobacillus* (2.4%) (Supplementary Figure S2). However, 53.2% of digesta-associated *Firmicutes* could not be assigned to any genus. The jejunal mucosa-associated bacteria contained *Firmicutes* (36.2%), *Proteobacteria* (29.2%) and *Bacteroidetes* (23.6%). However, 61.7% of total sequences from the jejunal mucosa-associated community could not be assigned to any genus. The prevalence of *Burkholderia*, which belongs to *Proteobacteria* was high only in the small intestinal mucosa-associated communities (ileal issue – 11.8%, jejunal tissue – 10.3%). Moreover, 17 bacterial genera were only detected in the small intestinal mucosa-associated bacterial community (*Caulobacter*, *Variorax*, *Acinetobacteria*, *Lautropia*, *Diaphorobacter*, *Janithinobacterium*, *Staphylococcus*, *Microbacterium*, *Nocardiopsis*, *Thermoactinomyces*, *Facklamia*, *Enterococcus*, *Phascolarctobacterium*, *Atopobium*, *Bradyrhizobium*, and *Pelomonas*).

The prevalence of abundant bacterial genera throughout the GIT.
The analysis of bacterial prevalence by qPCR confirmed the results observed in the pyrosequencing of pooled samples from pre-weaned calves (Table 2). The prevalence of bacterial genera was significantly different among GIT regions with the exception of *Clostridium* cluster xiv. The highest prevalence of *Prevotella* was observed in colonic digesta, compared to other digesta-associated communities, while its prevalence was higher in the small intestinal mucosa-associated communities when compared to the respective digesta-associated communities. A higher *Bacteroides* prevalence was observed in the rumen communities, while *Lactobacillus* was highest in small intestine relative to other GIT regions. The highest prevalence of *Faecalibacterium* was detected in the large intestinal communities.

**Discussion**

The distribution of OTUs obtained via pyrosequencing of mucosal tissue and digesta samples, along the GIT of 3-week-old pre-weaned calves in the present study was similar to that of bacterial phylotypes obtained via PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) profiling of the same samples in our previous study (9). The taxonomy analysis conducted in the present study was based on pooled samples from 8 animals. Therefore, to confirm that pooling of samples did not bias the description of microbiome composition in the pre-weaned calf GIT, qPCR was used to compare the prevalence of 5 selected bacterial genera that belonged to the abundant category (relative abundance \( \geq 10\% \) in at least one sample). The bacterial prevalence estimated by qPCR was in agreement with the relative abundance revealed by the pyrosequencing of pooled samples. The analysis of qPCR data also confirmed the observed differences in bacterial prevalence among GIT regions as well as between mucosa- and digesta-associated communities. Although, pooling of samples prevents exploring individual
animal variations, the present study succeeded in describing the composition and distribution of the pre-weaned calf gut microbiota.

The analysis of digesta-associated bacterial community along the GIT of a 2-year-old beef steer, revealed a high prevalence of *Bacteroidetes* in the rumen and a high prevalence of *Firmicutes* in the small and large intestine (15). Similarly, *Bacteroidetes* dominated the rumen content of pre-weaned calves, while *Firmicutes* dominated the small intestine. In contrast to the adult beef steer, *Bacteroidetes* dominated the large intestine of pre-weaned calves. Despite the differences observed in the bacterial composition due to the host age, diet and breed (15), both studies revealed a regional dependent bacterial community along the GIT of cattle. Moreover, de Oliveira and colleagues (15) reported more diverse rumen and large intestinal bacterial communities than small intestinal communities and these communities clustered separately. Our current data for pre-weaned calves is consisting with these previous observations. The present study also revealed that all mucosa-associated gut communities, with the exception of jejunal tissue, contained more *Bacteroidetes* than *Firmicutes*, implying that *Bacteroidetes* tend to more readily colonize mucosal surfaces during early life.

The observed regional variations in the bacterial composition of pre-weaned calves between small intestine and large intestine further revealed that fecal sample based studies may fail to detect the true gut microbiome. However, the regional perturbation to the microbial composition may be associated with enteric diseases. For example, the biopsy samples from Crohn’s disease (CD) patients revealed that the association of *Faecalibacterium prasnitizii* with CD as a decrease in bacterial numbers in ileal CD patients (16) and as an increase in colonic CD patients (17) when compared to the healthy individuals. These observations demonstrate the importance of proper sampling when identifying biomarkers for the associations between
microbiota and enteric diseases. Therefore, these previous studies together with the present findings suggest that sampling of local mucosa and digesta is essential to understand enteric infections and diseases caused by the dysbiosis of gut microbiome.

The higher number of OTUs and higher diversity in the digesta of the rumen and colon implies a more complex microbial population has been established in those two GIT regions than the small intestine of pre-weaned calves. Rumen and colon are known to function as sites for microbial fermentation of undigestible dietary substrates and retain digesta for a longer interval than the small intestine (18). The increased retention time of digesta may facilitate the growth of a more complex and dense bacterial community. The milk-fed calves in the present study had access to supplemental calf starter, which may have accelerated the colonization of rumen with a more diverse bacteria population.

The rumen bacterial population of 2-week-old calves fed only milk replacer was reported to contain 45 bacterial genera belongs to 15 different phyla (19). Similarly, the present study reported 47 bacterial genera belong to 13 phyla in the rumen of 3-week-old calves. In addition, Jami and colleagues (20) reported a diverse rumen bacterial community in 1 – 3-day-old calves, which was dominated by *Streptococcus* species. *Bacteroidetes* present in the rumen content of adult cattle fed grain-based diet was reported to contain more *Prevotella* than *Bacteroides* (21), while that in young calves fed whole milk or milk replacer mainly contained *Bacteroides* (19, 20). The rumen content of 3-week-old calves used in the present study contained similar levels of *Prevotella* (15.1%) and *Bacteroides* (15.8%), suggesting that calf starter may have driven rumen microbiome development toward that of the adult cattle. This transition suggests an early development of the rumen microbiome to ferment a plant polysaccharide based diet.
Previous studies conducted in pre-weaned calves relied on the collection of rumen contents. However, a comparison between the rumen digesta- and mucosa-associated communities in the present study, revealed differences in the bacterial composition and the relative abundance of shared bacterial genera. Moreover, the observed rumen tissue-specific bacterial genera indicate that studies based only on rumen content fail to adequately describe the rumen microbiome. Bacteroidetes dominated the rumen tissue-associated bacteria of pre-weaned calves. However, the rumen tissue-associated bacterial community in beef steers fed with a grain-based diet and beef heifers fed with hay or grain-based diets consisted of more Firmicutes than Bacteroidetes (21, 22). The difference observed in the rumen epimural bacterial community might be due to the differences in age, diet of the host, as well as the breed of cattle (dairy vs. beef) (23). The high prevalence of phylum Proteobacteria, class Betaproteobacteria (25.1%) in the tissue-associated microbiome was another distinct feature in the rumen of pre-weaned calves. The Proteobacteria species associated with rumen epithelium of dairy cows were different from the species identified from ruminal fluid (24). Those species isolated from the rumen epithelium clustered along with other Betaproteobacteria, which are capable of oxidising ammonia (24). Therefore, we speculate that the high prevalence of Proteobacteria in the rumen tissue-associated community may play an important role in scavenging the oxygen that diffuses from the blood to the epithelium and oxidising ammonia produced in the rumen. This may facilitate rumen bacteria colonization and its functions by maintaining the anaerobic conditions required for microbial fermentation. However, the large proportion of unclassified tissue-specific Proteobacteria indicates that there are many unknown bacteria attached to the rumen tissue. Thus, future studies are necessary to understand epimural bacteria and their dynamics in response to rumen development and dietary changes in dairy calves.
A high abundance of genus *Sharpea* was observed in the small intestine of pre-weaned calves compared to other gut regions. *Sharpea* belongs to the order *Erysipelotrichales* and the predicted family *Coprobacillaceae* and this is the first time *Sharpea* has been detected in the GIT of pre-weaned calves. *Sharpea azabuensis* belongs to Clostridial cluster xvii and has been detected in the feces of thoroughbred horses (25). *S. azabuensis* ferments a wide variety of sugars including glucose, galactose, lactose and fructose (25). Thus, the observed high abundance of *Sharpea* in the jejunal digesta of pre-weaned calves may facilitate the fermentation of milk sugars. Since genus *Sharpea* is abundant in the GIT of pre-weaned calves, future studies may provide a better understanding of its role in host metabolism.

In contrast to the rumen, the small and large intestines revealed higher bacterial diversity (Shannon index) in the mucosa-associated community than that of the adjacent digesta. Besides, the species richness (Chao1) was also higher in the mucosa-associated communities of jejunum, ileum and cecum when compared to the respective digesta. The higher species richness observed for the mucosa-associated communities is in agreement with our previous study conducted with the same samples but using PCR-DGGE (9). The association of mucosal bacteria with the expression of TLR2 and TLR4 in the colon of mice (10) suggests that mucosa-associated bacteria may play an important role in stimulating host immune responses through pattern recognition receptors. The present study revealed small intestinal mucosa-specific bacteria that were not identified in other GIT regions, suggesting that such mucosa-specific bacteria may survive mucosal immune defense mechanisms and may be crucial for priming the host mucosal immune system. A previous study investigating the mucosal expression of TLRs along the GIT of 3-week-old calves, using the same mucosal tissue samples, revealed TLR1, 9 and 10 gene expression was highest in the ileum followed by the jejunum and then other GIT regions (26).
Even though, there are regional variations in immune cells distribution throughout the gut (27, 28), we speculate that regional differences in the gut bacteria may also contribute to the observed TLRs expression pattern. Moreover, the small intestinal bacterial density was negatively correlated with the mucosal expression of TLRs of the pre-weaned calves. For example, digesta-associated total bacterial density was negatively correlated with the expression of TLR2 and TLR6 in the jejunum and ileum, respectively, while tissue-associated total bacteria and lactic acid bacteria (LAB) density was negatively correlated with the expression of TLR2 in jejunum (26). This hyporesponsiveness of mucosal TLRs to predominant Gram-positive bacterial population (Firmicutes) present in the small intestine communities may be a host mechanism to avoid immune responses towards commensal microbes.

The pre-weaned calf large intestinal regions contained a similar bacterial composition when comparing mucosa- and digesta-associated communities, which differed only in their relative abundance. Unlike the small intestinal and ruminal tissue-associated communities, most of the sequences obtained from the large intestine were assigned to a genus level, with the exception of the cecal tissue (51% unassigned sequences). Cecum and colon shared nearly 55% of the bacterial genera observed in the large intestine. However, in comparison to bacteria shared between ileum and jejunum (~85%), these two large intestinal bacterial communities showed less similarity to each other. The high proportion of assigned genus information observed in the large intestinal communities may be due to their resemblance to the fecal bacterial community, which has been studied more extensively than the small intestine community. Uyeno and colleagues reported that the fecal microbiome of 3-week-old dairy calves consist of 39.2% Bacteroides-Prevotella group, 17.7% Clostridium coccoides-Eubacterium rectale group and 11% Faecalibacterium (8). Colon samples from the 3-week-old calves used in the present study...
revealed similar levels for the Bacteroides-Prevotella group (32.5%) and Faecalibacterium (12.1%). However, the relative abundance of Clostridium was only 3.2% and there were no Eubacterium observed in any of the gut regions. These observations once again indicate that fecal samples best represent the composition of the large intestinal bacterial community, but not that of the entire GIT. The present study revealed a significantly higher prevalence of Faecalibacterium in large intestinal communities compared to other GIT regions. A high abundance of F. prasnitizii in the feces was associated with increased weight-gain and a decreased incidence of diarrhea in dairy calves (4). The fermentation of F. prasnitizii produces butyrate, which is the major energy source for colonic epithelial cells and it also stimulates anti-inflammatory responses (4). Therefore, the observed higher prevalence of Faecalibacterium species in the large intestine of pre-weaned calves may be important for maintaining proper body weight and reducing enteric infections during the early life.

Conclusion

In conclusion, the present study revealed that the bacterial composition throughout the GIT of 3-week-old calves varied between mucosa and digesta communities as well as among GIT regions. The observed mucosa-specific bacterial genera further suggest that fecal samples do not adequately represent the complexity of the gut microbiome and potential relationships with the host. Moreover, the colonization of calf rumen starts early in life with a distinct segregation of bacteria between digesta and epithelial surfaces. This segregation suggests future studies should focus on both communities. The large number of sequences throughout the GIT with unidentified genus level information suggests that our knowledge of the ruminant gut microbiome is not adequate to explain its complexity and functions in the host. In particular,
analysis of tissue-attached bacteria may provide a better understanding of early rumen/gut development and functions. Previous studies of bacteria in digesta of pre-weaned calves reported large variation among individual animals and it will be important to determine if a similar level of variation is observed for tissue-attached bacteria. Understanding the full extent of gut microbiome diversity among individual animals will be critical for determining how it shapes the individual animal performances in terms of health and production.

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31. Delroisse JM, Boulvin AL, Parmentier I, Dauphin RD, Vandenbol M, Portetelle D.
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of inulin on the human gut microbiota: Stimulation of *Bifidobacterium adolescentis* and
Figure Legends

Figure 1. Phylum level composition of gut bacteria in mucosa- and digesta-associated communities throughout the gastrointestinal tract of dairy calves. RT - Rumen tissue, RC – Rumen content, IT – Ileum tissue, IC – Ileum content, JT – Jejunum tissue, JC – Jejunum content, CeT – Cecum tissue, CeC – Cecum content, CoT – Colon tissue, CoC – Colon content.

Figure 2. Relative abundance and distribution of 20 abundant (≥1% in at least one GIT region) bacterial genera detected throughout the GIT of 3-week-old pre-weaned calves. (a) Distribution of abundant genera in mucosa-associated tissue (b) Distribution of abundant genera in digesta.

Figure 3. Clustering of bacterial genera and OTUs present in the GIT of 3-week-old pre-weaned calves. (a) A heat map generated using the relative abundance (%) of 20 abundant bacterial genera. Heat map was generated using ‘package gplots’ in R by clustering GIT regions based on the distribution and relative abundance of bacterial genera. Heat map scale displayed the row Z score (Z score = [Actual relative abundances of a species in a specific GIT region – Mean relative abundance of the same species along the GIT]/ Standard deviation). (b) Clustering of all OTUs obtained from pyrosequencing of GIT samples. Unweighted pair group method with arithmetic mean (UPGMA) clustering based on all available data with Jackknifed beta diversity, which compares the diversity among the samples. (c) Comparison of the number of shared bacterial genera among rumen, small intestine and large intestine.
Malmuthuge et al., 2013 Figure 1
Malmuthuge et al., 2013 Figure 3
Table 1. Generated sequences, identified operational taxonomic units (OTUs), bacterial diversity and species richness along the GIT of pre-weaned dairy calves

<table>
<thead>
<tr>
<th></th>
<th>Rumen</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Cecum</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequences</strong></td>
<td>94116</td>
<td>96086</td>
<td>112404</td>
<td>81567</td>
<td>177978</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>OTUs</strong></td>
<td>5693</td>
<td>2632</td>
<td>2685</td>
<td>2893</td>
<td>4694</td>
</tr>
<tr>
<td><strong>Chao1</strong></td>
<td>6046</td>
<td>2522</td>
<td>3044</td>
<td>4372</td>
<td>4644</td>
</tr>
<tr>
<td><strong>Shannon</strong></td>
<td>8.6</td>
<td>6.2</td>
<td>6.1</td>
<td>7.2</td>
<td>7.1</td>
</tr>
</tbody>
</table>

|                  |       |         |       |       |       |
| **Sequences**    | 48639 | 75960   | 57783 | 49232 | 49119 |
| **Mucosa**       |       |         |       |       |       |
| **OTUs**         | 6051  | 3394    | 3744  | 4196  | 3950  |
| **Chao1**        | 3908  | 2970    | 3865  | 3836  | 3335  |
| **Shannon**      | 8.2   | 6.3     | 6.9   | 7.1   | 6.9   |

|                  |       |         |       |       |       |
| **Sequences**    | 46110 | 20796   | 55416 | 33292 | 129507|
| **Digesta**      |       |         |       |       |       |
| **OTUs**         | 7374  | 2362    | 2571  | 3251  | 7264  |
| **Chao1**        | 4608  | 2041    | 2036  | 3348  | 3968  |
| **Shannon**      | 8.8   | 6.1     | 5.2   | 6.9   | 6.9   |

*a Total represent values of mucosa and digesta samples together for each gut region  
b Chao 1 – an estimation of species richness  
c Shannon index – an estimation of diversity*
Table 2. Prevalence of abundant bacterial genera along the GIT of 3-week-old calves

<table>
<thead>
<tr>
<th>Genus</th>
<th>Rumen</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Cecum</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tissue</td>
<td>Digesta</td>
<td>Tissue</td>
<td>Digesta</td>
<td>Tissue</td>
</tr>
<tr>
<td><strong>Prevotella</strong></td>
<td>9.9±0.8</td>
<td>15.1±0.4</td>
<td>10.8±6.8</td>
<td>0.5±0.2</td>
<td>10.8±2.2</td>
</tr>
<tr>
<td><strong>Bacteroides</strong></td>
<td>29.0±19.6</td>
<td>15.2±0.7</td>
<td>0.01±0.002</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Lactobacillus</strong></td>
<td>0.1±0.04</td>
<td>5.9±0.3</td>
<td>5.5±0.6</td>
<td>2.1±0.1</td>
<td>6.4±2.8</td>
</tr>
<tr>
<td><strong>Faecalibacterium</strong></td>
<td>0.2±0.03</td>
<td>0.2±0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Clostridial siv</strong></td>
<td>6.7±6.2</td>
<td>1.3±0.6</td>
<td>0.2±0.1</td>
<td>0.5±0.3</td>
<td>0.2±0.1</td>
</tr>
</tbody>
</table>

*Prevalance (%) = (16S rDNA genes copy number of each genus / Total bacterial 16S rDNA genes copy number) x 100%*

**Prevotella P_G*S = 0.02; Bacteroides P_G = 0.01; Lactobacillus P_G*S < 0.01; Faecalibacterium P_G*S < 0.01; G – Gut region, S – Sample type (tissue vs. digesta)**

**ND – not detected represents the prevalence of <0.001%**