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Anemia May Mask the Effects that Stunting has on the Dysbiosis of the Infant Gut Microbiota

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SUMMARY Anemia, the most common nutritional deficiency affecting ~25% of the world's population, and stunting, defined as hindered growth which impacts an estimated 149 million children under the age of 5 worldwide, have been strongly associated with each other in literature. One connection is through the dysbiosis of the gut microbiome, caused by conditions such as environmental enteropathy, which leads to micronutrient deficiencies, inflammation, and hormonal imbalances that prevent proper growth. The relationship between the infant gut microbiome and the conditions of anemia and stunting has important clinical relevance; however, research examining this relationship is limited. As both anemia and stunting have been found to affect microbiota composition, we investigated the effects of anemia and stunting on infant gut microbiomes. We found that infants with anemia were 2.57 times more likely to be stunted than healthy controls. Our Chao1 alpha diversity analysis revealed significant diversity differences only in the stunted healthy infants, but not in non-stunted healthy, non-stunted anemic, or stunted anemic infants. Stunting was shown to have a larger proportion of unique ASVs compared to anemia based on core microbiome analysis. Likewise, our indicator species analysis identified 22 significant genera indicative of stunting in only healthy infants. Lastly, anemia appeared to reduce the upregulation of differential taxa abundance in stunted infants. Taken together, these results suggest that anemia may conceal the effects of stunting on the infant gut microbiome. Overall, our study provides insight into the complex relationship between anemia, stunting, and the infant gut microbiome.

INTRODUCTION

Anemia is a condition characterized by low blood concentrations of hemoglobin that affects approximately a quarter of the world's population (1). The most common cause of anemia is iron deficiency (ID), which is the most prominent micronutrient deficiency worldwide amongst developed and developing countries (2). It is particularly concerning in infancy due to its lasting negative impacts on growth, cognitive development, and metabolism (1, 2). In addition to iron deficiency, inflammation is another common cause of anemia (3). As a micronutrient deficiency, anemia has been linked to impaired growth resulting from malnutrition (3). Stunting, defined as a height-for-age z-score at least two standard deviations below the World Health Organization (WHO) Child Growth Standards median (4), has also been found to be associated with higher risks for morbidity and mortality, cognitive impairment, and worsened educational outcomes in 6-month-old infants (5). Poor nutrition, repeated infection, and chronic inflammation of the small intestine have been postulated to be underlying causes of stunting (6, 7). In 2018, a cross-sectional study in Pakistan found that being male gender, having low maternal education, and being unvaccinated were significant risk factors for stunting in children less than 5 years of age (8). The relationship between anemia and stunting in infancy has important clinical implications, as treatment options addressing both issues concurrently would streamline interventions (9).

Existing evidence regarding the association between anemia and stunting is limited and contradictory, with some studies finding the two conditions to be largely independent of each other with dissimilar determinants (10, 11). Other studies have found a strong association between anemia and stunting, suggesting anemia is connected to impaired growth (1, 5, 9). One association involves environmental enteropathy, or enteric dysfunction (EED), a small intestine disorder characterized by chronic villous atrophy and crypt hyperplasia (11). EED can cause micronutrient deficiencies such as anemia in children due to gut inflammation and

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permeability, and has also been associated with stunting in both children and adults (12–16). Gut inflammation in EED disrupts iron homeostasis by reducing iron absorption and diverting iron from circulation to reticuloendothelial storage sites, limiting iron availability for erythropoiesis (17), as well as reducing plasma retinol, an essential component to erythropoiesis (3). Gut inflammation can therefore lead to anemia as iron uptake by erythroblasts is inhibited (3). Specifically in children under 5 years of age, pediatric environmental enteropathy (PEE) has been proposed as an underlying cause of stunting (18, 19). It is postulated that fecal contamination induces morphological changes in the small intestine, resulting in increased epithelial damage and microbial translocation. In the case of children with PEE and stunting, the resources normally directed to growth and development are reallocated to triggering local and systemic inflammation in the intestine, disrupting hormonal regulation of growth plate activity in long bones (18). As PEE is associated with undernutrition, there may be a cyclic relationship between undernutrition and repeated enteric infection (7). Furthermore, inflammatory and anti-inflammatory molecules have been associated with certain microbes, as some structural components can trigger inflammatory pathways while some metabolic byproducts, such as specific short-chain fatty acids, can have anti-inflammatory effects (20). Due to the relationship among enteropathy, anemia, and stunting, it is important to compare gut microbiome alterations in infants with anemia and stunting to further delineate the impact of the microbiome on these disease states, and vice versa.

Additionally, a study in sub-Saharan Africa found that enteropathogenic microbes such as *Escherichia coli*, *Shigella flexneri,* and *Campylobacter concisus* were more prevalent in the fecal samples of 2 - 5 year olds with stunting compared to healthy controls (7). They identified many oropharyngeal species to be more prevalent in stunted children, including *Lactobacillus salivarius, Prevotella histicola,* and *Porphyromonas asaccharolytica*. From their data, they proposed a model wherein the gastrointestinal tract undergoes "decompartmentalization," resulting in oropharyngeal bacteria readily colonizing the stomach and duodenum (7). This challenges previous literature stating that stunting is caused solely by small intestine infections of enteric pathogens.

Despite recent advances, research examining the co-occurrence of anemia and stunting and its associations with the infant gut microbiome is relatively limited. Our study aims to contribute to this research gap and build upon previous work by determining how the presence of stunting and anemia can affect gut microbiomes of infants against healthy controls and whether those effects are synergistic. In addition, we will look into the dysbiosis of the gut microbiota, which has been linked to inflammation, anemia, and stunting with recent research looking into sex-specific effects of dysbiosis on infant development (1, 21). This study aims to gain insights into how anemia and stunting interplay to affect the infant gut microbiota. As well, we aim to investigate whether these two conditions may result in distinct infant gut microbiome profiles and assess how these profiles might influence each other through interactions within the gut microbiome. Examining these relationships may have important implications for optimizing interventions to improve infant health.

METHODS AND MATERIALS

Dataset and metadata. The dataset used in this study was generated by McClorry *et al*. (2018) and consisted of data from fecal samples from a total of 193 infants (95 at 6-months of age and 98 at 12-months of age). Though McClorry *et al*. (2018) excluded data from the 6-month-old infants in their analyses, we included data from both age stages in our study. Microbial DNA was extracted from the fecal samples then the 16S ribosomal RNA segments were sequenced following V4-region amplification (1). Further information about the subjects was collected including sex and stunting status. Details about the study are accessible at clinicaltrials.gov under the ID NCT03377777. The fecal microbiome data is available from the European Nucleotide Archive under the accession ERP104978. We generated an additional metadata column to indicate disease state which comprised both anemia status and stunting status. This was performed to assess the sample sizes for each combination of the two variables to determine sample rarefaction depth in Quantitative Insights into Microbial Ecology 2 as well as to simplify further downstream analysis (22).

Preliminary data processing. QIIME2 was used to perform the following initial data processing steps, as documented in the QIIME2 script found on our associated GitHub (https://github.com/aejessen/MICB475_Group5) (22). Raw sequencing data was imported into our QIIME2 pipeline along with the associated metadata table, then demultiplexed, and denoised. The reads were truncated to a sequence length of 252 base pairs, which kept >98% of the data and ensured the Phred score remained above 35 for all reads. A sampling depth of 16,888 was applied for rarefaction, as it kept the maximum number of samples (95.85%), and was within the plateau range for total ASVs found. QIIME 2 outputs from preliminary data processing steps were then assembled into a phyloseq object using the phyloseq package in R studio (23). In R, data was filtered to exclude non-bacterial sequences, ASVs with less than 5 counts, and samples with less than 100 reads (24).

Alpha and beta diversity analyses. Alpha and beta diversity tests were performed using the QIIME2 diversity core-metrics-phylogenetic function (22). Faith's phylogenetic diversity index, observed index, and evenness index were used for alpha diversity analyses. Jaccard, unweighted unifrac, weighted unifrac, and Bray-Curtis indices were used for beta diversity analyses.

Logistic regression. A logistic regression model was created to evaluate the association between the categorical, binary variables of stunting and anemia. The tidyverse package was used for this analysis, with anemia and stunting serving as the independent and dependent variables, respectively (25). First, the stunting variable was converted to integers, with "0" and "1" representing "normal" or "stunted" infants, respectively. Next, the anemia column was converted to a factor and assigned the reference for the model. Finally, a linear regression model was fitted to the data to obtain an odds ratio which represents the likelihood of infants being stunted if they are anemic versus non-anemic. A mosaic plot graphically representing the frequencies of anemia and stunting was made in R using the ggmosaic package (26).

Alpha and beta diversity analyses. Alpha and beta diversity analysis were performed using our phyloseq object in RStudio. Alpha and beta diversity metrics were analyzed and plots were made in R using the tidyverse, phyloseq, picante, ggsignif, and ape packages (23, 25, 27–29). Shannon's diversity index and Chao1 diversity index were used for alpha diversity calculations. Statistical analyses were performed using the Kruskal-Wallis test.

Core microbiome analysis. Core microbiome analysis was performed using our phyloseq object in R. The core microbiome was defined by Amplicon Sequence Variants (ASVs) that were present in at least 20% of samples with a detection threshold of 0.000. All four disease state groups were compared and visualized using a 4-way Venn Diagram.

Indicator species analysis. Indicator species analysis was performed in R with the tidyverse, phyloseq, and indicspecies packages (23, 25, 30). Taxonomic data for each disease state was grouped to the genus level and only genera with p-values <0.05 were selected as indicators.

Differential expression sequence analysis (DESeq). DESeq2 differential expression analysis was performed in R with the tidyverse and phyloseq packages (23, 25, 31). Significant expression changes between conditions were determined to be those with a log2 fold change above 2 or below -2 and an adjusted p-value under 0.01.

RESULTS

Infants with anemia were more likely to exhibit stunted growth. The association between anemia and stunting was assessed using a logistic regression model. Compared to individuals without anemia, the adjusted odds of being stunted were 2.57 (95% CI: 1.12, 6.44, $p = 0.032$) times higher in those with anemia (Figure 1). These results indicate a significant positive relationship between anemia and stunting, by which anemic infants are more than twice as likely to be stunted than those without anemia.

Stunting increased gut microbiome diversity more in healthy infants compared to anemic infants. To compare the microbial diversities of the stunted healthy, stunted anemic,

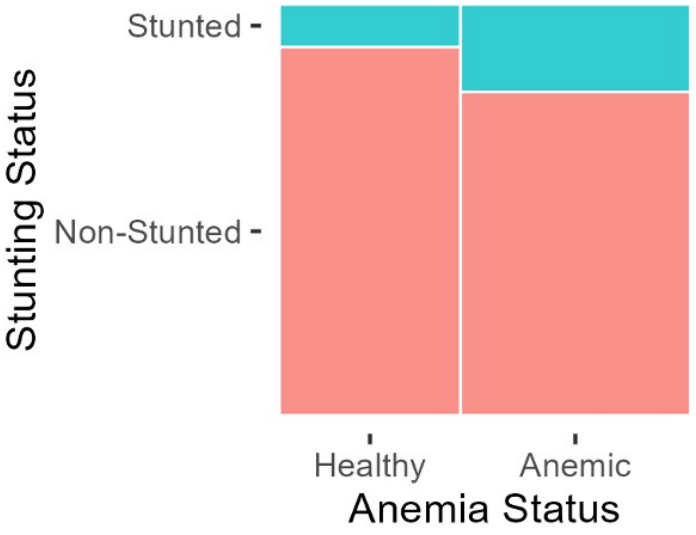

FIG. 1 Anemic infants are 2.57x more likely to be stunted than healthy infants. Mosaic plot showing the frequencies of anemia status (x-axis) and stunting status (y-axis). Non-stunted individuals are shown in red, and stunted individuals are shown in blue.

non-stunted healthy, and non-stunted anemic groups, alpha and beta diversity analyses were performed. Chao1 alpha diversity analysis showed the highest diversity in stunted healthy infants compared to the other groups (Figure 2), and the diversity of this group was found to be significantly different from the non-stunted anemic infants. Though the diversity differences between the stunted healthy group and the other two groups were non-significant, the Chao1 diversity index of the stunted healthy group was visually higher than all the other groups. Moreover, for other diversity tests, the groups showed similar levels of diversity, and no other significant differences were found (Supplemental Figures S2 & S3).

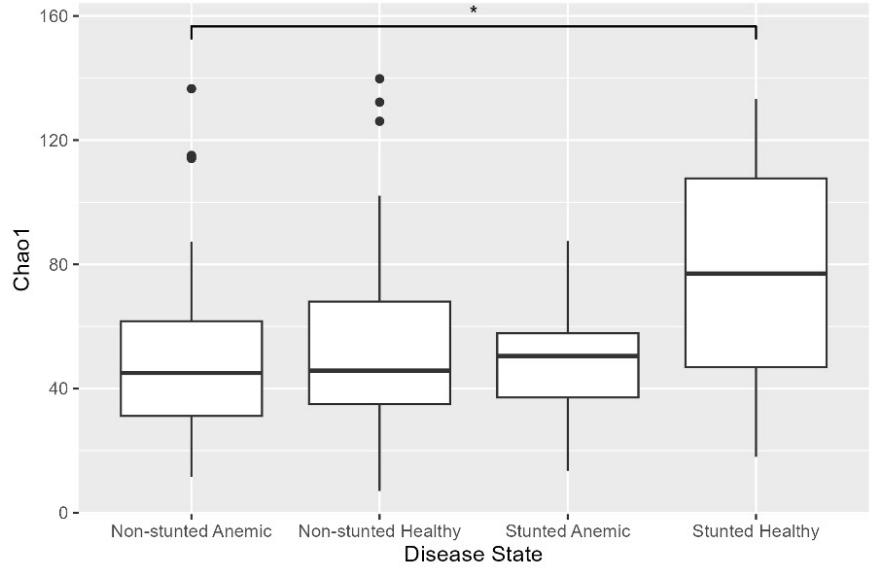

FIG. 2 Stunting increased gut microbiome diversity more in healthy infants compared to anemic infants. Chao1 diversity analysis of the gut microbiome of non-stunted anemic (n = 84), non-stunted healthy (n = 70), stunted anemic (n = 20), and stunted healthy (n= 7) infants. Differences were assessed by Kruskal-Wallis test, $*p < 0.05$.

Stunting had a larger proportion of unique ASVs than anemia. Core microbiome analysis was performed on infants with and without anemia or stunting, and a Venn diagram was created to visualize the number of unique and shared ASVs (Figure 3). Most ASVs (Amplicon Sequence Variants) were commonly shared between all individuals in the study regardless of whether they had anemia or stunting. A larger proportion (17%) of ASVs were uniquely associated with gut microbiotas of stunted infants, with or without anemia, compared to a smaller proportion (6%) of ASVs were unique to infants without anemia, regardless of whether they were stunted. All 4 study groups possessed only a minimal proportion of unique ASVs between 1-3%. These results suggest that there are no substantial differences between disease states for either anemia or stunting in ASV measures.

Significant indicator genera are only associated with stunting in healthy infants. To identify species indicative of anemia, stunting, or the combined disease state of anemia and stunting, we performed indicator species analysis grouped to the genus level (Table 1). We identified 22 significant genera indicative of stunting in healthy infants which were not found

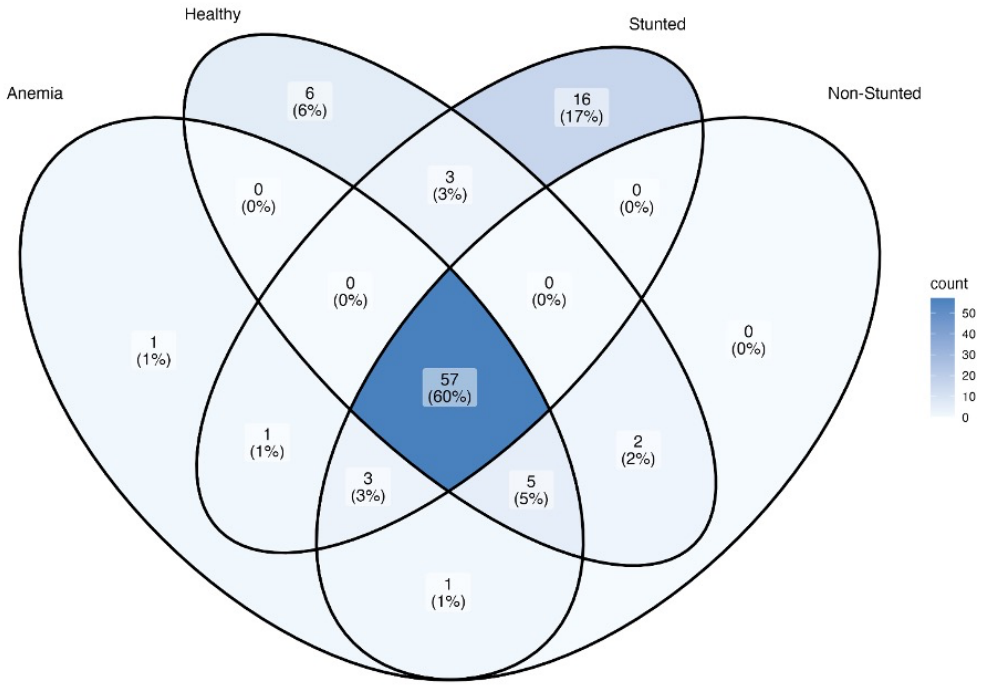

FIG. 3 Stunting had a bigger proportion of unique ASVs than anemia. Core microbiome analysis of the gut microbiome of infants with and without anemia as well as with and without stunting. Four-way Venn diagram showing the frequency of common and unique ASVs associated with each disease state. Prevalence = 0.2 and detection threshold = 0.000.

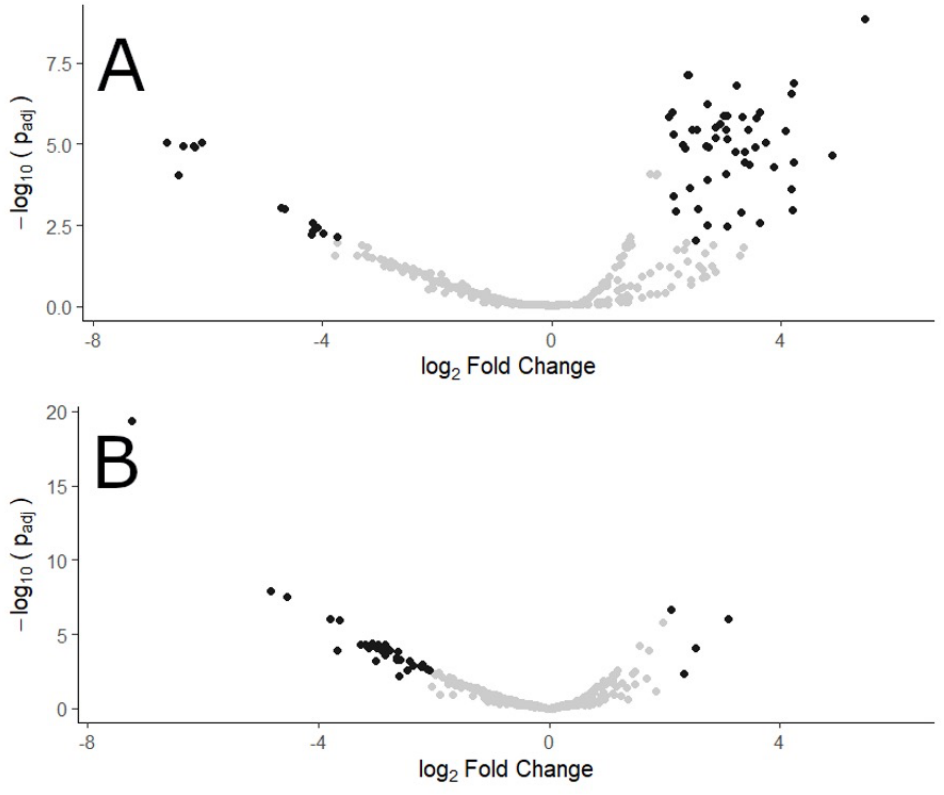

FIG. 4 Anemia masked the upregulation of differentially abundant taxa in stunted infants. Taxa found in the microbiome of infants in stunted versus non-stunted infants. Black dots represent taxa with significantly different abundance, split into healthy infants (A) and anemic infants (B). Analysis performed using DESeq2 (31). Significance was determined to be taxa up or down-regulated with a $|\log_2$ Fold Change$| > 2$ and adjusted p-value < 0.01.

in any other disease states. Additionally, we identified one significant genus, *Libanicoccus*, associated with the non-stunted healthy, non-stunted anemic, and stunted-anemic disease states. These results show that the 22 genera are indicative only of stunting, and there were no true indicator genera found for any of the other health outcomes.

TABLE. 1 There were 22 significant indicator genera for the stunted healthy infant group. The data were grouped at the genus level and the significance threshold was set at $p < 0.05$.

Genus	Indicator Value	p-value
Family XIII UCG-001	0.506	0.010
Anaerococcus	0.467	0.010
Leuconostoc	0.423	0.045
Dysgonomonas	0.494	0.010
Uncultured	0.476	0.035
Merdibacter	0.524	0.005
Faecalitalea	0.419	0.040
Candidatus Stoquefichus	0.350	0.030
Christensenellaceae R-7 group	0.409	0.040
Negativibacillus	0.493	0.015
Colidextribacter	0.476	0.020
UCG-005	0.365	0.045
[Eubacterium] hallii group	0.559	0.035
[Eubacterium] ruminantium group	0.509	0.005
Lachnospiraceae UCG-001	0.484	0.015
[Eubacterium] ventriosum group	0.482	0.015
Frisingicoccus	0.513	0.005
Lachnospiraceae ND3007 group	0.464	0.015
Marvinbryantia	0.472	0.020
CAG-56	0.479	0.025
Lactonifactor	0.488	0.015
Uncultured	0.454	0.015

Anemia appears to reduce the upregulation of differential taxa abundance in stunted infants. To determine trends in taxa abundance changes between the infant groups, DESeq2 differential taxa abundance analysis was performed comparing the change in abundance of taxa between the stunted and non-stunted anemic population and stunted and non-stunted healthy population (Figure 4). The analysis showed a large decrease in the number of significantly upregulated taxa found in stunted infants: 51 upregulated taxa in the healthy group versus 4 upregulated taxa in the anemic group, suggesting that the anemic infants on a microbiome level show a decrease in similarity to comparable infants which are stunted, because of the downregulation of these taxa.

DISCUSSION

In this study, we aimed to elucidate how anemia and stunting interact to alter the composition of the infant gut microbiota. We first created a logistic regression model and found a significant positive relationship between the two variables, indicating that infants with anemia are 2.57 times more likely to be stunted than healthy infants. In their original study, Mclorry *et al*. (2018) did not identify a difference in the prevalence of stunting across the anemic and healthy infants in this data set; however, they did not include the data from the 6-month-old infants in their analysis, which may explain this discrepancy. Other studies have revealed links between anemia and stunting in infants; for example, one study conducted in Ethiopia found the prevalence of concurrent anemia and stunting to be 23.9% and highlighted their overlapping risk factors (9). Another study conducted in Ethiopia also found a strong correlation between anemia and stunting in children under 5 years of age (32). They found that poor dietary diversity is significantly associated with both stunting and anemia in the children, which is a potential reason for the positive association we found between stunting and anemia (32). Other health factors, such as breastfeeding practices, household

environment, and unsafe drinking water, can be potential causes of both stunting and anemia (32).

Upon establishing a positive relationship between anemia and stunting, we conducted a series of diversity tests to investigate their potential effects on the infants' gut microbiomes. It was found that alpha diversity was higher in stunted healthy infants relative to the other groups. In particular, Chao1 alpha diversity analysis revealed significantly higher microbiome diversity in stunted healthy infants, suggesting that stunting does affect gut microbiome diversity in infants; however, higher gut microbiome diversity was not observed in stunted anemic infants. Thus, we suggest that the effects of anemia may be masking the effects of stunting on the gut microbiome. This is supported by previous studies which have linked anemia in infants with lower diversity of the gut microbiota, meaning that we may only be observing greater gut microbiome diversity in stunted-healthy infants given that anemia is reducing diversity in stunted-anemic infants (33). In other words, the effects of anemia on the microbiome are stronger than those of stunting, so anemia overrides any reflections of stunting in the gut microbiome. The Chao1 index is also an abundance-based estimator of species richness, which takes the number of low-abundance species into account (34). As such, the larger Chao1 index of the stunted healthy infants suggests a higher number of rare species in the group, which supports the idea that stunted healthy infants might have a more diverse gut microbiome. This increased diversity may indicate that certain microbial species are associated with stunting, which is supported by the 22 indicator genera for stunted-healthy infants identified through indicator species analysis. However, this trend is not observed in stunted anemic infants, indicating the complexity of the relationships between anemic status, microbial diversity, and stunting. Further research is needed to determine the specific factors and reasons for these observations.

Furthermore, we conducted a core microbiome analysis with the aim of identifying the number of ASVs both commonly shared between all disease states and those unique to anemic and stunting statuses. Using this information, we could determine what proportion of ASVs consistently occur across all disease states and whether stunting or anemia had a greater impact on unique microbial taxa to infer differences in microbial function between disease states. Our results revealed that most ASVs were shared across disease states, and more ASVs were unique to the presence of stunting than the presence of anemia. Supporting this, our indicator species analysis highlighted 22 genera indicative of stunting in healthy infants. Notably, the *[Eubacterium] hallii group, Merdibacter, Frisingicoccus, [Eubacterium] ruminantium group*, and *Family XIII UCG-00*1 were the five genera with the highest indicator values (>0.5). However, none of these genera are well-characterized in terms of anemia or stunting in the literature. On a similar note, our indicator species analysis results did not return any of the indicator species of anemia or stunting commonly identified by other studies. For example, anemia has been linked to higher abundances of *Actinomycetales* and *Streptococcus*, while stunting has been linked to higher abundances of *Ruminococcus 1* and *2*, *Clostridium sensu stricto*, and *Collinsella* (21, 35). Another study in sub-Saharan Africa found that enteropathogenic microbes such as *Escherichia coli*, *Shigella flexneri* and *Campylobacter concisus* to be more prevalent in the fecal samples of 2-5 year olds with stunting than in healthy controls (7). Again, none of these species or genera were determined to be indicators for anemia or stunting in our dataset, which may suggest a great degree of variation across sample and age groups. These results may also indicate that there are no true indicators of anemia or stunting in the gut microbiome across populations, and that reflections of both disease states are difficult to generalize.

DESeq differential abundance analysis showed a large decrease in upregulated taxa of stunted individuals between the healthy and anemic groups. The only significantly upregulated taxa in the anemic group in this analysis were *Holdemanella, Bacteroides Stercoris, Streptococcus,* and *Subdoligranulum*. Due to the fact that only one of the taxa is resolved down to the species level, the largest consensus amongst the literature on these species can all be found in normal gut microbiota (36, 37). This suggests that it is not the identity of the species that matters as much as the general observation that anemia appears to generally mask the upregulation of taxa in the stunted condition.

Limitations Several limitations to our study relate to the dataset that was analyzed. McClorry *et al.* (2018) only collected data from infants in Iquitos, Peru, which limits the generalizability of the findings. Additionally, confounding factors known to affect the infant gut microbiome such as infant dietary habits, antibiotic use, maternal diet and microbiome composition, degree of anemia, and environmental exposures were not accounted for, potentially introducing bias (38). Many of these factors, particularly infant diet and maternal diet, are known to have significant impacts on the gut microbiome of infants but these variables were not taken into consideration when filtering our data (39). Furthermore, during sample collection, factors including time between defecation and sample freezing were not standardized, which may have introduced variation in samples.

For our study, the stunted anemic group comprised only seven samples, which limited the power of our statistical analysis. This limitation could potentially be one reason as to why we did not find much significance for microbiome diversity tests. Additionally, our study did not consider sex or age (6 months and 12 months) differences. Differences between the gut microbiome of infants at the age of 6 months and infants at the age of 12 months have been found in previous studies, making age a confounding variable in our study (38). Likewise, differences between the gut microbiome of male and female infants have also been found in previous studies, and was noted by McClorry *et al.* (2018) for our specific dataset (1, 40). Ignoring these potential confounding variables may introduce bias into the results.

Unlike the Chao1 diversity test, no other diversity tests resulted in significant differences found between the study groups. For example, the Bray-Curtis diversity test (Supplemental Figure 1) and the Shannon diversity test (Supplemental Figure 2) did not find significant differences between the groups. Chao1 focuses on the rare species of the group, which could be the reason why a significant difference was observed using the test; however, the lack of significant differences from other tests is a limitation that could be due to factors including sample size, data quality, and the relationship between anemia, stunting, and the infant gut microbiome.

Conclusions Our study examines the relationship between anemia and stunting and their impact on the infant gut microbiome, as previous studies found contradicting and inconclusive relationships between stunting and anemia. While the effects of anemia and stunting on the gut microbiome have been studied, their effects as a pair on the gut microbiome are not well understood. As such, we set out to fill this gap in knowledge. First, we found a positive association between anemia and stunting from our logistic regression analysis. Our microbiome diversity, core microbiome, and DESeq2 analyses then revealed potential masking effects of anemia on stunting, as a stunting was found to have a larger effect on ASVs, and anemia was found to have a reducing effect on the differential taxa abundance in stunted infants. These findings led us to perform indicator taxa analysis that revealed 22 genera indicative of stunting. These results highlight the effect of stunting on the infant gut microbiome and the possibility that anemia masks the effects of stunting in infants. Our findings are a step forward in understanding the complex relationships between stunting, anemia, and gut microbial composition during infancy.

Future Directions Future studies should further explore the relationship between anemia and stunting in the infant gut microbiome by applying the methodology used in our study to other sample groups to determine whether similar patterns can be identified. The infant gut microbiome varies widely and is known to be influenced by a wide range of factors including mode of delivery, gestational age, and diet, and this diversity means it is important to determine whether the trends observed in our study can be reproduced (38). Groups of infants of different ages and from various geographical locations should be tested to evaluate the generalizability of our findings. Given McClorry *et al*.'s (2018) findings that anemia affects the infant gut microbiome in a sex-specific manner, future studies could analyze whether a sex-specific difference can be observed in terms of our finding that anemia conceals the effects of stunting, such as whether male or female infants might experience this to a greater extent. In addition, the 22 indicator genera previously identified with stunting should be further investigated to elucidate the mechanism by which they may be implicated in stunting. Current research is limited on these genera in terms of their relationship to stunting; further

study may reveal whether these genera have predictive value for stunting, which can have important clinical implications for critical early-life interventions. To further investigate the potential masking effect anemia may have on stunting in the infant gut microbiome, animal studies could be performed to analyze these conditions in a more controlled environment. Nutrient conditions can be manipulated to mimic anemia, stunting, or both conditions, and the gut microbiomes of the animal models can be examined to identify any gut microbial differences. The use of animal models could provide stronger causal evidence for the masking effect of anemia.

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CONTRIBUTIONS

All authors participated equally in the study conception, design, experimentation, writing, and revisions. Co-authorship credit should be considered equal for all authors.

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