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CHARACTERIZATION OF THE GUT MICROBIOTA AND SYSTEMIC INFLAMMATION IN HIV-EXPOSED UNINFECTED INFANTS FROM A RESOURCE-LIMITED SETTING AT 6 WEEKS OF AGE

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Abstract – *Objective:* Infants born to mothers infected with human immunodeficiency virus (HIV) experience heightened morbidity and mortality, likely due to systemic immune dysregulation potentially linked to gut microbial dysbiosis. However, the degree of variation in biomarkers and systemic inflammation between HIV-exposed uninfected (HEU) infants and their HIV-unexposed uninfected (HUU) peers remains uncertain.

Materials and Methods: A total of 78 infants, including one set of twins, were enrolled in this study at approximately 6 weeks of age. We collected sociodemographic and clinical data, along with stool samples, to characterize the infant gut microbiota using 16S rRNA gene sequencing. Additionally, whole blood samples were obtained from the infants, and plasma was isolated for MesoScale Discovery (MSD) V-Plex assays to quantify plasma inflammatory and endothelial dysfunction biomarkers.

Results: Among the 78 infants investigated, 35.9% were exposed to HIV in utero and during breastfeeding. At 6 weeks of age, 84.6% of the infants were exclusively breastfed, while 15.4% were mixed-fed with fluids and semi-solids. The gut microbiota comprised predominantly of *Bifidobacterium* (56.6%), *Streptococcus* (21.8%), *Bacteroides* (5.4%), *Collinsella* (2.8%) and *Parabacteroides* (2.7%). We did not observe significant differences in infant stool Shannon (p=0.760) and Simpson (p=0.510) indices by infant exposure to maternal HIV. The gut microbiota composition (Bray-Curtis dissimilarity) did not differ between HEU and HUU infants. Furthermore, plasma inflammatory and endothelial dysfunction biomarker levels did not significantly differ between HEU and HUU infants (p>0.05 after multiple test corrections). Notably, several significant positive correlations were observed between inflammatory and endothelial dysfunction biomarker levels (p<0.05). However, no significant associations were found between gut microbial taxa relative abundances and plasma inflammatory or endothelial dysfunction biomarker levels.

Conclusions: Exposure to maternal HIV did not result in any discernible alterations in diversity of the infant gut microbiota, nor in levels of systemic inflammation and endothelial dysfunction biomarkers at 6 weeks of age. Additionally, breastfeeding practice had no significant impact on diversity and composition of the infant gut microbiota. Furthermore, the lack of significant association between taxa relative abundances and systemic inflammation suggests that exposure to HIV and different feeding practices did not significantly influence these parameters in this population.

Keywords: Early life HIV exposure, Infant gut microbiota, Systemic inflammation, Resource-limited setting.

INTRODUCTION

Maternal and environmental factors, such as breastfeeding practice and hygiene, are significant determinants of the gut microbiota during early life[1]. The early-life gut microbiota is crucial for infant health and immune maturation by providing protection against enteric infections[2,3]. Commensals, such as *Bifidobacterium*, *Bacteroides* and *Lactobacillus* are some of the first colonizers of the infant gut, which are beneficial to infant nutrition and immune development[3-5]. During early life, the development of the gut microbiota is constantly changing depending on infant exposures such as breastfeeding practices, pathogens, and antibiotics[6]. It has been shown that breastfeeding favors an increased abundance of commensals, whereas formula feeding causes the development of a more diverse, non-beneficial infant gut microbiota[7].

The majority of infants born to human immunodeficiency virus (HIV) infected women escape infection with HIV despite having an immature immune system, and they have possible prolonged exposure to HIV *via* breastfeeding[8,9]. Infant exposure to maternal HIV results in altered immune regulation with increased susceptibility to infection, reduced growth, morbidity, and mortality compared to HUU infants[10-12]. Some of the main phenotypical findings in HEU infants include increased immune activation with increased apoptosis as well as fewer naïve T lymphocytes[8,13]. For instance, in a Canadian study, exposure to high levels of HIV-1 viremia in utero caused low infant CD4+ T-lymphocyte counts at two months of age without perinatal transmission[14].

Moreover, in a Brazilian study with antiretroviral therapy (ART) controlled HIV, infants had increased IL-6 at birth, which persisted up to 6 months of age when compared to HUU infants[15]. Similarly, in Zimbabwean HEU infants at 6 weeks of age, C-reactive protein (CRP) was increased for up to 6 months after birth when compared to HUU infants[16]. However, findings were heterogeneous, necessitating a larger, well-designed study with appropriate control groups[11]. Several mechanisms have been suggested regarding the role of the gut microbiota in immune dysregulation, with some researchers suggesting induction of systemic inflammation mainly ascribed to lipopolysaccharide (LPS) activity[17].

Although the influence of gut microbiota on host health is well documented, its composition during early life remains insufficiently understood, particularly in the context of maternal HIV exposure during the era of lifelong ART. We aimed to determine the effects of maternal HIV exposure on the infant gut microbiota diversity and composition, including plasma biomarkers of systemic inflammation and endothelial dysfunction in HEU and HUU Zimbabwean infants at 6 weeks after birth. In addition, the effects of infant feeding practices and antibiotic use on the infant gut microbiota were explored in association with systemic inflammation biomarkers.

PATIENTS AND METHODS

Study Design

This cross-sectional study is part of the University of Zimbabwe Birth Cohort Study (UZBCS), which has been previously detailed and is registered at www.clinicaltrials.gov (NCT04087239)[18]. All infants received postnatal care service at four primary healthcare centers (Budiriro, Glenview, Kuwadzana, and Rujeko) in high-density areas of Harare, Zimbabwe.

Inclusion and Exclusion Criteria

The study participants were enrolled while seeking postnatal care services at the study sites. All mothers gave written informed consent and parental consent for the participation of their babies in the study. Women with health disorders, such as mental health problems impairing them from participating, were excluded from the study. We included all infants of HIV-infected and HIV-uninfected women enrolled in the UZBCS in 2019 who came to the study at 6 weeks of age.

Data Collection

Infant sociodemographic and clinical data were collected using IRB-approved questionnaires. The data was entered into a Research Electronic Data Capture (REDCap) database, a secure platform designed to support data capture for research studies[19]. All infants underwent infant physical examination, including anthropometric assessment, by trained and qualified nurses.

Infant HIV Screening

Whole EDTA blood was used for dry blood spots collected on Whatman filter paper and used for early HIV diagnosis as described previously[18].

Infant Fecal Sample Collection, DNA Extraction and 16S rRNA Sequencing

Fresh infant stool was collected from diapers using sterile tongue depressors into sterile cups, then aliquoted into 2 ml tubes prior to storage at -80°C. Infant stool samples were thawed on ice, and approximately 250 mg were used for DNA extraction using a QIAamp PowerFecal Pro DNA kit (Qiagen, Dusseldorf, Germany) as previously described[20,21]. Briefly, bacterial DNA was eluted in 70 µl of elution buffer and stored at -20°C prior to amplification. Amplification of the V5-V6 regions of the 16S ribosomal RNA (rRNA) gene was carried out with bacteria-specific primers (forward 5' CCATCTCATCCCTGCGTGTCTCCGACTCAGC-barcode-ATT-AGATACCCYGGTAGTCC 3' and reverse 5' CCTCTCTATGGGCAGTCGGTGATA CGAGCT-GACGACARCCATG-3') as previously described[22]. A polymerase chain reaction (PCR) cycle included an initial 5-minute denaturation step at 94°C, then 35 cycles of denaturation at 94°C for 1 minute, annealing for 20 seconds at 46°C, initial elongation for 30 seconds at 72°C and a final elongation for 7 minutes at 72°C[23].

PCR products were run on 1% agarose gels, and a product with approximately 350 base pairs (bp) was expected. Amplicons were further purified using the QIAQuick Gel Extraction Kit (Qiagen, Dusseldorf, Germany), and a Qubit double-stranded DNA (dsDNA) high sensitivity (HS) Assay Kit (Invitrogen, Carlsbad, CA, USA) was used to determine pooled DNA concentrations on a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). A sequence library was prepared with amplicon concentrations set to 26 pM. Amplicon sequencing was done using an IonTorrent PGM™ System (Thermo Fisher Scientific Waltham, MA, USA) and Ion PGM™ Sequencing kit as described previously[24].

Infant Systemic Inflammatory Biomarker Assessment

The mesoscale discovery (MSD) multi-spot V-plex assays (Rockville, MD, USA) were used to quantify pro-inflammatory and vascular injury/endothelial dysfunction immune markers in 60 µl of plasma. The assays were conducted adhering to manufacturer instructions as previously described[25]. The plates were read individually and in series after selecting standards and assigning dilution factors and sample names. The standard curve plots were verified, and the sample concentration output was captured. We analyzed two panels: the pro-inflammatory and vascular injury/endothelial dysfunction V-plex panels.

The pro-inflammatory panel included 10 immune biomarkers: interferon-gamma (IFN-γ), interleukin-1-beta (IL-1β), interkeukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12p70 (IL-12p70), interleukin-13 (IL-13) and tumor necrosis factor (TNF). The vascular injury/endothelial dysfunction panel included 4 immune biomarkers: serum amyloid A (SAA), C reactive protein (CRP), vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). The biomarker SAA was excluded from our analyses due to a calibration failure in one of the assays.

Statistical Analysis

Participant sociodemographic and clinical characteristics

Data was analyzed using R software version 4.2.2[26]. The Shapiro-Wilk test was used to check for normalcy in all continuous variables, which were summarized using either a median and interquartile range (IQR) or a mean ± (standard deviation; SD), depending on the distribution of the data. Comparison of continuous data by groups was carried out using the Mann-Whitney U test, Kruskal Wallis test, or Student *t*-test where appropriate. Counts and percentages were reported for categorical data, and associations were determined using the Chi-squared test or the Fisher's exact test where appropriate. From infant anthropometric weight, height, and age, z-scores were calculated using the R package z-scorer. Z-scores for weight for height, weight for age, and height for age were calculated to determine infant wasting, underweight, and stunting status.

Analysis of infant stool 16S rRNA gene sequence data

The generated fastq sequencing files from the Ion Torrent PGM™ System were processed in the Quantitative Insights into Microbial Ecology 2 (QIIME2) version 2021.11 pipeline as previously described[22,27,28]. Taxonomy was assigned to Amplicon sequence variants (ASVs) at a 97% sequence identity threshold using the *q2-feature-classifier* plugin and a Naïve Bayes classifier in QIIME2. Taxonomic assignment was done with the SILVA reference database[29].

A phyloseq object was generated using the feature table and mapping file in the R package *phyloseq*[30,31]. Samples with fewer than 2000 sequence reads were excluded from the analysis, while those with more than 2000 reads were retained for further investigation. Alpha diversity was assessed using the Simpson and Shannon indices, and Beta diversity was evaluated based on Bray-Curtis dissimilarity[30]. The Kruskal Wallis and Mann-Whitney U tests were used to compare alpha diversity by groups. Permutational Multivariate Analysis of Variance (PERMANOVA) and pairwise Adonis were used to test the significance of beta diversity comparison by groups[32].

The Multivariable Association with Linear Models (MaAsLin2) package was used to determine associations of the gut microbiota with categorical and continuous variables[33]. Multiple testing corrections for q-value were calculated using Benjamini-Hochberg (BH) false discovery rate (FDR) correction. Taxa were considered significant at $q < 0.05$. Plots and figures were generated using R with further color and font editing in Adobe Illustrator version 27.3.1. Spearman rho (ρ) correlation was used to determine the correlation between pro-inflammatory and vascular injury immune biomarkers. Correlations with a p-value < 0.05 were considered significant.

Analysis of infant plasma pro-inflammatory and vascular injury biomarkers

The Shapiro-Wilk test of normality was used to check for normalcy in the levels of pro-inflammatory and vascular injury/endothelial dysfunction biomarkers. Levels of biomarkers were compared as medians and interquartile range (IQR) between HEU and HUU infants. The Mann-Whitney U test was used to determine significance of any differences between biomarker levels. Further, Bonferroni correction was used to correct for multiple testing.

RESULTS

Infant Sociodemographic and Clinical Characteristics at 6 Weeks Postpartum

A total of 78 infants, including one set of twins, were enrolled in this study at 6 weeks postpartum. The socio-demographic and clinical characteristics of the infants are summarized in Table 1. The proportion of female infants was slightly higher in both groups, comprising 57.1% of the HEU (HIV-exposed uninfected) group and 60% of the HUU (HIV-unexposed uninfected) group. The age of the infants did not differ significantly between HEU and HUU groups. Overall, 84.6% of the infants were exclusively breastfed at 6 weeks of age, while 15.4% received additional feeding to breastfeeding. Within the mixed-fed group, all infants received additional fluids, such

TABLE 1. INFANT SOCIO-DEMOGRAPHIC AND CLINICAL CHARACTERISTICS AT 6 WEEKS POSTPARTUM (N=78) STRATIFIED BY HIV EXPOSURE STATUS. DATA IS PRESENTED AS MEDIAN (IQR) FOR CONTINUOUS VARIABLES AND COUNTS AND PERCENTAGES [N (%)] FOR CATEGORICAL VARIABLES.

Variable	HIV-exposed uninfected (HEU, n=28), including 1 set of twins	HIV-unexposed uninfected (HUU, n=50)	*p*-value
Sociodemographic data			
Age (days) [median (IQR)]	42 (39-45)	43 (42.3-46)	0.045
Infant sex			0.163
Male	12 (42.9%)	20 (40%)	
Female	16 (57.1%)	30 (60%)	
Infant currently living with a smoker			0.530
Yes	6 (21.4%)	7 (14.3%)	
No	22 (78.6%)	42 (85.7%)	
Infant feeding practices at 6 weeks after birth			
Infant breastfeeding status			0.521
Exclusive	25 (89.3%)	41 (82%)	
Mixed	3 (10.7%)	9 (18%) (missing = 1)	
Infant fed formula milk			0.649
Yes	1 (3.6%)	4 (8%)	
No	27 (96.4%)	46 (92%)	
Mixed fed other fluids besides breast milk			0.521
Yes	3 (10.7%)	9 (18%)	
No	25 (89.3%)	41 (82%)	
Mixed fed liquids and semisolids other than breast or formula milk			0.316
Yes	2 (7.1%)	8 (16%)	
No	26 (92.9%)	42 (84%)	
Mixed fed adult diet and solids			–
Yes	0	0	
No	28 (100%)	50 (100%)	
Clinical and anthropometric data			
Mode of delivery			0.065
Spontaneous normal	25 (89.3%)	50 (100%)	
Caesarean section	3 (10.7%)	0	
Current weight (kg) [median (IQR)]	5 (4-6)	4.6 (4-5)	0.424
Current length (cm) [median (IQR)]	53 (51.3-56)	54 (52-56)	0.559
Current head circumference (cm) [median (IQR)]	38 (37-39)	38 (37-39)	0.232

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as water and juice, alongside breast milk, and 83.3% had also been introduced to semisolids, such as porridge. Only 6.4% of the infants were receiving formula milk, and none had been exposed to an adult diet or solid foods (Table 1).

Anthropometric measurements of the infants showed no significant differences when stratified by maternal HIV exposure status. Only one infant had been hospitalized for jaundice since birth. Nutritional indicators, including wasting, underweight, and stunting, did not vary by HIV exposure status. Overall, wasting was observed in 12% of the infants, representing 11.1% of HEU and 12.5% of HUU infants. All HIV-exposed infants were confirmed to be uninfected with HIV at the time of the study visit.

The Infant Gut Microbiota Showed a Dominance of Bifidobacterium at 6 Weeks Postpartum

At 6 weeks postpartum, the infant gut microbiota was predominantly characterized by the presence of *Bifidobacterium*. Stool samples from 76 infants were successfully analyzed, with two samples unavailable on the day of the study visit. In total, 2,255,453 sequence reads were obtained, with individual samples ranging from a minimum of 9,723 to a maximum of 79,236 reads. The infant stool microbiota was composed mainly of the phyla Actinomycetota (58.7%), Bacillota (21.3%), Pseudomonadota (15.2%), and Bacteroidota (4.72%). At the genus level, the microbiota was dominated by *Bifidobacterium*, *Streptococcus*, *unclassified_Enterobacteriaceae*, *unclassified_Coriobacteriaceae*, *Bacteroides*, *unclassified_Lachnospiraceae*, *Clostridium_sensu_stricto_1*, *Parabacteroides*, and *Lactobacillus*, with decreasing relative abundance (Figure 1A).

TABLE 1 *(CONTINUED)*. INFANT SOCIO-DEMOGRAPHIC AND CLINICAL CHARACTERISTICS AT 6 WEEKS POSTPARTUM (N=78) STRATIFIED BY HIV EXPOSURE STATUS. DATA IS PRESENTED AS MEDIAN (IQR) FOR CONTINUOUS VARIABLES AND COUNTS AND PERCENTAGES [N (%)] FOR CATEGORICAL VARIABLES.

Variable	HIV-exposed uninfected (HEU, n=28), including 1 set of twins	HIV-unexposed uninfected (HUU, n=50)	*p*-value
Hospital admission since birth			1.000
Yes	0	1 (%)	
No	27 (100%) (missing = 1)	48 (%) (missing = 1)	
Wasted (weight for height) [median (IQR)]	0.8 (-0.12-2.45)	0.49 (-1.11-1.90)	0.249
Wasted			1.000
Yes	3 (11.1%)	6 (12.5%)	
No	24 (88.9%) (missing = 1)	42 (87.5%) (missing = 2)	
Underweight (weight for age) [median (IQR)]	2.98 (1.6-4.59)	2.48 (1.55-3.31)	0.401
Underweight			–
Yes	0	0	
No	27 (100%) (missing = 1)	48 (100%) (missing = 2)	
Stunted (height for age) [median (IQR)]	1.55 (1.17-3.13)	2.47 (1.44-3.33)	0.392
Stunted			–
Yes	0	0	
No	27 (100%) (missing = 1)	48 (100%) (missing = 2)	

Abbreviations: HIV – Human Immunodeficiency Virus, HEU – HIV-exposed uninfected, HUU – HIV-unexposed uninfected, IQR – Interquartile range, kg – kilograms, cm – centimeters.

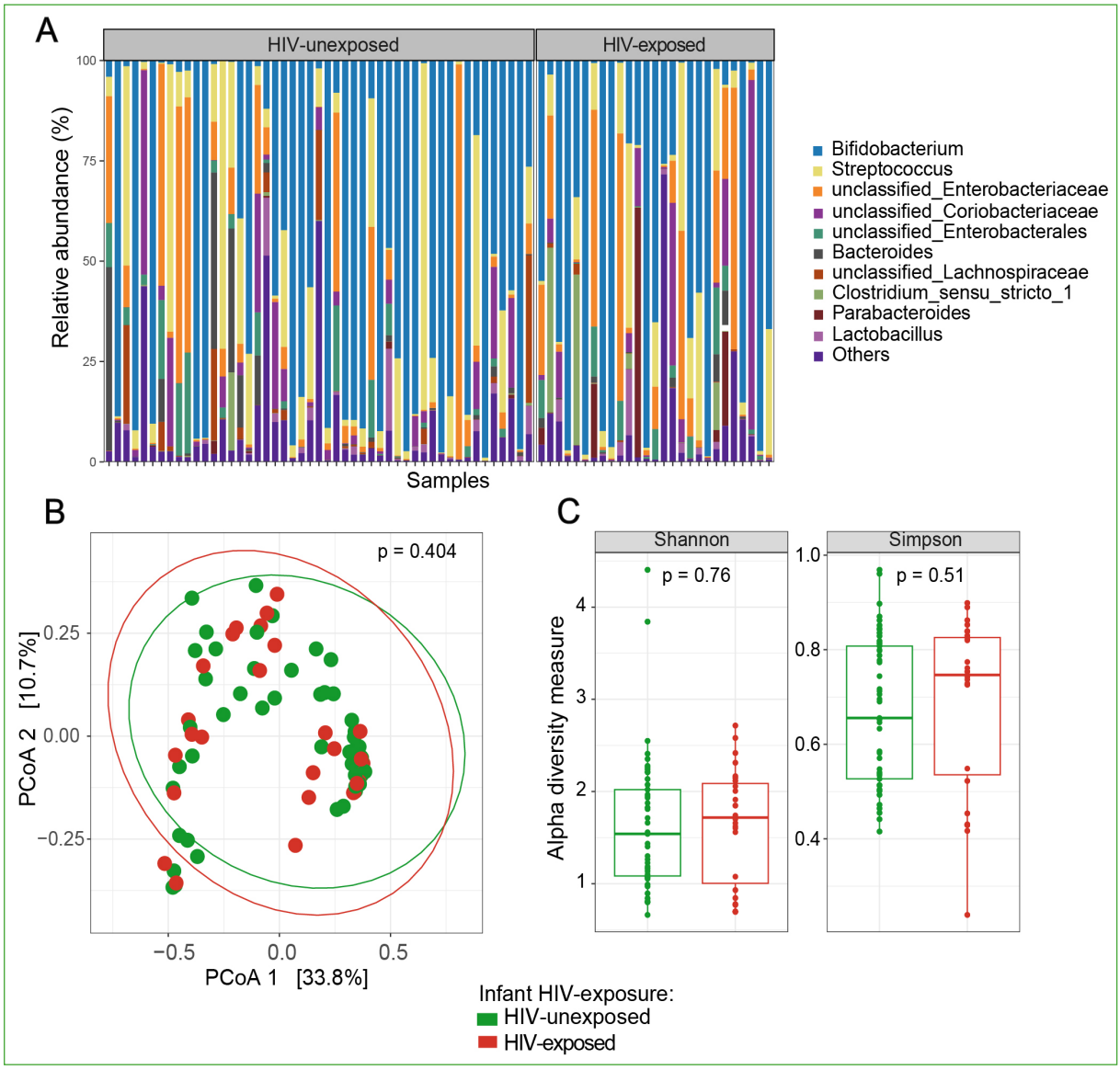

Figure 1. Diversity differences in infant stool. **A**, Relative abundance bar plot for top 10 genera compared by HIV-exposure status. **B**, Beta diversity (Bray-Curtis) comparison by HIV-exposure status. **C**, Alpha diversity comparison by HIV-exposure status.

Focusing on the identified and classified genera, the infant stool microbiota was primarily composed of *Bifidobacterium* (56.6%), *Streptococcus* (21.8%), *Bacteroides (*5.4%), *Collinsella* (2.8%), *Parabacteroides* (2.7%), *Clostridium_sensu_stricto_1* (2.3%), and *Lactobacillus* (2.0%). The relatively high abundance of *Streptococcus*, a taxon typically found on the skin, may be attributed to the transfer from the breast areolar skin during suckling.

The Infant Gut Microbiota Diversity and Composition Were Similar in HEU and HUU Infants at 6 Weeks of Age

We then compared the infant gut microbiota diversity by maternal HIV exposure status to determine the effects of exposure to HIV on microbial diversity and composition. There were no significant differences in alpha diversity indices (Shannon and Simpson) when comparing HEU *vs.* HUU infants (Figure 1B). Similarly, we did not find any significant differences in stool microbial composition when comparing the same groups (Figure 1C).

Furthermore, we compared infant gut microbial relative abundances of identified and classified genera by maternal HIV exposure, and none were significantly different as well ($p > 0.05$).

We further evaluated the impact of infant feeding practices on gut microbiota diversity by comparing exclusively breastfed infants with those receiving mixed feeding. The analysis revealed no significant differences in alpha diversity between the two groups, as indicated by similar Shannon (p=0.520) and Simpson (p=0.390) indices. Additionally, beta diversity, assessed using Bray-Curtis dissimilarity, showed no significant differences in gut microbial composition between exclusively breastfed and mixed-fed infants (p=0.296). The potential effects of antibiotic exposure on the gut microbiota could not be thoroughly investigated, as only one infant had received antibiotic treatment within one month prior to stool collection.

Infant Immune Biomarkers of Systemic Inflammation and Vascular Injury Are Similar When Compared by Maternal HIV Exposure

A subset of the infants (n=56) were randomly selected for assessment of systemic inflammation and endothelial dysfunction biomarkers with equal representation in both groups. There were no differences in the plasma pro-inflammatory and vascular injury/endothelial dysfunction biomarkers between HEU and HUU infants ($p > 0.05$). However, VCAM-1 levels were increased in the HEU group (p=0.033) but lost significance following Bonferroni multiple test correction (Figure 2).

Further, we computed correlations between the plasma immune biomarkers to determine any association between them. We noted positive significant correlations between CRP and IL-12p70 (ρ=0.34, p=0.009), IL-2 (ρ=0.28, p=0.036), VCAM-1 (ρ=0.39, p=0.002), ICAM-1 (ρ=0.65, $p < 0.001$), IL-4 (ρ=0.28, p=0.033) as well as IFN- γ (ρ=0.31, p=0.017), (Figure 3). Further IL-13 (ρ=0.32, p=0.014), IL-6 (ρ=0.34, p=0.009) and IL-8 (ρ=0.62, $p < 0.001$) correlated positively with IL-1β. VCAM-1 correlated positively with ICAM-1 (ρ=0.69, $p < 0.001$). TNF correlated positively with IL-1β (ρ=0.66, $p < 0.001$), IL-10 (ρ=0.50, $p < 0.001$) and IFN- γ (ρ=0.49, $p < 0.001$) (Figure 3).

Finally, we performed a MaAsLin analysis to investigate potential correlations between the relative abundances of infant gut microbial taxa and systemic pro-inflammatory and vascular injury/endothelial dysfunction biomarkers. The analysis revealed non-significant associations between the gut microbial taxa and systemic inflammatory or endothelial dysfunction biomarkers. However, the ability to detect associations was limited in certain cases due to the low prevalence of non-zero counts for many microbial taxa, which may have impacted the statistical power of the analysis.

DISCUSSION

In early life, the infants' gut microbiota is susceptible to perturbations from maternal factors. Our findings indicate that maternal HIV exposure, as well as mixed breastfeeding, did not significantly alter the infant gut microbiota at 6 weeks of age. We also observed similar levels of biomarkers of systemic inflammation when compared between HEU and HUU infants. However, the infant microbiota, as well as maternal characteristics and environmental factors, were not evaluated longitudinally, and their effects at other time points of the trajectory of infant microbiota and immune system development cannot be excluded.

Generally, the infants of our cohort were growing well, with none reported to be underweight or stunted. Wasting was prevalent, affecting 12% of infants, but without significant differences in weight for height z-scores when compared by HIV-exposure status. These results are inconsistent with a Nigerian study that found lower weight for age z-scores in HEU compared to HUU infants at 1 and 6 months of age[34]. In a rural Zimbabwean study done in 2020, HIV exposure in children aged 6-59 months resulted in a 3.6 times likelihood of stunting[35].

Overall, infants in our study demonstrated an increased relative abundance of the phylum Actinomycetota, comprising over 50% of the identified bacteria.

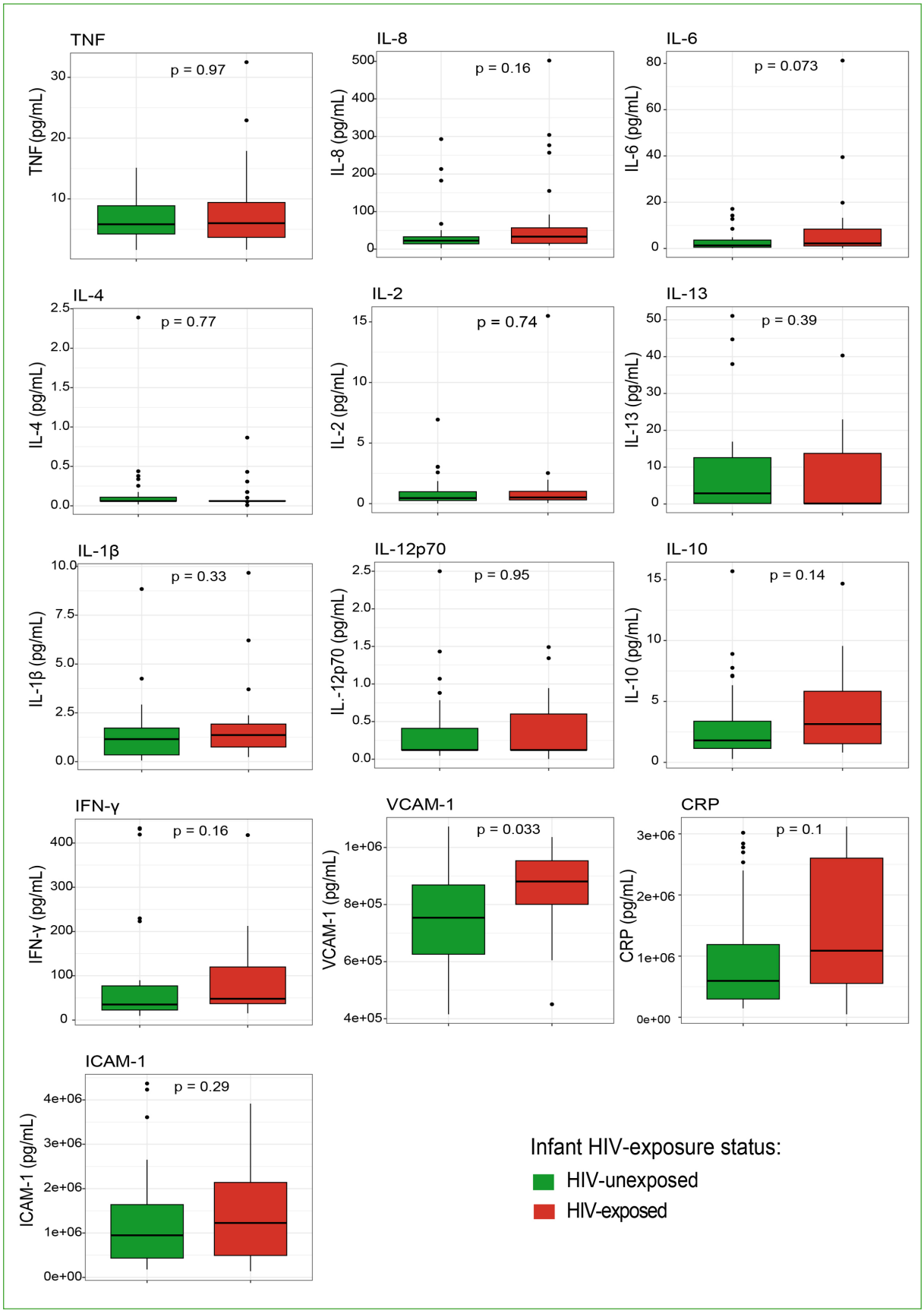

Figure 2. Infant plasma pro-inflammatory and endothelial dysfunction biomarker comparison by HIV exposure. Mann-Whitney-U test was used to test for significance. p-value < 0.05 was considered significant.

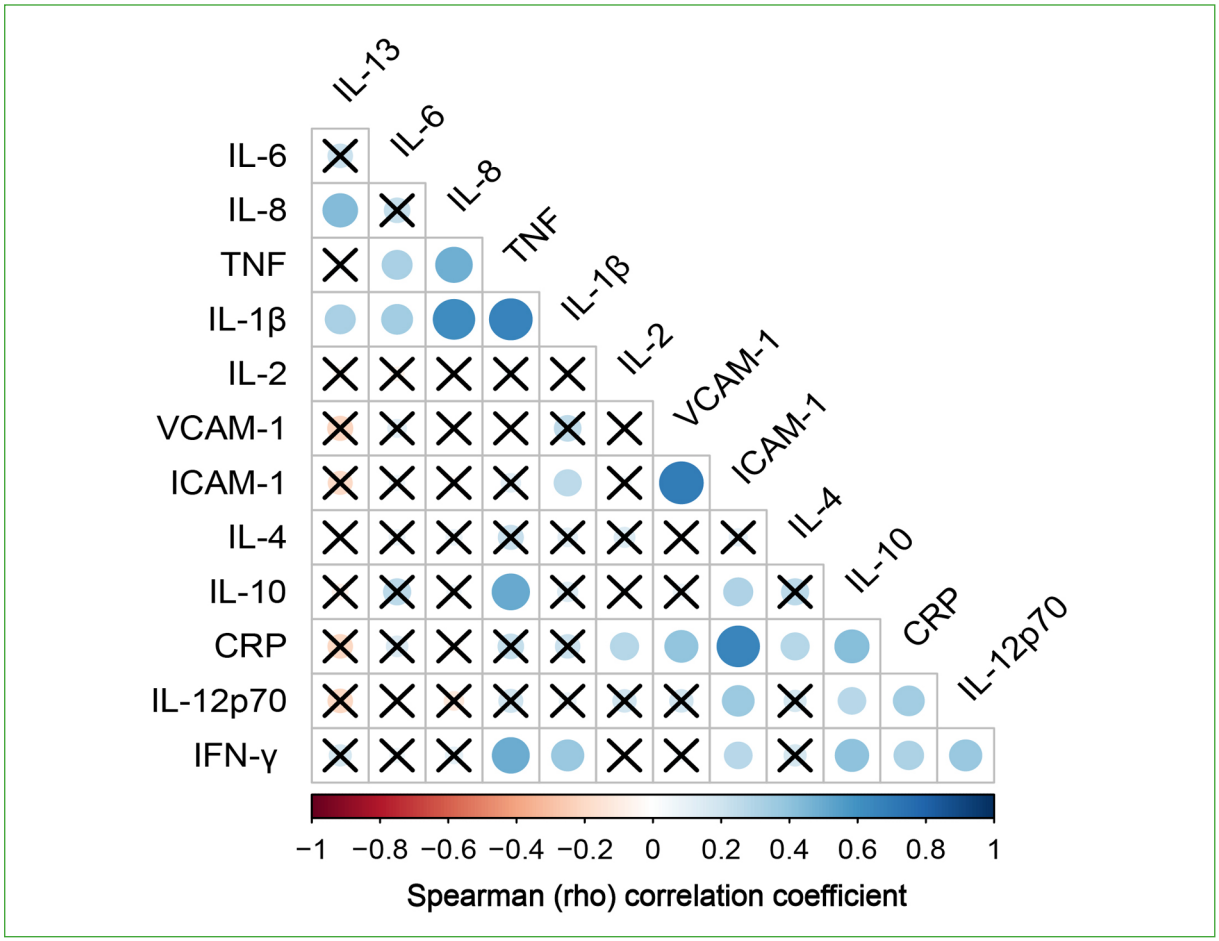

Figure 3. Correlation of infant plasma pro-inflammatory and endothelial dysfunction biomarkers. A correlation matrix of plasma pro-inflammatory and vascular injury biomarkers. Correlations marked with an X were non-significant ($p \geq 0.05$). Circle size indicates the magnitude of the correlation coefficient.

This was consistent with findings from a Chinese study in infants aged 1 month[36]. There was an increased relative abundance of commensals, such as *Bifidobacterium*, *Bacteroides*, and *Lactobacillus* in infant stool at 6 weeks postpartum. Our findings were consistent with studies in African and European infants where dominance of *Bifidobacterium* was observed in infant stool at 1 and 15 weeks of age[5,37]. However, a Nigerian study[34] found a lower relative abundance of *Bifidobacterium* species in HEU infants compared to HUU infants over the first 6 months postpartum. These findings might have significance for infant health since *Bifidobacterium* has been shown to enhance the absorption of essential nutrients and promote immune response to pathogens with anti-inflammatory effects[4,5].

Similar Infant Gut Microbiota Diversity and Composition Compared By Infant HIV-Exposure Status and Breastfeeding Method

We did not find any significant differences in infant stool alpha diversity indices when compared to HIV exposure status. Similarly, there were no significant differences in the infant gut microbial composition at 6 weeks postpartum. Our study results were consistent with studies in Brazil and South Africa conducted at 6 weeks postpartum[38,39]. Our findings were also comparable with findings from a study on Nigerian and South African infants, which found that HIV exposure minimally altered the infant gut microbiota[37]. In contrast, in a Haitian study[40] with well-controlled HIV in most mothers, HEU infants had lower stool alpha diversity compared to HUU infants aged 2 months.

Differences in the study population might explain some of the differences since the infants studied were born to mothers with various responses to HIV. Further, a much larger sample size in all settings would be desirable.

Furthermore, we compared infant gut microbiota diversity and composition by infant feeding mode (exclusive versus mixed breastfeeding) and found non-significant differences in both stool alpha and beta diversity. Our findings were inconsistent with those of an American study where higher alpha diversity was noted in the stool of exclusively breastfed infants compared to mixed breastfed infants at 6 months postpartum[41]. None of the infants in this study were exclusively receiving formula milk, and its independent effects on the infant gut microbiota could not be investigated. Moreover, in another American study[42], there was an increased abundance of *Bifidobacterium* in exclusively breastfed compared to exclusively formula-fed infants, demonstrating the benefits of breastfeeding towards a healthy infant gut microbiota.

Infant Systemic Biomarker Levels Were Similar by HIV Exposure Status

To investigate the effects of HIV exposure on the infant systemic immune environment, plasma biomarkers of systemic inflammation and vascular injury/endothelial dysfunction were compared by infant HIV-exposure status. We did not find significant differences in all immune biomarkers after multiple-test correction. We observed a non-significant trend of higher VCAM-1 and CRP in the HEU compared to the HUU group. Though not or only nominally significant, our findings were consistent with a Brazilian study that found increased CRP in HEU infants at birth[15]. In a Kenyan study[43] in HEU and HUU infants at 6 and 10 weeks, IFN-γ, IL-1β, IL-6, IL12p70, and TNF were similar when compared between the two groups. However, some of the effects in this study and other studies may be too small to reach significance for some of the correlations due to the small population investigated.

In contrast, other studies identified stronger effects of HIV exposure on the infant's immune system. For instance, in a South African study at 6-10 weeks postpartum lower serum IFN-γ and IL-1β levels were observed in HEU compared to their HUU counterparts[12]. Moreover, Brazilian HEU infants were observed to have increased IL-6 and VCAM-1 at birth with increased IL-6 persisting up to 6 months postpartum when compared to HUU infants and with no association to maternal inflammation status[15]. Further, in a Zimbabwean study of infants at 6 weeks postpartum, HEU infants had increased CRP compared to HUU infants which persisted up to 6 months[16]. These findings were inconsistent with our results, and we wish to highlight that the South African, Brazilian, and Zimbabwean infants were born to mothers with preserved immune function (undetectable viral loads and high CD4+ T-lymphocyte counts).

Furthermore, no infant gut microbial taxa relative abundance is significantly associated with infant plasma systemic inflammation and endothelial dysfunction biomarker levels, suggesting minimal to no effects of the infant gut microbiota on the systemic environment in 6-week-old infants. These findings are assuring as we did not find any red flags at 6 weeks of age. However, a longitudinal assessment could be carried out in the future, as well as an investigation of other maternal factors and infant outcomes such as neurodevelopment.

Strengths and Limitations

Our study was cross-sectional, and a longitudinal approach would likely have provided more comprehensive insights, especially if maternal factors, which can influence the systemic immune environment of infants, had been included. Nevertheless, our research is among the few to examine both gut microbiota and the systemic immune environment in HIV-exposed, uninfected infants within a resource-limited setting. Although our study initially included 50 HUU and 28 HEU infants, only 56 infant plasma samples were analyzed for systemic inflammation and endothelial dysfunction biomarkers. This limited subset may have introduced selection bias, potentially affecting the robustness and representativeness of our findings. Moreover, the small sample size constrains the extent to which our results can be generalized to the broader infant population in our region.

CONCLUSIONS

Our study found no discernible impact of HIV exposure or feeding practice on the diversity and composition of the infant gut microbiota at 6 weeks after birth. Moreover, HIV exposure did not significantly influence the systemic immune environment in HIV-exposed uninfected (HEU) infants compared to their HIV-unexposed uninfected (HUU) counterparts. These findings suggest that early HIV exposure does not have a detectable effect on the gut microbial landscape or on systemic immune parameters within the first six weeks of life. Furthermore, our data indicates that the infant gut microbiota at this early stage is not strongly associated with the systemic immune environment. This suggests that any potential interactions between the gut microbiome and systemic immunity may develop later in life or are influenced by factors beyond the first six weeks of life. This underscores the need for longitudinal studies to explore how these relationships may evolve over time and to determine whether later-life immune or microbial alterations might arise from early HIV exposure or other factors.

Ethics Approval

The study was conducted in line with the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the University of Zimbabwe, The Joint Parirenyatwa Hospitals, and the University of Zimbabwe Research Ethics Committee (JREC), JREC/114/20. The study was also approved by the Medical Research Council of Zimbabwe (MRCZ), MRCZ/A/2663.

Informed Consent

All mothers gave written informed consent and parental consent for the participation of their infants.

Availability of data and materials

The datasets generated and/or analyzed during the current study have been deposited in Zenodo https://zenodo.org/records/13829104?preview=1&token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6ImMxZWUzMDQ5LThmZDAtNDVi-My1hNTQ5LWY1MTI2NTliNDgyNCIsImRhdGEiOnt9LCJyYW5kb20iOiJkZmQwNWM2Y2I0OWUzMjEyYzBlNTIyYT-BlNjQyZGIyZCJ9.3PzJxpB_fIBbc0B82fMRGR-5EfOdk7MkFfHmQvX9IEiyaeajIaZuGmSIbVBgGBnm7ZZ4dh7q2ZOUlB-FAccH8Eg).

Conflict of Interest

The authors declare that they have no competing interests.

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Authors' Contributions

The study was conceived by BM, KD, BY, and PTM and designed by PTM, BY, and LK. Data collection, entry, and validation were done by PTM and AJM. PTM carried out the assays under the supervision of BY. PTM, SJ, and JW conducted the statistical analysis. PTM wrote the first draft, and all authors contributed to revisions of the manuscript. All authors approved the final manuscript.

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