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Non-breast milk diet increases gut microbial diversity and inflammation in six-month-old infants

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SUMMARY The interplay between the gut microbiome, inflammation, and diet is established. Previous research has explored the connections between the gut microbiome and inflammation. However, there is a lack of research evaluating this interplay within anemic and non anemic infant populations. This study investigates how anemia and diet, specifically breast milk (BM) and non-breast milk (non-BM) diets (lacking breast milk), affect inflammation and the gut microbiome. At first we found that diet plays a significant role on gut microbial diversity and inflammation, with a non-BM diet showing a significant increase in both gut microbial diversity and inflammation level. We then found that for BM and non-BM diets, anemic status of six-month-old infants does not significantly alter inflammation and the gut microbiota. Further analysis of the core microbiome then showed a higher number of indicator species in the gut microbiome of infants on a non-BM diet and those with elevated inflammation levels. An indicator species analysis of the gut microbiota revealed the presence of potentially pro-inflammatory microbes that were common in infants on the non-BM diet and those with elevated inflammation. These data suggest that a non-BM diet can lead to an increased risk of inflammatory diseases due to an increase in abundance of proinflammatory microbes. Overall, this study highlights the importance of using breast milk when considering an infants' diet, especially in situations where they are exposed to potentially higher inflammation levels.

INTRODUCTION

The short and long-term health status of infants can be affected by the gut microbiome and their inflammatory status (1, 2). Alterations in the microbiota during infancy are associated with diseases, such as inflammatory bowel disease (IBD) and asthma (1, 3). Furthermore, it has been reported that systemic inflammatory responses, marked by C-reactive protein (CRP), during infancy are associated with impaired postnatal growth (1, 4). Considering the importance of both inflammation and gut microbiome during infancy, exploring the factors that influence each and the interplay between them is crucial.

Effect of diet on inflammation and microbiome. Diet is one of the factors that significantly affects the infant's gut microbiome, and its interplay with inflammation. Different diets can modulate the gut microbiota composition differently. For instance, human milk from a healthy mother's diet selects for *Bifidobacteria* and *Lactobacilli*, which can promote gut health in infants (5). Further, research has shown that weaning of infants with adult foods, typical of a Mediterranean diet, leads to an eventual increase in gut microbial diversity, which can also lead to a prevention of inflammatory diseases (6). Additionally, a diet containing high fiber selects for gut microbiota with anti-inflammatory effects (1). Moreover, protein-rich diets can increase the number of *Proteobacteria* phylum, and are associated with gut microbiota dysbiosis within the general population (7). Therefore, diet

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can influence the alteration of gut microbiota and inflammation throughout life. It is important to study how diet affects these two factors in infants, who are already struggling with conditions that impact gut microbiome and inflammation.

Effect of anemia on inflammation and microbiome. After birth, infants are exposed to a reduction in hemoglobin, resulting in anemia with different severities (8). According to the World Health Organization, around 40% of children aged 6-59 months experienced anemia in 2019 (9). Due to its prevalence in infants, it is important to explore the effects of anemia for this age group. Research has found that anemia during infancy can impair growth and development, and increase the risk of disease and mortality (10, 11). Additionally, some forms of anemia, such as IDA, can alter gut microbial composition (11). Anemia is also found to affect the inflammation level in one-year-old infants through reducing the number of anti-inflammatory bacteria in the gut microbiome (11). Moreover, a recent study suggests that anemia can result in an elevation of gut inflammation and injury, increasing the risk of necrotizing enterocolitis (NEC). NEC is an acute inflammatory disease of the intestine, and it is a common cause of mortality among infants (12, 13). Thus, past studies have suggested that gut microbiome and inflammation level in infants are affected by anemia; however, more studies are needed to confirm if they are actually impacted in six-month-old infants, considering their different diets. Such studies are essential for highlighting the importance of diet during infancy, especially for infants who are already struggling with anemia, which is not uncommon in this age category.

To address the current knowledge gaps surrounding anemia, inflammation, and diet, we assessed the inflammation status and gut microbial diversity in healthy and anemic infants on varying diets. We hypothesized that the non-breast milk diet in six-month-old anemic infants promotes inflammation through increasing the pro-inflammatory microbial species in the gut.

First, we aimed to examine how different diets affect the alpha diversity and inflammation in six-month-old infants. We assessed microbiome alpha diversity using Shannon diversity index, and evaluated inflammation using C-reactive protein (CRP) levels, which is a plasma protein whose level rises in response to inflammation (1, 4). We found that the alpha diversity of the gut microbiome and inflammation level are significantly higher in the six-month-old infants on a non-breast milk diet compared to a breast milk diet. Therefore, we continued our study focusing on only two diets, breast milk and non-breast milk. Second, we explored the effect of anemic status on diet and inflammation. Our results show that anemia does not significantly change the gut microbial alpha diversity and inflammation levels in six-month-old infants on either BM or non-BM diet. Third, we analyzed gut microbial composition in infants with the non-BM diet, and in those with elevated inflammation, independently of their anemic status. We found three potentially pro-inflammatory indicator genera of *Erysipelatoclostridium, Tyzzerella,* and *Clostridium_sensu_stricto_1* shared between infants with an elevated CRP and those on the non-BM diet. This suggests that a non-BM diet in six-month-old infants can lead to an increase in pro-inflammatory bacterial genera. Overall our study emphasizes the significance of diet in promoting healthy gut microbiota and inflammation in six-month-old infants.

The main purpose of our analysis was to explore the impact of different diets on gut microbial alpha diversity and inflammation levels in six-month-old infants. Specifically, we wanted to investigate whether infants on an exclusive breast milk (BM) diet exhibit different levels of gut microbial diversity and CRP compared to those on a non-breast milk (non-BM) diet. Our analysis suggests that diet and inflammation are closely linked, and further research is needed to understand the potential long-term implications of these findings.

METHODS AND MATERIALS

Dataset. We utilized a dataset originally created by McClorry et al., which consists of samples collected from 101 infants (6-7 months old) at the Moronacocha Health Center in Iquitos, Peru (11). All subjects were healthy, born at term, and had a birth weight of ≥2500 g. Fecal samples from these infants underwent 16S ribosomal RNA gene Illumina sequencing for microbiome analysis (11, 14).

Data processing. We used the Quantitative Insights Into Microbial Ecology (QIIME 2) platform for data sequencing (15). The sequences were imported, quality-controlled using DADA2 method, and truncated to 291 bases (16). Taxonomic analysis and metadata filtering were performed, and rare amplicon sequence variants (ASVs), non-bacterial sequences, and features containing mitochondria were removed using q2-feature-table plugin. Taxonomic classifications for each ASV were generated using q2-feature-classifier plugin (17). This classifier was used to generate a taxonomy artifact containing information regarding the taxonomic classifications for each ASV. A phylogenetic tree was constructed using QIIME2 phylogeny align-to-tree-mafft-fasttree (18). The resulting files were exported for analysis in R.

R Analysis. We loaded necessary libraries into R studio (19-31) and filtered the metadata for six-month-old infants. CRP and AGP were converted from character to numeric variables after removing 'NA' values.

Diets that did not contain breast milk were combined into a “Non-Breast Milk” category, and diets with less than four samples were excluded. Diets containing breast milk only were categorized as “ExclusiveBM”. Diets with soup or broth in addition to breast milk were grouped into “BM.Soup.Broth”. The breast milk diet that was supplemented with solid food made up the category of “BM.Solids”. Diets containing juice and water in addition to the breast milk were classified as “BM.Juice.H2O”. Low-abundance reads (<5 reads total) and samples with less than 100 reads were removed. Sample rarefaction and standardization were performed to adjust for library size differences. Shannon diversity metrics were calculated, and boxplots were generated for different diet categories, CRP, and AGP levels. Wilcoxon Rank Sum test was used to assess statistical significance.

The core microbiome analysis was performed on “Exclusive Breast milk” and “Non-Breast Milk” diets with a detection threshold of 0.001 and a prevalence threshold of 0.1. The core microbiome analysis with the same detection and prevalence threshold was also performed on “elevated” versus “normal” CRP. Indicator species analysis was conducted on the “Exclusive Breast Milk” and the “Non-Breast Milk” and on the “elevated” CRP and AGP levels.

RESULTS

Six-month-old infants on the BM diet have significantly decreased gut microbial diversity and inflammation compared to infants on non-BM diet. To explore the effect of diet on gut microbial alpha diversity and inflammation level in six month old infants, Shannon diversity metrics and CRP level were quantified. Our findings revealed that infants on the exclusive BM diet recorded the lowest levels of both gut microbial diversity and CRP among all five diets studied (Fig. 1). In contrast, infants on the non-breast milk diet had the highest level of gut microbial diversity and CRP level (Fig. 1). Further, exclusive BM and non-BM diets were the only diets that had significant differences in both inflammation and gut microbial diversity, highlighting their impacts on infant health. Therefore, we decided to concentrate the rest of our study on these two diets to better understand their unique influences on the gut microbiome and inflammation in infants.

Anemia does not significantly change the gut microbial diversity and CRP level in six-month-old infants on BM and non-BM diets. To explore the effect of anemia on gut microbial alpha diversity and inflammation level in six-month-old infants, the Shannon diversity metrics and CRP levels were measured. It was crucial to investigate if there were any correlations or differences in the gut microbial diversity and inflammation levels between anemic and nonanemic infants because anemia can affect various physiological processes (10, 11). However, our analysis did not reveal any significant differences in the inflammatory levels or gut microbial diversity when comparing anemic and nonanemic six-month-old infants on either BM or non-BM diets (Fig. 2). This suggests that anemia does not significantly impact the gut microbial diversity and inflammation state in six-month-old infants. Therefore, to increase our sample size and reliability in our results, we decided to continue the data analysis by pooling anemic and nonanemic samples for BM and non-BM diets.

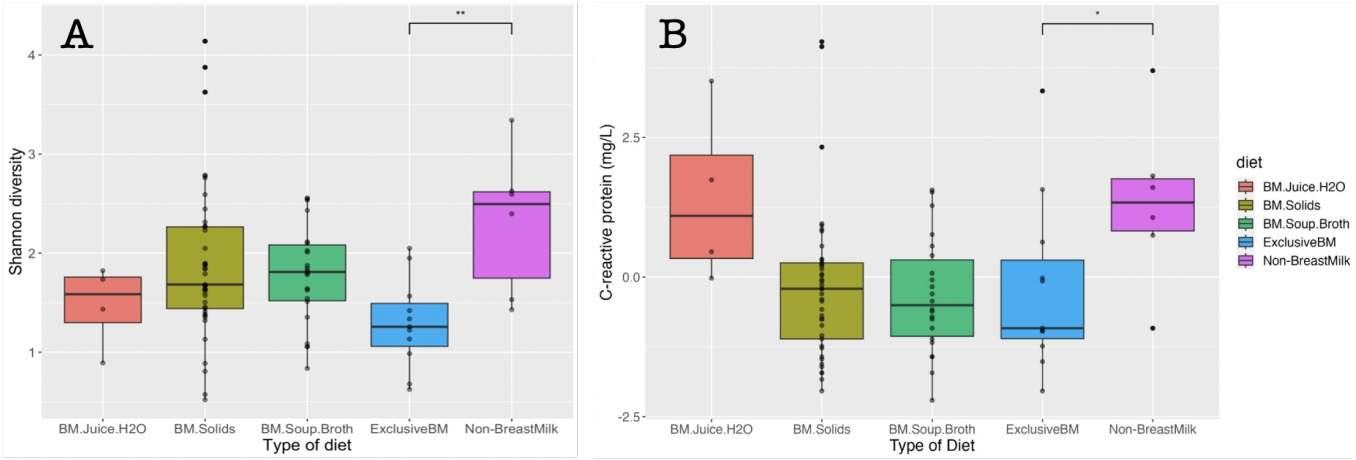

FIG. 1 Shannon alpha diversity and CRP levels are significantly increased in six-month old infants in non-breast milk diets only in comparison to those on an exclusive breast milk diet. A) Microbial alpha diversity across six-month-old infants taking different diets (P-value = 0.0048) (B) CRP levels across six-month-old infants taking different diets (P-value = 0.0346). For statistical significance, all the diets were compared to the non-breast milk diet. A Wilcoxon Rank Sum test was performed on the data in both panels. Error bars indicate standard error. * indicates a significant difference in either Shannon diversity or CRP level compared to non-breast milk diet (* =p<0.05, **= p<0.01). BM.Juice.H2O indicates a diet containing breast milk diet, juice, and water. BM.Solids indicates a diet containing breast milk and solid food. BM.Soup.Broth indicates a diet containing breast milk, soup, and broth. ExclusiveBM indicates a diet containing only breast milk. Non-BreastMilk indicates a diet does not contain breast milk. BM.Juice.H2O n = 4, BM.Solids n = 41, BM.Soup.Broth n = 22, ExclusiveBM n = 11, Non-BreastMilk n = 6.

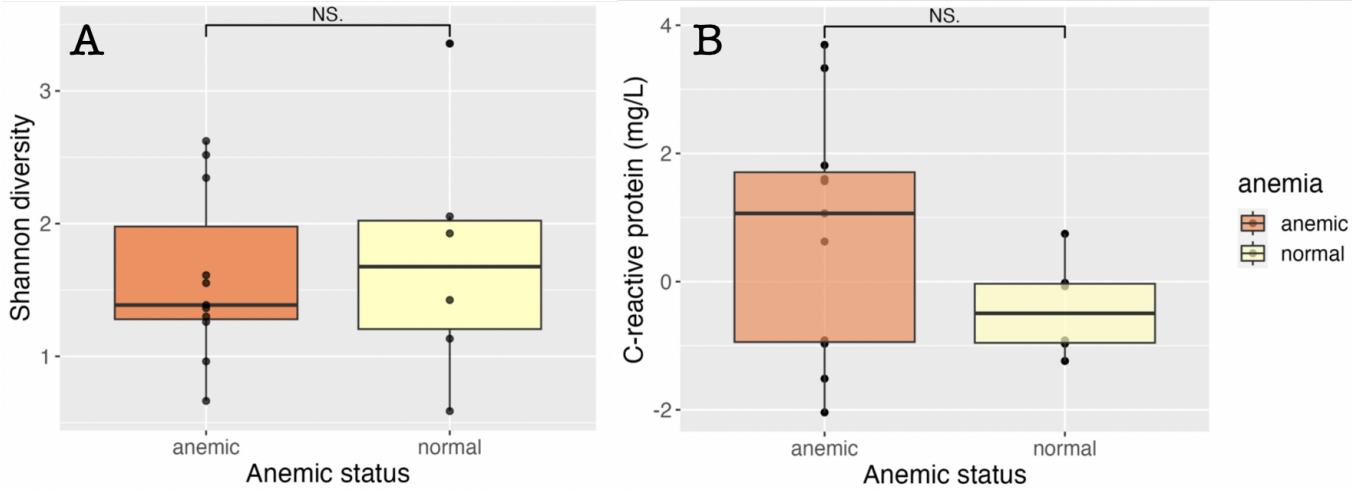

FIG. 2 There was no significant difference observed in Shannon diversity and CRP level between anemic and non-anemic six-month-old infants with exclusive BM or non-BM diets. A) Microbial alpha diversity between anemic and non-anemic infants measured by Shannon diversity (P-value = 0.08). B) CRP level between anemic and non-anemic six-month-old infants (P-value = 0.24). A Wilcoxon Rank Sum test was performed on the data. Error bars indicate standard error. NS = not significant. Anemic n = 11, Normal n = 6.

The number of unique microbial taxa were higher in the gut microbiota of six-month-old infants on a non-BM diet. Core microbiome analysis was performed on infants on the BM and non-BM diets (Fig. 3A). It was found that 75% of ASVs (Amplicon Sequence Variants) were uniquely associated with infants on a non-BM diet while only 6% were associated with infants on a BM diet. Additionally, core microbiome analysis was also performed on infants based on CRP levels (Fig. 3B) The results indicated that 47% of ASVs are uniquely associated with elevated CRP levels, while only 24% were uniquely associated with normal CRP levels.

This discrepancy highlights the correlation between inflammation, as indicated by CRP levels, and variations in the gut microbiota. Consequently, these differences in ASV

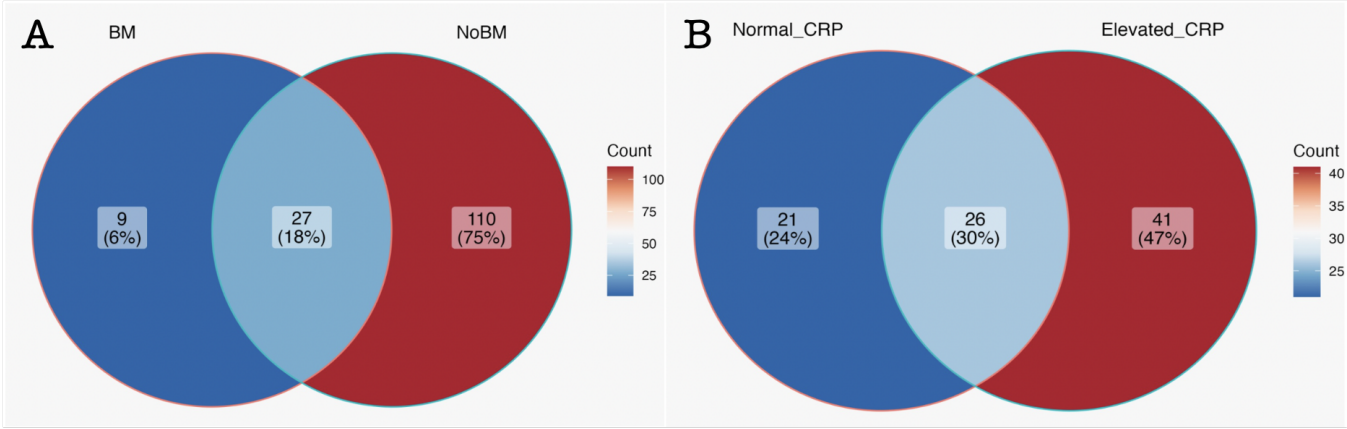

FIG. 3 There is a higher number of unique microbial taxa associated with six-month-old infants on the non-breast milk diet and six-month-old infants with elevated CRP. Core microbiome analysis of the gut microbiome in different diet groups and CRP-level groups. A) Venn diagram showing unique and shared ASV counts associated with BM and Non-BM diet groups. B) Venn diagram illustrating unique and shared ASV counts associated with different CRP level groups. In core microbiome analysis, the prevalence = 0.1, detection threshold = 0.001.

distribution led us to further investigate if there were any overlapping indicator genera in the gut microbiota of infants with a non-BM diet and those with elevated CRP levels.

Common gut microbiota between infants with elevated CRP and on a non-BM diet. Based on our previous findings, we observed that diet significantly increases microbial composition and CRP level (Fig 1). In addition, the number of unique taxa were also increased in both infants on a non-BM diet and those with an elevated CRP (Fig 3). To investigate potential overlapping of proinflammatory genera, we performed an indicator species analysis. The results identified 14 indicator species in the non-BM diet and five indicator species in the elevated CRP group (Table 1 and 2). Further, we found that three of the indicator genera

TABLE. 1 Indicator genera found in gut microbiota of infants with non-breast milk diets. Significant results are indicated by an asterisk (* = P<0.05, ** = P<0.01, *** = P<0.001). n = 17. The bold genera are the common indicator genera between non-breast milk diet and elevated CRP.

Genus	Diet	Observed Indicator Value (IV)	Significance
[Clostridium]_innocuum_group	Non-breast milk	0.8129489	*
Haemophilus	Non-breast milk	0.7876783	*
Bacillus	Non-breast milk	0.7071068	*
Lactococcus	Non-breast milk	0.7071068	*
Intestinibacter	Non-breast milk	0.7005555	*
Butyricicoccus	Non-breast milk	0.8109510	*
Subdoligranulum	Non-breast milk	0.9121717	**
Faecalibacterium	Non-breast milk	0.9082479	**
Anaerostipes	Non-breast milk	0.7071068	*
Roseburia	Non-breast milk	0.8164966	**
Blautia	Non-breast milk	0.9118964	*
Tyzzerella	**Non-breast milk**	**0.8164966**	***
Erysipelatoclostridium	**Non-breast milk**	**0.9810258**	*
g__Clostridium_sensu_stricto_1	**Non-breast milk**	**0.8103137**	*

TABLE. 2 Indicator genera found in gut microbiota of infants with elevated CRP. Significant results are indicated by an asterisk (* = P<0.05, ** = P<0.01, *** = P<0.001). n = 17. The bold genera are the common indicator genera between non-breast milk diet and elevated CRP.

Genus	Inflammation status	Observed Indicator Value (IV)	Significance
Romboutsia	Elevated	0.8164966	*
Howardella	Elevated	0.8164966	*
Tvzzerella	**Elevated**	**0.8153323**	*****
Erysipelatoclostridium	**Elevated**	**0.9810258**	*****
Clostridium_sensu_stricto_1	**Elevated**	**0.8103137**	*****

overlapped between elevated CRP and non-BM diet groups: *Erysipelatoclostridium, Tyzzerella,* and *Clostridium_sensu_stricto_1* (Table 1 and 2). All three have been shown to be potentially associated with pro-inflammatory effects as their increased abundance is correlated with inflammatory diseases like IBD (32-37). This overlap indicates a shared pro-inflammatory characteristic between the microbiota of infants on a non-BM diet and those with elevated CRP levels.

DISCUSSION

From our analysis, we noted a significant difference in alpha diversity and CRP levels between the BM and non-BM diets. However, within the BM and non-BM diet, there was no significant difference in gut diversity and CRP levels when comparing the anemic and nonanemic infants. Furthermore, we found an overlap of three indicator genera between infants on the non-BM diet and those with elevated CRP level. These suggest that the non-breast milk diet in six month old infants promotes inflammation, potentially by increasing pro-inflammatory microbial species in the gut.

Compared to BM diet, non-BM diet is associated with increased inflammation and alpha diversity. The increased diversity could be due to the introduction of various types of nutrients by non-BM diets, allowing for the growth of a more diverse range of microorganisms. This is while BM diet provides certain nutrients and selects for a less diverse range of species (38). Many studies in the field have observed breast milk to be a strong indicator of both microbial changes and development of the infant gut microbiome, potentially providing positive health benefits for developing infants (39, 40). In one study published by Mat et al., the gut microbial changes for infants who were fed exclusively breast milk versus those who fed on formula milk were investigated. They found a similar trend of infants on breast milk diet having decreased gut diversity than those on formula at early time points. However, there were no significant differences for 3 month and 6 month old groups (41). This contradiction to our result may be explained by the lack of information we have regarding the diet intake of participants within the dataset. While in the Ma et al., the authors took action in ensuring that the infants were fed exclusively the chosen diet from birth (41). The dataset we used collected their samples between August and November of 2014, and likely did not follow a long term study on the infant's dietary intake (14). The authors in the Ma et al. paper also indicated that in most research studies the infants are not fed exclusively one diet, while they also incorporated solid foods during ages 4-6 months, which can also affect gut microbial diversity (41).

There have been a number of studies that show a link between dietary intake and its effect on inflammation (42, 43, 44). These findings also extend to different milk diets, where researchers have shown that breast milk is associated with decreases in diseases that can induce severe inflammation (45, 46). This is congruent with our results shown through CRP levels. Past literature has also found that infants being breastfed for a longer duration of time generally show lower levels of CRP and inflammation (47, 48). This could be due to bioactive components in breast milk that can interact with the gut microbiome, leading to different inflammatory responses (46).

After looking into the effects of diet on microbial diversity and inflammation, we explored the effect of anemia on gut diversity and inflammation in 6 month old infants. We found that anemia did not significantly change the alpha diversity for all of the diet options,

including breast milk and non breast milk. Consistent with our results, in a study looking at the effects of iron deficiency anemia (IDA) on adult women, alpha diversity was not significantly different between normal, IDA and iron supplemented IDA patients (49). However, it should be noted that some studies have shown that anemia, such as IDA, can affect microbial composition, including increasing the abundance of certain bacteria such as *Enterobacteriaceae* (13).

Additionally, we observed that anemic patients had significant alterations in CRP levels when looking at all diet options. However, this effect was not significant when comparing our selected diets of only breast milk and non-breast milk**.** Similar to what we observed, when it comes to inflammation, the literature contains varied results. Multiple studies have shown that anemia is more likely present when a patient is infected by a disease that leads to higher rates of inflammation (50, 51). Righetti et al. also showed that there is a significant association between anemia and inflammation in children (52). While for all diets this significant association between anemia and inflammation was shown, a reason why it was not present for breast milk and non-breast milk diets can be due to a limited number of samples. Variation we saw can also be due to differences in types of anemia, as the type of anemia was not specified within the dataset we used, and also possible confounding variables, as factors like iron deficiency does not only lead to anemia (53). More research needs to be done on association of anemia and diet in an infant population.

At first, we found that both elevated CRP and the non-BM diet are associated with a higher number of unique microbial genera within the gut microbiota (56% and 75% respectively). Three overlapping indicator genera were found in the gut microbiota of infants with elevated CRP and those on a non-BM diet. These genera include *Tyzzerella*, *Erysipelatoclostridium* and *Clostridium_sensu_stricto_1*, which are interestingly associated with pro-inflammatory effects (32-37). Past Studies have found an overrepresentation of *Tyzzerella* in patients with Crohn's Disease (37). While research into this genus is limited, *Tyzzerella* has also been shown to be enriched among people with higher cardiovascular disease risk and ulcerative colitis (47,48). Additionally, *Erysipelatoclostridium* has been observed in both Crohn's Disease and clostridium difficile infection as a microbial gut biomarker (54), and among samples shown to be dramatically higher present in CD patients (34). Finally, *Clostridium_sensu_stricto_1* has also been associated with inflammatory diseases. *Clostridium_sensu_stricto_1* has been shown to be significantly increased in UC patients compared to the healthy controls, and is actually reduced to normal abundance once patients are treated with Mesalamine (35). Mesalamine, apart from its use as a treatment option for UC, can also potentially reduce the inflammatory response (35). It can be hypothesized that infants feeding on non-BM diets are likely increasing the presence and abundance of these proinflammatory genera relative to infants on a BM diet, and are possibly at an increased risk of certain inflammatory diseases. This finding is something we expected to see in our original hypothesis. The presence of these proinflammatory microbes within the 6 month infant population shows how a non-BM diet can promote inflammation through changes in microbial composition. However more research is recommended to see how these three microbes are present in IBD, and if non-BM diets elevate the risk of developing an inflammatory-mediated disease.

Limitations While most of our hypothesis was congruent with our results, there are a number of limitations that can affect our confidence in our analysis. One major limitation was our uneven sample distribution within the dataset. While there were 203 infants recruited (11), different filtering such as age, presence of anemia, and our narrow selection of BM and non-BM diets resulted in both a smaller and uneven number of samples, like BM.solids versus non-BM. This also led us to combine the solid and non-solid other milk categories, which prevented us from looking at multiple different milk categories and comparing them to the non-BM diet.

Another source of limitation was the lack of detail about the diets and the type of anemia in the dataset. While the dataset indicates if an infant is anemic (11), it does not provide detail on the type of anemia. This can limit the type of conclusion we can make, and once again also limits us in seeing if different types of anemia have varied effects on gut microbial diversity and inflammation. This same limitation is also present in the diet categories as the

dataset does not provide much information in both how long the infant has been exposed to a certain diet, or if the infants have also had any other forms of the diets. As previously mentioned, in most studies infants do not exclusively intake one type of diet (44), and not controlling for this can have an impact on the derived results.

Another potential limitation can be on how the data was collected, and the difference in time between when the serum sample and stool sample were collected by the researchers. In the original paper, McClorry et al. stated that they only evaluated stool samples that were collected less than or equal to 14 days of the serum sample time to control for different factors such as change in age or anemic status of infants (11). However, a difference of potentially 14 days can still mean that there is a lack of uniformity within the collected data, which can result in potential variation in the result. Furthermore, the authors themselves stated that they were unaware of when sample freezing occurred after defecation, and that collection of blood samples among participants were not uniform timewise, which can both affect results (11). In general, future research is needed to compare to our findings, and certain controls are needed to ensure uniformity across the collected data.

Conclusions Our paper aimed to evaluate the effect of diet and anemia on inflammation status and gut microbial composition in 6 month old infants. We found that diet significantly affects the alpha diversity of the gut microbiome and inflammation, marked by CRP. We found that anemic status did not significantly affect alpha microbial diversity and inflammation level among 6 month old infants for BM and non-BM diets. Further analysis of BM and non-BM diets revealed that in both elevated CRP and non-BM diets, there is a higher abundance of unique microbes. Indicator species analysis also revealed overlapping of the genera *Tyzzerella*, *Erysipelatoclostridium* and *Clostridium_sensu_stricto_1* among non-BM and elevated CRP groups, which have also been shown to be present in IBD. Thus, our research provides a foundation to further analyze these microbial genera, and see if they are also present within the gut microbiota in certain anemic or diet based groups also associated with higher inflammation.

Future Directions In future studies on this topic, it is essential to control for extraneous variables that can affect the gut microbial composition, such as iron supplement, and antibiotics. Iron supplements in general can directly affect the gut microbial composition. The subjects in the dataset used for this study were given different iron supplements which can have confounding effects on the result of the study. Gender might also affect how anemia impacts the gut. Therefore, future studies using this dataset need to consider both iron supplement usage and age of the infants. Moreover, in this study we focused on six month old infants. Other studies can also look at the 12 month old infant population of the dataset. They can also analyze how different metabolites, such as butyrate, are associated with inflammation status and gut microbial composition of infants. Additionally, this study focused on anemia in general. Future research can investigate the effect of different types of anemia on inflammation and gut microbiota in infants. Furthermore, this study illustrates the importance of diet for maintaining a healthy gut microbiome and inflammation level in infants. This shows the need for generating non-BM diets that maintain healthy gut microbiome and inflammation level in infants.

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CONTRIBUTIONS

Overall, all authors contributed equally to both the data analysis and writing process and placement of authorship should be viewed as equal. Negarin contributed in writing the abstract and introduction. Soroush and Pooya were involved in making figures and writing the results. Sourena contributed in writing the discussion, limitation and conclusion, with Negarin helping write future directions. During the data analysis, Sourena and Negarin were involved with QIIME2, and Sourena, Soroush and Pooya worked on R. Pooya was mainly involved in the latter core microbiome and indicator species analysis on R. All were heavily involved in the editing process of the manuscript.

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