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A breast milk exclusive diet promotes dysbiosis in the gut microbiome of six-month-old anemic infants

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SUMMARY Iron deficiency is the most common nutritional deficiency worldwide, with recent studies reporting the prevalence of iron deficiency anemia being 19% in Canada alone. The microbiome is increasingly recognized as a key regulator of immunity, and imbalances have been linked to a range of diseases. As a result, there is great interest in understanding the association between the microbiota and the onset of anemia. While there have been steady improvements in this regard, the impact of various types of diets on the gut microbiota in the context of anemia is not well-established. Since diet significantly affects the microbiota composition, we investigated effects of breast milk exclusive diet versus a complete diet on microbiota composition in anemic and normal infants. We found significant differences in alpha and beta diversity across diets among anemic six-month-old infants, but not in normal infants. In addition, our core microbiome analyses revealed certain genera that were unaffected by diet or anemic status, while others were characteristic of the anemic groups, such as *Actinomyces* and *Bacteroides*. Lastly, we observed decreased abundance of several key commensal genera in breast milk fed infants as compared to those fed a complete diet, along with an upregulation of enzymes associated with hemoglobin synthesis and iron scavenging. Overall, our study provides fundamental understanding into how diet affects the gut microbiota of anemic infants.

INTRODUCTION

Anemia is a condition characterized by a reduction in red blood cell number and hemoglobin concentration below the age specific mean (1). Anemia at infancy has been found to experience negative cognitive, metabolic, and developmental effects, some of which can persist into adulthood (2).

Previous studies have shown that infants who experience iron deficiency within the first year of life (6-12 months) are likely to suffer from delayed development of the central nervous system due to alterations in morphology, neurochemistry, and bioenergetics (3). This translates to hampered neurocognitive and neurobehavioural development in adulthood. In addition, iron acts as an important nutrient for the growth and colonization of bacteria, playing an important role in the establishment of a healthy gut microbiota (2). The gut microbiota is a key regulator of immunity, and a dysbiotic microbial composition associated with iron deficiency has been linked to a myriad of conditions including depression, autism spectrum disorder, neurodegeneration, and anxiety (2). Thus, iron-deficiency anemia could result in an altered gut-microbiota composition, which may have significant consequences on overall community homeostasis and subsequent mental and physical health. Several key pathogenic microbes are iron-dependent for colonization and infection, including *Salmonella Typhimurium* and enterosensitive *Escherichia coli/Shigella spp.* (EIEC/Shigella) (3). These pathogenic microbes typically cause enteric infections and contribute to iron-deficiency anemia in children by hindering the host's ability to absorb sufficient amounts of iron while also promoting systemic inflammation (4).

To investigate the link between diet and the microbiota composition in anemic and healthy infants, we explored the variation in the gut microbial composition across two diet types- a breast milk exclusive diet (referred to as BM exclusive diet hereafter) and a diet composed of breast milk in addition to soup and/or broth (referred to as a complete diet hereafter). Breast milk has shown to impart several benefits on developing infants. In addition

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to supplying the optimal amount of required macronutrients (fats, proteins), micronutrients (vitamins A, B1, B2, B12, and D) for growth, breast milk is a source of commensal bacteria that colonize the gut lining and promote pathogen exclusion (6). This is consistent with the findings of previous studies which demonstrated that formula-fed infants have less developed immune systems and a subsequent increased incidence of gastrointestinal diseases like diarrhea, acute respiratory infections, and higher mortality rates (7, 8).

Despite these advantages, breast milk has a very low iron content, so exclusive breastfeeding after 6-months is correlated with increased incidence of iron deficient anemia (IDA) (7). Previous studies have shown that iron-deficient diets have been associated with increased proportions of opportunistic-pathogens in the gut microbiota including *Escherichia/Shigella* despite being iron-requiring bacteria (7, 9). Similarly, a decrease in commensal beneficial genera including *Prevotella* (involved in carbohydrate and protein metabolism), some strains of *Bacteroidetes*, and non-pathogenic members of *Clostridium* has been observed in an iron deficient microbiota compared to healthy ones in rodent models, suggesting that iron is a key modulator of gut microbiota balance and homeostasis (9).

In this study, we investigated the relationship between anemic status and breast milk exclusive diets through analyses of infant gut microbiome alterations. While our analyses showed no significant differences in diversity metrics across anemic status, significant differences were observed across diets regardless of anemic status. Subsequently, we found that this difference was only significant within anemic infants. Our core microbiome analyses suggest that *Actinomyces* and *Bacteroides* were exclusively present in anemic infants fed a BM exclusive and complete diet respectively. Relative abundance analysis revealed a significant decrease in commensal genera in anemic infants fed BM only as compared to those fed a complete diet. Finally, we observed an upregulation of enzymes involved in iron scavenging and hemoglobin synthesis, alongside a downregulation of those associated with amino acid catabolism and bacterial cell wall synthesis in anemic infants fed BM only.

Previous research on the impact of anemic status on the microbiome composition of infants revealed significant differences in microbiome differences between anemic and normal infants (2). Interestingly however, the analysis of the microbial community composition between anemic and normal infants did not consider the impact of diet on gut-microbial community. Given that diet is a key factor that influences the composition of the gut microbiota, analyzing the variation in the microbial community across diets in anemic infants can offer key insights regarding the core microbiome associated with anemia compared to normal infants, and how the presence and absence of specific bacterial genera correlate to disease prognosis (5). Despite our knowledge and understanding of the role of diets on infants' developing gut microbiome and immune system, very few studies have investigated the tri-directional relationship between diet, the gut microbiome, and anemic status in infants.

The results of this study provide a deeper understanding of how diet and anemic status interact with and affect each other, thereby aiding the design of infant diets that facilitate the development of a healthy gut microbiota and minimize the risk of iron-deficiency anemia. This is highly relevant as iron-deficiency anemia in infants can have long-lasting impacts on cognitive, socio-emotional, and adaptive physiological functions, as previously described (10).

METHODS AND MATERIALS

Dataset. This project utilizes the dataset generated by McClorry *et al.* from the University of California, Davis. The dataset was generated by examining the microbial composition of stool samples and the metabolite composition of serum samples obtained from 102 infants aged 6-12 months. This was a mixed group, containing both normal infants and those that had iron-deficiency anemia. This data was collected to elucidate the gut microbial structure and function associated with anemic infants in comparison to normal infants. In addition, this study was carried out to identify serum metabolite/metabolome markers associated with anemia that can be used for diagnosis of the disease. Cases in which the fecal sample was provided more than 14 days after the associated serum sample was taken, and/or had abnormally low read counts, were not used for analysis.

The trial associated with this dataset is publicly available under clinicaltrials.gov and is registered as NCT03377777.

Processing in QIIME 2. Demultiplexed single-end sequence data was imported into Quantitative Insights into Microbial Ecology Version 2 (QIIME2) using the manifest format approach, and denoised using DADA2, which corrects errors made during amplicon sequencing (11). Reads were truncated at 252 bp to maintain an average Phred score of 38 (Figure S1). The quality of the reads was consistently high throughout, and thus the entire sequence length was retained. The denoised dataset was trained using a pre-trained classifier for the F515(5′-CACGGTCGKCGGCGCCATT-3′)-R806 (5′-GGACTACHVGGGTWTCTAAT-3′) primer pair using the SILVA 128 reference database for taxonomic classification of the ASVs in the samples (silva-138-99-515-806) (12–16). To account for unequal sample sequencing depth and capture a sufficient number of ASVs, samples were rarified at a depth of 25090 sequences per sample (Figure S2). A total number of 139 (72.02%) samples and 3,487,510 (62.68%) ASVs were retained for downstream analyses.

Diversity analysis in R & statistical testing. The rarefied dataset, including the metadata, feature table, taxonomy file, and rooted-phylogenetic tree, was imported into R using the *qiime tools export* command for subsequent alpha and beta diversity metrics. The data was then filtered to exclude bacterial and chloroplast DNA, followed by the removal of ASVs that occurred less than two times (i.e. rare ASV's). The rarefied data was further subsetted to only include 6-month-old infants who were given diets of interest, including one subset for infants fed BM only and one subset for infants fed a complete diet (Figure 1). To perform diversity metrics, the following R packages were installed and loaded: tidyverse, vegan, ggplot2, phyloseq, and ape (17-21).

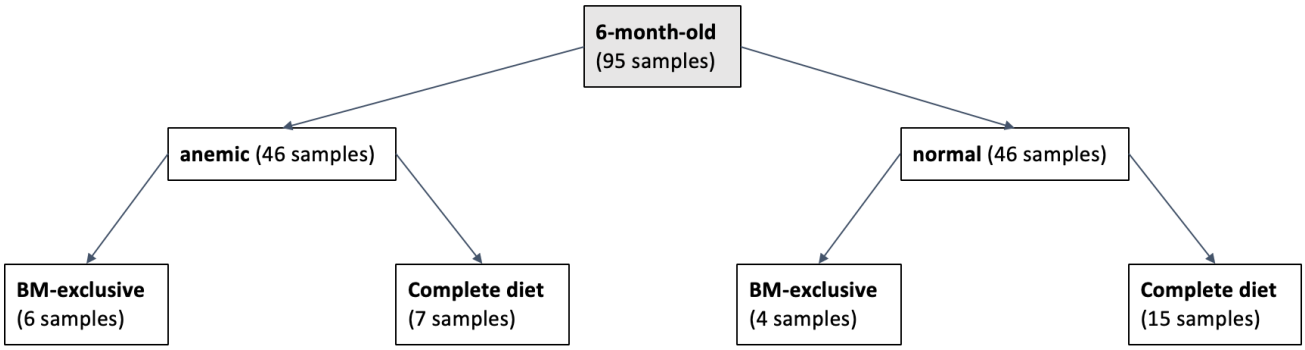

FIG. 1 Subsetting scheme used in the present study. The samples from the study conducted by McClorry *et al.* were subsetted as shown for subsequent analyses.

Alpha diversity was assessed for the four subgroups using Shannon's diversity index and the Chao1 diversity metric. A Wilcoxon rank sum test was carried out to determine statistical significance between anemic and normal individuals within each diet group, as well as across diets within anemia statuses. Taxa bar plots were generated to analyze differences in the abundances of phyla within each subset and to compare across subsets. Beta diversity was assessed through Jaccard distance, Bray-Curtis distance and the unweighted unifrac distance matrices. To assess if the taxonomic composition significantly varies across anemia status and/or diet composition, PCoA plots were generated to cluster the data points. Statistical significance was assessed using the PERMANOVA test from the ape package. A summary of the statistical tests is shown below in Table 1.

TABLE. 1 Summary of statistical tests conducted in the present study. P-values of Wilcoxon and PERMANOVA statistical tests tabulated. Groups compared shown under column 'Group A' and 'Group B'. Asterisks (*) indicate statistical significance ($p < 0.05$).

Groups compared		Wilcoxon (p-value)		PERMANOVA (Pr>F)		
Group A	**Group B**	**Shannon**	**Chao1**	**Unifrac unweighted**	**Bray-Curtis**	**Jaccard**
Anemic	Normal	0.949	0.200	0.270	0.871	0.925
BMonly	BM.Soup.Broth	0.008*	0.023*	0.072	0.023*	0.048*
Anemic BMonly	Anemic BM.Soup.Broth	0.002*	0.064	0.171	0.007*	0.005*
Normal BMonly	Normal BM.Soup.Broth	0.536	0.772	0.286	0.864	0.76
BMonly Anemic	BM only Normal	0.516	0.254	0.721	0.622	0.455
BM.soup.broth Anemic	BM.Soup.Broth Normal	0.150	0.878	0.186	0.154	0.186

Differential taxon abundance (Core Microbiome Analysis and Differential abundance analyses). Following the import of the QIIME 2 outputs (i.e. the taxonomy table, DNA fasta sequences file, phylogenetic tree and the metadata file (11, 12, 22–24), a phyloseq object was created using R (V 2022.12.0+353) with CRAN packages tidyverse, vegan, and Bioconductor package phyloseq. QIIME2 artifacts for ASVs (filtered to remove mitochondrial and chloroplast sequences) with assigned taxonomy, metadata, and phylogenetic tree were combined. The remaining samples were stratified by diet type (BM exclusive diet and complete diet). Relative abundance was calculated to normalize taxon abundance across samples.

The core microbiome was identified by first setting various sample prevalence thresholds 0.5 and an abundance frequency of > 0.1% at the genus level. We utilized the microbiome package for analysis of taxonomic profiling data, ggplot2 for data visualization, and ggVennDiagram for generation of Venn diagrams in our core microbiome analysis (25).
To identify differentially abundant genera across the various subgroups (Table 1), Differential gene expression analyses were carried out in R using the DESeq2 (26) package and a negative binomial distribution. The criteria we used for significantly differentially abundant species was based on previously established standards, considering only those species with a $\log_2$ fold change > 1.5 and a statistical significance at $P \leq 0.05$. The various comparisons done in the core microbiome analyses and DESeq2 analyses shown in Table 2.

TABLE. 2 Various groups compared for core microbiome and DESeq2 analyses. All groups under Group A were compared to groups listed in Group B. The metadat from McClorry *et al* was filtered to only include 6 month old infants (Broader filtering criteria). The 6-month-old infants were then grouped according to anemic status (Filtering criteria), and by diet (Group A and Group B).

Broader filtering criteria	Filtering criteria	Group A	Group B
6-month-old	Anemic	BM only	BM.Soup.Broth
	Normal	BM only	BM.Soup.Broth
	BM only	Anemic	Normal
	BM.Soup.Broth	Anemic	Normal

Differential gene expression analysis using PICRUSt2. To analyze differential enzyme expression across the various subgroups, PICRUSt2 analyses were conducted across diets within anemic infants on QIIME2 using the PICRUSt2 analysis pipeline (11). Changes in relative expression of PICRUSt2-predicted Enzymes (EC) were analyzed by converting the PICRUSt2 output into a DESeq2 object R v. 4.2.2 using the DESeq2 package. Using the results function under the DESeq2 package, the changes in enzyme expression between the BM exclusive diet and the complete diet were assessed using the $\log_2$ fold change and the Wald test adjusted p value with the Benjamini–Hochberg false discovery rate (FDR) correction (p_{adj}) (28). Enzymes with a $\log_2$ fold change of < 1.5 and a $p_{adj} > 0.05$ were considered not significantly differentially expressed and were filtered out.

RESULTS

Diet significantly affects microbial community diversity, while anemia status does not. To investigate the effects of anemic status and diet on gut microbial composition, we conducted alpha and beta diversity analyses across anemic status and diets independently. In 6-month-old anemic (n=15) and normal (n=21) infants, our Shannon and Chao1 alpha diversity analysis revealed no statistical differences between the two treatment groups, as substantiated by a Wilcoxon rank sum test (p=0.949) (Figure 2A). Subsequently, we conducted identical analyses across BM exclusive and complete diets, where we found significant differences in alpha diversity using both Shannon (p=0.008) and Chao1 (p=0.020) diversity metrics (Figure 2B). We further supported these findings by generating taxonomy barplots, which qualitatively showed no significant differential abundances between anemic and normal infants (Figure 2C), but noticeable differences across the diet groups (Figure 2D). Finally, we performed beta diversity testing across the two treatments. Across anemia status, we saw no significant difference when using all of the distance matrices including the Bray-Curtis diversity metric (p=0.871) (Figure 2E) the Jaccard, and the unweighted unifrac metrics (Figure S3A & S3B). However, there was a significant difference across diets, as per the Bray-Curtis diversity metric (p=0.048) (Figure 2F). Overall, these results suggest that anemic status alone has no significant impact on gut microbial composition, whereas diet has a significant effect.

Breast milk exclusive diet decreases alpha diversity in anemic infants only. Given that diet significantly affects the gut microbiome composition, we proceeded to investigate whether this difference was more profound in either anemic or normal infants. The four groups analyzed were as follows: anemic infants fed BM exclusive diet (n=6), anemic infants fed a complete diet (n=8), normal infants fed BM exclusive diet (n=4), and normal infants fed a complete diet (n=15). Significantly lower alpha diversity was observed in anemic infants fed BM only compared to a complete diet (p=0.002), while no difference was seen across diets within normal infants (p=0.536) (Figure 3A). To qualitatively support these findings, we generated a taxonomy barplot, which illustrates differential abundances in phyla including *Actinobacteria, Firmicutes*, and *Bacteroides* across the diets (Figure 3B). Finally, we conducted beta diversity analysis, the results of which showed significantly less diversity in anemic infants fed BM exclusively versus those fed a complete diet when using the Bray-Curtis diversity metric (p = 0.007) (Figure 3C) and Jaccard metrics (p=0.005) (Figure S4A). The unweighted unifrac metric did not show a significant difference (Figure S4B). Overall, these results strongly support that diet influences gut microbial diversity in anemic infants, but not normal infants.

Shared genera across anemic and diet groups reveal core microbial community involved in maintaining gut health, as well as genera unique to anemic infants. We conducted a core microbiome analysis to identify the microbial genera that were consistently present across all samples, as well as those that were unique to specific dietary and anemic status groups (Figure 4). The Venn diagram reveals the five core microbial genera that were unaffected by either anemic status or diet: *Streptococcus, Enterococcus, Escherichia-Shigella, Bifidobacterium,* and *Eggerthella*. Additionally, we observed that anemic infants fed either a complete diet or BM exclusive diet each had unique microbial genera. Specifically, *Bacteroides* was only present in anemic infants fed a complete diet, while *Actinomyces* was only found in anemic infants fed a BM exclusive diet.

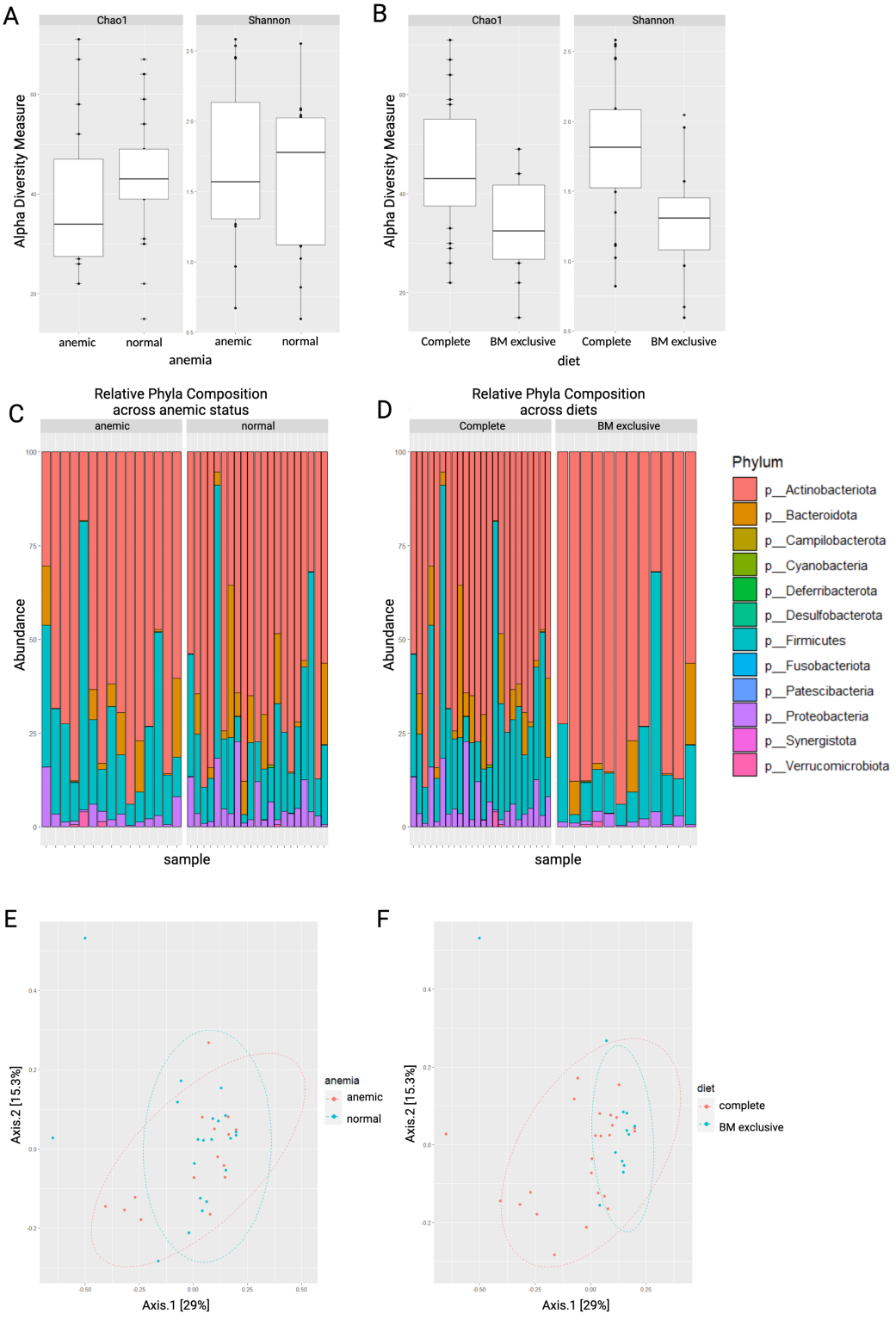

FIG. 2 Diet results in a significant difference in alpha and beta diversity, while anemia status does not. (A) Microbial alpha diversity across anemic and normal infants (Shannon; p=0.949 & Chao1; p=0.200), and (B) across infants fed BM exclusively or a complete diet (Shannon; *p=0.008431 & Chao1; *p=0.02332). Statistical testing for alpha diversity was done through a Wilcoxon rank sum test with continuity correction. Taxonomy bar plot comparing relative phylum abundances between (C) 6-month-old anemic and normal individuals, and (D) infants fed a BM exclusive or complete diet. (E) Bray-Curtis beta diversity metrics between anemic (red ellipses) vs. normal (blue ellipses) infants (p=0.871), and (F) between infants fed a BM exclusive (blue ellipses) vs. complete (red ellipses) diet (*p=0.048). Statistical testing for beta diversity was conducted using a PERMANOVA test.

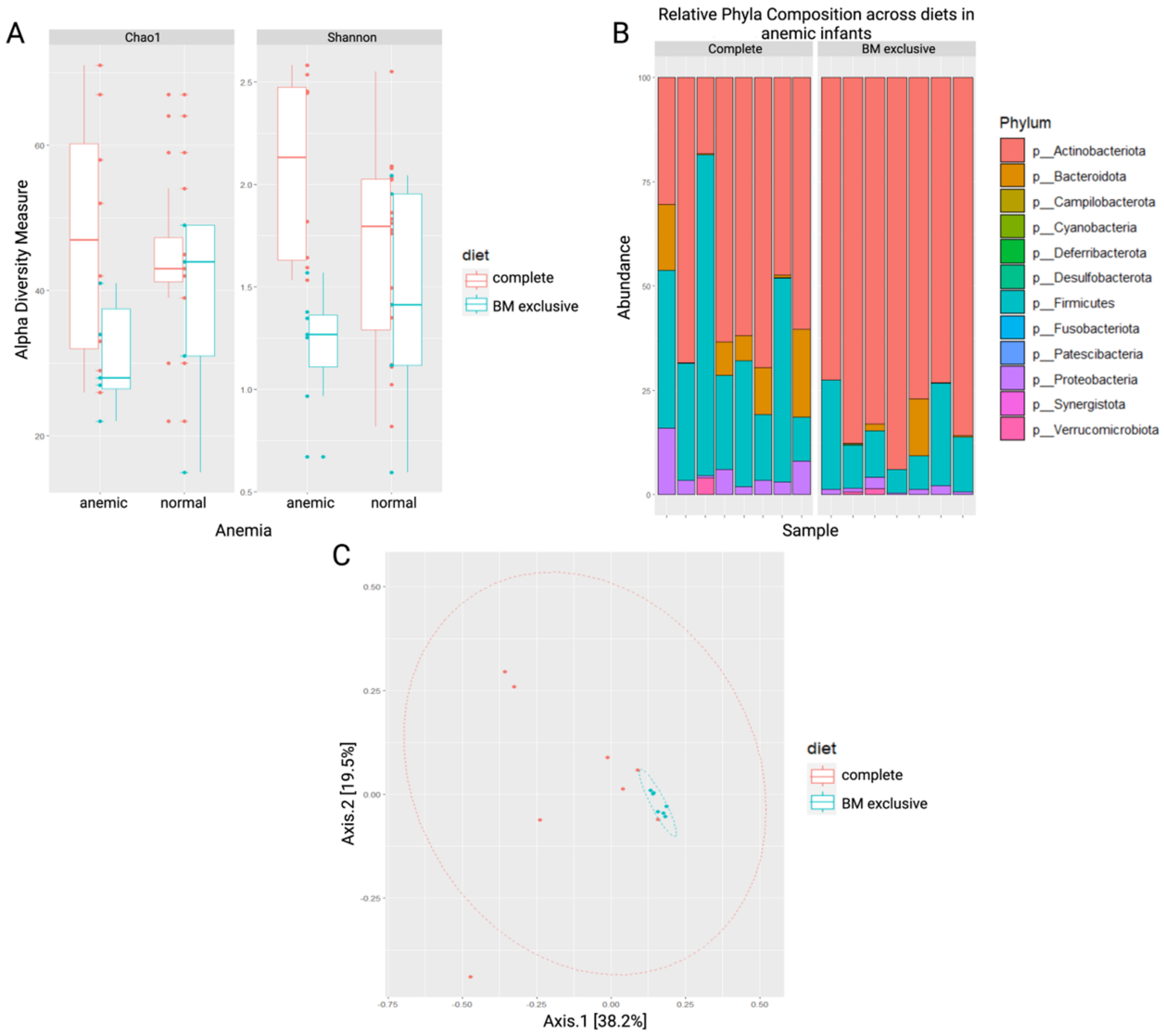

FIG. 3 A breast milk exclusive diet decreases diversity in anemic infants only. (A) Microbial alpha diversity across anemic and normal infants, with coloured bars indicating diet status for each group. Within anemic infants across diet, p = *0.002164 (Shannon) & p = 0.0636 (Chao1); within normal infants across diet, p = 0.5357 (Shannon) & p = 0.7718 (Chao1), as per a Wilcoxon rank sum test with continuity correction. (B) Taxonomy bar plots comparing relative phyla abundances in 6-month-old anemic infants fed a complete diet (left) or BM exclusive diet (right). (C) Bray-Curtis analysis for differences in beta diversity between anemic infants fed a complete diet or BM exclusive diet (p = 0.007, as per PERMANOVA test). Blue ellipses show clustering of infants fed BM exclusively and red ellipses show clustering of infants fed a complete diet.

Commensal gut bacteria significantly decreased in anemic patients fed breast milk exclusive diets

Given that the microbial community composition varies significantly across diets with anemic patients, we sought out to determine which bacterial genera are significantly differentially abundant in infants fed a BM exclusive diet compared to those fed a complete diet. To do this, we conducted a DESeq2 analysis. We observed six genera that were significantly decreased ($\log_2$ fold change >1.5 and $p < 0.05$) in infants fed breast milk only, including *Blautia, Bifidobacterium, Tyzezerella, Bacteroides, Clostridiales, and Feacalimonas*. Among the six genera, *Blautia* exhibited the greatest $\log_2$ fold change (Table 3). However, there were no bacterial genera that were significantly increased.

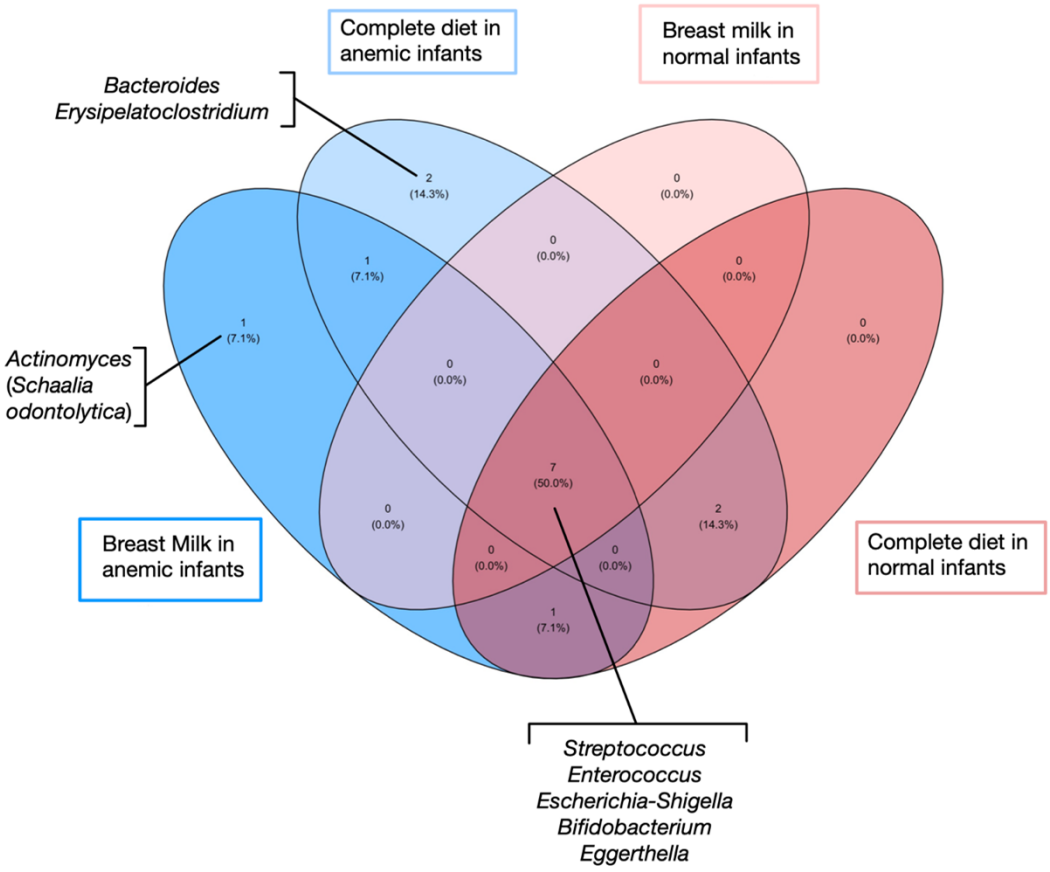

FIG. 4 Core microbial genera present in all subgroups, and unique bacterial genera found in anemic infants fed either a BM exclusive diet or a complete diet. Four-way Venn diagram displaying the core and unique genera present in each subgroup, based on a minimum abundance threshold of 0.1% and prevalence of 50%. Numbers represent the number of samples with genera above these thresholds. Blue ellipses: Anemic infants, Red ellipses: Normal infants.

Several metabolic enzymes significantly differentially expressed in anemic infants fed breast milk only.

To determine differentially expressed enzymes across diets within anemic individuals, we conducted a PICRUSt2 analysis. From the significantly differentially expressed enzymes within the BM exclusive diet (Figure 5), we observed a significant upregulation of enzymes involved in nucleotide catabolism, amino acid synthesis, and amino acid salvage (Figure 5; Figure S3). Similarly, we observe an increase in siderophore-associated enzymes involved in iron scavenging in this group. In terms of the down regulated enzymes, we see a decrease in those involved in tryptophan catabolism, serine protease, and lactocepin. These processes have been linked to an increase incidence of inflammatory bowel disease. In addition, we observed a downregulation of cell wall peptidoglycan synthesis enzymes, suggesting decreased growth of the bacterial microbiota in response to the decreased nutrient levels in the BM exclusive diet as compared to a complete one (Figure S3).

DISCUSSION

This study was conducted to investigate how differences in diet can impact the gut microbial community in anemic and normal 6-month-old infants. Our study found that diet significantly affects community composition in anemic infants, but not in normal infants. Specifically, a BM exclusive diet results in a significantly decreased diversity and relative abundance of commensal bacteria compared to those fed a complete diet. Interestingly, we also found a significant upregulation of siderophore synthesis and nitrogen metabolism

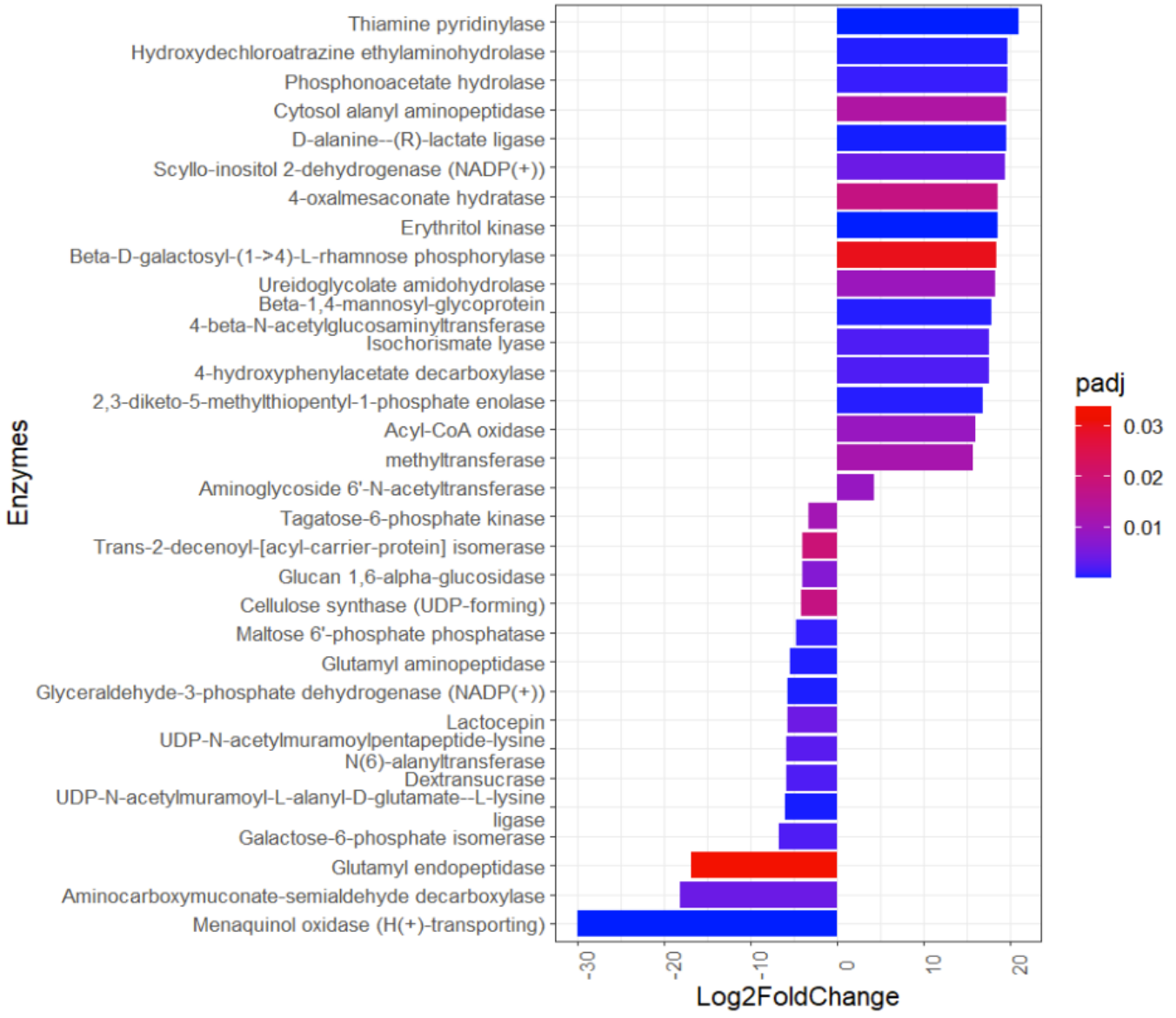

FIG. 5 Metabolic enzymes significantly up/downregulated in anemic infants fed breast milk exclusive diets. The samples were filtered to include only 6-month-old anemic individuals fed BM exclusive or complete diets. The associated fasta files and taxonomy files were used to conduct a PICRUSt2 analysis. Only significantly differentially expressed enzymes (padj <0.05 and $\log_2$ fold change >1.5) shown along Y axis. X axis is $\log_2$ fold change. Legend shows the coloring scheme based on padj values, which are p values adjusted for multiple testing with the Benjamini-Hochberg method.

enzymes in anemic infants fed BM exclusively. Overall, our study explored the tri-directional relationship between diet, anemia status, and gut microbial diversity in 6-month-old infants.

We first conducted alpha and beta diversity metrics analyses between anemic and normal infants to determine if anemic status affects the microbial community composition. We found no significant changes in alpha diversity (Figure 2A) or beta diversity (Figure 2E; Figure S2A, S2B) between anemic and normal infants, which contradicted our hypothesis. Our taxonomy barplot further supports this result, as the abundance of each phylum was similar across anemic and normal infants (Figure 2C). It is possible that no difference was seen between these two sample groups due to a variety of confounding variables including sex and diet, both of which are known to influence the microbiome composition (4). Subsequently, we analyzed differences across the two diet types (BM exclusive vs. complete diet) through alpha and beta diversity metrics analyses identical to those conducted across anemia status. We saw that infants fed a diet composed exclusively of breast milk had decreased alpha

diversity compared to those fed a complete diet, using the Chao1 and Shannon's diversity metrics (Figure 2B). These results suggest decreased evenness, abundance (microbes per species), and number of species present in the gut microbiome of infants fed exclusively breast milk. This downward shift in microbial diversity can be linked to the relatively low iron content in breast milk (9). This is supported by the corresponding taxonomy bar plot that illustrates differential abundances across diets. Specifically, we observed decreased relative abundance of *Bacteroides* and *Proteobacteria* in infants fed BM exclusively. (Figure 2D). Prior studies have corroborated the decrease in *Bacteroides* in infants fed BM exclusively (6,8). Ho *et al.* (2018) reported that an increase in Bacteroides in infants fed a complete diet is correlated with and increased body-mass index and risk of diabetes, suggesting that a BM exclusive diet may be beneficial for 6-month-old infants (7,8). Interestingly, previous studies have reported that a decrease in the abundance of the *Proteobacteria* phylum is observed in individuals with ulcerative colitis as compared to a healthy control (9). This is consistent with the known role of *Proteobacteria* in regulating gut homeostasis, where it helps maintain the anoxic gut environment required for heaty gut function consequently prevents onset of IBD (9,10). Since we observed a decreased proteobacteria in anemic infants fed BM diets exclusively, it implies that these infants may be at higher risk of developing gut microbial imbalance and IBD. Iron-deficient diet has been linked to a decrease in commensal bacteria populations in the gut (9), and could thus promote dysbiosis in the gut microbial community.

Given that diet significantly impacts microbial community composition, we sought to place this in the context of anemia; thus, we grouped the normal and anemic infants into those fed either a BM exclusive diet or complete diet. We discovered that the decrease in gut microbial diversity due to a BM exclusive diet was significant only in anemic infants (Figure 3A, 3B, & 3C). This is consistent with previous findings in mouse models that have shown that an iron-deficient diet is linked to long-term reductions in gut microbial diversity (5). This decreased diversity is especially important in the context of anemia, as this condition can lead to immune system impairments including lower phagocytic activity and oxidative burst from neutrophils (29), which in turn can result in dysregulation of the gut microbial composition (30). Taken together, we can infer the possibility of the gut microbiota of anemic infants being more volatile to diet alterations than the microbiota of normal infants.

In addition to alpha and beta diversity metrics, we conducted a core microbiome analysis to identify shared genera across all diets and anemic status, as well as those which were unique to a particular subgroup. Our analysis revealed five core genera between all infants regardless of anemic status or diet: *Streptococcus*, *Enterococcus*, *Escherichia-Shigella*, *Bifidobacterium*, and *Eggerthella*. The high prevalence of *Streptococcus* and *Bifidobacterium* is consistent with previous studies that have reported their abundance in human milk microbiota (31). As all infants in our study were fed breast milk, the presence of these genera in all the subgroups is consistent with literature. We propose that the high prevalence of *Enterococcus*, *Streptococcus*, and *Bifidobacterium* may be due to their ability to sequester iron, which is important for survival in the low-iron environment of breast milk (32). *Eggerthella,* which was also identified as a core member, is a beneficial genus that produces short chain fatty acids and plays a crucial role in bile acid conversion (33, 34). The high specificity and adaptability of *Eggerthella* to the human intestinal tract make its presence in all subgroups expected (33, 34). However, the presence of *Escherichia-Shigella* in all subgroups regardless of anemic status or diet is particularly striking, as this genus is typically associated with gut dysbiosis (34). Its presence may reflect the early instability of infant gut microbiota.

We also found distinctive genera in anemic infants who were fed either the breast milk exclusive diet or the complete diet, which were not observed in normal infants. Specifically, *Actinomyces* were more abundant in anemic infants fed BM exclusively, while *Bacteroides* were enriched in anemic infants fed a complete diet. This difference could be attributed to the fact that breast milk contains simple carbohydrates that *Actinomyces* can utilize as an energy source, while complex carbohydrates in a complete diet are more favorable for *Bacteroides* metabolism (35). Overall, our study provides insights into the core and unique genera of the infant gut microbiome across various diets and anemic statuses, shedding light on the potential impact of both factors on the composition of the gut microbiota. Our findings not only highlight the importance of considering diet and anemic status when investigating the

early-life microbiota but also provide a basis for developing interventions that optimize the infant gut microbiota to promote healthy growth and development.

To determine the differentially abundant genera between anemic infants with a BM exclusive diet and a complete diet, we conducted a differential abundance which revealed six genera that were significantly downregulated in these infants that were fed breast milk only. Of these, *Blautia*, *Bifidobacterium, Bacteroides*, *Clostridiales*, and *Feacalimonas* are known to be commensal microorganisms, and thus their downregulation implies disruption of regular gut homeostasis (36). In particular, *Blautia* and *Bacteroides* have been characterized for their anti-inflammatory effects (37), and their decrease could increase the chances of gut inflammation. Previous studies have shown that a decrease in *Blautia* has been linked to age-related immunosenescence, which is associated with chronic low-level inflammation in the gut (38). This suggests anemic infants fed a BM exclusive diet could be at risk for colitis or inflammatory bowel disease in the long term. Further, a decrease in *Bifidobacteria,* which play a vital role in competitive exclusion of pathogens in the gut, leaves individuals susceptible to opportunistic pathogens (39). Anemic infants fed a BM exclusive diet would benefit from probiotic supplement to counter this loss of protection. The *Clostridium* genus has been coined as an indispensable regulator of gut homeostasis, predominantly due to their production of short-chain fatty acids which provide energy to colonic epithelial cells, and for their role in reducing the solubility of bile salts (40). However, without attenuation by other commensal bacteria that is seen in the healthy gut environment, opportunistic pathogens such as *Clostridium difficile* can damage the large intestine through enterotoxin production (40). Due to the dual role of this genus, it would be beneficial to go a step further to investigate the exact species within this genus that are downregulated. The last genus that was downregulated was the pathogenic *Tyzzerella*, which is known to promote colorectal carcinogenesis (41) and cardiovascular disease (42). Specifically in children, this genus has been associated with adiposity, an increase in fatty tissue in the body (43). This is an interesting finding, as it suggests that a BM exclusive diet confers protection from a variety of conditions. However, there is limited research surrounding the role of this genus in infancy, making it difficult to determine the reason for its downregulation in the present study. Overall, a breast milk exclusive diet results in the downregulation of key commensal bacteria that work to prevent inflammation and promote homeostasis.

Finally, we conducted a PICRUSt2 analysis for functional characterization of the differentially expressed enzymes across diets within anemic infants. Within the significantly upregulated enzymes in infants fed BM only diets were those involved in salicylic acid synthesis, methionine salvage and isoprenoid synthesis. Bacterial salicylate biosynthesis has been linked to the biosynthesis of small ferric-ion chelating molecules known as salicyl-derived siderophores under conditions with limited iron (44). Further, the methionine salvage pathway is implicated with hemoglobin synthesis, which also aligns with the low iron conditions within anemic individuals. Finally, upregulation of isoprenoid synthesis is notable as it is a pathway that is exploited by pathogens (45). This suggests that anemic infants may be more susceptible to colonization by pathogenic species and its associated disease phenotype. In addition to upregulation of siderophore synthesis genes, we also observed an upregulation of nucleotide catabolism and amino acid savage pathways (Table S1). The upregulation of nitrogen metabolism genes suggest that a BM exclusive diet does not contain sufficient protein for the growth of 6-month-old anemic infants.

Similarly, significantly downregulated genes of interest include enzymes involved in Vitamin K biosynthesis, tryptophan catabolism, and galactose metabolism. Vitamin K is an important cofactor required for the optimal function of Vitamin-K dependent proteins such as blood coagulation proteins and Gla-rich proteins that are involved in modulating bone mineralization (46). Decreased Vitamin K biosynthesis in anemic infants fed BM exclusively could be at a higher risk of developing blood clotting or bone metabolism impairments, manifesting as clinical conditions such as excessive bleeding or osteoporosis, respectively (46). Interestingly, our results indicate a downregulation of tryptophan catabolism or the kynurenine pathway (KP). Previous studies have suggested that these metabolites exert either pro- or anti-inflammatory effects based on the cell types, suggesting that further investigation is required regarding their mechanistic effects within anemic patients (47). In addition, we see a downregulation of galactose metabolism enzymes, which suggests decreased overall

energy metabolism. This is consistent with the results of our diversity metrics and DESeq2 analyses, where the significantly decreased diversity and abundance of commensal bacteria could be the result of insufficient sugar/energy sources needed to facilitate the growth of these key, beneficial microbes compared to a complete diet. In addition, the downregulation of this enzyme in anemic infants is supported by the findings of previous studies showing that a deficiency in galactose metabolism can promote hemolytic anemia in infants (48).

Significance. This study provides insight on the development of an ideal infant diet that promotes healthy gut microbiota development and minimizes the risk of iron-deficiency anemia. Additionally, the study highlights how despite the many nutritional benefits of breast milk in microbiota formation and immune system development, a BM exclusive diet may not be adequate for promoting the optimal growth and health of developing 6-month-old infants.

Limitations Some of the limitations of the present study include the sample size, confounding variables, and uncertainties regarding diet compositions. Our study contained a relatively small sample size for the conditions analyzed upon filtering for diet and anemic status. Therefore, generalizability of the patterns from the results should be validated with not only a larger sample size, but also with a greater variety of infant diets. Further, there were various confounding variables that were not filtered for, such as ferritin levels and sex of the infants. For example, the effect of diet may be mediated through fluctuations in ferritin levels, which in turn may be observed as differences in alpha or beta diversity within anemic infants. Similarly, previous studies have highlighted that there are differences in the gut microbiota due to sex, with greater a-diversity (Chao, Shannon) within females (49). Therefore, it is essential to analyze the effects of diet on anemic infants for each sex individually. Lastly, McClorry *et al.* provided minimal information on the constituents of diets, and therefore we were unable to deduce which specific components of the complete diet were contributing to the differences in diversity within anemic infants.

Conclusions Our study highlights the impact of anemia and diet on the composition of the infant gut microbiota, revealing the volatility of anemic infants' microbiota and the dysbiosis associated with a breast-milk exclusive diet. Our study found that a breast-milk exclusive diet promotes gut microbiota dysbiosis, characterized by a significant decrease in key commensal bacteria. The dysbiosis is accompanied by an upregulation of genes responsible for hemoglobin and siderophore synthesis, as well as a downregulation of enzymes involved in energy metabolism and protection against onset of inflammation.

Future Directions Due to the limited information regarding the constituents of the complete diet from McClorry *et al*.'s dataset, future groups should explore the nutrients present within the diets they are investigating prior to performing subsequent analyses. For example, glucosinolates, which are biologically active compounds found within vegetables such as cabbage, broccoli, and cauliflower, are actively metabolized by human gut microbiota (50, 51). Confirming the presence of these compounds within a complete diet would suggest differential selection of microbial species that metabolize these compounds, accounting for differences in alpha and beta diversity within anemic infants fed each diet. Future studies could also focus on investigating how factors including sex contribute to changes in microbial community composition and function between anemic and normal infants. While our study found no significant differences in diversity metrics across anemic status, it is possible that this is due to sex being a confounding variable. Future studies could be undertaken to analyze if the differences seen between diets is reflected to a greater extent in males or females. Additionally, given that our study focused primarily on 6-month-old infants, future studies could explore how anemic status and diet affect microbiota composition in older age groups. Lastly, it would be interesting to develop a comprehensive understanding of how the effects of anemic status and diet on the gut microbiome will influence immune system development in the long term.

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CONTRIBUTIONS

AS performed preliminary QIIME2 data analysis, PICRUSt2 analysis for enzyme expression pathways, DESeq2 analysis for differential abundance of bacterial genera, and contributed to writing of the abstract, introduction, methods, results, and conclusion. **HK** performed core microbiome analysis in R, and contributed to writing of the methods, results, and discussion of the manuscript. **JH** performed alpha and beta diversity analyses in R, and contributed to writing of the methods, results, and discussion. **ES** performed alpha and beta diversity analyses and contributed to writing of results, discussion, and significance of the manuscript. All authors contributed equally to writing limitations, future directions, and significance of the project, along with revising and editing of the manuscript.

REFERENCES

1. **Kett JC.** 2012. Anemia in infancy. Pediatr Rev 33:186–187.
2. **McClorry S, Zavaleta N, Llanos A, Casapía M, Lönnerdal B, Slupsky CM.** 2018. Anemia in infancy is associated with alterations in systemic metabolism and microbial structure and function in a sex-specific manner: an observational study. *The American journal of clinical nutrition*, **108**, 1238–1248.
3. **Lambrecht NJ, Bridges D, Wilson ML, Adu B, Eisenberg JNS, Folson G, Baylin A, Jones AD**. 2022. Associations of bacterial enteropathogens with systemic inflammation, iron deficiency, and anemia in preschool-age children in southern Ghana. *PLOS ONE* **17**:e0271099.
4. **Lyons KE, Ryan CA, Dempsey EM, Ross RP, Stanton C**. 2020. Breast Milk, a Source of Beneficial Microbes and Associated Benefits for Infant Health. *Nutrients* **12**.
5. **Leeming ER, Johnson AJ, Spector TD, Le Roy CI**. 2019. Effect of Diet on the Gut Microbiota: Rethinking Intervention Duration. *Nutrients* **11**:2862.
6. **Ballard O, Morrow AL**. 2013. Human Milk Composition: Nutrients and Bioactive Factors. *Pediatr Clin North Am* **60**:49–74.
7. **Muleviciene A, D'Amico F, Turroni S, Candela M, Jankauskiene A**. 2018. Iron deficiency anemia-related gut microbiota dysbiosis in infants and young children: A pilot study. *Acta Microbiol Immunol Hung* **65**:551–564.
8. **Ippolito JR, Piccolo BD, Robeson MS, Barney DE, Ali J, Singh P, Hennigar SR.** 2022. Iron deficient diets modify the gut microbiome and reduce the severity of enteric infection in a mouse model of S. Typhimurium-induced enterocolitis. *J Nutr Biochem* **107**:109065.
9. **Knight LC, Wang M, Donovan SM, Dilger RN**. 2019. Early-Life Iron Deficiency and Subsequent Repletion Alters Development of the Colonic Microbiota in the Pig. *Front Nutr* **6**.
10. **Dalili H, Baghersalimi A, Dalili S, Pakdaman F, Hassanzadeh Rad A, Abbasi Kakroodi M, Rezvany SM, Koohmanaei S**. 2015. Is there any relation between Duration of breastfeeding and anemia? I*ran J Pediatr Hematol Oncol* **5**:218–226.
11. **Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP.** 2016. DADA2: High-resolution sample inference from Illumina amplicon data. 7. *Nat Methods* **13**:581–583.
12. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2 | Nature Biotechnology. https://www.nature.com/articles/s41587-019-0209-9. Retrieved 16 April 2023.
13. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin | Microbiome | Full Text. https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-018-0470-z. Retrieved 16 April 2023.
14. **Robeson MS, O'Rourke DR, Kaehler BD, Ziemski M, Dillon MR, Foster JT, Bokulich NA**. 2020. RESCRIPt: Reproducible sequence taxonomy reference database management for the masses. bioRxiv https://doi.org/10.1101/2020.10.05.326504.
15. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB - PubMed. https://pubmed.ncbi.nlm.nih.gov/17947321/. Retrieved 16 April 2023.
16. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools - PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3531112/. Retrieved 16 April 2023.
17. **Paradis E, Schliep K.** 2019. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* **35**:526–528.
18. **Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen TL, Miller E, Bache SM, Müller K, Ooms J,**

Robinson D, Seidel DP, Spinu V, Takahashi K, Vaughan D, Wilke C, Woo K, Yutani H. 2019. Welcome to the Tidyverse. *J Open Source Softw* **4**:1686.

19. **Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Solymos P, Stevens MHH, Szoecs E, Wagner H, Barbour M, Bedward M, Bolker B, Borcard D, Carvalho G, Chirico M, Caceres MD, Durand S, Evangelista HBA, FitzJohn R, Friendly M, Furneaux B, Hannigan G, Hill MO, Lahti L, McGlinn D, Ouellette M-H, Cunha ER, Smith T, Stier A, Braak CJFT, Weedon** J. 2022. vegan: Community Ecology Package (2.6-4).
20. **Wickham H.** 2016. Programming with ggplot2, p. 241–253. *In* Wickham, H (ed.), ggplot2: Elegant Graphics for Data Analysis. Springer International Publishing, Cham.
21. **McMurdie PJ, Holmes S.** 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS One* **8**:e61217.
22. **McDonald D, Clemente JC, Kuczynski J, Rideout JR, Stombaugh J, Wendel D, Wilke A, Huse S, Hufnagle J, Meyer F, Knight R, Caporaso JG**. 2012. The Biological Observation Matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome. GigaScience 1:2047-217X-1–7.
23. FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments | *PLOS ONE.* https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0009490. Retrieved 16 April 2023.
24. **Katoh K, Standley DM**. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Mol Biol Evol* **30**:772–780.
25. **Gao C-H, Yu G, Dusa A.** 2022. ggVennDiagram: A "ggplot2" Implement of Venn Diagram (1.2.2).
26. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2 | Genome Biology | Full Text. https://genomebiology.biomedcentral.com/articles/10.1186/s13059-014-0550-8. Retrieved 16 April 2023.
27. **Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, Huttenhower C, Langille MGI.** 2020. PICRUSt2 for prediction of metagenome functions. *Nat Biotechnol* **38**:685–688.
28. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing - Benjamini - 1995 - Journal of the Royal Statistical Society: Series B (Methodological) - Wiley Online Library. https://rss.onlinelibrary.wiley.com/doi/10.1111/j.2517-6161.1995.tb02031.x. Retrieved 16 April 2023.
29. **Hassan TH, Badr MA, Karam NA, Zkaria M, El Saadany HF, Abdel Rahman DM, Shahbah DA, Al Morshedy SM, Fathy M, Esh AMH, Selim AM**. 2016. Impact of iron deficiency anemia on the function of the immune system in children. *Medicine (Baltimore)* **95**:e5395.
30. **Belkaid Y, Harrison OJ.** 2017. Homeostatic immunity and the microbiota. Immunity 46:562–576.
31. **Mueller NT, Bakacs E, Combellick J, Grigoryan Z, Dominguez-Bello MG.** 2015. The infant microbiome development: mom matters. *Trends Mol Med* **21**:109–117.
32. **Malesza IJ, Bartkowiak-Wieczorek J, Winkler-Galicki J, Nowicka A, Dzięciołowska D, Błaszczyk M, Gajniak P, Słowińska K, Niepolski L, Walkowiak J, Mądry E**. 2022. The Dark Side of Iron: The Relationship between Iron, Inflammation and Gut Microbiota in Selected Diseases Associated with Iron Deficiency Anaemia—A Narrative Review. 17. *Nutrients* **14**:3478.
33. **Li L, Chen L, Yang Y, Wang J, Guo L, An J, Ma X, Lu W, Xiao Y, Wang X, Dong Z**. 2022. Characteristics of Gut Microbiome and Its Metabolites, Short-Chain Fatty Acids, in Children With Idiopathic Short Stature. *Front Endocrinol* **13**:890200.
34. **Godlewska U, Bulanda E, Wypych TP.** 2022. Bile acids in immunity: Bidirectional mediators between the host and the microbiota. *Front Immunol* **13**:949033.
35. **Flint HJ, Scott KP, Duncan SH, Louis P, Forano E.** 2012. Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* **3**:289–306.
36. The essential genomic landscape of the commensal Bifidobacterium breve UCC2003 | Scientific Reports. https://www.nature.com/articles/s41598-017-05795-y. Retrieved 16 April 2023.
37. **Zhou Y, Zhi F.** 2016. Lower Level of Bacteroides in the Gut Microbiota Is Associated with Inflammatory Bowel Disease: A Meta-Analysis. *BioMed Res Int* **2016**:5828959.
38. **Liu X, Mao B, Gu J, Wu J, Cui S, Wang G, Zhao J, Zhang H, Chen W**. Blautia—a new functional genus with potential probiotic properties? *Gut Microbes* **13**:1875796.
39. **O'Callaghan A, van Sinderen D**. 2016. Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Front Microbiol* **7**:925.
40. **Guo P, Zhang K, Ma X, He P**. 2020. Clostridium species as probiotics: potentials and challenges. *J Anim Sci Biotechnol* **11**:24.
41. **Zhang Y, Lu M, Lu B, Liu C, Ma Y, Liu L, Miao X, Qin J, Chen H, Dai M.** 2021. Leveraging Fecal Microbial Markers to Improve the Diagnostic Accuracy of the Fecal Immunochemical Test for Advanced Colorectal Adenoma. *Clin Transl Gastroenterol* **12**:e00389.
42. **Ascher S, Reinhardt C.** 2018. The gut microbiota: An emerging risk factor for cardiovascular and cerebrovascular disease. *Eur J Immunol* **48**:564–575.
43. **Chen L-W, Xu J, Soh SE, Aris IM, Tint M-T, Gluckman PD, Tan KH, Shek LP-C, Chong Y-S, Yap F, Godfrey KM, Gilbert JA, Karnani N, Lee YS.** 2020. Implication of gut microbiota in the association between infant antibiotic exposure and childhood obesity and adiposity accumulation. 7. *Int J Obes* **44**:1508–1520.

44. **Meyer JM, Azelvandre P, Georges C**. 1992. Iron metabolism in Pseudomonas: salicylic acid, a siderophore of Pseudomonas fluorescens CHAO. *BioFactors Oxf Engl* **4**:23–27.
45. **Heuston S, Begley M, Gahan CGM, Hill C**. 2012. Isoprenoid biosynthesis in bacterial pathogens. *Microbiol Read Engl* **158**:1389–1401.
46. Vitamin K and Osteoporosis - PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7760385/. Retrieved 16 April 2023.
47. **Ramprasath T, Han Y-M, Zhang D, Yu C-J, Zou M-H**. 2021. Tryptophan Catabolism and Inflammation: A Novel Therapeutic Target For Aortic Diseases. Front Immunol **12**:731701.
48. Galactosemia - PerkinElmer. https://rh.perkinelmer.com/disorders/galactosemia/. Retrieved 16 April 2023.
49. Sex Differences in Gut Microbiota - PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6920072/. Retrieved 16 April 2023.
50. Glucosinolates From Cruciferous Vegetables and Their Potential Role in Chronic Disease: Investigating the Preclinical and Clinical Evidence - PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8575925/. Retrieved 16 April 2023.
51. **Sikorska-Zimny K, Beneduce L.** 2021. The Metabolism of Glucosinolates by Gut Microbiota. Nutrients **13**:2750.
52. PubChem. 2.-.-.- Transferases | 2.5.-.- Transferring alkyl or aryl groups, other than methyl groups | 2.5.1.- Transferring alkyl or aryl groups, other than methyl groups. https://pubchem.ncbi.nlm.nih.gov/protein/EC:2.5.1.2. Retrieved 16 April 2023.
53. Information on EC 3.5.4.43 - hydroxydechloroatrazine ethylaminohydrolase - BRENDA Enzyme Database. https://www.brenda-enzymes.org/enzyme.php?ecno=3.5.4.43. Retrieved 16 April 2023.
54. **Balmer ME, Sulzberger B.** 1999. Atrazine Degradation in Irradiated Iron/Oxalate Systems: Effects of pH and Oxalate. *Environ Sci Technol* **33**:2418–2424.
55. **Agarwal V, Borisova SA, Metcalf WW, van der Donk WA, Nair SK**. 2011. Structural and Mechanistic Insights into C-P Bond Hydrolysis by Phosphonoacetate Hydrolase. *Chem Biol* **18**:1230–1240.
56. **Starnes WL, Behal FJ**. 1974. Human liver aminopeptidase. Amino acid and carbohydrate content, and some physical properties of a sialic acid-containing glycoprotein. *Biochemistry* **13**:3221–3227.
57. **Morinaga T, Ashida H, Yoshida K-I.** 2010. Identification of two scyllo-inositol dehydrogenases in Bacillus subtilis. *Microbiol Read Engl* **156**:1538–1546.
58. **Kang D-M, Michon C, Morinaga T, Tanaka K, Takenaka S, Ishikawa S, Yoshida K.** 2017. Bacillus subtilis IolQ (DegA) is a transcriptional repressor of iolX encoding NAD+-dependent scyllo-inositol dehydrogenase. *BMC Microbiol* **17**:154.
59. PubChem. 4.-.-.- Lyases | 4.2.-.- Carbon-oxygen lyases | 4.2.1.- Hydro-lyases. https://pubchem.ncbi.nlm.nih.gov/protein/EC:4.2.1.83. Retrieved 16 April 2023.
60. **Miallau L, Alphey MS, Kemp LE, Leonard GA, McSweeney SM, Hecht S, Bacher A, Eisenreich W, Rohdich F, Hunter WN**. 2003. Biosynthesis of isoprenoids: Crystal structure of 4-diphosphocytidyl-2C-methyl-d-erythritol kinase. *Proc Natl Acad Sci* **100**:9173–9178.
61. **Percudani R, Carnevali D, Puggioni V**. 2013. Ureidoglycolate hydrolase, amidohydrolase, lyase: how errors in biological databases are incorporated in scientific papers and vice versa. *Database J Biol Databases Curation* **2013**:bat071.
62. **DeClue MS, Baldridge KK, Künzler DE, Kast P, Hilvert D**. 2005. Isochorismate pyruvate lyase: a pericyclic reaction mechanism? *J Am Chem Soc* **127**:15002–15003.
63. **Selvaraj B, Buckel W, Golding BT, Ullmann GM, Martins BM**. 2016. Structure and Function of 4-Hydroxyphenylacetate Decarboxylase and Its Cognate Activating Enzyme. *J Mol Microbiol Biotechnol* **26**:76–91.
64. **Selvaraj B, Pierik AJ, Bill E, Martins BM.** 2013. 4-Hydroxyphenylacetate decarboxylase activating enzyme catalyses a classical S-adenosylmethionine reductive cleavage reaction. *J Biol Inorg Chem JBIC Publ Soc Biol Inorg Chem* **18**:633–643.
65. **Sekowska A, Ashida H, Danchin A**. 2018. Revisiting the methionine salvage pathway and its paralogues. *Microb Biotechnol* **12**:77–97.
66. InterPro. https://www.ebi.ac.uk/interpro/entry/InterPro/IPR012258/. Retrieved 16 April 2023.
67. Acyl-CoA Oxidase - an overview | ScienceDirect Topics. https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/acyl-coa-oxidase. Retrieved 16 April 2023.
68. **Yi SM, Narasimhulu KV, Samoilova RI, Gennis RB, Dikanov SA**. 2010. Characterization of the Semiquinone Radical Stabilized by the Cytochrome aa3-600 Menaquinol Oxidase of Bacillus subtilis*. *J Biol Chem* **285**:18241–18251.
69. ACMSD aminocarboxymuconate semialdehyde decarboxylase [Homo sapiens (human)] - Gene - NCBI. https://www.ncbi.nlm.nih.gov/gene/130013. Retrieved 16 April 2023.
70. **Kainer D, Templeton AR, Prates ET, Jacboson D, Allan ERO, Climer S, Garvin MR**. 2022. Structural variants identified using non-Mendelian inheritance patterns advance the mechanistic understanding of autism spectrum disorder. *Hum Genet Genomics Adv* **4**:100150.
71. **Demidyuk IV, Chukhontseva KN, Kostrov SV**. 2017. Glutamyl Endopeptidases: The Puzzle of Substrate Specificity. *Acta Naturae* **9**:17–33.
72. **van Rooijen RJ, van Schalkwijk S, de Vos WM**. 1991. Molecular cloning, characterization, and nucleotide sequence of the tagatose 6-phosphate pathway gene cluster of the lactose operon of Lactococcus lactis. *J Biol Chem* **266**:7176–7181.

73. **Anderson RL, Bissett DL**. 1982. [95] d-Galactose-6-phosphate isomerase, p. 562–565. *In* Methods in Enzymology. Academic Press.
74. **Gordon E, Flouret B, Chantalat L, van Heijenoort J, Mengin-Lecreulx D, Dideberg O**. 2001. Crystal structure of UDP-N-acetylmuramoyl-L-alanyl-D-glutamate: meso-diaminopimelate ligase from Escherichia coli. *J Biol Chem* **276**:10999–11006.
75. **Hörmannsperger G, Schillde M-A von, Haller D**. 2013. Lactocepin as a protective microbial structure in the context of IBD. *Gut Microbes* **4**:152.
76. GAPDH glyceraldehyde-3-phosphate dehydrogenase [Homo sapiens (human)] - Gene - NCBI. https://www.ncbi.nlm.nih.gov/gene/2597. Retrieved 16 April 2023.
77. **McNamara JT, Morgan JLW, Zimmer J**. 2015. A Molecular Description of Cellulose Biosynthesis. *Annu Rev Biochem* **84**:895–921
78. **Ohya T, Sawai T, Uemura S, Abe K**. 1978. Some Catalytic Properties of an Exo-1, 6-α-glucosidase (Glucodextranase) from *Arthrobacter globiformis* I42. *Agric Biol Chem* **42**:571–577.