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Region-specific establishment of bacterial communities in the small intestine of neonatal calves from birth

Nilusha Malmuthuge[1,2], Yanhong Chen[1], Guanxiang Liang[1,3], Anna Widenmann[1] and Le Luo Guan[1,4]

[1]Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB, Canada; [2]Lethbridge Research and Development Center, Agriculture Agri-Food Canada, Lethbridge, AB, Canada; [3]Center for Infectious Disease Research, School of Medicine, Tsinghua University, Beijing, China and [4]Faculty of Land and Food Systems, The University of British Columbia, Vancouver, BC, Canada

Research Article

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Corresponding author: Le Luo Guan;
Email: leluo.guan@ubc.ca

Abstract

Initial microbial colonization plays an important role in neonatal gut health. However, studies on gut microbial composition at birth are challenging, due to the limited access to accurate sampling. Here, we characterized the jejunal and ileal bacterial composition (epimural and luminal) of neonatal calves within 30 minutes after birth, and compared it with maternal (birth canal and rectum) and birth environments. RNA-based quantification along with amplicon sequencing revealed the colonization of active, dense ($1.1–9.4 \times 10^8$ 16S rRNA copy/g of sample), and diverse bacteria in the calf small intestine at birth. *Pseudomonadaceae* and *Propionibacteriaceae* dominated epimural communities, while *Propionibacteriaceae, Prevotellaceae, Ruminococcaceae*, and *Lachnospiraceae* dominated luminal communities. The composition of calf gut bacteria at birth was significantly different from maternal bacteria, especially for beneficial bifidobacteria. The bacterial communities of calf body habitats were similar to those of the birth environment, which was again divergent from gut microbiota. This study suggests an establishment of small intestinal-specific microbiota from birth, which is considerably deviated from maternal microbiota. In corollary, we further propose that small intestinal microbiota colonization could be mainly modulated by host selection.

Introduction

A dynamic microbial population colonizes the mammalian gut after birth; under the deliberate modulation of the host, microbial (Van den Abbeele et al. 2011), and environmental factors during pre- and postnatal period (Adlerberth and Wold 2009; Arrieta et al. 2014; Dominguez-Bello et al. 2010; Fanaro et al. 2003; Renz et al. 2017). The composition of neonatal microbiota plays a vital role in modulating the trajectories of the early gut colonization process (Song et al. 2021) as well as the growth and development of the host (Nabhani and Eberl 2020; Robertson et al. 2019). For example, *Ruminococcus obeum* (a commensal bacteria in the neonatal gut) has been reported to be associated with normal gut microbial establishment in children and to restrict the colonization of *Vibrio cholerae* in humanized mice (Subramanian et al. 2015). Besides, the alterations in normal microbial succession patterns, such as delayed colonization of *Bacteroidetes* in the gut, are associated with reduced Th1 responses (Jakobsson et al. 2014), suggesting that the succession process is also equally important as the early composition.

Due to its long-term impact on host health, early gut microbiome (composition, colonization process) has been well studied in human and mouse models. However, studies on gut microbiota at birth are challenging, due to the limitations in sampling. Therefore, the first-pass meconium is often used as a proxy. The collection time of the first-pass meconium can vary from a few hours to a day after birth in human infants (Hansen et al. 2015; Jimenez et al. 2008), which may not necessarily represent the gut microbiome at birth. Nevertheless, fecal sample-based studies in humans have suggested that the neonatal microbial community is mainly influenced by the maternal microbiota (Bogaert et al. 2023). However, fecal sample-based studies in cattle were not successful in identifying such a strong linkage between cow and calf microbiota (Barden et al. 2020; Klein-Jobstl et al. 2019).

The establishment of a gut microbiota regulates the intestinal epithelium in a region-specific manner (Sommer et al. 2015). However, there is a lack of understanding of the establishment of the regional microbiota, especially the small intestinal microbiota, which closely interact with the developing mucosal immune system.

Therefore, this study investigated the small intestinal bacterial composition of calves at birth and compared it with that of calf body habitats, maternal (birth canal and rectum), and the birthing environment (calving pen floor) to comprehend the initial microbial composition and its inoculum, aiming to provide the fundamental knowledge needed to improve gut health of young ruminants.

Materials and methods

Animal experiment and sampling

All experimental protocols were approved by the Livestock Care Committee of the University of Alberta (AUP00001012) and were conducted following the guidelines of the Canadian Council on Animal Care. All calves and dams were obtained from the Dairy Research and Technology Center, University of Alberta (Edmonton, AB). Multiparous Holstein cows with male fetuses were transferred into calving pens a week before the predicted due dates and closely monitored through cameras. Newborn calves ($n = 6$) were removed from dams soon after birth using cleaned plastic containers to prevent calves from reaching the floor, transferred to a surgery room immediately, and humanly euthanized within 5 minutes. Small intestinal tissue and content samples were collected as closed segments at predetermined regions, within 30 minutes after euthanization. All the samples were snap-frozen in liquid nitrogen and stored at −80°C. Briefly, the esophagus and rectum were first ligated to occlude the lumen and prevent environmental contamination of the intestine. Proximal jejunum was defined as 100 cm distal to the pylorus sphincter; distal jejunum was defined as 30 cm proximal to the collateral branch of the cranial mesenteric artery; and ileum was defined as 30 cm proximal to the ileocecal junction (Malmuthuge et al. 2019a). Ten-centimeters-long intestinal segments were collected from each region in the middle of each segment aligned with the abovementioned measurements.

Rectal and birth canal swab samples from the respective dams were collected using BD Screw Cap Single SWUBE™ Applicators (polyester) (Becton, Dickinson and Company, NJ), immediately following calving. Similarly, calf body habitat swabs (nose, mouth, and skin) were also collected before euthanasia. Litter materials/shavings from the calving pens were also collected immediately following the calving.

Nucleic acid isolation

Total genomic DNA was extracted from small intestinal tissue and content samples using the repeated bead-beating plus column method (Yu and Morrison 2004). Total DNA from environmental samples (swabs and litter material) was extracted using GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, MA), following the manufacturer's instructions for Gram-positive bacteria genomic DNA purification protocol. DNA quality and quantity were measured using an ND 1000 spectrophotometer (NanoDrop Technologies, DE). RNA was extracted from small intestinal epithelium tissue and content together using mirVana™ miRNA isolation kit (Ambion, Carlsbad, CA) to be used in quantitative real-time polymerase chain reaction (PCR) to estimate active/alive bacteria population.

Sequencing and data analysis

Diluted total DNA (25 ng/μl) from all the samples was used to amplify the V1–V3 region using universal bacterial primers (9F – 5′ACACTGACGACATGGTTCTACAGAGTTTGATCMTGGCTCAG3′ and 519R – 5′TACGGTAGCCAGAGACTTGGTCT-CCGCGGCKGCTGGCAC3′) with added tags. PCR products (∼500 bp) were purified using 1% agarose gel and QIAEX II gel extraction kit (QIAGEN Sciences, MD), following the manufacturer's instructions. Barcodes were added to the purified PCR products and subjected to pyrosequencing using the Roche GS-FLX System with Titanium chemistry at Genome Quebec, McGill University (Quebec, Canada). Phylogenetic assignments and the analysis of bacterial diversity (alpha- and beta-diversity) were performed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.8.0 (Caporaso et al. 2010). Briefly, the raw sequences were first filtered through a quality control pipeline to retain sequences >200 bp and bases with Phred quality score >25. Then, the chimeric sequences were filtered out using usearch61 within the QIIME platform and the remaining high-quality sequences were used in the taxonomic assignment using Greengenes database 13_8 (2013 July release). Raw sequences were deposited in NCBI sequence read archive under the bioproject PRJNA309079 and biosamples SAMN04505066-SAMN04505134.

Co-occurrence analysis of identified bacterial families

To explore the interaction among initial bacteria colonized the small intestine, a co-occurrence analysis was performed using the relative abundance of bacterial families and Spearman's rank correlation analysis. A positive correlation ($\rho > 0.5$, $P < 0.05$) between two families was considered as a co-occurrence incidence, and the distance was calculated using Spearman's rank correlation value (distance $= 1 - \rho$). Distance values were used to generate a co-occurrence network using Gephi (Version 0.10.01).

Quantification of bacterial density

Diluted intestinal tissue and content DNA and RNA were used to estimate total bacteria, Lactobacillus, and *Bifidobacterium* densities using SYBR green chemistry (Fast SYBR® Green Master Mix, Applied Biosystems) with StepOnePlus real-time PCR system (Applied Biosystems) using universal bacterial primers and genus-specific primers (Liang et al. 2014). Standard curves for total bacteria, *Lactobacillus*, and *Bifidobacterium* were generated using purified 16S rRNA genes of *Butyrivibrio hungatei, Lactobacillus acidophilus* ATCC4356, and *Bifidobacterium longum*, respectively. Bacterial density (DNA- and RNA-based) was then calculated using the equation described by Li et al. (2009).

Statistical analysis

Effects of sample type (tissue vs. content) and small intestinal region were evaluated using group_significance.py function and Kruskal–Wallis nonparametric analysis with P value corrected by the Benjamini–Hochberg false discovery rate (FDR) procedure for multiple comparisons within QIIME. Significant comparisons were declared at FDR <0.05. Beta-diversity was computed using beta_diversity.py, and the weighted UniFrac distance matrices were used to perform analysis of similarity (ANOSIM) using

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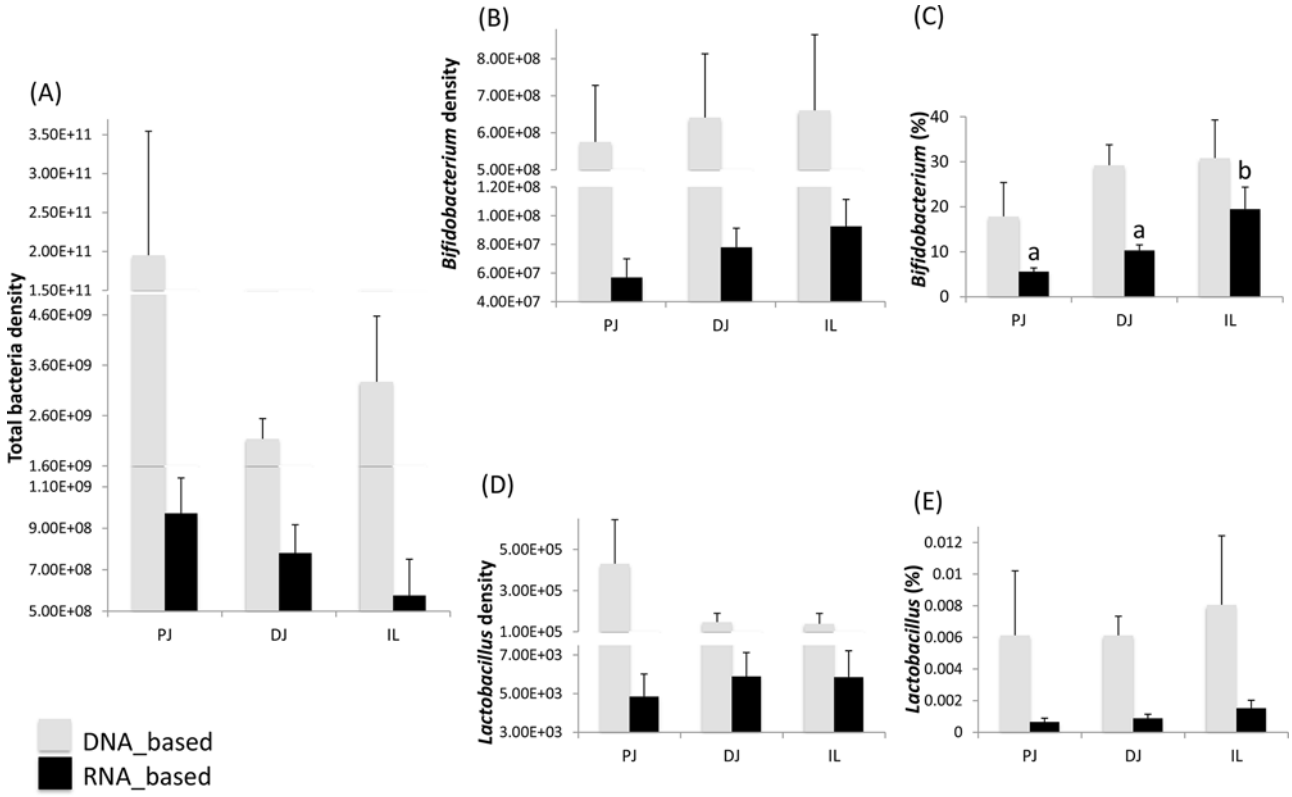

Figure 1. Estimation of the small intestinal bacterial density at birth using DNA (16S rRNA gene copy/g of fresh sample) and RNA (16S rRNA copy/g of fresh sample) extracted from small intestinal tissue and content. (A) Total bacterial density, (B) *Bifidobacterium* density, (C) Proportion of *Bifidobacterium* (density of *Bifidobacterium* /total bacteria) × 100%, (D) *Lactobacillus* density, (E) Proportion of *Lactobacillus* (density of *Lactobacillus* /total bacteria) × 100%. PJ – proximal jejunum, DJ – distal jejunum, IL – ileum.

compare_category.py function to declare bacterial communities (intestinal, body habitat, maternal, and birth environmental communities) different from each other (ANOSIM R 0.5-1, $P < 0.05$).

Bacterial population data (total bacteria density, density/proportion of *Bifidobacterium*, density/proportion of *Lactobacillus*) were analyzed using SAS version 9.4 (SAS Inc., Cary, NC) and analysis of variance. A repeated-measures experimental design was used with small intestinal region as the repeated measurement and individual animal as the experimental unit. Compound symmetry covariance structure was selected as the best fit by the lowest Bayesian information criteria, and the following statistical model was fitted to test the effect of small intestinal region and sample type on bacterial populations: $Y\text{ijk} = \mu + Ri + Sj + TS(\text{ij}) + \text{eijk}$, where Y = bacterial density/proportion (total bacteria, *Lactobacillus, Bifidobacterium*); μ = mean; R = small intestinal region; S = sample type (tissue, content); and e = residual error. Differences in least square means were declared at $P < 0.05$ using the PDIFF option in SAS when applicable.

Results

Active and dense bacterial population at birth

DNA- and RNA-based estimations revealed dense and active bacterial colonization in the small intestine of neonatal calves at birth (Fig. 1). Alive/active bacterial densities detected using RNA-based quantification were lower than those of DNA-based estimations (Fig. 1). Bacterial densities (total, *Bifidobacterium, Lactobacillus*) were not different among the small intestinal segments, but the proportion of *Bifidobacterium* was higher in the ileum than that in jejunum (Fig. 1).

Taxonomic assessment of gut microbiota at birth

The small intestinal epimural (tissue-attached) and luminal (content-associated) communities of calves at birth were already colonized with diverse bacteria belonging to 12 phyla (Supplementary Fig. 1A, Supplementary Table S1). *Proteobacteria* ($40.4 \pm 4.9\%$), followed by *Firmicutes* ($23.2 \pm 3.0\%$), *Actinobacteria* ($20.5 \pm 3.7\%$), and *Bacteroidetes* ($10.7 \pm 1.8\%$) dominated the calf small intestine at birth. In total, 89 bacterial families and 122 genera were identified from all the small intestinal regions at birth (Supplementary Table S1). Among the bacterial families, *Pseudomonadaceae, Propionibacteriaceae*, and *Ruminococcaceae* were predominant, while *Pseudomonas, Propionibacterium, Prevotella*, and *Bacillus* were the predominant genera (Fig. 2, Supplementary Table S1). However, the relative abundance of these bacterial taxa varied among individuals (Supplementary Table S1). For example, the relative abundance of *Pseudomonas* ranged from 0.3% to 83.8%; *Propionibacterium* ranged from 0% to 86.4%; and *Prevotella*, the third highly abundant genera, ranged from 0% to 20.8% in six newborn calves.

The small intestinal luminal bacteria were dominated by *Firmicutes* ($28.6 \pm 4.2\%$), followed by *Proteobacteria* ($27.4 \pm 5.3\%$), *Actinobacteria* ($20.2 \pm 5.1\%$), and *Bacteroidetes* ($15.9 \pm 2.8\%$). However, these observed relative abundances were numerically

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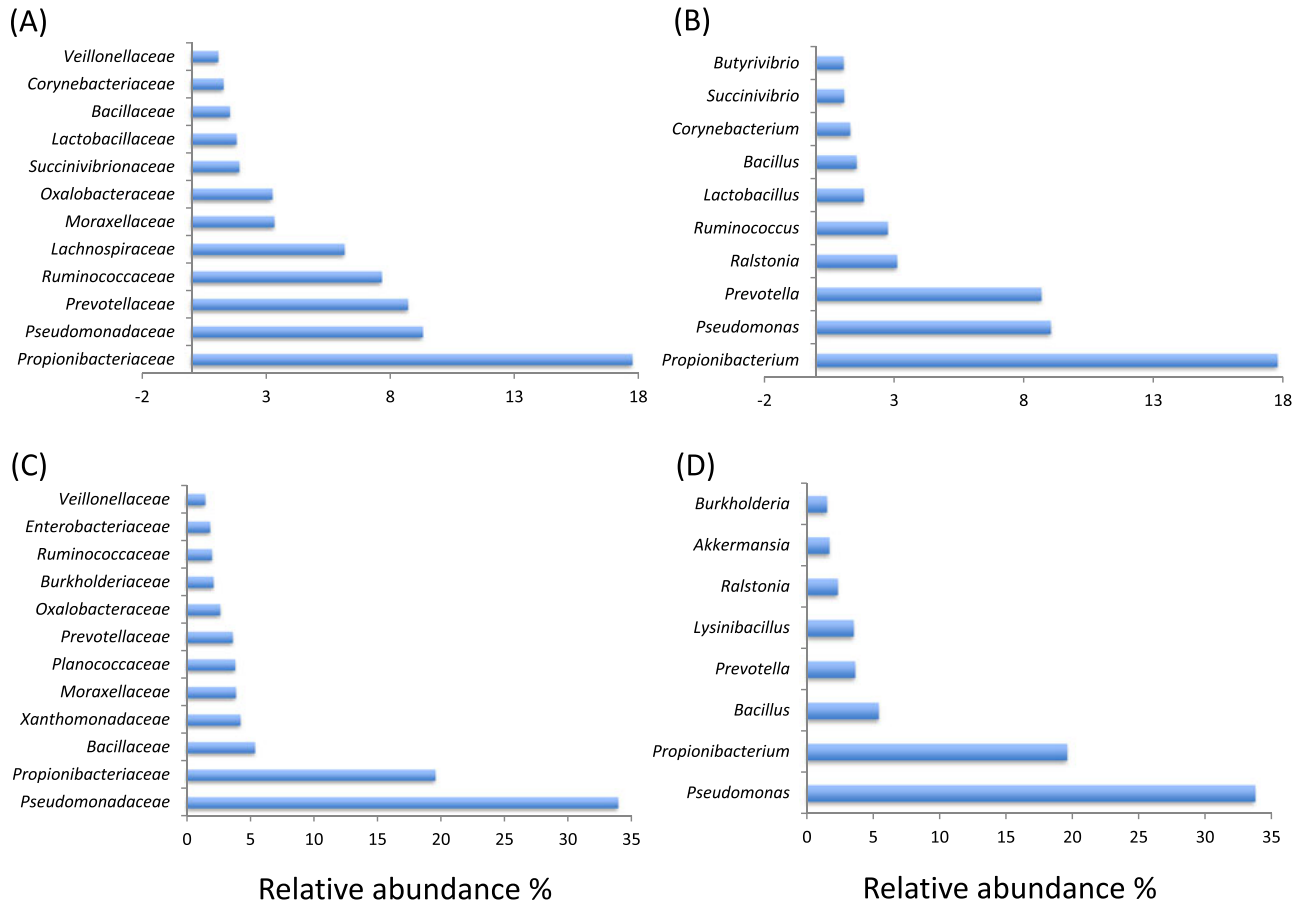

Figure 2. Relative abundance of predominant bacterial families and genera. (A) Predominant bacterial families in the luminal communities, (B) Predominant bacterial genera in the luminal communities, (C) Predominant bacterial families in the epimural communities, (D) Predominant bacterial genera in the epimural communities.

different among the small intestinal regions (Supplementary Table S1). For example, *Firmicutes* dominated the ileal lumen (36.7 ± 7.4%), while *Actinobacteria* dominated the distal jejunal lumen (37.3 ± 11.3%). Although *Firmicutes* were abundant in luminal communities, the majority of them were only assigned *Ruminococcaceae* (3.5 ± 1.1%), *Lachnospiraceae* (4.9 ± 1.9%) families, and Clostridiales (6.8 ± 2.0%) order. Similar to the phylum level composition, the abundance of these dominant bacterial genera was numerically different among the three small intestinal regions (Supplementary Table S1). For example, the relative abundance of *Prevotella* was higher in the lumen of the ileum (14.2 ± 1.4%) than in the jejunal communities [proximal jejunum – (6.9 ± 3.5%); distal jejunum – (5.0 ± 1.7%)]. In contrast, the abundance of the aerobic bacterium *Pseudomonas* is lower in the lumen of the ileum (1.4 ± 0.7%) than that in the jejunal communities [proximal jejunum – (15.8 ± 13.5%); distal jejunum – (9.9 ± 4.3%)].

The small intestinal epimural bacteria were dominated by *Proteobacteria* (53.4 ± 7.2%), *Firmicutes* (17.8 ± 3.9%), *Actinobacteria* (20.8 ± 5.6%), and *Bacteroidetes* (5.6 ± 1.7%). However, *Actinobacteria* was second predominant in the jejunal epimural communities (proximal jejunum – 34.0 ± 14.9%; distal jejunum – 20.8 ± 4.2%; Supplementary Table S1). Similar to the luminal bacteria, the abundance of the observed dominant bacterial genera was numerically different among the three small intestinal regions (Supplementary Table S1). *Pseudomonas* was the most abundant genus in epimura of the distal jejunum (32.4 ± 12.3%) and ileum (39.2 ± 12.9%); however, *Propionibacterium* (30.6 ± 15.8%) dominated the proximal jejunum (Supplementary Table S1). Moreover, the abundance of *Bacillus* was higher in the distal jejunum (11.9 ± 7.3%), than in proximal jejunum (2.5 ± 1.9%) and ileum (1.8 ± 1.7%).

When the bacterial composition was compared between luminal and epimural communities, a higher relative abundance of *Bacteroidetes* was observed in the small intestinal lumen (proximal jejunum – 11.7 ± 5.0%; distal jejunum – 9.4 ± 3.4%; ileum – 26.6 ± 2.7%), than the respective epimural communities (proximal jejunum – 0.6 ± 0.2%; distal jejunum – 6.6 ± 2.6%; ileum – 9.5 ± 3.8%) (Supplementary Table S1). At genus level, *Propionibacterium* (17.8 ± 5.0%), *Pseudomonas* (9.0 ± 4.7%), *Prevotella* (8.7 ± 1.6%), *Ralstonia* (3.1 ± 1.7%), *Ruminococcus* (2.8 ± 1.2%), *Lactobacillus* (1.8 ± 0.6%), *Bacillus* (1.6 ± 1.2%), *Corynebacterium* (1.3 ± 1.0%), *Succinivibrio* (1.1 ± 0.6%) and *Butyrivibrio* (1.0 ± 0.6%) were abundant (>1% relative abundance) in luminal communities (Fig. 3). Whereas, the epimural communities were dominated by *Pseudomonas* (33.8 ± 7.6%), *Propionibacterium* (19.6 ± 5.6%), *Bacillus* (5.4 ± 2.7%), *Prevotella* (3.6 ± 1.4%), *Ralstonia* (2.3 ± 0.9%), *Lysinibacillus* (3.5 ± 1.3%), *Akkermansia* (1.7 ± 1.7%) and *Burkholderia* (1.5 ± 0.9%) (Fig. 3). Among the observed predominant genera, *Burkholderia* and *Akkermansia* were not identified in any of the luminal communities; while *Coprococcus, Succinivibrio, Staphylococcus*, and

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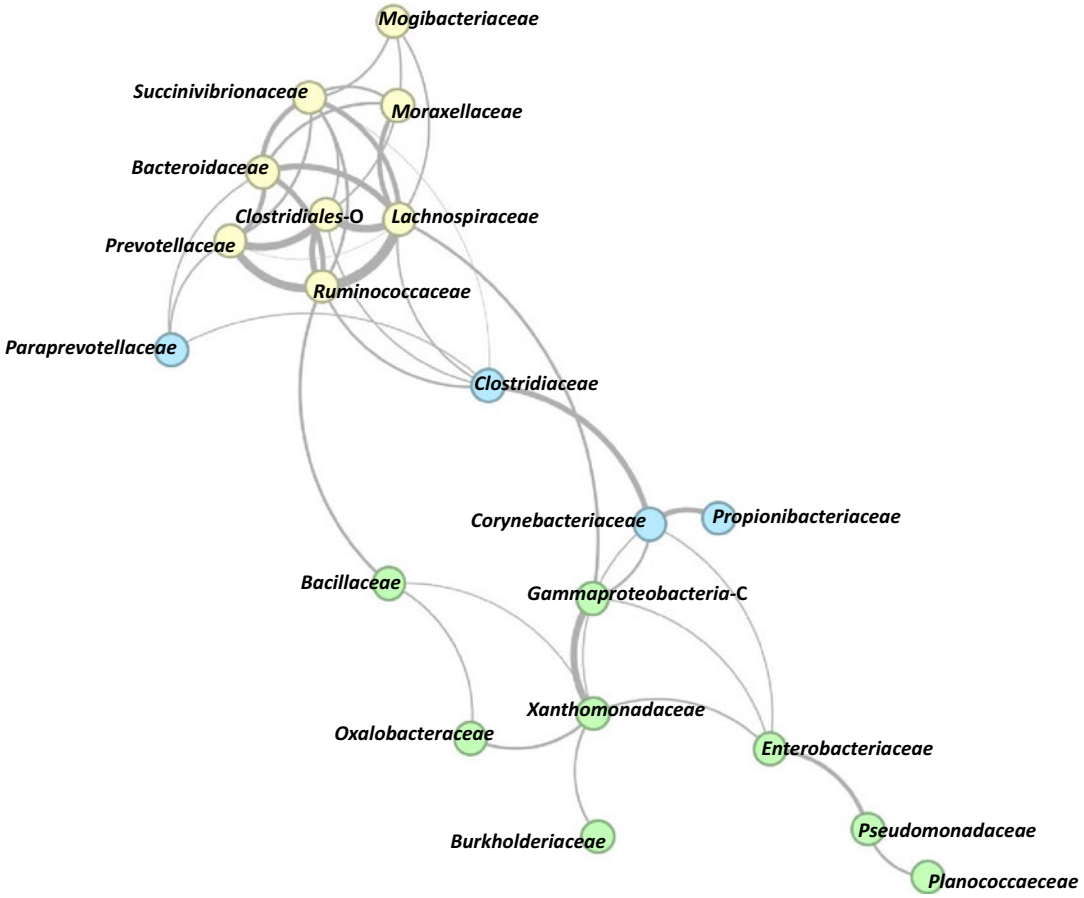

Figure 3. Co-occurrence network of bacterial families detected from the calf small intestine. Positive correlations ($\rho > 0.5$, $P < 0.05$) among bacterial families are defined as co-occurred families and the distance between two families is calculated using Spearman's correlation coefficient (distance = 1 – ρ). Lower distance and thick edges connecting two bacterial nodes represent a higher correlation and vice versa. All the co-occurrence incidents in all three gut regions were plotted in one network using the Gephi (version 0.10.01).

Clostridium were absent in epimural communities (Supplementary Table S1).

Besides the composition, bacterial richness (number of families/genera) was also different among small intestinal regions, between the epimural and luminal communities as well as among individual animals (Table 1). The number of families and genera in the ileal lumen was numerically higher than in jejunal communities (Table 1). Similarly, a significantly higher bacterial diversity was observed in the ileum (phylogenetic distance, observed species, Shannon index), when comparing to two jejunal regions, regardless of the gut environment (epimura vs. lumen) (Table 1). However, the bacterial diversity and richness were lower in small intestinal epimural communities at birth, compared to that of luminal communities (Table 1).

Firmicutes and Bacteroidetes families co-occur at birth

Despite the observed higher abundance of *Proteobacteria* in all calves, families from *Firmicutes* and *Bacteroidetes* phyla mainly co-occurred at birth, except for *Succinivibrionaceae* with 10 positive correlations identified from all three small intestinal regions (Fig. 3). The hubs of the co-occurrence network mainly consisted of families belonging to phylum *Firmicutes*. *Lachnospiraceae* (14 positive correlations), *Clostridiaceae* (8 positive correlations), *Ruminococcaceae* (5 positive correlations), and an unidentified family belonged to the order Clostridiales (13 positive correlations) were identified among these main hubs. Whereas, *Bacteroidaceae* (9 positive correlations) belonging to *Bacteroidetes* was identified as another hub of the co-occurrence network.

Comparison of maternal, environment, and calf body habitat microbial communities

The microbial compositions of the maternal environment (rectum and birth canal of dams), calving pen floor/birth environment, and calf body habitats (mouth, nose, and skin) were also explored to understand the impact of exposure to dam and environment on early gut bacterial colonization. There were 10 phyla identified from rectal and birth canal communities of dams that were dominated by *Firmicutes* (Supplementary Fig. S1B). Eight out of 44 bacterial families identified from the birth canal (*Streptococcaceae, Aerococcaceae, Ruminococcaceae, Clostridiaceae, Peptostreptococcaceae, Lachnospiraceae, Bacteroidaceae*, and *Corynebacteriaceae*) accounted for 77.2% of birth canal bacteria. Seven predominat families (*Ruminococcaceae, Clostridiaceae, Peptostreptococcaceae, Lachnospiraceae, Bacteroidaceaei Veillonellaceae*, and *Streptococcaceae*) accounted for 59.2% of the total (44) bacterial families identified from the cow rectum. The main genera identified from maternal communities are *Streptococcus, Facklamia, Clostridium*, and *Ruminococcus* (Table 2). Calf body habitats (10 phyla) and birth environment (9

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Table 1. Bacterial diversity and richness in the calf small intestine at birth

	All regions		Proximal jejunum		Distal jejunum		Ileum	
	Tissue	Content	Tissue	Content	Tissue	Content	Tissue	Content
No. of families	19 ± 1	33 ± 3	12 ± 1	14 ± 2	9 ± 1	13 ± 2	10 ± 1	22 ± 3
No. of genera	20 ± 2	33 ± 4	12 ± 1	12 ± 2	9 ± 1	11 ± 1	9 ± 1	20 ± 4
Unassigned sequences at family level (%)	1.0 ± 0.5	7.1 ± 2.0	0.1 ± 0.05	2.6 ± 1.8	1.5 ± 1.3	4.1 ± 2.6	1.4 ± 0.5	14.5 ± 3.6
Unassigned sequences at genus level (%)	15.7 ± 3.2	33.6 ± 4.1	14.3 ± 4.8	30.1 ± 6.9	15.1 ± 7.5	27.5 ± 8.8	17.6 ± 5.0	43.2 ± 4.2
Good's coverage	0.98 ± 0.002	0.96 ± 0.004	0.97 ± 0.004	0.97 ± 0.01	0.98 ± 0.002	0.97 ± 0.002	0.97 ± 0.003	0.94 ± 0.01
Shannon*#	3.4 ± 0.1	4.3 ± 0.2	3.2 ± 0.5	3.8 ± 0.6	3.4 ± 0.2	3.7 ± 0.5	3.8 ± 0.1	5.6 ± 0.6
Chao 1#	80.8 ± 6.4	125.4 ±10.7	79.4 ± 9.3	97.6 ± 29.4	77.6 ± 7.7	103.2 ± 8.0	82.2 ± 8.7	175.3 ± 43.2
Observed species*#	47.1 ± 2.2	81.2 ± 8.8	46.3 ± 20.0	58.7 ± 3.9	44.8 ± 2.1	61.8 ± 5.9	50.2 ± 4.1	123.3 ± 30.7
Phylogenetic distance*#	4.7 ± 0.3	9.3 ± 0.8	4.7 ± 0.4	7.4 ± 2.1	4.5 ± 0.3	6.9 ± 1.0	5.0 ± 0.3	13.5 ± 2.8

*Alpha diversity in the ileum was higher ($P < 0.05$) than that in the proximal jejunum and distal jejunum regardless of the sample type.
#Alpha diversity of the content-associated community was higher ($P < 0.01$) than that of the tissue-associated community regardless of the small intestinal region.

Table 2. Maternal, calf body habitats, and birth environment bacteria

	Birth canal	Rectum	Mouth	Nose	Skin	Floor
No. of families	19 ± 3	29 ± 4	28 ± 5	27 ± 5	29 ± 4	28 ± 8
Unassigned sequences (%)	17.9 ± 4.9	32.6 ± 4.0	4.8 ± 2.8	7.7 ± 4.9	10.4 ± 5.6	14.3 ± 8.3
No. of genera	21 ± 3	26 ± 4	33 ± 8	31 ± 4	29 ± 6	31 ± 9
Unassigned sequences (%)	44.3 ± 9.6	72.5 ± 3.5	35.1 ± 8.2	47.6 ± 10.3	24.7 ± 9.3	44.1 ± 9.6
Main families (%)	*Streptococcaceae* (25.5 ± 13.2) *Aerococcaceae* (16.7 ± 13.1) *Ruminococcaceae* (11.4 ± 4.6) *Clostridiaceae* (9.6 ± 3.4)	*Ruminococcaceae* (24.6 ± 1.1) *Clostridiaceae* (13.3 ± 2.7) *Peptostreptococcaceae* (6.6 ± 2.6) *Lachnospiraceae* (6.4 ± 0.6)	*Staphylococcaceae* (17.9 ± 10.6) *Aerococcaceae* (16.2 ± 8.5) *Xanthomonadaceae* (11.7 ± 6.4) *Caulobacteraceae* (10.6 ± 6.5)	*Xanthomonadaceae* (14.3 ± 14.2) *Staphylococcaceae* (12.3 ± 11.1) *Aerococcaceae* (8.4 ± 7.3)	*Aerococcaceae* (32.6 ± 10.5) *Staphylococcaceae* (12.5 ± 12.3) *Methylobacteriaceae* (11.8 ± 4.8)	*Ruminococcaceae* (16.9 ± 13.9) *Aerococcaceae* (16.2 ± 10.8) *Staphylococcaceae* (13.6 ± 13.0) *Xanthomonadaceae* (7.9 ± 7.3)
Main genera (%)	*Streptococcus* (22.5 ± 13.2) *Facklamia* (11.6 ± 10.6) *Corynebacterium* (2.9 ± 1.2) *Ruminococcus* (2.4 ± 0.7)	*Clostridium* (5.3 ± 0.4) *Ruminococcus* (4.2 ± 3.2) *5 – 7N15* (3.7 ± 2.1) *Dorea* (2.2 ± 0.3)	*Jeotgalicoccus* (17.8 ± 10.6) *Facklamia* (9.6 ± 5.6) *Streptococcus* (3.8 ± 3.7) *Brevundimonas* (2.8 ± 1.7)	*Jeotgalicoccus* (12.2 ± 11.2) *Facklamia* (4.2 ± 4.1) *Corynebacterium* (3.9 ± 3.0) *Bacillus* (2.9 ± 2.3)	*Facklamia* (19.8 ± 9.1) *Methylobacterium* (12.3 ± 12.2) *Jeotgalicoccus* (10.7 ± 4.4) *Aerococcus* (6.5 ± 5.9)	*Facklamia* (12.1 ± 7.6) *Jeotgalicoccus* (11.8 ± 11.3) *Stenotrophomonas* (5.3 ± 5.0) *Corynebacterium* (3.5 ± 3.1)

phyla) bacteria mainly consisted of *Firmicutes* and *Proteobacteria* at the phylum level (Supplementary Fig. S1B). The main families identified from these communities were *Aerococcaceae, Staphylococcaceae*, and *Xanthomonadaceae*, wherea, the main genera observed were *Jeotgalicoccus* and *Facklamia* (Table 2).

When the calf bacterial communities were compared with those of dams and floor pens (Fig. 4, Table 3), the small intestinal communities at birth were significantly different from those of maternal, body habitat, and birth environments (Table 3, Fig. 4A–C). Although *Firmicutes* was one of the main phyla in all of these communities, the composition was significantly different at lower levels of the taxonomical hierarchy. *Ruminococcus* was mainly found in the calf gut (Supplementary Table S1), while *Streptococcus, Fackalmia, Clostridium, Jeotgallicoccus*, and *Aerococcus* dominated maternal and calf body habitats (Table 2). Moreover, the body habitat communities were also different from that of maternal microbiota (Table 3, Fig. 4E), consisting of a higher abundance of facultative anaerobes, such as *Jeotgallicoccus* and *Aerococcus* on body habitats. Calf body habitat communities were not different from that of the birth environment (Table 3, Fig. 4D), which were dominated by *Jeotgallicoccus* and *Fackalmia*. Besides, the birth environment could not be differentiated from the rectum ($R = 0.074$, $P = 0.50$), but was different from the birth canal ($R = 0.401$, $P < 0.01$). Dam's rectal community and the

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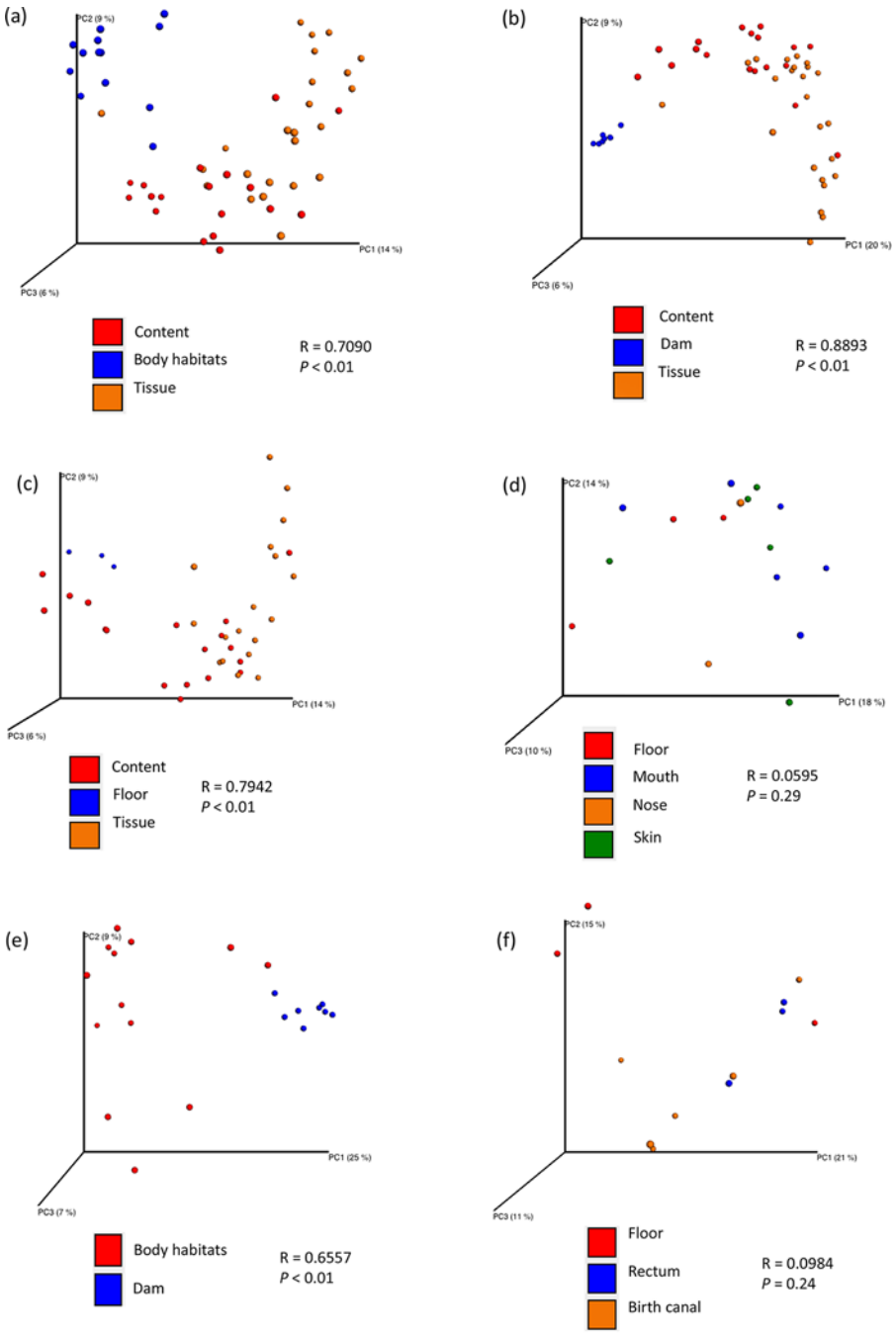

Figure 4. Comparison of calf gut, calf body habitats, maternal and birth environment bacterial communities. OTU profiles are compared using an unweighted UniFrac distance matrix within QIIME platform.

birth environment community of one of the calves had more similar microbial profiles to each other than other rectal and pen floor samples (Fig. 4F). *Ruminococcaceae* was the most abundant bacterial family in these two samples (rectum – 26.8%, pen floor – 44.7%) and the relative abundances were higher than the other rectal or pen floor samples.

Discussion

The early microbiota, which plays a crucial role in the development of the mucosal immune system, subsequent microbial succession, and host health, is well studied in human and murine models; however, such understanding in ruminants is very limited. This is the first attempt to report the colonization of the small intestinal epimural and luminal communities with active bacterial populations immediately after birth and their link to the maternal, birth environment, and body habitat microbiota. Next-generation sequencing of the small intestinal tissue and content samples revealed a dynamic microbial population that mainly consisted of *Proteobacteria* in the newborn calf gut. Profiling of the fecal microbiota immediately after birth has also showed a higher abundance of *Proteobacteria* in dairy calves (Barden et al. 2020;

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Table 3. Comparison of calf, dam, and environmental bacterial communities

Comparison	ANOSIM R	*P*-value
Calf gut vs. Dam	0.8893	<0.01
Calf gut vs. Birth canal	0.9049	<0.01
Calf gut vs. Rectum	0.9130	<0.01
Calf gut content vs. Birth canal	0.7187	<0.01
Calf gut content vs. Rectum	0.6788	<0.01
Calf gut tissue vs. Birth canal	0.7574	<0.01
Calf gut tissue vs. Rectum	0.6133	<0.01
Calf gut vs. Body habitats	0.7090	<0.01
Calf gut vs. Floor	0.7942	<0.01
Content vs. Tissue	0.2652	<0.01
Body habitats vs. Floor	0.0595	0.29
Body habitats vs. Dam	0.6557	<0.01
Floor vs. Dam	0.0984	0.24
Floor vs. Rectum	0.0741	0.50
Floor vs. Birth canal	0.4012	0.04

Klein-Jobstl et al. 2019). The jejunal and ileal digesta-associated bacteria of 3-week-old calves fed milk and calf starter were dominated (>97%) by *Firmicutes* (Malmuthuge et al. 2014). However, *Bacteroidetes* were predominant in jejunal (23.6%) and ileal (33.7%) tissue-attached communities at 3 weeks (Malmuthuge et al. 2014). In contrast, the present study revealed a lack (<10%) in these obligate anaerobes at birth. Studies on fecal bacterial communities have also revealed a higher abundance of *Firmicutes* in older calves (Barden et al. 2020; Oikonomou et al. 2013; Uyeno et al. 2010). Thus, these studies suggest that the obligate anaerobes establish gradually and become predominant with increasing calf age. The facultative anaerobe pioneer bacteria (Proteobacteria) observed at birth plays a role in creating anaerobic conditions in the gut for obligate anaerobes to succeed. In addition, the newborn calf gut microbiota differed from the meconium bacterial composition of human infants. In contrast to human infants gut microbiota that are dominated by *Lactobacillus, Bacteroides, Prevotella, Bifidobacterium, Enterobacteriaceae*, and *Enterococcaceae* within the first 24 hours (Dominguez-Bello et al. 2010; Hansen et al. 2015), we observed a higher abundance of *Propionibacterium, Pseudomonas, Prevotella, Bacillus*, and *Ralstonia* at birth. However, whether these differences indicate species specificity or differences in sampling time and location needs to be further understood. *Actinobacteria* dominates 3- to 4-month-old infant fecal microbiota (Azad et al. 2013; Turroni et al. 2012), while *Firmicutes* dominates calf fecal microbiota within the first 7 weeks of life (Oikonomou et al. 2013). Thus, these observed differences at birth may indicate the establishment of a species-specific microbial communities starting from birth.

Besides the differences in the abundance of detected bacteria, the diversity of the bacterial communities at birth differed from those of 3-week- and 6-month-old calves (Malmuthuge et al. 2012). In contrast to the high diversity and richness observed in the epimural communities of older calves (Malmuthuge et al. 2014, 2012), we observed a less diverse epimural community in the small intestinal at birth. The low diversity may be mainly due to the higher abundance of *Pseudomonas*, an aerobic bacterial genus, observed in all tissue communities. *Pseudomonas* as a transient species may play a role in creating the anaerobic environment for autochthonous gut bacteria through tissue oxygen scavenging, which is similar to the roles of *Enterococcus*, and *Streptococcus* observed in infant gut (Fanaro et al. 2003; Jost et al. 2012). It may also be the reason for the absence of *Psuedomonadaceae* in co-occurrence networks. Although the role of aerobes and facultative anaerobes during gut colonization is defined, the role of anaerobic bacteria present at birth on subsequent microbial colonization is not yet understood. We observed a higher prevalence of *Prevotellaceae, Ruminococcaceae*, and *Lachnospiraceae* families in the calf small intestine at birth, which were the hubs of co-occurrence networks. These bacterial families belong to *Firmicutes* and *Bacteroidetes*, the two main phyla in the small intestinal content and tissue communities of preweaned calves (Malmuthuge et al. 2014), may play essential roles in the succession of small intestinal bacterial communities. Moreover, these anaerobic bacterial families observed at birth may be the inoculum for the obligate-anaerobic, autochthonous microbial population in the gut.

DNA- and RNA-based estimations revealed a higher abundance of *Bifidobacterium* in calf small intestinal communities, especially in tissue. This beneficial bacterium has been reported as one of the main bacteria to first colonize neonatal calf gut (3 to 7-day-old calves) and remain predominant until 6–9 months of age (Rada et al. 2006; Vlkova et al. 2006). The presence of conserved solute binding proteins can facilitate the binding of *Bifidobacterium* to mucin-glycans (LoCascio et al. 2007), which may also enhance their population along the gut of calves as reported by previous studies (Rada et al. 2006; Uyeno et al. 2010; Vlkova et al. 2006) and its preferable rapid colonization on tissue (Malmuthuge et al. 2015). Moreover, *Bifidobacterium* contains a highly conserved milk oligosaccharide utilization gene region and is capable of utilizing sialylated oligosaccharides (LoCascio et al. 2007), which is the major oligosaccharide in bovine colostrum and milk (ten Bruggencate et al. 2014). These calves were fed colostrum during the first 3 days of life, followed by exclusive milk feeding until the sample collection. Sialylated oligosaccharides, the main oligosaccharides present in bovine colostrum and milk (ten Bruggencate et al. 2014), may have boosted the population within the first week of life. However, later its abundance decreased drastically at 3 weeks (0.4%) and could not be detected at 6 weeks, when calves were supplemented with *ad libitum* calf starter. Additionally, the timed feeding of heat-treated colostrum boosts *Bifidobacterium* density (tissue-attached) within 6 hours of life compared to that of fresh colostrum or no colostrum feeding (Malmuthuge et al. 2015), indicating that early feeding management strategies can be optimized to increase their colonization. Based on human and mouse studies, *Bifidobacterium* plays a vital role in the development of mucosal immune system, host health, and in the prevention of pathogen colonization (Hart et al. 2004; Hidalgo-Cantabrana et al. 2014). Human studies have also suggested the vertical transmission of *Bifidobacterium* species from mother to infant (Makino et al. 2013). However, we could only detect *B. longum* and *B. pseudolongum* in maternal communities by PCR (Supplementary Figure S2). Thus, future research is necessary to understand its roles in calf gut development and health to prevent neonatal calf diarrhea, the major cause of calf death. Nonetheless, we speculated that *Bifidobacteria* transmitted from the dam might become predominant in the calf gut at birth and during early life, due to its ability to adapt well in a neonatal gut environment enriched with mucin-glycans and milk oligosaccharides.

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In humans, the fecal microbiota of vaginally delivered infants highly resembles maternal microbiota (Backhed et al. 2015; Bogaert et al. 2023; Dominguez-Bello et al. 2010). In contrast to humans, our data revealed the establishment of notably different bacterial communities in the calf small intestine at birth, compared to those of the maternal bacteria (birth canal and rectal). Our comparisons further revealed that calf body habitat communities are similar to that of birth environment (calving pen floor) microbiota, which are dominated by facultative anaerobes (*Jeotgalicoccus, Facklamia*) and aerobes (*Corynebacterium*). Jami et al. (2013) reported a higher abundance of *Streptococcus* in the rumen fluid of 1-day-old calves, while we observed a higher abundance of *Veillonella, Bacteroides, Streptococcus, Clostridium*, and *Ruminococcus* in rumen content at birth (Malmuthuge et al. 2019b), which were the main bacterial genera observed in the dams (rectum, vagina) following calving in the present study. This suggests a possible vertical transmission of birth canal bacteria to the newborn calf rumen at birth, but not to the small intestine. Our previous studies (Malmuthuge et al. 2014, 2012) and a study in humans (Romano-Keeler et al. 2014) revealed region-specific bacterial composition along the gut that could not be detected in the large intestinal regions or feces. We have also observed a complex rumen microbiome that was colonized with a dynamic anaerobic bacterial population [*Veillonella* ($23.3 \pm 14.1\%$), *Prevotella* ($23.0 \pm 4.0\%$), *Bacteroides* ($21.6 \pm 5.6\%$), *Eubacterium* ($7.0 \pm 0.7\%$)] at birth (Malmuthuge et al. 2019b). Thus, the observed unique bacterial community in the calf small intestine within a few minutes after birth suggests the colonization of gut region-specific bacteria from the commencement.

The small intestinal bacterial community was not only different from the dam but also lacked a core microbiome. The absence of core bacterial taxa among individual calves implies that the impact of the host on the small intestinal microbiota establishment may be stronger than that of environmental or microbial effects. The small intestine of ruminants, the major site of the mucosal immune system, contains Peyer's patches that are developed prenatally (Griebel et al. 1996; Yasuda et al. 2004). Microbiota colonizing the small intestine have largely undergone comprehensive scrutiny by the host immune system (Hansen et al. 2012; Van den Abbeele et al. 2011), which may result in a higher impact of the host on the small intestinal microbiota than environmental or microbial factors. The observed similarity between the rumen fluid- (Jami et al. 2013) as well as content-associated (Malmuthuge et al. 2019b) bacteria and maternal bacteria also implies a higher impact of dam on the pioneer rumen microbiota and endorses our speculation on high impact of the host on the small intestinal microbial establishment. Besides, the observed differences between the luminal and epimural communities also suggest a segregation of microbial colonization from birth, which could also be the result of the host effect. Although it is not clearly understood the activity level of gut-associated lymphoid tissues of calves at birth, the establishment of a unique microbial population indicates an active mucosal immune system that can influence its microbial composition in the small intestine of newborn calf.

Conclusion

The present study revealed the colonization of the calf small intestine with a dense, diverse, and active microbiota at birth. However, the composition of small intestinal bacteria within a few minutes after birth was markedly different from that of the maternal microbiota (birth canal and rectum) composition. This suggests that small intestinal-specific bacteria establishment may be deliberately scrutinized by mucosal immune components. Although aerobic bacteria (*Pseudomonadaceae*) were the most abundant, we discovered a higher presence of anaerobic bacterial families (*Prevotellaceae, Ruminococcaceae*, and *Lachnospiraceae*) at birth, which were generally present in adult gut environment. These anaerobic families were the hubs of co-occurrence networks; however, their roles in the region-specific microbial succession are yet to be discovered. Although a high prevalence of *Bifidobacterium* in maternal and calf small intestinal microbial communities was observed, the composition was significantly different between calves and dams. The microbial colonization process in the small intestine needs to be carefully modulated during early life since newborn gut permeability is high at birth to absorb colostrum components. Such meticulous modulation will prevent unnecessary inflammatory responses toward the establishing commensal microbiome and will not exert stress on the naïve immune system. Thus, studies to explore microbial colonization, immediately postnatal, with varying management practices (different colostrum/milk feeding methods) may be valuable to understand the role of initial microbiota on subsequent succession and gut development. This will also provide means to manipulate such processes via modifying early management practices.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/anr.2024.4.

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