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Increase in gut microbiota taxonomic sensitivity to inflammation from 6 to 12 month infants

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SUMMARY Inflammation is a biological response typically indicative of a diseased state, and long-term inflammation is also potentially pathological. Recent studies have highlighted the important relationship between the gut microbiota and inflammation in the human body, identifying a potential avenue of new therapeutics. However, this relationship remains to be comprehensively explored in infants. As such, the objective of this study is to investigate the relationship between gut microbial composition and inflammation in 6 and 12 month old infants. In this study, inflammation markers C-reactive protein and α1-acid glycoprotein were used to examine the overall diversity differences as well as taxonomic changes between infants with high and low inflammation levels. It was found that overall diversity of infants is not significantly impacted by inflammation levels in both 6 and 12 month old infants. By contrast, taxonomic changes reflect an increasing association of specific taxa with inflammation levels as infants age from 6 to 12 months old. These results suggest a potential age-related correlation between the gut microbiome of infants and inflammation status. Overall, this study demonstrates the need to pursue further research in this area to allow for the development of potential early diagnostic tools vital for early intervention and treatment for inflammation in infants.

INTRODUCTION

Unlike most adults, infants have an underdeveloped gut microbiome and an immature immune system (1). When an infant is born, microbial colonizers that initially settle in the gut originate from the mother's vaginal microbiome (1). During their early years, infants are exposed to different factors that affect microbial composition and diversity in the gut, such as varying feeding patterns and antibiotic and environmental exposures (1). These exposures also influence the development of an infant's immune responses against pathogens particularly in the mucosal tissues as the establishment of host-microbiota symbiosis helps in the maturation of the immune system (2). When an infant reaches an age of approximately 2 to 3 years, a set of microbial taxa is established in their gut, and they eventually develop an adult-like gut microbiome (3). Importantly, while the majority of microorganisms in the human gut are non-pathogenic and play an integral role in metabolism and intestinal barrier function (4,5), several studies have also successfully linked dysbiosis, the disruption of the gut microbiota, with inflammatory diseases including Crohn's disease, irritable bowel syndrome, and allergic asthma (6-8). Nevertheless, understanding of the exact relationship between the gut microbiota and inflammatory disease progression is still not robust (7,8).

Inflammation is a defense mechanism typically activated in response to harmful stimuli and supports the survival and homeostasis of tissues upon infection or injury (9). Nonetheless, long-term inflammation leads to reduced tissue function and possible disease states when improperly managed (9). While the exact inflammatory response can vary depending on the

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location and type of stimuli, inflammation processes frequently share inflammatory markers as a common mechanism to recruit inflammatory cells, and these biomarkers can be measured to predict inflammation levels (10-13). Specifically, acute-phase proteins C-reactive protein (CRP) and α1-acid glycoprotein (AGP) are often used clinically as markers of inflammation (13,14).

CRP is a protein synthesized in the liver typically found at concentrations < 10 mg/L but will rapidly rise and can peak within 48 hours of initiating an inflammatory disease state (14). AGP is a plasma protein that complements CRP as an inflammatory biomarker; while CRP rises and falls rapidly in response to stimuli, AGP levels change more gradually, reflective of recovery or long-term inflammation (12,14,15). Investigation of the relationship between gut microbiota composition and inflammation may lead to new early diagnostic tools to address inflammatory diseases in infants.

Studies on the relationship between the gut microbiome and inflammation are limited and conflicting. Research in animal models and of non-infant humans have noted that gut dysbiosis can lead to changes in intestinal barrier permeability and can cause low grade inflammation, indicating that inflammation in infants may be correlated to a different microbiome composition (16). Nevertheless, a similar study by Kamng'ona et al. notes that although microbiota diversity may be linked to inflammation, their findings were overall inconsistent (17). The absence of consistent results highlights the necessity of more in-depth studies on infant microbiomes and the importance of this research as a contribution to the field. This study aims to investigate the relationship between the gut microbiome and inflammation in infants. CRP and AGP serum concentrations and fecal microbiome sample data from McClorry et al. were analyzed to assess the difference in gut microbial composition correlating to the presence or absence of inflammation at two different timepoints: 6 months and 12 months. The results of this study will address the knowledge gap in infant microbiome studies by elucidating the potential role of the microbiome as an early diagnostic tool to assess infant health (18).

METHODS AND MATERIALS

Dataset used in this study. The 'Anemia in Infancy' dataset was downloaded from the European Nucleotide Archive (accession: ERP104978) (18) and was originally published by McClorry et al. (18). The subjects analyzed included 82 non-anemic 6 and 12 months old infants used as controls in the original study (18). Subjects were residents of Moronacocha, Iquitos, Loreto and recruited at the Moronacocha Health Center in Iquitos, Peru. Fecal samples underwent 16S ribosomal RNA sequencing. The V4 region of the 16S ribosomal RNA was amplified using polymerase chain reaction (PCR) and the F515-R806 primer pair. Sequencing was completed by the University of California, Davis, Genome Center (DNA Technologies Core) with the Illumina MiSeq platform (Illumina). Venous blood samples were collected without anticoagulants and stool samples were collected by caregivers from diapers at home. Blood serum samples were shipped to the laboratory of Jurgen Erhardt for assessment of a range of serum biomarkers, including C-reactive protein and α1-acid glycoprotein.

Initial data processing using the Quantitative Insights into Microbial Ecology 2 (QIIME2) pipeline. Raw 16S ribosomal RNA (rRNA) sequences were imported using a manifest file and demultiplexed using the bioinformatics tool QIIME2 (v2021.11) (19). For quality control purposes, a denoising step was performed on the demultiplexed sequences using the Divisive Amplicon Denoising Algorithm 2 (DADA2) (20) pipeline, in which the sequences were truncated to a length of 250 bp, and low-quality reads were removed. After denoising, sequences were clustered into amplicon sequence variants (ASVs), and a table showing relative frequencies and number of reads of the ASVs per sample was generated. Taxonomic classification was conducted on the ASVs using a Naïve Bayes classifier trained after alignment with the SILVA database (SILVA 138 SSU Ref NR 99) (21,22). Multiple sequence alignments by the Multiple Alignment using Fast Fourier Transform (MAFFT) (23) program were performed to create a rooted phylogenetic tree required for further downstream

analyses (Figure 1). Non-bacterial ASVs were filtered out from the samples by excluding taxa that do not have chloroplasts or mitochondria.

The samples were further filtered to include only healthy infants with no anemia and no parasites in 6-month-old and 12-month-old cohorts. The ASV table, taxonomy file, and rooted tree were exported from QIIME2 into RStudio for downstream analysis. The QIIME2 workflow that mentions specific tools used in this processing step is detailed in the QIIME2 script.

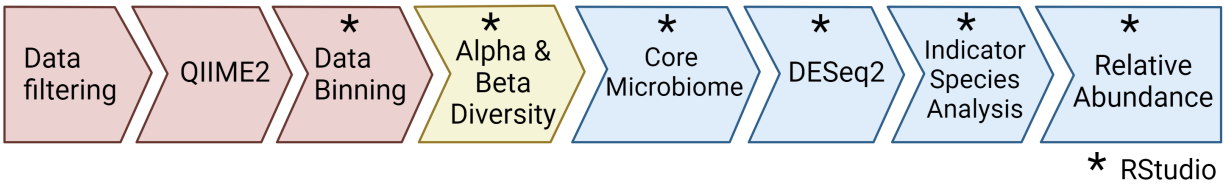

FIG. 1 The workflow for this project was designed to address three different goals. Data preparation, filtering, and binning (red) were conducted prior to diversity analysis (yellow) and individual taxa changes between conditions (red). Created with BioRender.com.

Initial data binning and processing using RStudio. The original dataset was first filtered to remove infants with anemia or parasitic infections, and all other infants were classified as healthy. This metadata was then separated into 6 and 12 month age groups, which were then further divided based on inflammation biomarker levels. AGP and CRP serum concentrations were classified as high or low whether they were above or below the clinical guidelines (1 g/L for AGP and 5 mg/L for CRP) or the median serum concentration across all healthy infants within the given age category, respectively. A phyloseq object was then created using the phyloseq package (v1.42.0) (24) for each age group using the metadata from McClorry et al.'s study, the exported ASV table, taxonomy file, and rooted tree from the QIIME2 pipeline. Further filtering was applied to remove non-bacterial sequences, ASVs with less than 5 total counts, samples with less than 100 reads, and samples with no CRP/ AGP level.

Alpha and beta diversity analysis. Each phyloseq object was rarefied on RStudio at a depth of 15000 sequences per sample to maximize sample richness and to preserve enough samples for further analyses. Alpha and beta diversity metrics were produced with the following R packages in R: tidyverse (v1.3.2) (25), vegan (v2.6-4) (26), phyloseq (v1.42.0) (24), ggplot2 (v3.4.1) (27), and ggsignif (v0.6.4) (28). For each cohort, Shannon alpha diversity and beta diversity metrics were calculated and statistical significance ($p<0.05$) was determined using the Wilcoxen rank sum test and Permutational Analysis of Variance (PERMANOVA). Clustering was completed at a 95% confidence interval.

Differential abundance analysis. To conduct the differential abundance analysis (DESeq2) on AGP and CRP groupings on two timepoints of 6 months and 12 months, the following R packages were used: tidyverse (v1.3.2) (25), vegan (v2.6-4)(26), phyloseq (v1.42.0)(24), ggplot2 (v3.4.1) (27), ape (v.5.7) (29), and DESeq2 (v1.34.0) (30). From the non-rarefied phyloseq object, the samples were manipulated to add 1 read pseudocount to achieve minimum nonzero values. Where the log2FoldChange ratio of either AGP or CRP was High/Low, statistical significance was defined as an adjusted p-value of < 0.05, and only significant ASVs that had a log2FoldChange greater than 2 were retained. The ASVs were mapped to their genus taxonomic level for ease of interpretation, and the results were plotted using a matrix to include both timepoints for each panel in Figure 5.

Indicator species analysis (ISA). To further elucidate on the differences in the gut microbiome composition between infants with and without inflammation at 6 months and 12 months of age, an indicator taxa analysis was performed in RStudio. The R packages used in this analysis were dplyr (v1.1.0) (31), phyloseq (v1.38.0) (24), and indicspecies (v1.7.12) (32). Using the phyloseq object, ASVs were agglomerated to the genus level, and bacterial counts were converted into relative abundance. Then, the indicator taxa were determined using the multipatt function, which identifies genera that are particularly prevalent/abundant

in one or more groups with a p-value of < 0.05. A detailed outline of the methodology of this analysis can be seen in AGP master and CRP master scripts.

Core microbiome analysis. To determine the shared taxa between different groups in each cohort, a core microbiome analysis was performed. In each age bracket, detection and prevalence parameters were set to 0 and 0.80 respectively to account for the small sample size of the study. R packages tidyverse (v1.1.0) (25), phyloseq (v1.38.0) (24), and microbiome (v1.16.0) (33,37) were used to conduct the analysis, while ggVennDiagram (v1.2.2) (34), ggeasy (v0.1.4) (27), RColorBrewer (v1.1.3)(35), and ggpubr (v0.6.0) (36) were used to create a four-way Venn diagram visualizing the core taxa for each age group. The core microbiome workflow indicating specific functions used is presented in the Core Microbiome script.

Relative abundance. Relative abundance plots were generated using RStudio for both Indicator Species Analysis and DESeq2 significant results independently. Bacterial count data were transformed to relative abundance using transform() within the microbiome R package (v1.16.0) (33,37) and filtered to only include significant indicator taxa ($p<0.05$). A pseudocount equal to the minimum nonzero abundance value was added to all abundances to improve plot readability. Statistical analysis was performed using the Wilcoxen rank sum test under the function stat_means_compare in the ggpubr package (36).

RESULTS

6 and 12 month infant cohort data was binned according to CRP and AGP levels independently. Metadata for 77 infants was organized into age groups, where there were 46 infants at 6 months, and 31 infants at 12 months of age (Figure 2). For both age groups, AGP levels were classified as high or low according to the clinically significant serum concentration seen in inflammation, which was 1 mg/L. Binning based on AGP classification resulted in four bins, including 6 month high AGP (10 infants), 6 month low AGP (36 infants), 12 month high AGP (14 infants), and 12 month low AGP (17 infants), as shown in Figure 2. Classifying the data according to the clinical CRP serum concentration (5 g/L), however, had very few infants with high CRP levels in both age groups. As a result, the median of the CRP serum concentrations for each age group were used to allow for large enough groups. The median concentrations were then implemented into classification, such that 6 month and 12 month thresholds for high CRP were > 0.50 g/L and ≥ 0.95 g/L, respectively. Binning based on CRP classification were 6 month high CRP (23 infants), 6 month low CRP (23 infants), 12 month high CRP (15 infants), and 12 month low CRP (16 infants) (Figure 2).

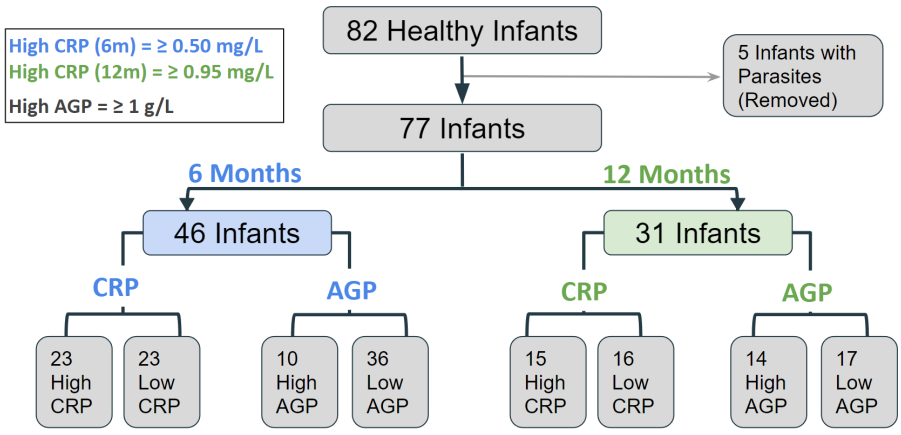

FIG. 2 Infant metadata was filtered and binned according to age and levels of the inflammatory markers. Healthy infant data were categorized into low and high CRP using an identified threshold for 6 month (0.50 mg/L) and 12 month (0.95 mg/L) age groups. Data for each age group was also categorized into low or high AGP using the clinical threshold concentration for inflammation (1 g/L). Infants with parasites were discarded.

Overall diversity of the gut microbiome does not show significant correlation to inflammation in infants between 6 and 12 months old. To explore how inflammation contributed to gut microbiome diversity in 6 and 12-month-old infants, we evaluated alpha diversity using Shannon's diversity index, a measure of community richness and abundance. With the AGP markers, results showed p values of 0.5274 and 0.9263 for 6 and 12 months old infants respectively, indicating that there was no significant difference in evenness and abundance of gut microbial composition between the low and high inflammation conditions (Figure 3a,c). Alpha diversity analysis of infants binned using CRP reflected similar results of insignificance (Figure 3b,d) where p = 0.6915 for 6 months old infants and p = 0.1781 for 12 months old infants. These trends of non-significance were further continued in beta diversity analysis as there were no obvious clustering patterns related to inflammation level for any of the cohorts (Figure S1, Table S2).

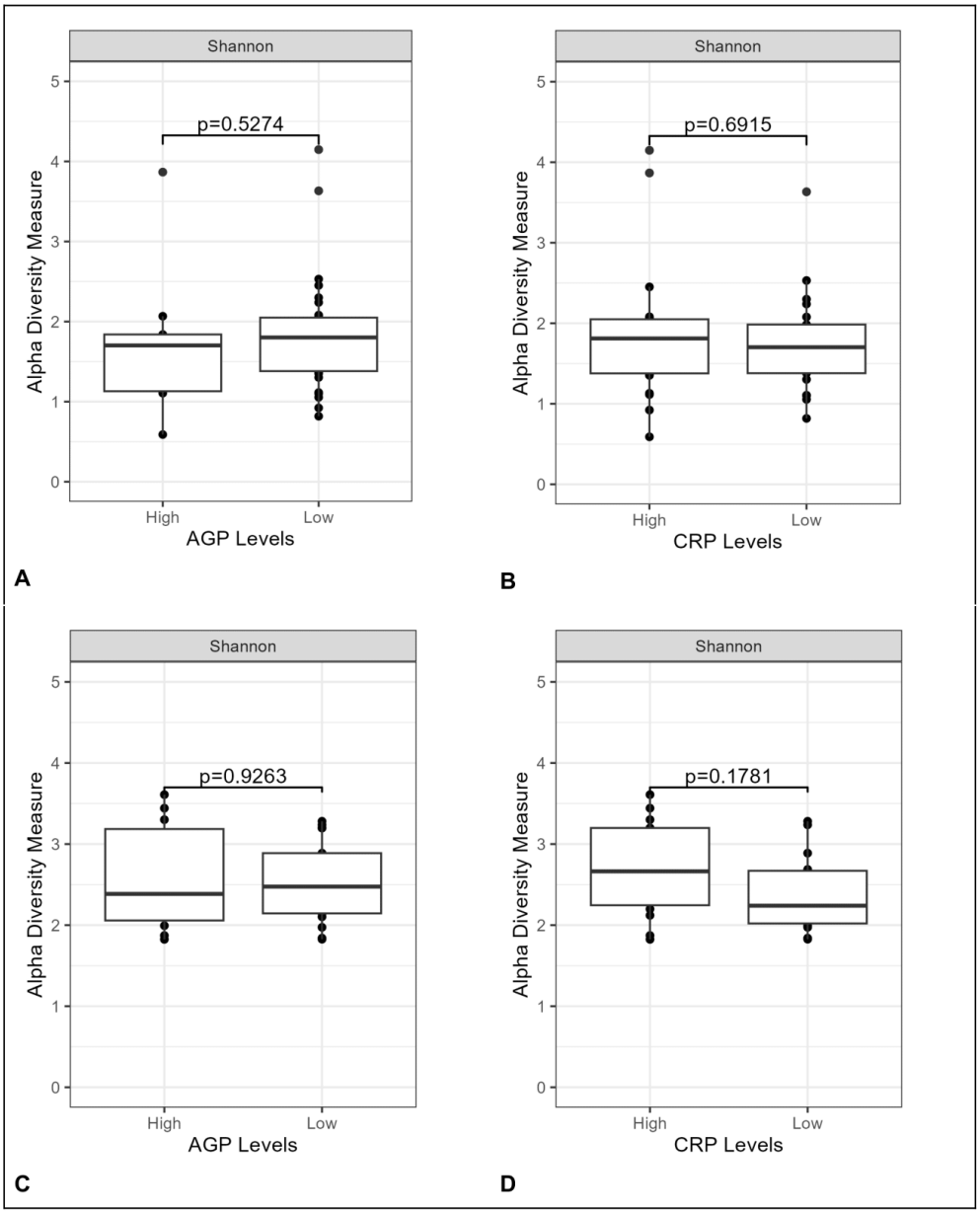

FIG. 3 Alpha diversity of infants is not significantly different between high and low inflammation levels. Boxplot visualization of Shannon diversity analysis correlating inflammation levels in infants and diversity within the cohort a) Comparison of 6 months old infants with high or low AGP levels b) Comparison of 6 months old infants with high or low CRP levels c) Comparison of 12 months old infants with high or low AGP levels d) Comparison of 12 months old infants with high or low CRP levels.

Core microbiome analysis shows an increase in the core taxa in infants from 6 to 12 months of age. Upon conducting core microbiome analysis on the 6-month and 12-month age groups, a higher number of shared taxa was observed in the 12-month-old infants (Figure 4). One taxon that is consistent in all groups in both age brackets is *Bifidobacterium*, which is a common genus in the infant gut. We further explored the ASV and performed a BLAST search, after which it was found that the ASV may be *Bifidobacterium longum* or *Bifidobacterium breve*, which are species that are known as 'infant-type' bacteria that dominate the infant gut in its early years (38). More taxa unique to specific groups were also observed at 12 months (Figure 4b) compared to 6 months of age (Figure 4a). We found *Actinomyces*, *Streptococcus*, *Anaerostipes,* and *Clostridium innocuum*, in infants with high CRP, low CRP, high AGP, and low AGP levels, respectively.

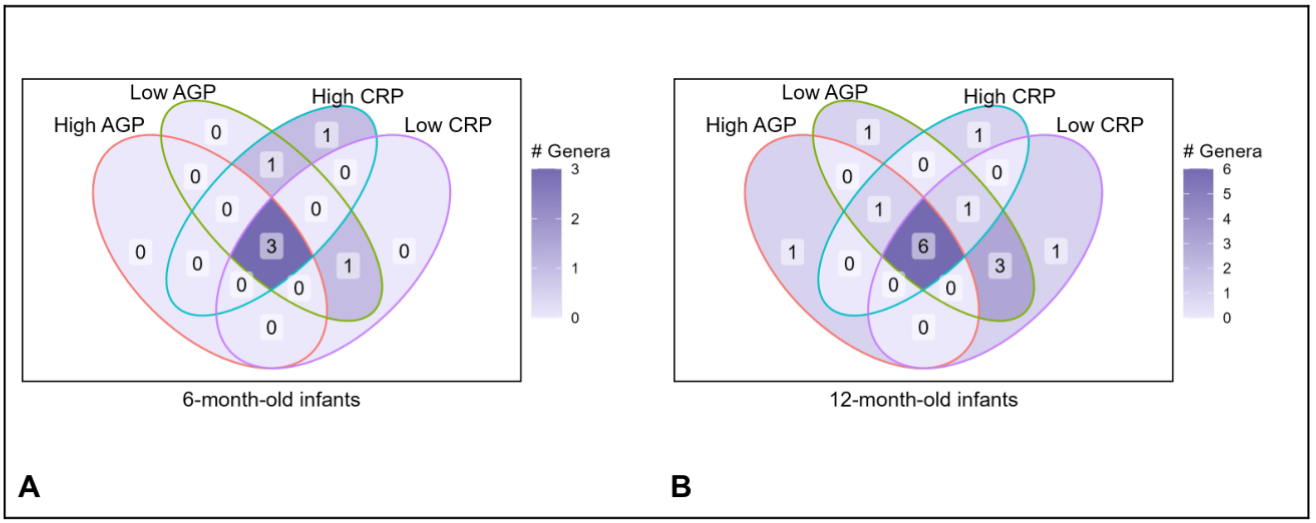

FIG. 4 An increase in the number of shared genera was observed in 12-month-old infants compared to 6-month-old upon conducting core microbiome analysis. Infants at A) 6 months of age showed fewer taxa prevalent in one or more groups (high/low AGP and high/low CRP) than at B)12 months of age.

More differentially abundant taxa is found in 12 month old vs 6 month old infants for both CRP and AGP cohorts. DESeq2 analysis was conducted to compare the differentially abundant taxa at the genus level between 6-month and 12-month-old infants using the stratified AGP and CRP cohorts (Figure 5). It was observed that a higher number of significant, differentially abundant genera was found in the 12-month-old infants when compared to the 6-month-old infants for both CRP and AGP groups. Specifically, the large increase of differentially abundant genera was found to be primarily associated with the 'High' level of CRP, and for both the 'High' and 'Low' levels of AGP.

Indicator species analysis identified more genera that are prevalent/abundant in 12-month-old infants than in the 6-month cohort. After conducting an indicator species analysis in each group per age bracket, only the 'High AGP' group was found to have genera that are indicative of its inflammatory condition in 6-month-old infants (Table 1). In contrast, all four groups in the 12-month cohort were observed to have indicator taxa.

Relative abundance shows more consistent results with DESeq2 and ISA in 12 months than 6 months. Relative abundance analysis of the seven significant indicator species elucidated how taxa were distributed for high and low inflammation biomarker levels. Both *Coprococcus* and *Gemella* showed increased relative abundance in high CRP conditions compared to low CRP in the 12-month age group (Figure 6b). *Eubacterium eligens* showed significantly increased relative abundance in high AGP conditions (Figure 6c). Conversely, *Lachnospiraceae UCG-004* had significantly higher relative abundance in low AGP conditions. There were no significant changes in relative abundance in either CRP or AGP level comparisons in the 6-month age group (Figure 6a).

Relative abundance was determined for all significant differentially expressed taxa found using DESeq2 (Figure 7). No significant taxa were identified when determining relative

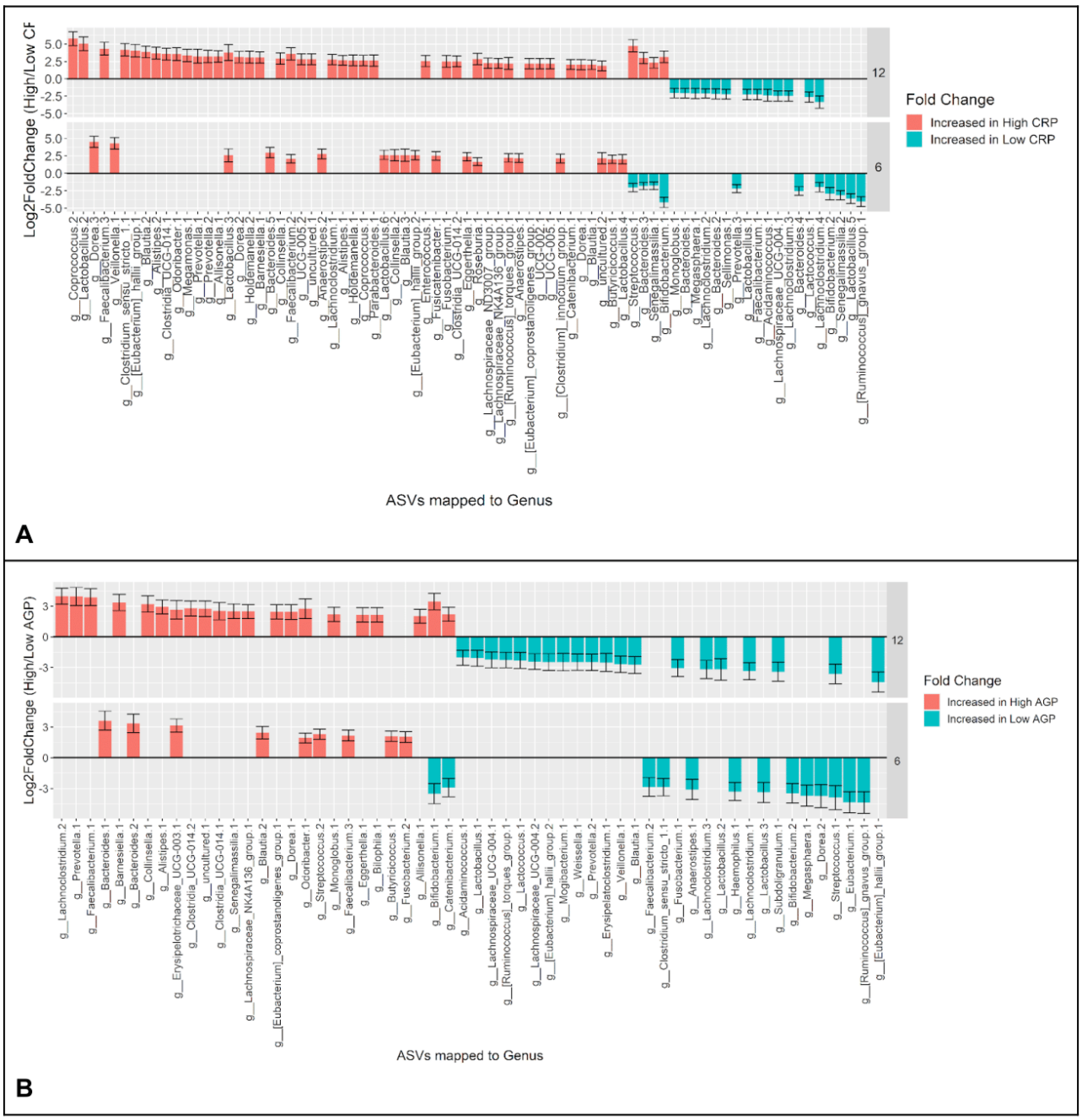

FIG. 5 Genera are more differentially abundant in 12 month infants when compared to 6 month old infants. Differential abundance analysis showing the log2fold change between different genera of the gut microbiome of infants at two different age timepoints, 12-months and 6-months. A positive log2foldchange value indicates greater abundance of a genus in the 'High' grouping while a negative log2foldchange value indicates a greater abundance of a genus in the 'Low' grouping of a) CRP and b) AGP respectively. Only statistical results of lower than 0.05 for adjusted p-value, and that had greater than 2 log2foldchange were included. The error bars correspond to the DESeq2 standard error.

abundance for both CRP and AGP conditions in the 6-month age group (Figure 7a,b). *Coprococcus* showed increased relative abundance in 12-month infants with high CRP levels compared to low CRP (Figure 7c). This result was consistent with relative abundance analysis of indicator species (Table 1, Figure 6). Regarding AGP levels at 12 months of age, *Erysipelotrichaceae UCG-003* showed higher abundance in infants with high AGP (Figure 7d). ASVs of these genera were searched using BLAST, but was unsuccessful in providing any further information on the associated species.

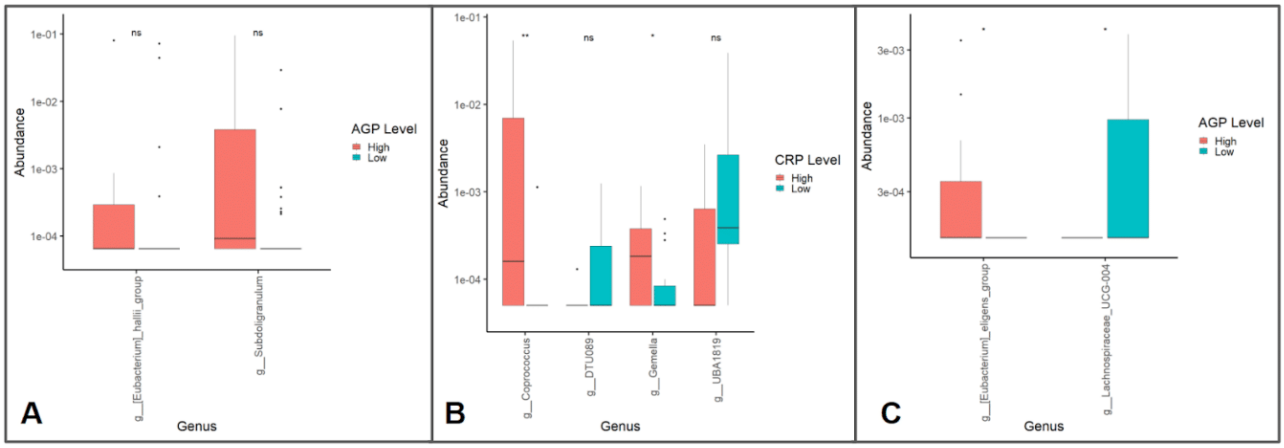

FIG. 6 Relative abundance of some species identified using indicator species analysis are significant in 12 month infants. Relative abundance plots were generated for results from indicator species analysis for 6 month infants with a) high vs low CRP, and 12 month infants with b) high vs low AGP and c) high vs low AGP. Plots were created using ggplot2 in RStudio. Statistics were performed using the Wilcoxen rank sum test. * $p \leq 0.05$, ** $p \leq 0.01$, ns = not significant.

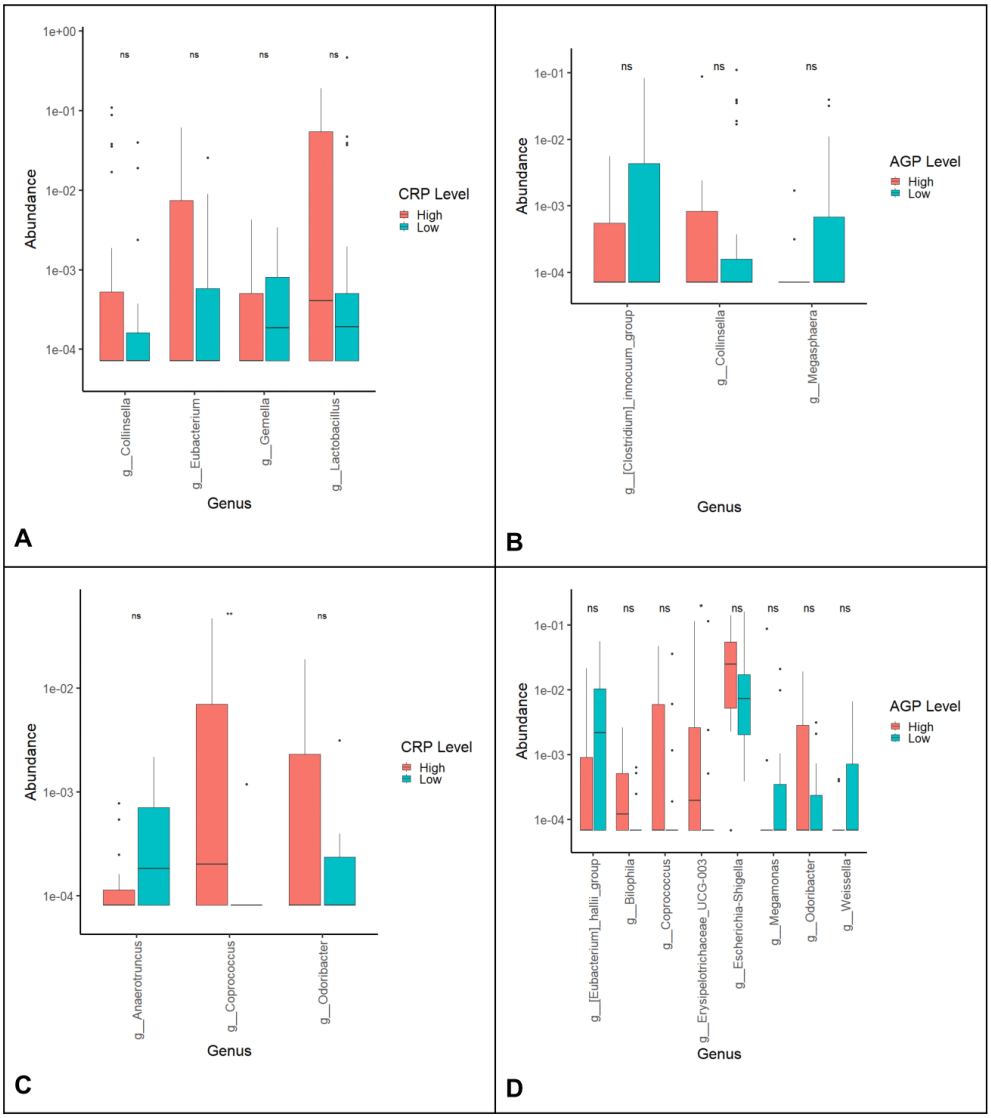

FIG. 7 Relative abundance of species identified through DESeq2 was significant for Coprococcus and Erysipelotrichaceae UCG-003 in 12 month infants. Relative abundance plots were generated for results from differential expression analysis for 6 month and 12 month age groups. Plots were generated using 6 month data for relative abundance in a) high vs low CRP and b) high vs low AGP conditions. The 12 month age group was plotted for b) high vs low AGP and c) high vs low AGP. Plots were created using ggplot2 in RStudio. The Wilcoxen rank sum test was used for statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, ns = not significant.

DISCUSSION

Differences in inflammation levels have no significant effect on gut microbial diversity of 6 and 12-month-old infants (Figure 3, S1). Interestingly, another study by Malawi et al. reported that microbial diversity was associated with high AGP at 6 months but not CRP (17). One possibility for this discrepancy is that AGP can be raised for different reasons; AGP is associated with both chronic inflammatory conditions and in individuals who have recently recovered from inflammatory conditions and are in the convalescent stage. While Malawi et al. does not specify if the infants from their study had chronic inflammation, the infants used in this study were healthy with no parasites, which may explain why AGP levels would not correlate with gut microbiome composition in our experiment (15). However, our study does corroborate Malawi et al.'s results for CRP; our experiment demonstrates that high CRP levels do not correspond to diversity changes in the gut microbiome. One possibility for this result is because our study stratified infants using CRP medians of 0.95 mg/L and 0.50 mg/L for 6-month-old and 12-month-old infants, respectively, both of which are noticeably lower than the clinical cut-off of 5 mg/L (13). Using the CRP median thresholds, many infants with high CRP were potentially only recently exposed to mild inflammation-inducing conditions or offending agents, so the inflammation level induced is subclinical, consequently resulting in non-significant data.

Interestingly, although the gut microbiota in infants demonstrates healthy maturation overall, there were increasing taxonomic changes associated with inflammation from 6 to 12 months old. Core microbiome analysis of 6 and 12-month-old infants show an increase in the genera shared by all infant cohorts regardless of inflammation level (Figure 4). Of the listed genera, *Bifidobacterium* was found in all cohorts and shared between 6 and 12-month-old infants (Table S2). *Bifidobacterium* is one of the genera that are drastically more abundant in infants than adults as they can utilize human milk oligosaccharides to produce short-chain fatty acids (SCFA) and is one of the most overrepresented bacterial genera in healthy infants, suggesting their importance in infant development (39,40). Specifically, SCFAs such as butyrate have been reported to have an anti-inflammatory impact in the gut by decreasing proinflammatory cytokine expression via inhibition of NFκB activation and IκBα degradation (41,42). Resultantly, *Bifidobacterium* dominance in infants has been linked to reduced colonization by organisms with antimicrobial genes, while its loss has been shown to increase the prevalence of obesity, diabetes, and metabolic disorders (43). As *Bifidobacterium* presence is primarily determined by consumption of milk, regardless if it is formula or breast milk, and has anti-inflammatory effects, we believe that diet may act as a confounding variable in the determination of the gut microbiome in infants with and without inflammation (39,44).

This reasoning is further verified in the examination of the 12 months core microbiome (Table S2). Diversification of the infant gut microbiome at 12 months begins to reflect normal adult gut microbiomes with the addition of the *Blautia, Streptococcus, and Ruminococcus gnavus* from the Firmicutes phylum and the *Bacteroides* from the Bacteroidetes phylum (45-47). Previous studies report that the introduction of Firmicutes and Bacteroidetes typically occurs with the introduction of solid food as bacteria in these phyla are able to digest plant polysaccharides and are also associated with omnivorous diets in various species (39,47-50). The overall impact of these new genera are varied. *Bacteroides* has been implicated in the modulation of obesity, inflammatory bowel disease (IBS), and neurodevelopment, while genera like *Streptococcus* are reported to downregulate pro-inflammatory activity in disease (51-54). Similarly, depletion of *Blautia* species has been associated with intestinal inflammation, while mouse model studies have demonstrated that the administration of certain *Blautia* species has been shown to reduce obesity and type 2 diabetes (55,56). By contrast, genera such as the *R. gnavus* can produce inflammatory polysaccharides implicated in inflammatory bowel diseases (57,58). The genera identified in the core microbiome analysis of 12-month-old infants are demonstrated to be associated with established trends expected of healthy infants transitioning from a milk diet to solid food rather than inflammation, further confirming infant diet as potential confounding variable in this study.

Nonetheless, we begin to see a general trend where the infant gut microbiota is more influenced by inflammation levels at 12 months compared to 6 months (Figure 4,5). There were a greater number of ASVs mapped to genera that were differentially abundant in 12

months old infants when compared to 6-month-old infants for both of the CRP and AGP groups (Figure 5). The trend was prominent in the 'High' CRP and in both 'Low' and 'High' AGP groupings of the DESeq2 suggests that the strength of the relationship between the gut microbiome and systemic inflammation was consistent regardless of the inflammation marker, but instead depends primarily on time. The increase of differentially expressed genera in 12 months corroborates the literature in that the gut microbiota is maturing and establishing itself during the first 2 to 3 years of life, increasing the abundance of different genera present in that natural process (8).

Furthermore, changes in relative abundance of *Lachnospiraceae UCG-004* and *Gemella* also reflect the development of taxonomic trends in response to inflammation from 6 to 12 months old. *Lachnospiraceae UCG-004* was found to be most prevalent in low AGP conditions at 12 months (Figure 6). *Lachnospiraceae* is a family of butyrate-producing bacteria, which are known to help inhibit inflammation in the intestine (59,60). Lower relative abundance of certain *Lachnospiraceae* bacteria has been confirmed in cases of inflammatory diseases such as inflammatory bowel disease (IBD) (61) and colitis (62), in addition to metabolic diseases, liver disease, multiple sclerosis syndrome, and chronic kidney disease (63). Research involving the *Lachnospiraceae UCG-004* genus specifically have also demonstrated similar findings, with decreased abundance in conditions involving inflammation including Parkinson's disease (64,65), coronary artery disease (66), and diabetes (67).

At 12 months, *Gemella* was found to be a significant taxon with increased relative abundance in inflammatory conditions of high CRP (Figure 6b). This is consistent with literature that has identified *Gemella* species, including *Gemella morbidillorum* and *Gemella haemolysans*, to be associated with several infections and diseases, such as cell carcinoma (68,69), sepsis (70), and endocarditis (71,72). Consequently, these opportunistic taxa support the results of the analyses performed in the 12 month cohort in this study.

However, several taxonomic changes in 6 and 12-month-old infant gut microbiota due to inflammation do not consistently reflect trends seen in literature. Specifically, several of the significant relative abundance taxonomic changes do not reflect what other studies have demonstrated, including *Coproccocus*, *Erysipelotrichaceae UCG-003,* and *Eubacterium eligens.* While there is little information linking *E. eligens* to AGP, current evidence shows that it has an anti-inflammatory effect (74). *E. eligens* has been shown to have a negative correlation with inflammatory markers, including IL-2 and CRP (75). This suggests that the results for the relative abundance observed were not likely a direct correlate of inflammation, which prompts the necessity of further research in the field to clarify the inconsistencies between different literature sources.

Coprococcus, which was significantly increased in high CRP conditions through both DESeq2 and indicator species analysis, is also known to have anti-inflammatory effects (Figure 6,7). Similar to *Lachnospiraceae UCG-004, Coprococcus* species produce butyrate, which can in turn be used to decrease hyperinflammation and increase anti-inflammatory responses (76). Studies have found that *Coprococcus* has a negative correlation with IBD in patients, and it has also been shown to protect the liver from inflammation in mice (77-80). While this information does not explain its increased abundance with high CRP, these samples may have contained different *Coprococcus* species that do not rely as heavily on butyrate-producing metabolic pathways.

Similarly, the genera *Erysipelotrichaceae UCG-003* shows conflicting research, despite showing higher prevalence in high AGP conditions at 12 months (Figure 7). Researchers have speculated that the inconsistent results seen in *Erysipelotrichaeceae UCG-003* may be due to different species in the genus having different immunogenicity profiles and inflammatory response mechanisms (81). Increased abundance of the genus is seen in the lumen of colorectal cancer patients in addition to patients with untreated infection, intestinal dysfunction, and bile acid metabolism disorders (82-84). Additionally, *Erysipelotrichaceae UCG-003* has a positive correlation with Th17 T helper cells, unique CD4+ T helper cells that produce interleukin-17, a highly inflammatory cytokine (85,86). This contradicts different evidence demonstrating that *Erysipelotrichaeceae UCG-003* bacteria are propionate producers, which often contribute to anti-inflammatory effects, as seen with lower abundance in cases of lung cancer and chronic atrophic gastritis (87-89). As shown with the

Erysipelotrichaceae UCG-003 genus, different results can arise from different species, which may have occurred in this case with *Coprococcus* and *Eubacterium eligens.*

Limitations The main limitation of this study is the low sample size of each group. As previously mentioned, the original dataset by McClorry et al. was used to study anemia so approximately half the subjects are anemic and were filtered out (18). Furthermore, infants undergo significant changes in their gut microbiome between 6 and 12 months of age, and the infants were further divided into their respective age groups to prevent age from acting as a confounding variable (3). The lack of infants with high CRP and AGP levels in each group reduces the capability to extrapolate conclusions with high statistical power (90).

One of the downstream consequences of the filtering and binning process is that there was a limited number of infants with clinically-significant levels of CRP, and the median CRP level was used for stratification instead. Since these median values were much lower than the clinical threshold, the results for CRP may not reflect true differences associated with presence or absence of inflammation. The median values for 6-month and 12-month data were also different from each other, which may have contributed to the differing results between age groups for CRP.

Conclusions This study analyzed the relationships between inflammation and gut microbial composition in 6 and 12-month-old infants. Our results revealed that although overall diversity of infant gut microbiomes is not significantly impacted by inflammation status, there begins to be a shift of more taxonomic changes being associated with inflammation as infants age from 6 to 12 months old. This demonstrates that as the gut microbiome ages and diversifies, it becomes more sensitive to and fluctuates with inflammation status. Determining the relationship between inflammation and infant gut microbiome composition provides a framework for future analysis using this dataset, however, it also identifies important trends that can be further explored in future studies to better understand relationships between infant gut microbiome and infant developmental processes. A better understanding of these dynamics could lead to the application of the infant gut microbiome to be used as an early diagnostic tool to quickly identify inflammation in infants for appropriate treatment.

Future Directions First and foremost, it is recommended that this analysis be repeated using a larger cohort of infants. This would allow for larger numbers of samples within each bin, which may reveal different and more significant results. A larger cohort size would also allow for stratification of infants according to clinically significant CRP levels.

Future studies using 18-month and/or 24-month-old cohorts can help determine whether this trend shows a continued increase in diversity in early infancy. Using an age group at approximately 2 or 3 years of age could also be used to see if this trend follows maturity of the microbiome, such that stabilization occurs when approaching an adult-like gut microbiome composition.

To conduct further analysis on the results determined in this study, it is recommended to perform functional analysis on species determined from core microbiome, DESeq2, and indicator species analysis. This analysis may elucidate why the specific genera identified in this study were associated with particular inflammatory conditions.

As mentioned in the discussion, diet may present a confounding variable in this study. It is recommended to bin the infants by diet categories to investigate how this variable impacts microbial composition in infants with and without inflammation.

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CONTRIBUTIONS

The initial conception and proposal for the project was determined by all authors. MAJ completed initial data processing using QIIME2 and core microbiome and indicator species analyses. CP performed binning of data, additional filtering and data processing in RStudio, as well as differential abundance analysis. ZM conducted the DESeq2 analysis, and JW completed calculation of diversity metrics and statistical analysis. All members participated in writing this manuscript, including editing and revisions.

DATA AVAILABILITY

Bash and R scripts developed for this project are available at the following GitHub repository: https://github.com/muradiz/MICB475_Project2

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