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Neonatal Diet Impacts Bioregional Microbiota Composition in Piglets Fed Human Breast Milk or Infant Formula

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ABSTRACT

Background: Early infant diet influences postnatal gut microbial development, which in turn can modulate the developing immune system.

Objectives: The aim of this study was to characterize diet-specific bioregional microbiota differences in piglets fed either human breast milk (HM) or infant formula.

Methods: Male piglets (White Dutch Landrace Duroc) were raised on HM or cow milk formula (MF) from postnatal day (PND) 2 to PND 21 and weaned to an *ad libitum* diet until PND 51. Piglets were euthanized on either PND 21 or PND 51, and the gastrointestinal contents were collected for 16sRNA sequencing. Data were analyzed using the Quantitative Insight into Microbial Ecology. Diversity measurements (Chao1 and Shannon) and the Wald test were used to determine relative abundance.

Results: At PND 21, the ileal luminal region of HM-fed piglets showed lower Chao1 operational taxonomic unit diversity, while Shannon diversity was lower in cecal, proximal colon (PC), and distal colon (DC) luminal regions, relative to MF-fed piglets. In addition, at PND 51, the HM-fed piglets had lower genera diversity within the jejunum, ileum, PC, and DC luminal regions, relative to MF-fed piglets. At PND 21, *Turicibacter* was 4- to 5-fold lower in the HM-fed piglets' ileal, cecal, PC, and DC luminal regions, relative to the MF-fed piglets. *Campylobacter* is 3- to 6-fold higher in HM-fed piglets duodenal, ileal, cecal, PC, and DC luminal regions, in comparison to MF-fed piglets. Furthermore, the large intestine (cecum, PC, and rectum) luminal region of HM-fed piglets showed 4- to 7-fold higher genera that belong to class Bacteroidia, in comparison to MF-fed piglets at PND 21. In addition, at PND 51 distal colon lumen of HM-fed piglets showed 1.5-fold higher genera from class Bacteroidia than the MF-fed piglets.

Conclusions: In the large intestinal regions (cecum, PC, and rectum), MF diet alters microbiota composition, relative to HM diet, with sustained effects after weaning from the neonatal diet. These microbiota changes could impact immune system and health outcomes later in life. *J Nutr* 2019;149:2236–2246.

Keywords: piglet, microbiota, human milk, infant formula, gastrointestinal bioregions

Introduction

Human breast milk (HM) contains bioactive components, some of which are highly conserved (such as lactoferrin, an iron-containing protein), and has evolved to nourish a developing infant. Epidemiologic evidence demonstrates that babies fed HM have improved short- and long-term immune health outcomes compared to their formula-fed counterparts (1–5). For example, preterm babies that receive HM are at a much lower risk of developing necrotizing enterocolitis, a potentially life-threatening disease (6). Bioactive components in HM are believed to sculpt the gut microbiota of neonates, which may infer immunologic protection (7–10).

HM- and cow milk formula (MF)-fed infants have been shown to have different microbiota profiles, measured in the stool (11, 12). There are many components of HM that may be driving this effect; however, human milk oligosaccharides (HMOs) may be among the most influential. HMOs are the third most abundant component of breast milk and are not utilized by the neonate, but instead are metabolized by specific bacteria in the gut (8). In clinical trials, a specific strain of *Bifidobacterium*, which is known to metabolize HMOs, has been shown to directly colonize the gut following co-exposure to HM (13). Additional components of HM, such as IgA (14), glycosylated proteins, and bacteria, may also modify the neonates' gut microbiota.

Currently, limited data exist on whether HM components influence the microbiota in region-specific ways in the gastrointestinal (GI) tract. Considering the ethical constraints of

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obtaining multiple bioregional samples from human infants, researchers must turn to animal models to examine this question. The piglet is a good animal model for the study of GI tract development, because there are many similarities with humans in terms of the digestive and metabolic functions of the gut (15–18). Additionally, the establishment of microbiota in the pig is similar to infants with respect to being influenced by the mode of delivery (19) and early postnatal diet and weaning (20). We have previously established this model for the examination of HM feeding's effects on the humoral and cell-mediated immune response (19). The current study complements and extends our prior work by determining the bioregional microbiota in response to HM versus cow milk formula (MF). The objective was to determine the effects of HM and MF neonatal diets at postnatal day (PND) 21, as well as the impact after weaning (PND 51) on luminal microbiota.

Methods

Animal experiments

The piglet study design has been previously described (19). Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research established and approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. White Dutch Landrace Duroc sows were fed a soy-free diet and artificially inseminated in a commercial farm setting. All sows were multiparous and the resulting litter sizes were 8–11; male piglets were used. Piglets were not from the same mother; however, all the mothers and piglets were housed in the same open area, but in separate pens in the same farm. Male piglets were allowed to suckle for 48 h at the farm, then received an intramuscular injection of iron (Iron Hydrogenated Dextran, 1 mL at 100 mg/mL) at postnatal day (PND) 2, prior to transfer to the Arkansas Children's Nutrition Center vivarium. At the vivarium, piglets were individually housed, which allowed for monitoring of food intake and separation in the case of diarrhea. The temperature was maintained at 27°C for the first week and transitioned to 25°C in the second week. Piglets could see and hear each other but otherwise had no direct contact. They were fed an isocaloric diet of either HM, provided from the Mothers' Milk Bank of North Texas, or a dairy-based MF (Similac Advance powder; Ross Products, Abbott Laboratories, Columbus, OH; $n = 12$/group) until PND 21. The feeding schedule was as follows: every 2 h in the first week of the study; every 4 h in the second week of study; and every 6 h in the third week of study. Each milk feeding was determined by a daily desired caloric intake (based on body weight), divided by the number of feeds. Piglets were trained to drink from nipples on a fixed schedule to provide 1.047 MJ/kg per day until PND 21. Dietary information has been published (19). At PND 14, a solid "starter pig food' was introduced until PND 21, where full weaning to an *ad libitum* diet occurred (Teklad diet 140,608; Harlan). From PND 21 until PND 51, piglets were fed *ad libitum* in accordance with the nutrient recommendations of the NRC for growing piglets (21). Piglets were immunized on PND 21 and PND 35 with 100 μg of cholera toxin (C8052, Millipore Sigma) and 100 μg of cholera toxin subunit B (CTB; C9903, Millipore Sigma) by gavage (total volume 2 mL saline, with 0.2 M sodium bicarbonate). Piglets received omeprazole (20 mg, orally; Arkansas Children's Hospital pharmacy) and were deprived of food 8 h prior to immunization and 1 h postimmunization. We administered the DAPTACEL vaccine (0.5 mL; Arkansas Children's Hospital pharmacy) intramuscularly in the shank on the same days. Control piglets also received omeprazole and deprived of food similarly to the treatment group (19). Piglets were euthanized 6–8 h after the final feeding period by anesthetizing with isoflurane, followed by exsanguination. Upon collection, GI contents were separated into 7 sections: duodenum, jejunum, ileum, cecum, proximal colon (PC), distal colon (DC), and rectum. DC contents were collected 50 cm from the end of the colon, and PC contents were obtained 25 cm from the cecum. Ileal contents were collected 50 cm from the distal end of the small intestine. The jejunal contents were collected 229 cm from the distal end, while the duodenal contents were designated at 50 cm from the proximal end of the stomach. For the cecum contents, a 10-cm section from the middle was utilized, and the rectal content was collected right before necropsy. Luminal contents from each section were collected within a scintillation vial, immediately snap-frozen in liquid nitrogen, and stored at –80°C until use.

16s Ribosomal RNA amplicon sequencing

Bioregional GI contents were homogenized using the QIAamp Fast DNA stool mini kit (Germantown, MD; Cat#51,604) for DNA extraction. Amplification of variable region 4 of bacterial 16s ribosomal RNA (rRNA) genes was carried out using 515F/806R forward and reverse primers (22). The pool of amplicons was paired-end sequenced (2 × 250 bp) with ~30% PhiX DNA using an Illumina Miseq. The data analysis was carried out by the Quantitative Insight into Microbial Ecology pipeline for operational taxonomic unit (OTU) clustering and taxonomic identification of amplicon sequences (23). We had a similarity threshold of 97% to cluster amplicon sequences for taxonomic annotation of OTUs using the Greengenes 16s rRNA database and a confidence threshold of 80%.

Statistical analysis

All tests utilized 16s rRNA gene sequencing data, and a P value of less than or equal to 0.05 was considered significant. All analyses were performed directly in R (version 3.5.2) or in the R-based HTML app, Dynamic Assessment of Microbial Ecology (24). Prior to the statistical analysis, sequencing counts were initially screened for sample depths <1000 to remove poorly sequenced samples. Following this screen, the mean sequencing depth for PND 21 and PND 51 samples were 24,822 and 14,958, respectively. We removed low abundant OTUs by filtering OTUs that had <10% of samples below 10 read counts. A 1-way ANOVA was used to identify diet and regional differences in Shannon and Chao1 measurements of alpha-diversity. Beta-diversity measurements were calculated using Bray–Curtis dissimilarities, and diet and regional differences were assessed by a permutational multivariate ANOVA (PERMANOVA). Dietary differences in individual taxa were assessed on shrunken log-fold differences by Wald's test for significance, as implemented in DESeq2 (25–27). Shrunken log2 fold estimates at the genera and phyla levels were calculated by DESeq2 with a false discovery rate. A P value <0.05 was considered significant. Standard log2 fold differences were also calculated and compared to the estimates derived from DESeq2. Taxa significantly altered by the diet with a mismatched DESeq2 log2 fold estimates and standard calculations were considered not significant. Immunization did not influence the measures of alpha- or beta-diversity; therefore, control and immunized animals were pooled in the analysis of the PND 51 piglet microbiota (**Supplemental Tables 1** and **2**). An analysis of the microbiota across time points had to be pulled from 2 miSeq runs. OTUs were summed across all samples to determine whether the total sums were different across the 2 data sets.

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Supplemental Tables 1–3 and Supplemental Figure 1 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
Address correspondence to LY (e-mail: vlyeruva@uams.edu).
Abbreviations used: CTB, cholera toxin subunit B; DC, distal colon; FF, formula-fed piglets without prebiotics; FP, formula-fed piglets with prebiotics; GI, gastrointestinal; HM, human breast milk; HMO, human milk oligosaccharide; MF, milk formula; PC, proximal colon; PERMANOVA, permutational multivariate ANOVA; PND, postnatal day; OTU, operational taxonomic units; rRNA, ribosomal RNA; SF, sow-fed; SR, sow-reared.

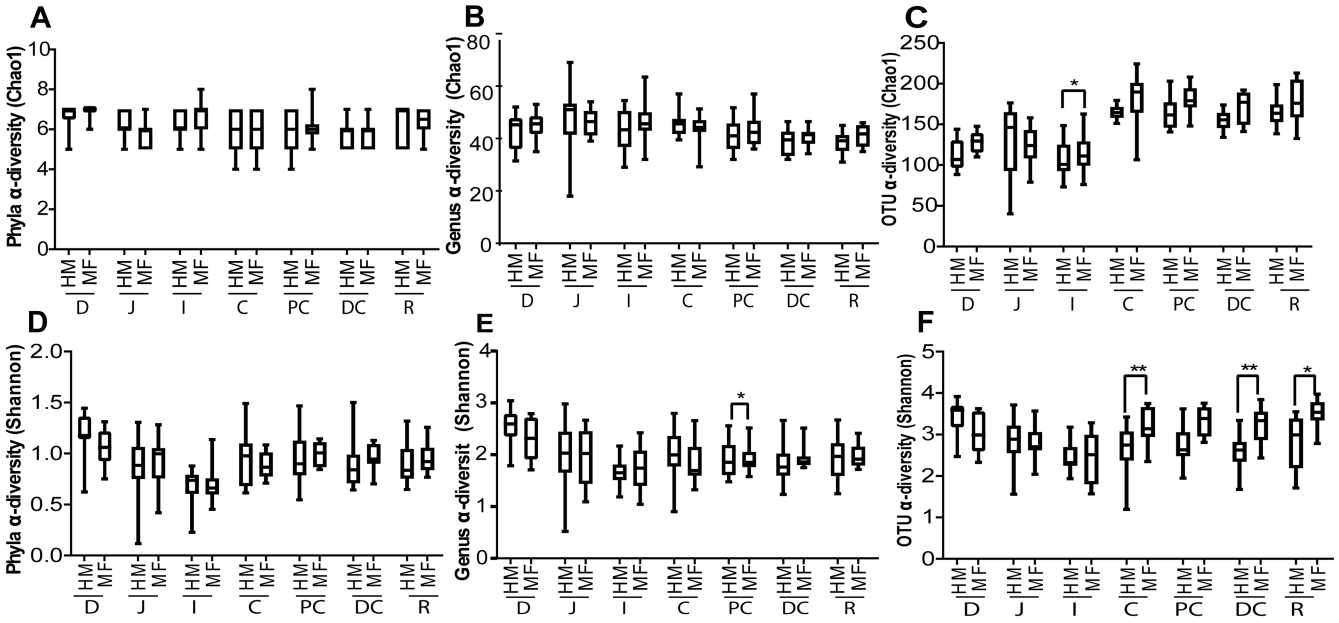

FIGURE 1 Dietary differences in alpha-diversity across luminal regions in piglets fed with HM or MF at PND 21. (A) Phyla Chao1, (B) genus Chao1, (C) OTU Chao1, (D) phyla Shannon, (E) genus Shannon, (F) OTU Shannon. Animal numbers were 9–11 for each diet group. Medians are shown with the 25th and 75th quartiles of Chao1 and Shannon diversity measurements, with significance between diets across the luminal regions. $^{*}P < 0.05$, $^{**}P < 0.01$. C, cecum; D, duodenum; DC, distal colon; HM, human milk; I, ileum; J, jejunum; MF, milk formula; PC, proximal colon; PND, postnatal day; OTU, operational taxonomic units; R, rectum.

No significant difference in total read counts was observed; thus, the data were rarefied to the lowest sample utilizing the Vegan package. The number of samples at PND 21 and PND 51 were 8 to 11 per group.

Results

Alpha-diversity is affected by diet in a region-specific manner

We compared PND 21 to PND 51 to determine microbial diversity (**Supplemental Table 3**). Statistical significance was noted for the interaction between time and tissue for Chao1 ($P < 0.001$). A 3-way interaction was observed for time, tissue, and diet for Shannon ($P < 0.05$; Supplemental Table 3) at the genera taxonomic level. Thus, we analyzed the 16s rRNA data for diet and region. At PND 21, HM- and MF-fed piglets showed similar diversity at phylum and genus taxonomic levels for Chao1 (**Figures 1**A and B). However, at the OTU level, the luminal region of the ileum of HM-fed piglets showed significantly lower diversity (Chao1 index) relative to MF-fed piglets (Figure 1C). For the Shannon index, no differences were noted at the phylum level between the diets across the luminal regions of the GI tract (Figure 1D). However, the HM group had a higher Shannon diversity index at the genera level in the PC lumen compared to MF-fed piglets (Figure 1E). Additionally, at the OTU level, HM-fed piglets had lower diversity (Shannon) in the luminal regions of the cecum, distal colon, and rectum compared to MF-fed piglets (Figure 1F).

At PND 51 (weaning neonatal diet), the luminal regions of PC and DC of HM-weaned piglets showed significantly lower diversity (Chao1) relative to those weaned from the MF diet (**Figure 2**A). Interestingly, the HM weaned group also had lower diversity (Chao1) at the genus level within the jejunum, ileum, PC, and DC lumen, relative to those weaned from MF (Figure 2B). Similarly, at the OTU level, piglets weaned from the HM diet had lower Chao1 measures in the cecal lumen, but higher Chao1 in the ileal lumen, relative to piglets assigned to MF (Figure 2C). No dietary differences were seen at PND 51 at the phyla level using Shannon diversity measurements (Figure 2D). At the genera (Figure 2E) and OTU (Figure 2F) taxonomic levels, piglets weaned from HM diet showed significantly lower diversity (Shannon) in the duodenum lumen and higher diversity in the ileum lumen compared to those weaned from MF. Overall, HM-fed piglets had lower alpha-diversity measurements within all regions and at both feeding periods.

Region-specific beta-diversity shifts due to diet

When we compared time differences from PND 21 to PND 51, the data demonstrated a clear separation of the microbiota by time at the genera taxonomic level (**Supplemental Figure 1**). Based on the PERMANOVA analysis, we observed an interaction between time and diet ($P < 0.01$) and time and tissue ($P < 0.01$), suggesting that differences by region and diet should be assessed within a given time (Supplemental Figure 1). Additionally, PERMANOVA at the phyla level shows a significant interaction between diet and tissue ($P < 0.05$). No interaction was seen at the OTU level; however, diet ($P < 0.01$) and tissue ($P < 0.01$) were independently significant (data are not shown). **Figures 3** and **4** display the beta-diversity at PND 21 and PND 51 across regions at the genera taxonomic level. On PND 21, diet effect in beta-diversity was seen at the genera level in the PC ($P < 0.01$), DC ($P < 0.01$), and rectal luminal regions ($P < 0.05$; Figure 3). At the phyla and OTU levels, there were also significant effects of diet (phyla $P < 0.01$; OTU $P < 0.01$) and tissue (phyla $P < 0.01$; OTU $P < 0.01$; data are not shown). Moreover, significant diet-specific differences were observed in the cecum, PC, DC, and rectum luminal regions (data are not

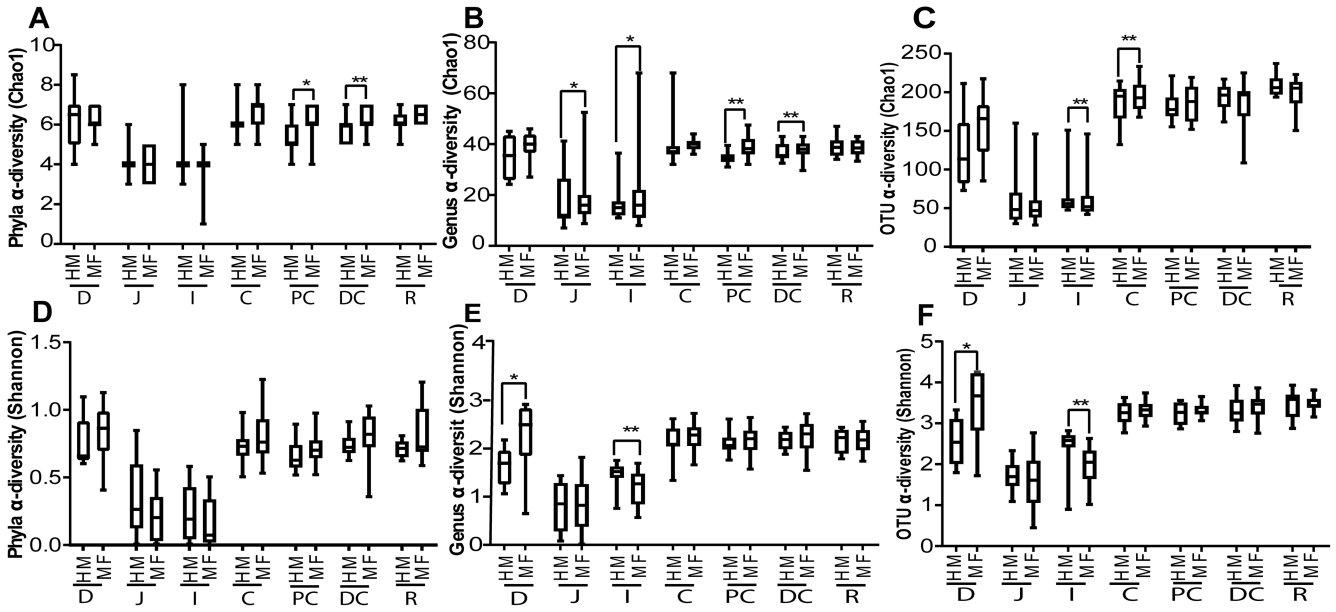

FIGURE 2 Dietary differences in alpha-diversity across luminal regions in piglets fed with HM or MF at PND 51. (A) Phyla Chao1, (B) genus Chao1, (C) OTU Chao1, (D) phyla Shannon, (E) genus Shannon, (F) OTU Shannon. Animal numbers were 8–15 for each diet group. Medians are shown with the 25th and 75th quartiles of Chao1 and Shannon diversity measurements, with significance between diets across the luminal regions. $^{*}P < 0.05$, $^{**}P < 0.01$. C, cecum; D, duodenum; DC, distal colon; HM, human milk; I, ileum; J, jejunum; MF, milk formula; PC, proximal colon; PND, postnatal day; OTU, operational taxonomic units; R, rectum.

shown) at the OTU level. In the PND 51 piglets, HM-fed and MF-fed piglets showed a clear separation of the community structure in DC and rectal (Figure 4) luminal regions at the genera taxonomic level.

Region-specific relative abundance shifts due to diet

Region-specific differences in relative abundance due to diet were seen across the GI tracts in both PND 21 (preweaned) and PND 51 (weaned) piglets. **Table 1** describes significantly altered phyla due to diet across intestinal regions at PND 21. Tenericutes phylum was more abundant in the cecum, PC, and rectal luminal regions of HM-fed piglets, relative to the MF-fed group. Moreover, Fusobacteria phylum was lower in the HM-fed group in the cecum, PC, and rectum regions, in comparison to the MF group. *Verrucomicrobia* was more abundant only on the rectal region of the HM-fed group, relative to the MF-fed piglets. We further determined significantly altered genera at PND 21 across all regions, and only 8 genera were significantly different between the diet groups in the small intestinal regions (**Table 2**). In the duodenal region, genus *Campylobacter* was more abundant in the HM group, while an unidentified genus from the Flavobacteriaceae family was lower in HM- compared to MF-fed piglets. In the jejunal region, *Campylobacter* was more abundant in HM-fed piglets, relative to the MF-fed group, while an unidentified genus from the Peptostreptococcaceae family was lower in the HM-fed group. In addition, *Veillonella*, *Clostridium*, *Turicibacter*, *Sarcina*, and an unidentified genus in the Clostridiaceae family were less abundant in the ileum region of the HM-fed group than in the MF-fed group. *Campylobacter* was more abundant in the HM-fed group than in the MF-fed group (Table 2). In contrast to the small intestine, 40 genera were altered across large intestinal regions between the HM- and MF-fed piglets (Table 2). In the cecal luminal region of HM-fed piglets, *Dorea*, *Actinobacillus*, *Prevotella*, *Campylobacter*, *Haemophilus*, *Anaerovibrio*, and an unassigned genus from the Prevotellaceae family, the S24-7 family, and the 2 classes Bacteroidia and Mollicutes were significantly more abundant, relative to MF-fed piglets. The genera *Fusobacterium* and rc4-4 were less abundant in the HM-fed group compared to MF fed-piglets. In the PC and DC regions of the large intestine, *Dorea* and *Actinobacillus* were significantly abundant in HM-fed piglets, relative to MF-fed piglets. In addition, *Anaerovibrio* and unassigned genera from families Prevotellaceae and S24-7 and class Bacteroidia were abundant in the PC luminal region of HM-fed piglets. In the DC region of HM-fed piglets, *Prevotella* and *Haemophilus* were more abundant, relative to MF-fed piglets. Additionally, *Turicibacter* was decreased in HM-fed piglets compared to MF-fed piglets, across all luminal regions of the large intestine. Interestingly, *Campylobacter* was found to be greater in the HM-fed group from the duodenum through the DC lumen compared to MF-fed piglets. Importantly, *Campylobacter* was most abundant in the duodenal lumen of the HM-fed group, at 2.11%, and decreased through the GI tract to the rectal lumen, with an abundance of 0.182%. Furthermore, in the rectal region, *Dorea*, *Akkermansia*, genera from class Bacteroidia, families S24-7 and Erysipelotrichaceae were more abundant in HM-fed piglets than in MF-fed piglets.

At PND 51, there were a smaller number of taxa significantly affected by diet; however, in the large intestinal lumen phyla taxonomic level changes were still observed. In HM-fed piglets, Proteabacteria phyla were lower in the cecum and distal colon lumen, while Tenericutes was more abundant in PC lumen, relative to the MF-fed piglets (**Table 3**). No dietary differences were observed in the duodenal lumen; however, several genera were significantly altered by diet throughout the remaining regions of the GI tract (**Table 4**). *Turicibacter*, which was significant across many luminal regions at PND 21, was not

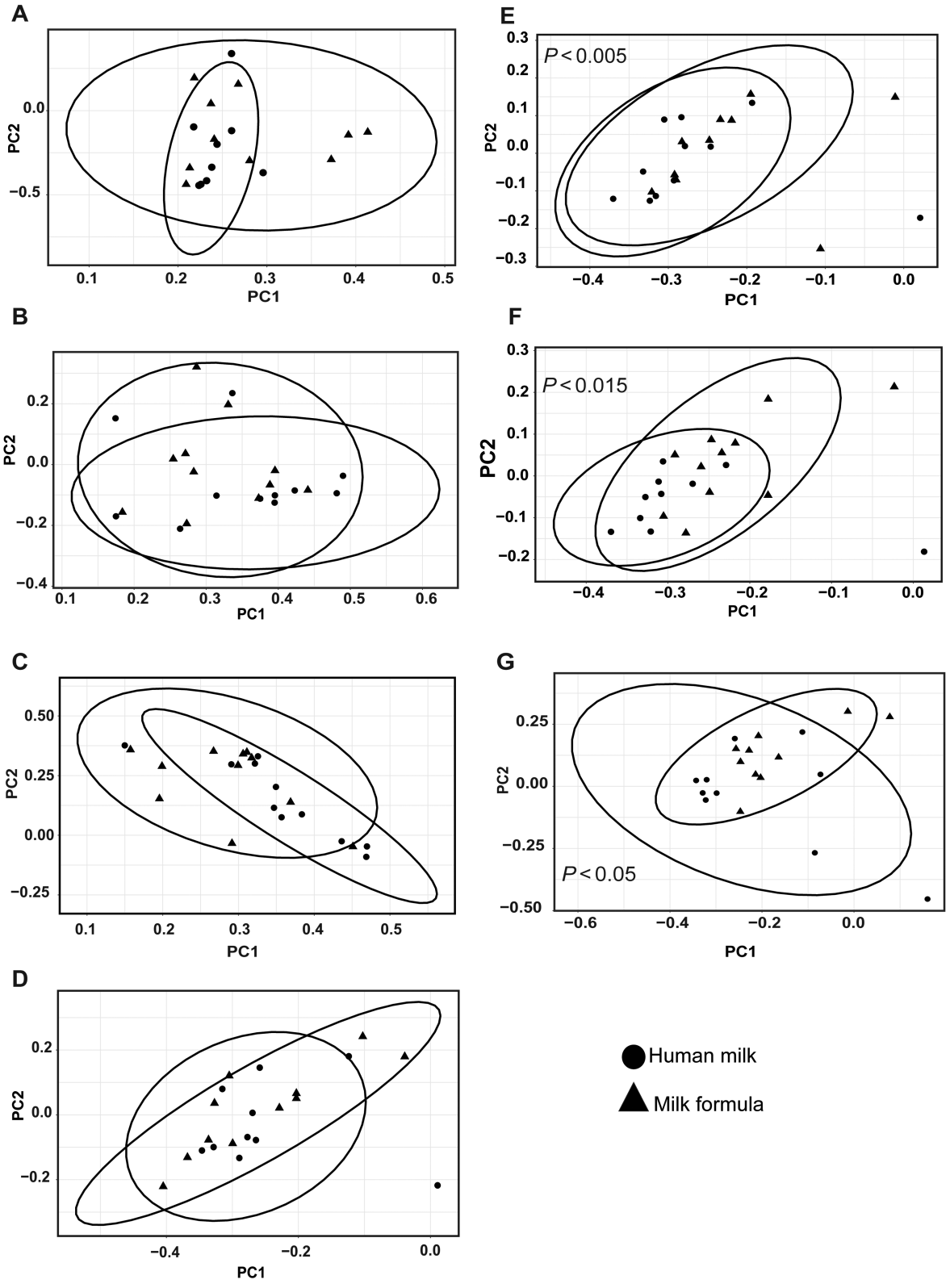

FIGURE 3 Impact of neonatal diet on bacterial community across luminal regions in piglets fed with HM or MF at PND 21. Distances created with Bray–Curtis show genera taxonomic communities across luminal regions. (A) Duodenum, (B) jejunum, (C) ileum, (D) cecum, (E) proximal colon, (F) distal colon, and (G) rectum. *P* values represent group differences among PCoA scores along component 1. Animal numbers were 8–11 for each diet group. HM, human milk; MF, milk formula; PC, principal component; PCoA, principal coordinates analysis; PND, postnatal day.

altered by diet in any region at PND 51. Within this time point, multiple unspecified genera were identified, which are indicated with family or class designations in Table 4. YRC22 and an unspecified genus within the 4C0d-2 class are decreased in the HM-fed group from the cecal to the rectal lumen. Furthermore, an unidentified genus from class Bacteroidia was more abundant in HM-fed piglets than in MF-fed piglets in the distal colon lumen (Table 4).

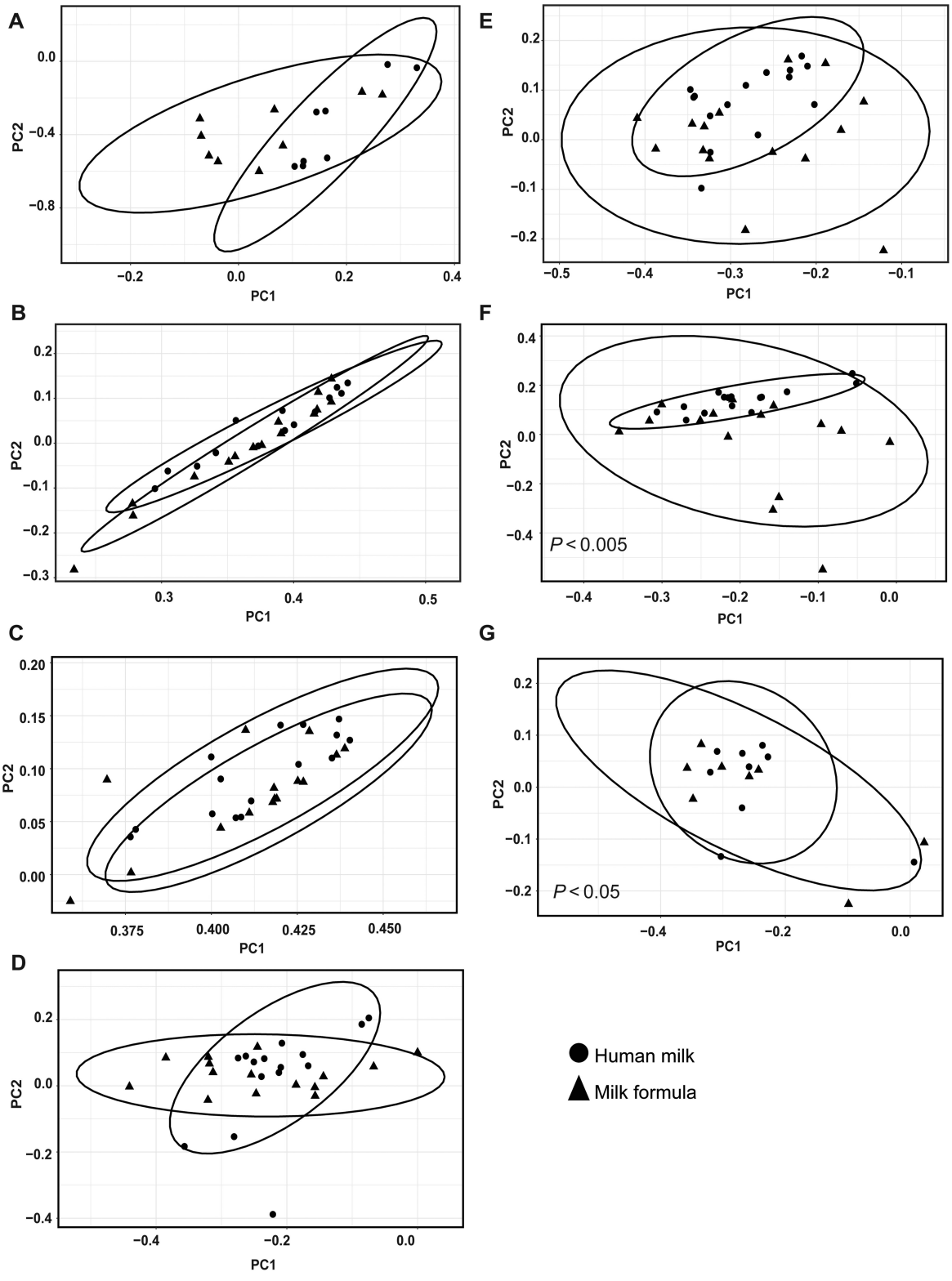

FIGURE 4 Impact of neonatal diet on bacterial community across luminal regions in piglets fed with HM or MF at PND 51. Distances created with Bray–Curtis show genera taxonomic communities across luminal regions. (A) Duodenum, (B) jejunum, (C) ileum, (D) cecum, (E) proximal colon, (F) distal colon, and (G) rectum. *P* values represent group differences among PCoA scores along component 1. Animal numbers were 8–15 for each diet group HM, human milk; MF, milk formula; PC, principal component; PCoA, principal coordinates analysis; PND, postnatal day.

Discussion

Several studies have shown differences in microbiota composition between HM-fed and formula-fed infants using fecal samples (11, 12, 28–31). The current study examined bioregional differences in the microbiota of piglets fed either a HM or MF diet. The piglet model has been described to be a good model for infant gut and immune system development (32) and, indeed, has been utilized to study the effects of different diets

TABLE 1 Relative abundance of phyla affected by human milk or milk formula in piglets at postnatal day 21[1]

Regions	Phylum	HM	MF	log2 FC	*P* adj
Cecum	Tenericutes	3.137 ± 6.1	0.153 ± 0.4	5.2	0.004
Cecum	Fusobacteria	0.546 ± 1.4	3.935 ± 7.3	−3.0	0.023
Proximal colon	Tenericutes	3.462 ± 6.5	0.178 ± 0.5	5.9	0.027
Proximal colon	Fusobacteria	0.631 ± 1.1	4.231 ± 4.3	−3.1	0.044
Rectum	Verrucomicrobia	2.623 ± 6.2	1.099 ± 3.4	5.5	0.001
Rectum	Tenericutes	2.622 ± 6.6	0.197 ± 0.4	3.5	0.020
Rectum	Fusobacteria	0.241 ± 0.6	3.739 ± 9.8	−3.4	0.001

[1] Values are mean relative abundance ± SEMs, n = 8–11 per diet group. Adjusted P values from Wald test indicate differences between log2 FC values, calculated by DeSeq2 where reference group is HM. Phyla that were significant with an adjusted P value < 0.05 are shown. FC, fold change; HM, human milk; MF, milk formula.

on the microbiota of various regions of the GI tract (11, 33–35). These studies have been limited, however, to 1 or 2 sections within the porcine GI tract. Moreover, when utilizing piglets for neonatal diet studies, sow-reared (SR) piglets are usually utilized as a control. Specifically relating to microbiota research, this poses a serious limitation, as SR piglets generally never leave the farm, whereas the treated piglets were transferred to a vivarium setting. The study we conducted is unique in that we utilized samples from piglets that received donor HM and at day 21 compared microbiota differences directly to piglets fed MF. Piglets used for this study were housed in the same location; thus, changes in microbiota composition are attributed to diet and not environmental influences. Interestingly, the microbiota composition differed 4 weeks after weaning from the neonatal diet (PND 51), suggesting persistent effects of a neonatal diet on the microbiota composition. Moreover, our results suggest that piglets fed HM are closer to infants in their microbiota composition.

The piglet microbiota across the 7 GI sections has been well-characterized by Crespo-Piazuelo et al. (32); thus, only dietary differences are discussed. Additionally, the bacterial and host proteins across the porcine GI tract has also been recently described (33). However, to our knowledge, the current study is the first exhaustive investigation of all 7 regions of the piglet GI tract following a neonatal diet intervention and during the neonatal versus postweaned conditions. We observed that MF-fed piglets had increased microbial diversity and richness across the luminal regions compared to HM-fed piglets, which is in agreements with infants who receive formula (11). For example, a study of Chinese infants showed that formula-fed infants have higher microbiota richness, as measured by the Shannon and Chao1 indices (28). Another study from a Canadian birth cohort also described an inverse relationship between breastfeeding and microbial diversity at 3 months of age (36). Finally, infants fed formula with added prebiotics and probiotics showed higher alpha-diversity (as measured by the Shannon index) at both 6 and 12 weeks of age compared to breast-fed babies (37). We have previously published on the small intestinal microbiota of piglets receiving either a dairy or soy formula compared to sow-fed (SF) piglets (38). When compared to the SF piglets, the formula-fed piglets displayed lowered richness and evenness within the duodenum. Additionally, within the jejunum, microbial diversity was also higher in the SF piglets than in their formula-fed counterparts (38). This was in agreement with a study by Wang et al. (39), which compared formula-fed piglets with (FP) or without prebiotics (FF) to SF piglets, and found that the SF group had the highest alpha-diversity at PND 7, the prebiotic-fed had the lowest, and the FF were in the middle. Thus, SF piglets have a higher alpha-diversity compared to FF piglets, whereas in our study HM-fed piglets had a lower alpha-diversity, which is similar to findings from breast-fed infants. This further highlights how our controlled environmental conditions of the HM-fed piglet model are closer to those of infants fed with human milk. The diversity observed in the FP group suggests that the FP diet interacts with the microbiota similar to HM-fed group. Most recently, higher microbial diversity at 4 months of life has been associated with an increased risk of developing celiac disease later in life (40). However, a different report indicated that higher microbial diversity at 6–9 months of age is associated with alleviating symptoms of atopic eczema, further suggesting that microbiota development during early life possibly plays a role in immune programming (41).

The Bray–Curtis measurements performed for beta-diversity utilized quantitative sequence abundances to map distances between samples by diet. We observed the clustering of bioregional microbiota between the small and large intestinal lumen. We also found the greatest dietary differences within the large intestinal lumen. These findings are in agreement with a study of a milk fat globule membrane and lactoferrin test diet fed to piglets, which found significant diet effects in the ascending colon and rectum (42). Indeed, the beta-diversity segregation of samples by early diet has been seen in human infants as well. Exclusively breastfed infants have been shown to segregate from those nonexclusively breastfed both prior to and after the introduction of solid foods (43, 44).

Specific bacteria were found to be significantly affected by diet across multiple luminal regions. Previous studies have demonstrated that microbes found within the small intestine are more likely to induce an IgA response than those found in the large intestine (colon) (45–47). The microbiota of the colon is likely to be involved in immune tolerance mechanisms (48, 49). Furthermore, adaptive immune responses were shaped by lymph node drainage to different compartments of the GI tract (45–47). Interestingly, in our previous publication, we have shown a CTB–IgA response in small intestinal contents (19) but no CTB–IgA response was observed in the colon (unpublished Yeruva laboratory data). Additionally, in the HM-fed piglets, *Turicibacter* was decreased, which has previously been found to be positively correlated with nutrient digestibility (50) and is significantly higher in SR piglets (51). In a rat hypertension model, *Turicibacter*-induced T-cell activation and polarization in mesenteric lymph nodes, resulting in an increased Th17/IL-17 and reduced Treg cell response (52). Interestingly, in HM-fed piglets, *Turicibacter* was decreased, in comparison to MF-fed piglets. Furthermore, a gut commensal, *Prevotella histicola*, has been shown to decrease Th1/Th17 cells and increase the CD4 + FoxP3 + regulatory T-cells (53). Our study shows that

TABLE 2 Relative abundance of genera affected by human milk or milk formula in piglets at postnatal day 21[1]

Regions	Genus	HM	MF	Log2 FC	*P* adj
Duodenum	*Campylobacter*	2.11 ± 3.5	0.128 ± 0.1	3.2	0.037
Duodenum	f__Flavobacteriaceae	0.12 ± 0.2	0.762 ± 0.9	−3.6	0.037
Jejunum	*Campylobacter*	1.72 ± 3.8	0.067 ± 0.1	4.7	0.023
Jejunum	f__Peptostreptococcaceae	0.36 ± 1.0	0.817 ± 1.7	−1.9	0.048
Ileum	*Campylobacter*	0.202 ± 0.3	0.018 ± 0.04	4.0	0.015
Ileum	*Veillonella*	1.161 ± 2.2	1.624 ± 2.4	−2.4	0.015
Ileum	*Clostridium*	0.73 ± 1.9	2.43 ± 5.2	−3.9	0.003
Ileum	f__Clostridiaceae	0.52 ± 0.6	4.727 ± 8.4	−3.3	0.005
Ileum	*Sarcina*	0.005 ± 0.02	0.091 ± 0.2	−4.5	0.015
Ileum	*Turicibacter*	0.079 ± 0.2	2.412 ± 4.5	−5.4	0.002
Cecum	c__Bacteroidia	5.194 ± 9.1	2.328 ± 7.7	7.8	0.001
Cecum	c__Mollicutes	3.137 ± 6.1	0.153 ± 0.4	6.4	0.001
Cecum	*Dorea*	2.637 ± 2.1	0.25 ± 0.3	3.5	0.001
Cecum	*Actinobacillus*	1.99 ± 1.7	0.495 ± 0.7	2.2	0.005
Cecum	*Prevotella*	1.553 ± 2.8	0.015 ± 0.1	5.9	0.009
Cecum	f__S24-7	1.505 ± 3.9	0.523 ± 1.7	5.7	0.005
Cecum	*Campylobacter*	0.575 ± 0.9	0.039 ± 0.1	5.5	0.005
Cecum	*Haemophilus*	0.428 ± 0.7	0.058 ± 0.1	2.7	0.013
Cecum	*Anaerovibrio*	0.176 ± 0.4	0.001 ± 0.002	7.6	0.015
Cecum	f__Prevotellaceae	0.069 ± 0.1	0.001 ± 0.002	6.0	0.045
Cecum	*Fusobacterium*	0.546 ± 1.4	3.935 ± 7.3	−3.4	0.016
Cecum	*Turicibacter*	0.022 ± 0.1	0.558 ± 0.8	−5.2	0.001
Cecum	rc4-4	0.016 ± 0.02	0.159 ± 0.3	−3.7	0.006
Proximal colon	c__Bacteroidia	3.634 ± 7.5	1.455 ± 4.6	5.9	0.008
Proximal colon	*Dorea*	3.305 ± 3.1	0.34 ± 0.3	3.8	0.001
Proximal colon	*Actinobacillus*	1.249 ± 1.9	0.178 ± 0.3	3.1	0.001
Proximal colon	f__S24-7	0.635 ± 1.3	0.608 ± 1.9	5.0	0.044
Proximal colon	*Campylobacter*	0.394 ± 0.8	0.01 ± 0.03	6.1	0.012
Proximal colon	f__Prevotellaceae	0.124 ± 0.3	0.00 ± 0.00	22	0.001
Proximal colon	*Anaerovibrio*	0.062 ± 0.1	0.00 ± 0.00	22	0.001
Proximal colon	*Turicibacter*	0.013 ± 0.03	0.67 ± 1.3	−5.4	0.001
Proximal colon	rc4-4	0.01 ± 0.02	0.148 ± 0.2	−4.0	0.019
Distal colon	*Dorea*	1.907 ± 1.3	0.577 ± 0.7	1.8	0.011
Distal colon	*Actinobacillus*	0.89 ± 1.6	0.02 ± 0.02	5.2	0.001
Distal colon	*Campylobacter*	0.743 ± 1.5	0.023 ± 0.1	5.1	0.004
Distal colon	*Prevotella*	0.341 ± 0.6	0.083 ± 0.3	8.7	0.001
Distal colon	*Haemophilus*	0.141 ± 0.3	0.004 ± 0.009	5.2	0.003
Distal colon	*Oscillospira*	1.151 ± 0.4	4.317 ± 5.8	−1.3	0.018
Distal colon	*Ruminococcus*	0.322 ± 0.7	0.86 ± 1.3	−2.6	0.002
Distal colon	*Turicibacter*	0.003 ± 0.005	0.381 ± 1.0	−4.6	0.003
Rectum	*Dorea*	3.103 ± 3.4	0.64 ± 0.6	2.5	0.005
Rectum	*Akkermansia*	2.623 ± 6.2	1.099 ± 3.4	5.7	0.001
Rectum	c__Bacteroidia	1.884 ± 3.8	0.102 ± 0.3	4.6	0.019
Rectum	f__S24-7	1.538 ± 2.5	0.067 ± 0.1	5.2	0.003
Rectum	f__Erysipelotrichaceae	0.762 ± 1.3	0.347 ± 0.8	2.7	0.017
Rectum	*Streptococcus*	0.622 ± 1.1	1.018 ± 1.1	−1.9	0.019
Rectum	*Fusobacterium*	0.241 ± 0.6	3.739 ± 9.8	−3.1	0.007
Rectum	*Eubacterium*	0.118 ± 0.1	1.222 ± 1.8	−2.7	0.028
Rectum	*Turicibacter*	0.006 ± 0.01	0.172 ± 0.5	−2.6	0.046
Rectum	f__Mogibacteriaceae	0.001 ± 0.003	0.056 ± 0.1	−5.3	0.001

[1] Values are mean relative abundance ± SEMs, $n = 8$–11 per diet group. Adjusted *P* values from Wald test indicate differences between log2 FC values, calculated by DeSeq2 where reference group is HM. Genera that were significant with an adjusted *P* value < 0.05 are shown. c__, Class; f__, Family; FC, fold change; HM, human milk; MF, milk formula.

Prevotella is more abundant in HM-fed piglets than in MF-fed piglets. Thus, it is possible that some of the commensal bacteria observed in the HM-fed group exhibit a protective immune response compared to the MF-fed group. We have previously shown that FF piglets have altered GI morphology compared to SR piglets, while we found no differences between the HM- and MF-fed groups (19, 54). Some of these bacteria, however, may be influencing the GI tract gene expression and immune cell composition, which is the scope of future studies. In this paper, we are reporting on the luminal microbiota, which may not interface with the intestinal epithelial cells. Bacteria within the mucosa, which can interface with the epithelial layer, may play

TABLE 3 Relative abundance of phyla affected by human milk or milk formula in piglets at postnatal day 51[1]

Regions	Phylum	HM	MF	log2 FC	*P* adj
Cecum	Proteobacteria	1.749 ± 1.7	4.879 ± 3.9	−1.4	0.011
Proximal colon	Tenericutes	0.478 ± 0.7	0.199 ± 0.2	1.5	0.024
Proximal colon	Actinobacteria	0.01 ± 0.01	0.05 ± 0.1	−1.8	0.024
Distal colon	Proteobacteria	1.569 ± 1.2	5.032 ± 4.7	−1.4	0.043
Rectum	Firmicutes	31.691 ± 11.0	27.24 ± 8.7	0.9	0.033
Rectum	Lentisphaerae	0.859 ± 1.2	0.103 ± 0.2	2.8	0.033

[1] Values are mean relative abundance ± SEMs, n = 8–11 per diet group. Adjusted P values from Wald test indicate differences between log2 FC values, calculated by DeSeq2 where reference group is HM. Phyla that were significant with an adjusted P value < 0.05 are shown. Animal numbers are n = 8–15 per diet group. FC, fold change; HM, human milk; MF, milk formula.

a larger role in the development of the GI immune system. The aim here is to describe the bioregional microbiota differences while examining the bacteria found within the mucosal layer would enhance our understanding of the diet effects on mucosal gut microbiota. In addition, the DNA isolation kit used in the study does not include the step for mechanical disruption of the bacteria. Thus, it is possible that some of the microbiota, such as *Lactobacillus* species, present in the small and large intestines of piglets were not detected. Future studies comparing the DNA isolation procedures are needed to determine the differences in microbiota composition.

The major differences found in this study were from the lumen of the large intestine, which appears to be similar to the infant fecal microbiota composition. Thus, examining the fecal material from infants can be a good proxy to the infant distal end of the GI tract. At 3 months of age, breastfed babies have a higher percentage of Bacteroidetes and a lower percentage of Firmicutes and *Verrucomicrobia* compared with FF infants (55). It is interesting to note that the opposite findings were noted in our study, as *Verrucomicrobia* was higher at the PND 21 time point in the HM-fed piglets. In a study by Poroyko et al. (51) in which MF-fed and SR piglets were studied, Bacteroides and Parabacteroides were significantly higher, whereas *Prevotella*, *Oscillibacter*, and *Turicibacter* were significantly lower, in the MF-fed group. *Turicibacter* was significantly higher in the MF-fed group at the weaning time point across multiple regions: the ileum, cecum, PC, DC, and rectum. *Prevotella*, in contrast, was significantly lower in the MF-fed group at this time point in the cecum and DC. In addition, we found unspecified genera in the Bacteroidia class were more abundant in the HM-fed group within the cecum, PC, and rectal lumen, relative to the MF-fed group. Studies conducted worldwide, such as in Norway (56), Sweden (57), Canada (58), Italy (59), Switzerland (60), Bangladesh (61), the United States (62), Malawi, and Finland (63), show that some of the healthy breast-fed infants have lower amounts of *Bifidobacterium* species than others. The most recent report suggests that at least 3 compositionally distinct neonatal gut microbiota might be present, with a high relative abundance of Bacteroides or *Bifidobacteria*, and higher abundances of *Bifidobacterium* and Bacteroides (64).

TABLE 4 Relative abundance of genera affected by human milk or milk formula in piglets at postnatal day 51[1]

Regions	Genus	HM	MF	log2 FC	*P* adj
Jejunum	*Streptococcus*	0.158 ± 0.4	0.58 ± 0.8	−3.0	0.034
Jejunum	*Moraxella*	0.044 ± 0.1	1.226 ± 3.2	−4.6	0.034
Ileum	f__Enterobacteriaceae	0.559 ± 0.9	1.25 ± 4.8	5.5	0.001
Ileum	*Streptococcus*	0.166 ± 0.4	1.234 ± 2.6	−4.0	0.001
Cecum	c__Chloroplast	0.337 ± 0.8	0.03 ± 0.05	3.1	0.010
Cecum	*Sutterella*	1.169 ± 1.3	3.37 ± 3.5	−1.7	0.035
Cecum	*Faecalibacterium*	0.152 ± 0.1	0.643 ± 0.9	−2.1	0.009
Cecum	CF231	0.003 ± 0.009	0.58 ± 1.2	−7.7	0.001
Cecum	YRC22	0.002 ± 0.007	2.915 ± 3.9	−10.1	0.001
Cecum	c__4C0d-2	ND	1.895 ± 3.6	−11.6	0.001
Proximal colon	*Faecalibacterium*	0.091 ± 0.1	0.307 ± 0.4	−1.7	0.048
Proximal colon	*Collinsella*	0.01 ± 0.01	0.05 ± 0.1	−2.2	0.048
Proximal colon	CF231	0.003 ± 0.008	1.289 ± 2.9	−7.9	0.007
Proximal colon	YRC22	0.001 ± 0.003	3.662 ± 5.3	−11.4	0.001
Proximal colon	c__4C0d-2	ND	0.386 ± 0.8	−7.6	0.002
Distal colon	c__Bacteroidia	13.468 ± 6.7	6.189 ± 5.1	1.5	0.012
Distal colon	c__RF3	0.029 ± 0.05	1.067 ± 3.4	−2.1	0.049
Distal colon	YRC22	ND	2.419 ± 3.5	−12.3	0.001
Distal colon	c__4C0d-2	ND	0.445 ± 0.9	−23.6	0.001
Distal colon	c__Alphaproteobacteria	ND	1.003 ± 3.2	−7.2	0.001
Rectum	YRC22	0.037 ± 0.1	2.893 ± 4.0	−6.7	0.011
Rectum	c__4C0d-2	0.003 ± 0.006	0.738 ± 1.0	−7.6	0.001

[1] Values are mean relative abundance ± SEMs, n = 8–15 per diet group. Adjusted P values from Wald test indicate differences between log2 FC values, calculated by DeSeq2 where reference group is HM. Genera that were significant with an adjusted P value < 0.05 are shown. c__, Class; f__, Family; FC, fold change; HM, human milk; MF, milk formula; ND, not detected.

Interestingly, when MF-fed piglets were compared to SR piglets, more abundant Bacteroides were observed in MF-fed piglets, which is the opposite of what is seen in infants. Moreover, we observed a higher abundance of genera from class Bacteroidia in the large intestine, and also Bacteroides genera in feces, in HM-fed piglets (19) relative to MF-fed piglets.

In summary, our data suggest that piglets fed with human milk are closer to infants fed breastmilk. These data further highlight Dutch microbiologist Lourens Baas Becking's hypothesis, "everything is everywhere, but the environment selects" (65). Finally, the complex mixture of breastmilk could be key to shaping the gut microbiota. Future studies are needed to understand how microbial colonization impacts immune programming and health outcomes later in life.

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