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Iron-deficiency anemia is associated with loss of gut microbial diversity in 6- and 12-month-old infants

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SUMMARY Iron-deficiency anemia (IDA) is the most common form of anemia, arising from an inadequate quantity of iron in the body. IDA is especially concerning for young children, as it is linked to neurological dysfunction, immunological deficiencies, and increased mortality. As early life is associated with changes in the human microbiome, we aimed to investigate the effects of IDA on the gut microbiome of 6- and 12-month-old infants. We used a previously generated dataset of 16S rRNA gene sequences to compare diversity metrics, core microbiome analyses, and indicator taxa between IDA and non-IDA reference infants of both age groups. We found that both 6- and 12-month-old infants with IDA had significantly lower microbiome diversities compared to controls. Additionally, the indicator taxa differed between 6- and 12-month-old IDA infants and reference infants. This may suggest IDA could have differential, age-associated impacts on infants. Loss of gut microbial diversity in infants with IDA offers a potential explanation for the cognitive deficits associated with the condition. However, the nature of this relationship remains to be further clarified.

INTRODUCTION

Anemia is a condition in which the number of red blood cells or the hemoglobin concentration within them is lower than normal (1). Iron-deficiency anemia (IDA) is the most common form of anemia and is caused by an insufficient quantity of iron in the body (2, 3). This results in poor development of hemoglobin in the red blood cells (2, 3). Anemia is common in children less than 5 years of age. The World Health Organisation (WHO) estimates as many as 42% of children are suffering from this condition (1). IDA has been shown to cause several health problems in infants, such as reduced growth, vulnerability to infections, and increased mortality (4–6). IDA during early life has also been associated with impaired cognitive abilities, which have been shown to persist even following iron repletion (4, 7).

For many bacterial species, iron is an essential nutrient for colonization and growth (8). Dietary iron is never completely absorbed and thus passes into the colon which can be used by microbial communities (9). Thus, the iron consumption of the host has the potential to impact microbial community structure. In humans, associations have been found between gastrointestinal microbes and anxiety, depression, autism spectrum disorder, and neurodegeneration (10–13).

To gain a better understanding of how IDA in infancy is associated with alterations in metabolism and microbial structure, McClorry *et al*. acquired stool and serum samples from 6- and 12-month-old infants from Iquitos, Peru (14). The researchers believed that IDA might be influencing changes in the microbial communities in infants which were driving symptoms associated with IDA, such as impaired cognitive abilities. From the fecal microbiome analysis

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of 12-month-old infants, McClorry *et al*. observed that IDA was associated with a decrease in several microbe genera (14). Some of these absent microbes are known to produce butyrate, which is an important molecule for myelination (14, 15). They postulated that a loss of these bacteria may lead to impaired neurological development.

While McClorry *et al.* collected samples from 6-month-old infants, they excluded these samples from their analysis. The microbiome in young infants is just being cultivated, thus infants even just a few months apart can show dramatically different microbial populations (16). This difference in microbial composition means these infants should be analyzed separately to elucidate the differential effects IDA has on microbial composition for 6- and 12-month-old infants. Another important variable impacting microbial composition is sex. It has been demonstrated that microbial compositions differ between the sexes, which has been shown to impact disease symptoms (17). Indeed McClorry *et al.* found that while IDA led to a reduction in butyrate-producing bacteria in both males and females, different genera were impacted between the sexes (14). With this knowledge in mind, we sought to determine how IDA impacts microbiome composition, and how this differs based on sex and age.

Further, while McClorry *et al*. were able to identify a loss of microbial diversity and the loss of specific butyrate-producing bacteria in infants with IDA, they did not find any bacteria species specifically associated with IDA. A reduction in bacterial diversity in the gut microbiome can lead to pathogenic bacteria taking up residence in the gut. *C. difficile* is a well-known opportunistic pathogen which induces colitis. It is often found in patients receiving antibiotic treatments. *C. difficile* takes advantage of the loss of commensal bacteria and reduced competition, such as that observed in IDA infants by McClorry *et al*. Further, C. *difficile* is highly resistant to iron deficiency, giving it an advantage over many other commensal microbes (18).

In early childhood development, the microbiome fluctuates in its composition. To discover the correlation between IDA and the gut microbiome of developing infants, we examined the data provided by McClorry et al. Taken together, we hypothesized that IDA would be associated with a decrease in microbiome diversity in infants. Specifically, we expected to see a decrease in butyrate- producing bacteria in infants with IDA, corroborating the results found by McClorry *et al*., and we expected to see pathogenic bacteria associated with IDA. Further, we hypothesized that IDA would have differential effects on microbiome diversity and indicator species based on the infants' age and gender.

METHODS AND MATERIALS

Dataset and metadata. The original dataset by McClorry *et al*. consists of both stool and serum samples acquired in Moronacocha, Iquitos, Loreto, Peru from 194 infants, including 96 6-month-old and 98 12-month-old infants (14). Serum samples were analyzed for hemoglobin concentrations and malaria status before further metabolome analysis, whereas the stool samples were sent to California for further analysis (14). McClorry *et al*. extracted DNA from patient-provided stool samples and the V4 region of the 16S ribosomal RNA gene was amplified using the F515-R806 primer pair (14). Additional information about each infant was also provided, including their age, sex, infection status, ferritin and hemoglobin levels. The dataset is publicly available on the European Nucleotide Archive (ENA) Browser under accession PRJEB23239. In their analysis of 12-month-old infants, McClorry *et al*. excluded data from subjects whose fecal samples were collected more than 14 days after blood sample collection. This data was excluded due to iron supplementation treatment possibly impacting the subject's microbiome. However, in our preliminary analysis, we found no significant difference in microbial diversity between infants with or without iron supplementation and thus decided to include these samples in our analysis. In their analysis of 12-month-old infants, McClorry *et al*. excluded data from subjects whose fecal samples were collected more than 14 days after blood sample collection. This data was excluded due to iron supplementation treatment possibly impacting the subject's microbiome. However, in our preliminary analysis, we found no significant difference in microbial diversity between infants with or without iron supplementation and thus decided to include these samples in our analysis.

Preliminary data processing in QIIME2. We first imported and demultiplexed our 16S rRNA sequences from the dataset using QIIME2 (19). The demultiplexed sequences underwent denoising and truncation through the use of the QIIME2-compatible plugin Divisive Amplicon Denoising Algorithm 2 (DADA2) (20, 21). This process spots and corrects potential sequencing errors, removes chimeric sequences and identifies unique amplicon sequence variants (ASVs). A truncation read length of 250 base pairs was retained to adjust for differences in sequence base quality. This data was then used to generate a representative sequences file as well as a feature table of sequences that were filtered using the q2- feature-table plugin (19).

Taxonomic analysis in QIIME2. The SILVA 138-99 database was used to generate the taxonomic classification for the samples (22, 23). The database was truncated to only reflect the variable regions V3 and V4, as collected by McClorry *et al*. in the original dataset. This successfully generated a taxonomy file for further analysis. QIIME2 was then used to remove any mitochondrial and chloroplast DNA sequences in the sample. Following this, a phylogenetic tree was made using the representative sequences file with QIIME2 FastTree and MAFFT alignment (24, 25).

Metadata filtering in QIIME2. Any samples from infants with detected parasites were filtered out of the dataset using the q2- feature-table plugin (19). Any samples which did not have associated ferritin levels gathered as well were also filtered out. From there, patients were divided by age (6-month-olds or 12-month-olds) and then further divided by sex (male or female). The resulting dataset and subsets were exported for further analysis.

Metadata filtering and grouping in R. To correct for unequal reads misrepresenting species richness in samples, we rarefied the dataset in R. Samples from 12- and 6-month-old infants were rarefied using a sampling depth of 10,000 to maximize the number of samples and features retained (Supplemental Table S1). The original paper rarefied the samples to a sampling depth of 10,000. As we are attempting to perform their process in a similar fashion, we elected to rarefy the dataset to 10,000 as well. To understand how IDA affects the gut microbial composition of 12- and 6-month-old infants, we used a definition that incorporated both hemoglobin and ferritin levels. Anemia was defined as having a hemoglobin concentration <110 g/L, and iron deficiency as having an adjusted ferritin level of < 12 µg/L, following WHO standards (26). In our analysis, we defined infants with IDA as those who are both anemic and iron-deficient (ID). Reference patients were defined as those who were not ID or anemic.

Alpha and beta diversity analyses. Alpha and beta diversity metrics were produced and analyzed using R version 4.2.2 and the following R packages: tidyverse 1.2.0, vegan 2.6-4, qiime2R 0.99.6, phyloseq 1.16.2, DESeq2 1.40.2, ggplot2 3.4.2, dplyr 1.1.1, indicspecies 1.7.13 and ape 5.0 (27–35). Chao1 was used for the analysis of overall alpha diversity between reference infants and infants with IDA, age groups, and sexes. For comparisons of Chao1 diversity between reference infants and infants with IDA, Wilcoxon Rank Sum and Kruskal-Wallis tests were performed. Results from beta diversity analysis were visualized by generating Principal Coordinates Analysis (PCoA) plots with the plot_ordination function in phyloseq using weighted UniFrac distances between infants with IDA and reference infants 6 and 12 months of age (30). Ellipses for all PCoA plots represent a 95% confidence interval. Significance of microbial compositional differences were determined with permutation multivariate analysis of variance (PERMANOVA).

Core microbiome analysis. To identify microbial taxa associated with IDA status and age, we conducted a core microbiome analysis using the microbiome R package (36). For 6- and 12-month-old infants, relative abundance for each age group was generated from the phyloseq object using the transform_sample_counts function. Core members from infants with IDA and reference controls were compared using the core_members function. Quantification of the core microbiome was performed using abundance and prevalence parameters of 0 and 0.5, respectively, to retain only microbial taxa present in more than 50% of the analyzed samples.

Results were visualized by generating Venn diagrams using the ggVennDiagram R package (37).

Indicator species analysis. Indicator species analyses were performed in R using the indicspecies package (38). Individually, indicator species analyses were conducted comparing IDA and reference infants in the following subsets: all 12-month-olds, 12-month-old males, 12-month-old females, all 6-month-olds, 6-month-old males, and 6-month-old females. The outputs of this process were deemed significant with P values less than or equal to 0.05.

RESULTS

Alpha and beta diversity were significantly different between 6- and 12-month-old infants. In order to determine the effect of IDA on infant microbiome diversity, a Chao1 diversity index was calculated for infants with IDA and reference infants 6 and 12 months of age. A similar level of richness was observed between IDA infants and reference infants (Fig. 1A). Clustering based on beta diversity (weighted UniFrac) also showed no difference between the microbial communities of the two groups (Fig. 1B). Alpha diversity analysis was also conducted on 6- and 12-month-olds to investigate the impact of age on microbiome diversity during early infancy. 12-month-old infants had significantly higher alpha diversity than 6-month-old infants (Fig. 2A). Clustering based on beta diversity showed a significant difference in microbial communities between 6- and 12-month-old infants (Fig. 2B).

Alpha diversity was significantly different between IDA and reference infants in the 6- and 12-month-old cohorts. As our results revealed significant age-related impacts on microbial communities of 6- and 12-month-old infants, we filtered our data by age and again assessed the impact IDA had on microbial diversity. The gut microbiome alpha diversity was

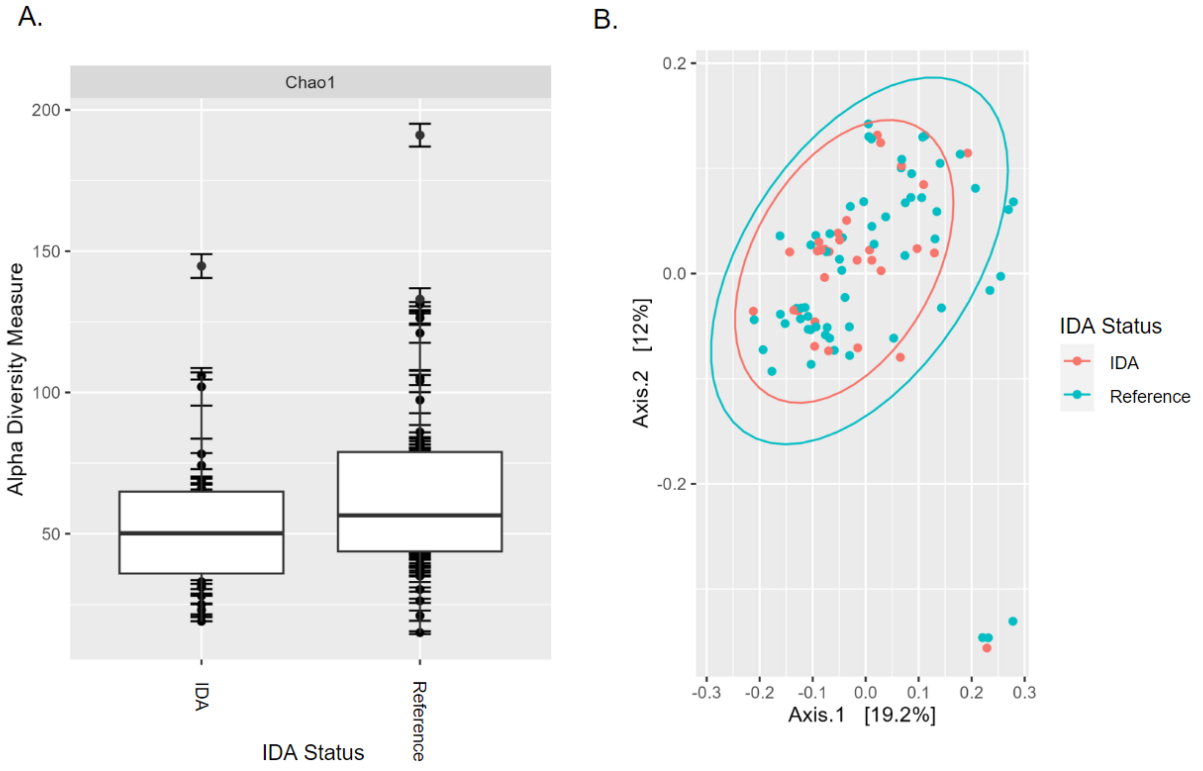

FIG. 1 No significant difference in alpha or beta diversity between infants with IDA and reference infants. (A) Chao1 group significance boxplot of both 6- and 12-month-old infants comparing the alpha diversity between infants with IDA and reference infants ($p = 0.097$, Wilcoxon Rank Sum test). (B) Principal Coordinate Analysis (PCoA) plot of beta diversity (weighted UniFrac) for infants with IDA and reference infants ($p = 0.393$, PERMANOVA). Ellipses represent a 95% confidence interval.

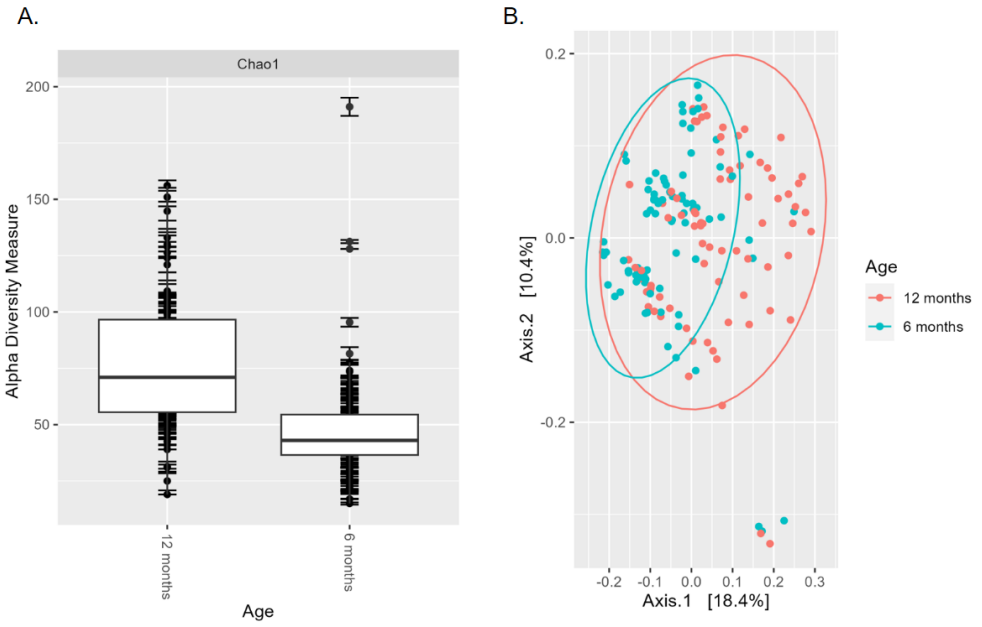

FIG. 2 Significant difference in alpha and beta diversity between 6- and 12-month-old infants. (A) Chao1 group significance boxplot comparing the alpha diversity between 6- and 12-month-old infants ($p < 0.001$, Wilcoxon Rank Sum test). (B) PCoA plot of beta diversity (weighted UniFrac) for 6- and 12-month-old infants ($p = 0.007$, PERMANOVA). Ellipses represent a 95% confidence interval.

calculated for IDA and reference control infants in the 12-month-old (Fig. 3) and 6-month-old (Fig. 4) age groups. For the 12-month-old cohort, infants with IDA had significantly lower alpha diversity when the sexes were combined as well as when females and males were looked at separately. In the 6-month-old cohort, infants with IDA had significantly lower alpha diversities compared to reference infants when the sexes were combined (Fig. 4A) and in female infants (Fig. 4B). However, there was not a statistically significant difference in

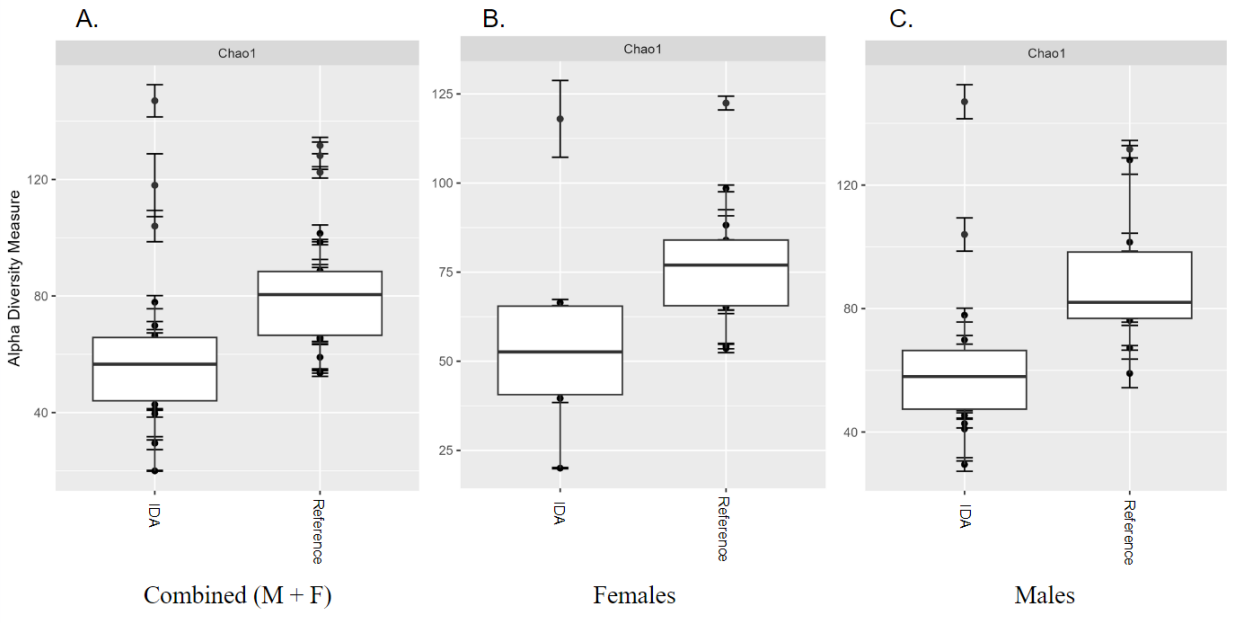

FIG. 3 Significant difference in Chao1 alpha diversity between infants with IDA and reference 12-month-old infants. Chao1 group significance boxplots comparing the alpha diversity between IDA and reference 12-month-old infants. Boxplots show alpha diversity when (A) sexes are combined ($p < 0.001$, Wilcoxon Rank Sum test), looking at (B) females ($p = 0.020$) and (C) males ($p = 0.005$) separately.

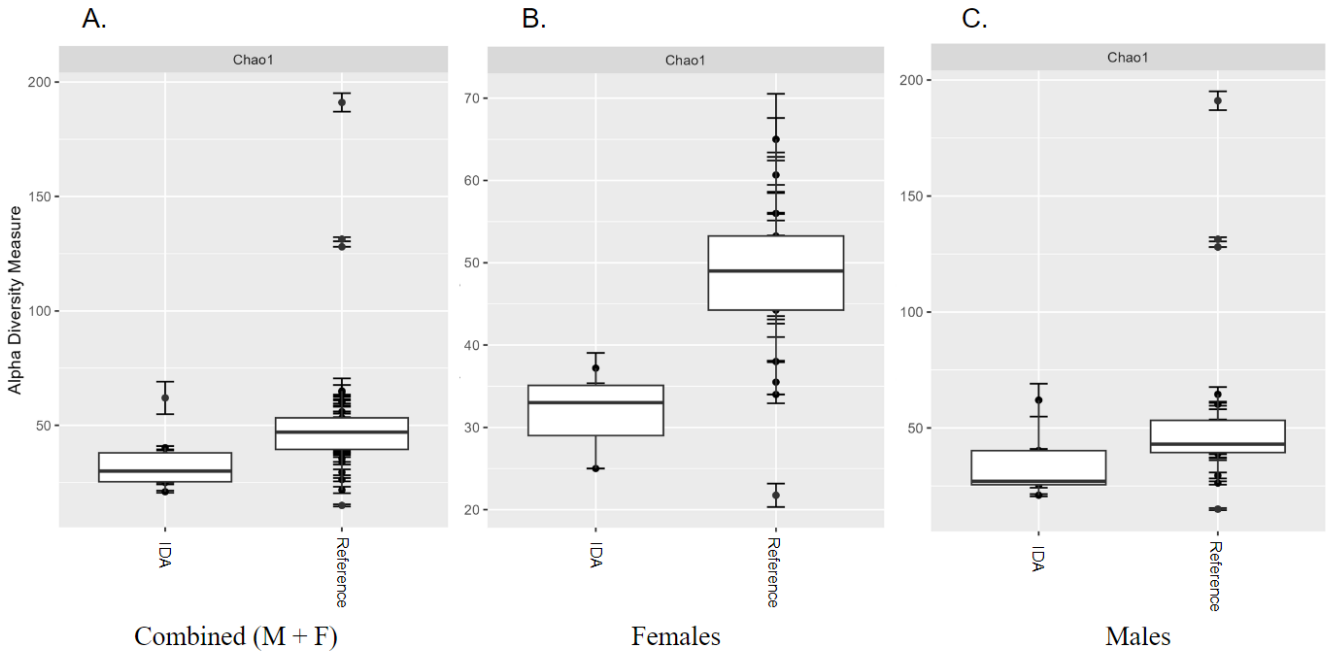

FIG. 4 Significant difference in Chao1 alpha diversity between infants with IDA and reference 6-month-old infants. Chao1 group significance boxplot comparing the alpha diversity between IDA and reference 6-month-old infants. Boxplots show alpha diversity when (A) sexes are combined ($p = 0.010$, Wilcoxon Rank Sum test), and when looking at (B) females ($p = 0.034$) and (C) males ($p = 0.144$) separately.

alpha diversity between 6-month-old male infants with and without IDA (Fig. 4C). Additionally, there was not a statistically significant difference in alpha diversity between males and females in either of the age groups (Supplementary Figure S1).

IDA is associated with reduced core members in 12-month-olds and an increased abundance of core members in 6-month-olds. Using abundance and prevalence parameters to retain only microbial taxa present in more than 50% of the analyzed samples, we found that IDA in 12-month-old infants was correlated with a lower number of core members compared to their reference counterparts (Fig. 5A). In particular, our results reveal 1 unique amplicon sequence variant (ASV) associated with 12-month-olds with IDA, whereas 15

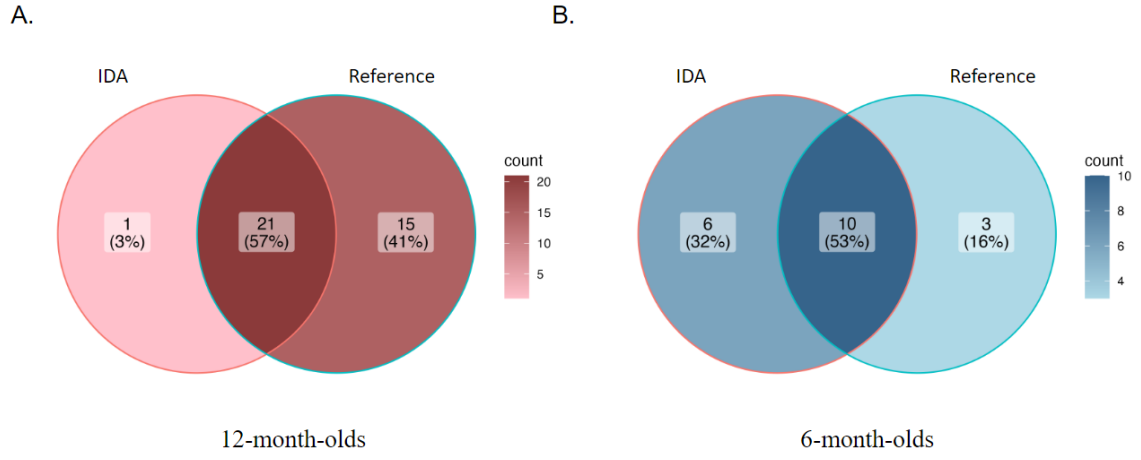

FIG. 5 Core microbiome analysis reveals loss of microbial taxa in 12-month-old infants with IDA but not 6-month-old infants. Venn diagram representing the percentage of unique taxa across IDA status in (A) 12-month-old and (B) 6-month-old infants. Core microbiome was quantified for 12-month-old infants with IDA (n = 23) and without IDA (n = 23) and 6-month-old infants with (n = 8) and without IDA (n = 37).

ASVs were unique to controls. In contrast, 6-month-olds with IDA were associated with a higher number of core members compared to reference infants (Fig. 5B). Together, these results indicate that the impact of IDA on core microbial species in infants is age-dependent as IDA in 12-month-olds is associated with a loss of core members whereas in 6-month-olds, IDA is associated with an increased abundance of core microbes.

Indicator taxa analysis of 12-month-old infants shows *C. difficile* as an indicator species in IDA patients. Indicator taxa analysis was performed to determine if there were any bacterial species that were correlated with IDA status. In 12-month-old infants, we found 6 significant genera associated with reference infants and only 1 genus associated with IDA when both sexes were analyzed together (Table 1). Similarly, when separated by sex, 12-month-old males had 6 significant genera that were associated with reference infants and only 1 genus associated with IDA (Table 1). There was a significant overlap in genera between 12-month-old reference males and combined 12-month-old reference infants, including *Eubacterium*, *Lachnospiraceae*, and *Ruminococcaceae*. However, for 12-month-old females, indicator taxa analysis identified 1 significant species associated with infants in the reference group. There were no associations with IDA (Table 1). In 12-month-old infants, *C. difficile* was observed as an indicator species for IDA. It was the only indicator species observed in 12-month-olds with IDA. Taken together, these results show that 12-month-old infants with IDA saw a loss in several bacterial species and only saw an increase in *C. difficile*, a notoriously harmful bacterium (52).

TABLE. 1 Indicator taxa analysis of 12-month-old infants shows more unique species among infants in the reference category. Results from indicator species analysis of 12-month-old infants based on their IDA status. Both sexes were analyzed together (n = 46) and separately as males (n = 25) and females (n = 21). P-values were determined by a permutation test.

Sex	IDA Status	Family	Genus species	P-Value
Both	**Reference**	*Akkermansiaceae*	*Akkermansia sp.*	0.02
		Butyricicoccaceae	*Butyricicoccaceae pullicaecorum*	0.03
		Eubacterium	*Coprostanoligenes sp.*	0.02
		Ruminococcaceae	*Anaerotruncus sp.*	0.03
		Lachnospiraceae	*Hungatella sp.*	< 0.01
		Lachnospiraceae	*Agathobacter sp.*	0.03
	IDA	*Peptostreptococcaceae*	*Clostridioides difficile*	0.04
Male	**Reference**	*Lachnospiraceae*	*Hungatella sp.*	0.02
		Lachnospiraceae	*Dorea sp.*	0.02
		Lachnospiraceae	*Roseburia sp.*	0.05
		Eubacterium	*Coprostanoligenes sp.*	< 0.01
		Ruminococcaceae	*Ruminococcus bicirculans*	0.04
		Lachnospiraceae	*NA*	0.03
	IDA	*Carnobacteriaceae*	*Granulicatella sp.*	0.04
Female	**Reference**	*Akkermansiaceae*	*Akkermansia sp.*	0.05

Indicator taxa analysis of 6-month-old infants show several indicator taxa in patients with IDA. Indicator taxa analysis was performed again on 6-month-old infants to determine if the bacterial species associated with IDA status would differ in this age group. In 6-month-old infants, we found 3 significant genera associated with IDA (Table 2). When separated by sex-status, 6-month-old males showed similar results, with only 2 indicator genera associated with IDA (Table 2). Of these 2 indicator genera, *Rothia mucilaginosa* is also found to be associated with IDA in 6-month-old infants overall, suggesting that this increase in *R. mucilaginosa* in males may be driving the increase of this species seen in the

Table 2: Indicator taxa analysis of 6-month-old infants shows more unique species among infants in the IDA category. Results from indicator species analysis of 6-month-old infants based on their IDA status. Both sexes were analyzed together (n = 45) and separately as (n = 25) and females (n = 20). P-values determined by permutation test.

Sex	IDA Status	Family	Genus species	P-Value
Both	**IDA**	*Micrococcaceae*	*Rothia mucilaginosa*	0.01
		Gemellaceae	*Gemella sp.*	0.03
		Lactobacillaceae	*Lactobacillus fermentum*	0.02
Male	**IDA**	*Micrococcaceae*	*Rothia mucilaginosa*	0.01
		Carnobacteriaceae	*Granulicatella sp.*	0.05
Female	**Reference**	*Lachnospiraceae*	*Ruminococcus sp.*	0.04

overall 6-month-old infants with IDA. Indicator taxa analysis of 6-month-old females revealed only one species, *Ruminococcus gnavus*, that was associated with the non-IDA reference infants, which indicates that this species had a comparatively reduced presence in IDA cases (Table 2). Interestingly, these bacteria weren't found to be indicator species in 12-month-old infants, indicating the differential effects of age and IDA.

DISCUSSION

Using previously published metadata by McClorry *et al*. (14), we aimed to investigate the difference in microbial diversity between infants with and without IDA at the ages of six and twelve months and explore how the abundance of different microbial genera changed as a result of IDA. Using alpha and beta diversity metrics, we found a significant difference in microbial gut diversity between infants with IDA and reference infants, which was age specific. We found that microbiome diversity was significantly higher in 12-month-old infants compared to 6-month-old infants and significantly lower in infants with IDA compared to reference infants. Furthermore, using indicator species analysis, we found that IDA was associated with a loss of beneficial bacteria and an increase in pathogenic *C. difficile* in 12-month-old infants. These changes in the microbiome may partially explain certain symptoms of IDA.

Loss of microbial diversity in infants with IDA compared to reference infants. When we first compared alpha and beta diversity between reference infants and those with IDA, we did not see a significant difference between groups. This was likely due to confounding variables such as age and sex. When we compared alpha and beta diversity between 6- and 12-month-old infants we found that 12-month-old infants had significantly higher microbial diversity. This result was consistent with previous research and reflects the gradual cultivation of the gut microbiome as infants age (39). After filtering our data by age, we were able to detect a significant difference in alpha diversity between reference infants and those with IDA in both age groups. This indicates that IDA is associated with a loss of microbial diversity in both 6- and 12-month-old infants. When we further filtered each age group by sex, we were able to find diversity differences between infants with and without IDA. Statistically significant differences were found in the alpha diversity of 12-month-old males and females and 6-month-old females when comparing infants with and without IDA. However, we did not find a difference in alpha diversity between reference 6-month-old males and those with IDA, likely due to a small sample size. To investigate if sex plays a role in the microbiome diversity of infants with IDA, we compared alpha diversity between male and female infants with IDA. No significant difference was detected in 6- or 12-month-old infants. Previous research has indicated that sex plays a role in microbiome diversity, and impacts disease symptoms between the sexes (17, 40–42). One reason we did not find a difference between males and females with IDA might have been due to confounding variables in our data set such as iron supplementation or diet.

Loss of beneficial bacteria in infants with IDA may lead to impaired neurological development. Results from the core microbiome analysis revealed that IDA is correlated with reduced core members in 12-month-olds and an increased abundance of core members in 6-month-olds, indicating that age-related factors play a role in the impact of IDA status on

microbial diversity. Using indicator species analysis to identify unique microbial taxa and build upon the core microbiome results, we observed both *Ruminococcaceae* and *Butyricicoccaceae* populations were both observed to be reduced in 12-month-olds with IDA in our indicator species analysis as seen in Table 1. Previous studies have found a loss of bacterial species from the *Ruminococcaceae* and *Butyricicoccaceae* families in iron-deficient environments (43, 44). Although we were not able to establish iron deficiency as the true cause for the loss of these bacteria, we found an association between these variables. The ID environment seen in IDA may result in this lost diversity. It is worth noting that both of these families have members which produce butyrate in the human colon. The original paper from which this dataset is derived discussed the implications of the loss of butyrate-producing bacteria (14). Butyrate has been shown to play an important role in several facets of human health (45–47). Among these benefits is its potential contribution to neurological development (46). Studies in mice have found that butyrate-producing bacteria led to an increase in blood-brain barrier structure, and can contribute to other brain health (48). Additionally, butyrate is an important source of nutrients for cells of the colon (45). Pharmaceutical studies have also found that butyrate improves memory, learning, and reduces neuronal damage in neurological diseases in murine models (49).

Lachnospiraceae members were also lost in patients with IDA in the dataset. This loss was observed when analyzing all 12-month-olds collectively, and when we analyzed 12-month-old males and females individually. Loss in *Lachnospiraceae* has been tied to iron-deficient environments in some studies (50). In previous studies, the loss of *Lachnospiraceae* has been correlated to reduced head circumference in infants (51). This lack of *Lachnospiraceae* could partially account for the decreased development of the brain observed in some patients with IDA.

IDA is associated with an apparent increase in the abundance of *Clostridiodes difficile* in the microbiota of 12-month-olds. Dysregulation of the microbiome not only results in the loss of beneficial bacteria but can also increase the presence of pathogenic bacteria. In our findings, *C. difficile* was found as an apparent indicator species in 12-month-old infants with IDA. *C. difficile* can be very detrimental to the host; symptoms of infection range from diarrhea to severe colitis, and can result in death (52).

C. difficile has been found to colonize individuals who have been undergoing antibiotic therapies. This is attributed to the loss of normal commensal bacteria allowing for *C. difficile* to establish itself (18). We have found that 12-month-old infants with IDA experience a decline in gut microbial diversity, and therefore *C. difficile* may take similar advantage of this reduction of normal gut bacteria. *C. difficile* is also found to be highly resilient and has several mechanisms to survive and out-compete commensal bacteria when faced with iron deficiency (53). Combined, these factors could result in the increased presence of *C. difficile* in iron-deficient conditions. This may put infants with IDA at a greater risk of *C. difficile* infections.

Limitations A noteworthy constraint of this study revolves around the exclusive reliance on the SILVA database for our taxonomic classification. The utilization of a singular database diminishes the robustness of our findings, especially considering that the SILVA database might not comprehensively represent all species due to potential lag in updates compared to databases like BLAST. Moreover, our taxonomic analysis, aiming to delineate species-level distinctions, is founded upon 16S sequencing data. This approach introduces a substantial element of uncertainty, as certain species could exhibit overlaps that hinder precise differentiation.

Another limitation of this study is the insubstantial sample size, especially for 6-month-olds. While the dataset was still large enough to acquire statistical significance, a larger sample size would increase confidence in our findings. The threshold for IDA was based on the adjusted ferritin level recommended by the World Health Organization (26). However, ferritin levels are subject to a variety of fluctuations during early stages of life, so this may not necessarily be a perfect diagnostic for IDA, particularly for infants (54). Soluble transferrin receptor (sTfR) is a better indicator of iron deficiency because it is not affected by inflammation (55). sTfR could be used as an indicator in future studies as a more reliable marker for iron deficiency (14).

Unlike the original paper, we elected to include iron-supplemented cases in with the rest of the analysis. When we compared alpha diversity between infants receiving $FeSO_4$ or multiple micronutrient powder (MNP), we did not see a significant difference (Supplementary Figure S2), which is why we decided to include these infants in our analysis. However, prior research has substantiated the influential role of iron supplementation in reshaping the overall composition of the microbiome (56, 57). Thus, the retention of these samples could potentially introduce a confounding variable that exerts an impact on our outcomes. We did not subdivide the iron supplementation groups by sex or anemia status. The separation and evaluation of these variables could reveal some significant findings and further elucidate the effect of various iron supplements. In our analysis of indicator species, several of the bacterial families which appeared as indicator species had members which could produce butyrate (7,9). However, the indicator species analysis oftentimes did not resolve the bacteria to the species level. Some of these bacteria are yet to be cultured. Many have little literature surrounding them. Additionally, though infants were deemed "reference" if they were not selected as having IDA, they may not necessarily have been without disease. The inherent variability of infant health outside of IDA was not controlled for and could alter the results between different groups.

Conclusions In our study, we aimed to investigate the impact of IDA on the gut microbiota of 6- and 12-month-old infants. The results of our study indicate that IDA status and age is associated with significant changes in gut microbial diversity and composition. Specifically, alpha and beta diversity analyses revealed significantly higher microbial diversity in 12-month-old infants compared to 6-month-olds. Furthermore, we also found that 6- and 12-month-old infants with IDA showed reduced gut microbial diversity compared to reference controls. Using indicator species analysis to identify changes in microbial taxa, we found that IDA in 12-month-olds was associated with a loss of bacteria from the *Ruminococcaceae* and *Butyricicoccaceae* families and an apparent increased abundance of pathogenic *C. difficile*. Overall, these findings suggest that IDA in 6- and 12-month-old infants is correlated with reduced microbial diversity and significant changes in the composition of the infant gut microbiome.

Future Directions Conducting our analysis with an additional database, like Greengenes or RDP, for taxonomic classification would be highly beneficial. Utilizing multiple databases can significantly enhance the confidence of our outputs, thereby reinforcing the robustness of our findings.

Future studies exploring the role of IDA on infant gut microbiome composition can build upon our current understanding by examining a larger and more diverse dataset in which potential confounding variables could be considered. Confounding variables such as geographical location, maternal diet, and mode of delivery during the infant's birth could be controlled to establish a clear relationship between IDA and gut microbiota (58). In particular, our beta diversity analysis comparing gut microbiota between 6- and 12-month-old infants revealed three distinct clusters in the PCoA plot (Figure 1B). As no indications for this observation were provided in the original dataset, it is possible that this distinct clustering represents a confounding variable. Therefore, it is essential for future studies to clarify this finding by conducting further analyses on this dataset to identify this confounding variable.

In our study, we found that IDA in 12-month-old infants is associated with a loss of indicator taxa from the *Ruminococcaceae* and *Butyricicoccaceae* families and increased abundance of pathogenic *C. difficile* bacteria. However, due to the limitations of our study, the true nature of the relationship between IDA and the infant gut microbial diversity and composition is unclear. Further research is required to establish a potential causal relationship between IDA and the loss of microbial diversity in infants.

In addition, future studies should aim to improve our understanding of age-related effects on gut microbial composition and diversity in infants with IDA using a longitudinal study design. The metadata used in this study consisted of samples from different 6- and 12-month-old infants with stool samples collected at a single point in time. Longitudinal cohort studies following infants with IDA from birth to early infancy would be helpful to further elucidate the relationship between IDA and diversity of gut microbiota (59).

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CONTRIBUTIONS

All authors contributed equally to the background research, planning, and design of this study, and should be credited as such. L.L.P contributed to the abstract, introduction, methods, results and discussion. M.J.H. contributed to the introduction, methods, discussion, study limitations, and conclusion. T.B. contributed to the methods, results, discussion, generated figures 1-4 and supplementary material. P.S. contributed to the abstract, methods, results, conclusion, and future directions. All authors contributed to the editing of this manuscript.

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