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Bacterial Diversity in Placentas from Complicated Pregnancies Using 16s rRNA Gene Sequencing

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Abstract

Introduction: The 'sterile womb paradigm' is currently under debate and the advent of next generation 16S rRNA gene sequencing is driving the characterization of microbes associated with the amniotic cavity during pregnancy.

Objective: To characterize the bacterial diversity in placentas from preterm and term births using next generation 16S rRNA gene sequencing in association with adverse pregnancy outcomes and histopathology studies.

Methods: In this prospective study, placentas were collected consecutively from patients attending a public tertiary referral hospital in South Africa, delivering preterm (n=42; 28-34 weeks gestational age) and term (n=20; >37 weeks gestational age). Placentas underwent histopathology tests and next generation 16S rRNA gene sequencing.

Results: Analysis of microbial diversity in the placenta showed a significantly higher alpha diversity in term placentas compared to preterm placentas (P=0.000075), in placentas with acute chorioamnionitis compared to placentas without acute chorioamnionitis (P=0.0061), between HIV negative term births and both HIV negative and HIV positive preterm births (P=0.0118 and P=0.0008), respectively. Beta diversity was significantly different between preterm and term births (unweighted UniFrac distance, P=0.003996; Jaccard distance, P=0.03696) and *Escherichia/Shigella*, *Shuttleworthia*, *Anaeroglobus* and *Megasphaera* were differentially expressed.

Conclusion: This is the first South African study to characterize the bacterial diversity in placentas from complicated pregnancies using next generation 16S rRNA gene sequencing in conjunction with full placental histology. Microbial diversity differs between preterm and term placentas where HIV may act as a cofactor associated with decreased bacterial alpha diversity in placentas from preterm birth.

Keywords: Next generation sequencing, 16S rRNA, Placenta, Preterm birth, HIV, Acute chorioamnionitis

Introduction

Intra-amniotic infection due to microbial invasion of the amniotic cavity has been implicated in various pregnancy complications; spontaneous preterm labor, preterm premature rupture of membranes, chorioamnionitis and adverse maternal and neonatal outcomes [1-4]. Infection may result from multiple routes of invasion including ascending infection from

the lower genital tract, hematogenous dissemination through the placenta, retrograde seeding through the fallopian tube and iatrogenic infection [1,5]. Bacterial colonization of the placenta has been reported in association with adverse pregnancy outcomes including preterm birth and chorioamnionitis [4,6]. A study demonstrated the presence of bacteria in the basal plate of the placenta, at the maternal-fetal interface, in association with early preterm birth and in the absence of chorioamnionitis [7].

The 'sterile womb paradigm' is currently under debate and the advent of next generation 16S rRNA gene sequencing is driving the characterization of microbes associated with the amniotic cavity during pregnancy [8-10]. In 2014, the sterile womb hypothesis was challenged by Aagaard *et al*. who reported the detection of bacterial DNA in placental samples [11]. However, a complication for these studies is the presence of low levels of microbial DNA in laboratory dust and commercial reagents which may produce signals from the highly sensitive molecular methods used. A further complication is that it is possible to generate germ-free neonates by sterile caesarean delivery which makes the sterile womb hypothesis appealing as pathogens can cross the placenta [12]. This contemporary area of research has led to the recent proliferation of microbiome studies of the amniotic cavity to increase understanding on this topic.

Major findings of this research include detection and identification of bacterial species from predominantly amniotic fluid and placenta samples and association of the microbiome with specific outcomes or pathology in study cohorts [6,11,13-15]. However, some placenta-based studies failed to detect microbial DNA [16-18]. This may be due to the low biomass of microbes reported in placental tissue as well as the difficulty in isolating genomic DNA from tissue rather than amniotic fluid [11]. Recent studies oppose the idea that there is a microbiome associated with the placenta, citing contamination or background signals [16,17,19,20]. In contrast, some studies offer insight into a uterine microbiome which is often described as unique and associated with specific factors such as birth weight, chorioamnionitis and pathology [5,6,11,14]. Cohort selection criteria also plays an important role in microbiome studies such as Kuperman *et al.* (2019) where placentas were delivered from women with preeclampsia, which is not typically associated with microbial infection, and the lack of detection of microbial DNA is expected [16].

However, the different regions of the placenta must also be considered when comparing subsets of placental microbiome studies. Variations have been described in microbial composition between the fetal amniotic membranes, the placental villi, and the basal plate, and these three regions of the placenta differ by fetal or maternal origin, structure, function, and barrier capacities as well as timing of infection [6,15,21]. In this current study, the placental samples submitted for next generation 16S rRNA gene sequencing comprised the placental parenchyma, including the chorionic plate, the intervillous space, and basal plate.

The clinical relevance of using DNA-based molecular detection of microbes has been questioned as it is known that DNA is able to persist for weeks following antibiotic usage and cell death and perhaps longer in a stable environment such as the uterine cavity [22,23]. The inability to differentiate between viable and non-viable cells is a major limitation in microbiome studies which characterize dynamic systems [31]. However, additional investigations such as quantification of microbes using qPCR or correlation of the presence of microbial DNA with clinical indications of infection or inflammation may provide a more detailed understanding of the specific microbiome being studied.

Other studies have employed a similar approach to this current study by using NGS of the 16S rRNA [11,14,17,19]. Zheng *et al*. detected microorganisms in the placenta in association with neonatal low (LBW) or normal (NBW) birth weight [14]. The relative abundance of *Lactobacillus*, *Clostridium*, *Cyanobacteria*, *Ruminococcus* and *Lawsonia* were significantly lower in LBW group and conversely *Megasphaera*, *Faecalibacterium*, *Jeotgalicoccus*, *Pediococcus*, *Sneathia*, and *Sphingobacterium* were significantly higher in LBW group, compared with the NBW group. Prince *et al*. found a higher abundance of *Ureaplasma parvum*, *Streptococcus agalactiae* and *Fusobacterium nucleatum* from fetal chorion and/or villous placental membranes in association with preterm birth with severe chorioamnionitis [6].

Despite recent advances in metagenomics, and perhaps because of the opposing results of microbiome studies, it is apparent that the microbial census of the amniotic cavity remains incomplete. Gaining understanding of the microbial diversity associated with pregnancy may propel research regarding the source of infection, pathogenicity, synergism of pathogens and adverse clinical outcomes. Microbiome data combined with clinical outcomes may significantly enhance the knowledge of the role of placental colonization and the effect on adverse pregnancy outcomes. The objective of this study was to characterize the microbial diversity in placentas from complicated pregnancies using next generation sequencing of the 16S rRNA gene in association with adverse pregnancy outcomes and histopathology studies.

Materials and Methods

Ethical clearance, study population and design

This prospective hospital-based study included patients delivering in the labor ward at a tertiary referral hospital in the Eastern Cape, South Africa from March 2016 to November 2017. This hospital services a large area and only patients who present with complicated deliveries are eligible for admission

to the maternity unit. Ethics approval was granted from the Nelson Mandela University Research Ethics Committee (Human) (reference: H15-SCI-BCM-001). Permission to conduct the study was obtained from the Eastern Cape Department of Health (EC_2015RP8_78) and from the acting Clinical Governance Manager at the hospital respectively. Patients were recruited and informed consent was obtained including permission for the collection of their placenta after delivery and for their medical records to be accessed for maternal and neonatal chart review. Patients received unique and anonymous study numbers for entry into a de-identified database. Placentas from preterm deliveries (n=42; 28 to 34 weeks gestational age) were collected as the test cohort, while placentas from term deliveries (n=20; >37 weeks gestational age) were collected as the control cohort. Placentas were collected consecutively according to confirmation of inclusion criteria of gestational age and if the mother was able and willing to give informed consent after delivery. The gestational age range was selected as, according to the World Health Organization, viability of preterm neonates reaches a 50% chance of survival at 34 weeks gestational age in low to middle income countries, compared to 24 weeks in high income countries [25].

Placentas

Following delivery, the placenta was immediately transferred to a sterile container and processed within 1 hour. The placenta was placed maternal side down on a sterile work table in a biosafety cabinet. The fetal surface was decontaminated using 70% ethanol to minimize contamination by maternal skin and vaginal flora depending on mode of delivery. Using sterile surgical implements, an incision was made in the amnion, at a point approximately halfway between cord insertion and placental edge, where a single full-thickness biopsy (approximately 1 cm^3) was removed from below the chorioamnion including the chorionic villi, syncytiotrophoblast and decidua and was stored in RNAlater (Life Technologies, Canada) in a cryogenic vial at -80°C. The remainder of each of the placentas underwent routine macroscopic examination, followed by fixation in 10% buffered formalin and histology at National Health Laboratory Services (NHLS), Gqeberha, Eastern Cape. All cases were evaluated histologically by a single pathologist using a standardized placental macroscopic evaluation protocol and histology template approved and validated by the NHLS laboratory. Prof CA Wright, anatomical pathologist, a specialist in placental histology, reviewed all the placenta specimens for this study and histopathology data was published in 2023 [27]. The pathologist was blinded to all information except gestational age and whether live-born or stillbirth. Based on the macroscopic and microscopic data, a histological diagnosis was made according to the Amsterdam Consensus Classification System [26,27].

Maternal and neonatal chart review

A maternal chart review was performed to gather demographic and obstetric characteristics such as maternal race, age, parity, smoking status, diabetes, preeclampsia, Human Immunodeficiency Virus (HIV) status and the mode of delivery (Caesarean section [CS] or normal vertex delivery [NVD]) (**Table 1**). Certain fetal/neonatal characteristics that were available shortly after delivery were recorded in the maternal chart before a neonatal chart was opened (**Table 2**). Demographic information was required to investigate patterns around different disease conditions in the study

Table 1. The distribution of placental lesions from preterm and term births.

Placental pathology*	Preterm, n (%) (*n*=42)	Term, n (%) (*n*=20)
ACAM	8 (19)	11 (55)
MVM	20 (48)	8 (40)
AP	18 (43)	7 (35)
FVM	4 (10)	0 (0)
VUE	4 (10)	0 (0)
ACAM with FIR	4 10)	0 (0)
Chorangiosis, chorangioma	1 (2)	0 (0)
Intravillous haemorrhage	2 (5)	0 (0)
TTS	1 (2)	0 (0)
RPH	2 (5)	0 (0)
Other	7 (17)	3 (14)

ACAM: Acute Chorioamnionitis; MVM: Maternal Vascular Malperfusion; AP: Abruptio Placenta; FVM: Fetal Vascular Malperfusion; VUE: Villitis of Unknown Etiology; FIR: Fetal Inflammatory Response; RPH: Retroplacental Hemorrhage; TTS: Twin Transfusion Syndrome.

*Placentas may have more than one lesion.

Table 2. Maternal and obstetric characteristics of the preterm study and term control groups.

	Preterm (*n*=42)	**Term (n=20)**
Numerical	**Mean (SD)**	**Mean (SD)**
Maternal age, years	28.88 (6.46)	27.35 (4.84)
Parity	2.05 (1.11)	0.90 (0.91)
Categorical	**Total, *n* (%)**	**Total, *n* (%)**
Ethnicity		
African	29 (69)	14 (70)
Mixed	13 (31)	6 (30)
Birth Mode		
NVD	19 (45)	10 (50)
CS	23 (55)	10 (50)
Substance abuse		
Smoking	9 (21)	15 (75)
Alcohol	6 (14)	10 (50)
HIV	18 (43)	4 (20)
Diabetes	1 (2)	3 (15)
Pre-eclampsia	14 (33)	1 (5)
AEDF	2 (5)	1 (5)
HELLP	4 (10)	0 (0)
PROM	9 (21)	2 (10)

SD: Standard Deviation; NVD: Normal Vertex Delivery; CS: Caesarean Section; AEDF: Absent End Diastolic Flow; HELLP: Haemolysis Elevated Liver Enzymes and Low Platelets Syndrome; PROM: Premature Rupture of Membranes.

population group [28]. A neonatal chart review was performed for each neonate from preterm and term delivery. General characteristics such as live birth/ still birth, neonatal gender, weight, and APGAR score were recorded at birth. Neonatal charts were later reviewed for outcomes, i.e., respiratory distress syndrome, sepsis, pneumonia, neonatal jaundice, and necrotizing enterocolitis. Data on histopathology is available (**Tables 1** and **2**).

DNA extraction and library preparation

The 16S rRNA gene is made up of nine hypervariable regions (designated V1-V9) [29]. The choice of which hypervariable region to target for microbiota studies is complex as the phylogenetic resolution and abundance estimate achieved with each region will differ for the different taxa within the community. While the V3-V4 regions have been used to examine cervical and vaginal communities, there is no agreement on the optimal region for cervical and vaginal microbiota studies [30]. The V3-V4 region of the 16S rRNA gene was targeted to characterize the bacterial community within preterm and term placenta samples. Next generation sequencing was performed by KRISP (Kwazulu-Natal Research Innovation and Sequencing Platform) Laboratories, Nelson R Mandela School of Medicine at the University of KwaZulu-Natal (http://www.krisp.org.za). Tissue samples from 42 preterm and 20 term placentas (50 mg) were excised from the original tissue biopsy of the chorionic villi, syncytiotrophoblast and decidua within a biological safety cabinet. Tissues were lysed using the Qiagen Tissue Lyser at 30 pulses for 45 seconds. Total nucleic acid was extracted using the PerkinElmer Chemagic 360 Automated system and DNA was quantified using the Qubit 3.0 instrument using the Qubit dsDNA Assay Kit (Thermo Fisher Scientific, South Africa) and normalized to 5 ng/µl in 10 mM Tris, pH 8.5. Negative kit controls were included for extraction, library preparation, and amplification.

The V3 and V4 regions of the bacterial 16S rRNA gene were amplified by PCR using Platinum Taq DNA Polymerase Kit (Invitrogen, California, USA) with Forward primer: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′ and Reverse primer 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATCC-3′ which result in a single amplicon of approximately ~460 bp

(Illumina 16S Metagenomic Sequencing Library Preparation Guide, California, USA) [31]. Sequencing included negative controls. PCR assays were performed in 12.5 µL reactions containing 5 µL of extracted DNA, 0.1 µL of DNA Polymerase, 1.2 µL buffer, 0.5 µL $MgCl_2$, 0.3 µL dNTP, 2 µL of each primer (1 µM) and sterile DNase/RNase-free dH_2O. The Nextera XT Index primers (Illumina, California, USA) were used to provide a unique barcode to each sample. Agencourt AMPure XP beads (Beckman Coulter, California, USA) were used to remove unbound adapters. DNA was quantified using the Qubit 3.0 instrument and Qubit dsDNA Assay Kit. A fragment size of 550 bp was verified on a 1% agarose gel. The libraries were normalized to equimolar 4 nM prior to sequencing.

Bioinformatics approach and statistical analysis

Statistical analyses and data visualization were performed using 'R' programming language and environment (R, 2018, https://www.r- project.org version 3.5.0). Raw reads were evaluated for sequence quality and trimming parameters using Fastqc [(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)] and MultiQC [32]. The Divisive Amplicon Denoising Algorithm (DADA) 2 [33] was then used to filter for quality, trim, and infer amplicon sequence variants (ASVs were taxonomically classified to genus or higher levels using a Naïve Bayes classification approach [33] and SILVA ribosomal RNA database [34]. A maximum likelihood phylogeny was inferred using the R package Phangorn. The resulting phylogenetic tree, ASV table, taxonomy table, and sample metadata were combined into a Phyloseq object using the R package phyloseq [35] to perform community compositional analyses and ordination. Prior to the compositional analyses, non-bacterial sequences, taxa that are not classified beyond the Phylum level, outliers and taxa which were not prevalent in at least 1% of the samples were removed. Rarefaction curves were generated using the R package vegan [35] to determine if sequencing depth was sufficient. These curves show the number of species as a function of number of samples. Information regarding sequencing depth and other descriptors are indicated in **Figures S1** and **S2**, and **Table S1**. The range was 108 – 23190. Samples with read counts less than 100 were considered too low and therefore dropped from subsequent analyses.

Community compositional analyses

Alpha diversity (within-group diversity) estimates for richness and evenness were calculated using Shannon indexes while presence-absence was performed using the Simpson indexes [36]. The effect of various parameters on alpha diversity was determined using the Kruskal Wallis and Wilcoxon rank sum tests after confirming that the data was not normally distributed using the Shapiro test. Interactions were modelled using the R base function, "interaction". The dunn test was used post-hoc analysis to determine the effect of interacting groups. These parameters assessed include histopathology, maternal characteristics, and neonatal outcomes.

Bacterial community dissimilarity or beta diversity was determined using; the Bray-Curtis index which accounts for ASV relative abundance between samples, the Jaccard index which accounts for ASV presence/absence between samples, the UniFrac which takes into consideration the phylogenetic relatedness of ASVs detected. The weighted UniFrac considers ASV relative abundance between samples while the unweighted UniFrac which considers ASV presence/absence between samples. Principal Coordinate Analysis (PCoA) ordinations were used to visualize sample relationships and statistical testing to confirm observed relatedness. Permutational multivariate analysis of variance (PERMANOVA) testing for statistical significance was performed using the adonis function from the Vegan R package (https://github.com/vegandevs/vegan), (https://cran.r-project.org/web/packages/vegan/index.html) [37].

Evaluating differential abundance

DESeq2 [38] was used to determine taxa that are differentially abundant between term and preterm deliveries. This implements the FDR/Benjamini-Hochberg method which ranks the genes by p-value, then multiplies each ranked p-value by m/rank. Prior to testing for differential abundance, an independent filter was used to exclude ASVs absent in at least 1% of the samples. ASVs were considered significantly differentially abundant between classes if their adjusted P value was <0.05 and if the estimated fold change was >1.5 or <1/1.5. The sequence data bioproject accession for the requested raw read files have been successfully submitted to NCBI SRA: PRJNA1047970.

Results

Library preparation and data assessment

DNA was isolated from the chorioamnion of placentas from preterm (n=42) and term (n=20) births and subjected to next generation sequencing. Obtained sequences were quality filtered and ASVs were evaluated. Following DNA extraction and quality assurance, one sample had a very low read count (<100 reads) and was thus eliminated. The remaining 61 samples (41 preterm and 20 term) comprised the phyloseq object containing 12029 taxa prior to filtering. Supervised prevalence filtering removed non-microbial sequences and taxa prevalent below 1%, before outlier detection. ASV outliers were removed, therefore leaving 38 preterm and 19 term samples.

Alpha diversity was higher among term births

Overall, there was a higher alpha diversity in term (n=19) samples than preterm (n=38) samples with a statistically

significant measure of the Shannon diversity index (**Figure 1A**; Kruskal-Wallis, P=0.000075). Term samples had higher abundance and richness of ASVs than preterm samples as observed by the higher Shannon diversity index.

Alpha diversity differed significantly between placentas with chorioamnionitis and those without (**Figure 1B**; P=0.0061) and placentas from HIV positive vs HIV negative mothers (**Figure 1C**; P=0.0089). Statistical significance was absent for mode of delivery (**Figure 1D**; P=0.75) and placentas with histological maternal vascular malperfusion (P=0.056), abruptio placenta (P=0.99), diabetes (P=0.39), smoking (P=0.44), preeclampsia (P=0.59), race (P=0.43), and live-birth vs stillbirth (P=0.16).

There was a statistically significant difference between HIV negative term births and both HIV negative and HIV positive preterm births, P=0.0118 and P=0.0008, respectively (**Table 3**). This indicates that there is a difference in Shannon diversity of preterm placentas in association with HIV status. In contrast, for term placentas there was no significant difference for HIV positive versus HIV negative status (P=0.2858). Therefore, the effect of HIV cannot be ruled out in preterm birth in this study where HIV is a cofactor and its presence is associated with decreased microbial alpha diversity in preterm birth.

Beta diversity revealed for between group clustering

Principal coordinate plots (**Figures 2A-2D**) showed differences in clustering between preterm and term beta diversity measures. Significant differences in beta diversity

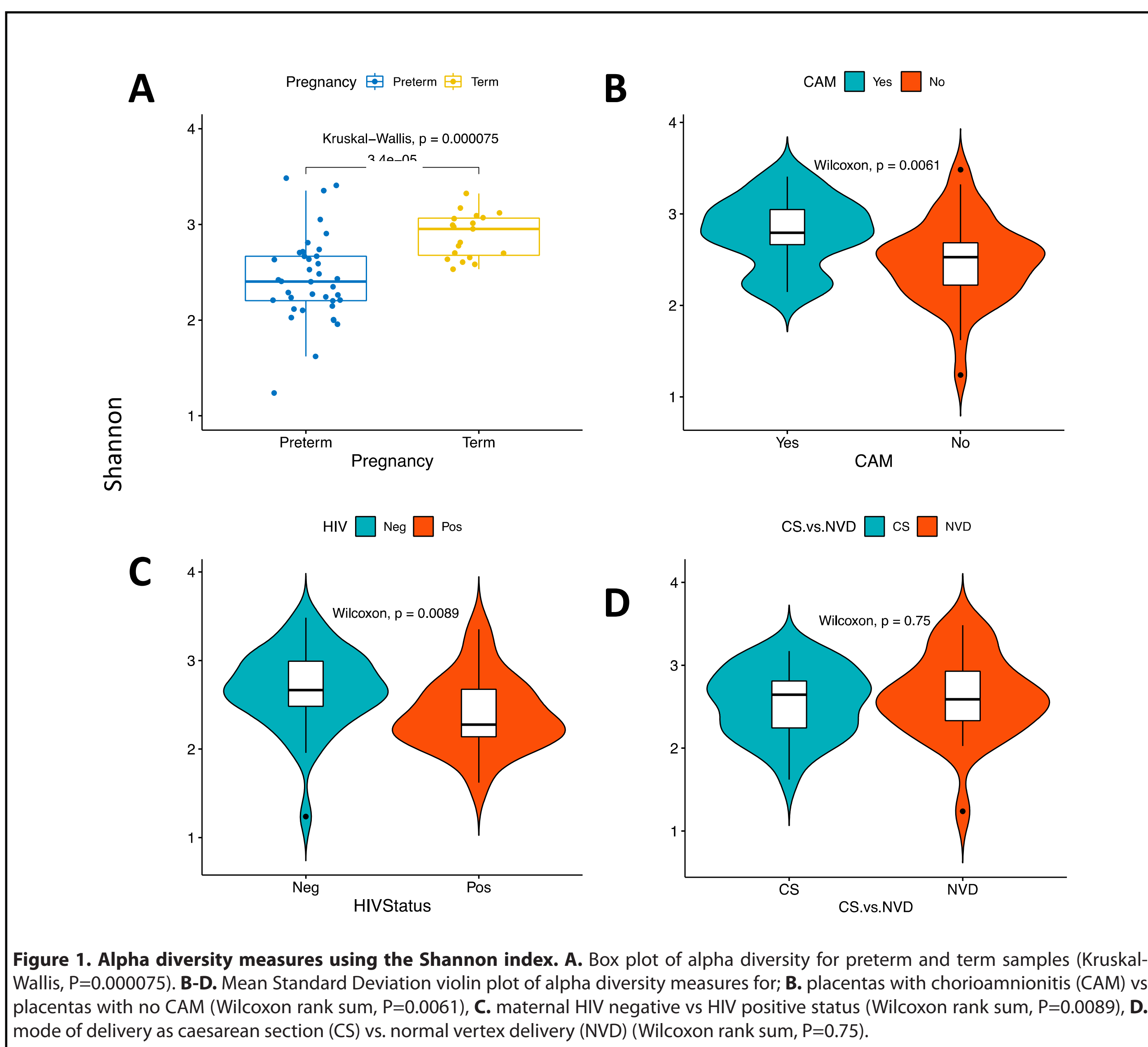


Figure 1. Alpha diversity measures using the Shannon index. A. Box plot of alpha diversity for preterm and term samples (Kruskal-Wallis, P=0.000075). **B-D.** Mean Standard Deviation violin plot of alpha diversity measures for; **B.** placentas with chorioamnionitis (CAM) vs placentas with no CAM (Wilcoxon rank sum, P=0.0061), **C.** maternal HIV negative vs HIV positive status (Wilcoxon rank sum, P=0.0089), **D.** mode of delivery as caesarean section (CS) vs. normal vertex delivery (NVD) (Wilcoxon rank sum, P=0.75).

Table 3. Kruskal-Wallis rank sum test cross tabulation of alpha diversity measured as the Shannon diversity index between preterm and term birth for HIV status.

Col Mean- Row Mean	HIV negative Preterm	HIV negative Term	HIV positive Preterm
HIV negative Term	-2.263258 **0.0118***		
HIV positive Preterm	1.160623 **0.01229***	3.169693 **0.0008***	
HIV positive Term	-0.808623 0.2094	0.565647 0.2858	-1.468421 0.0710

Bold*: P-values

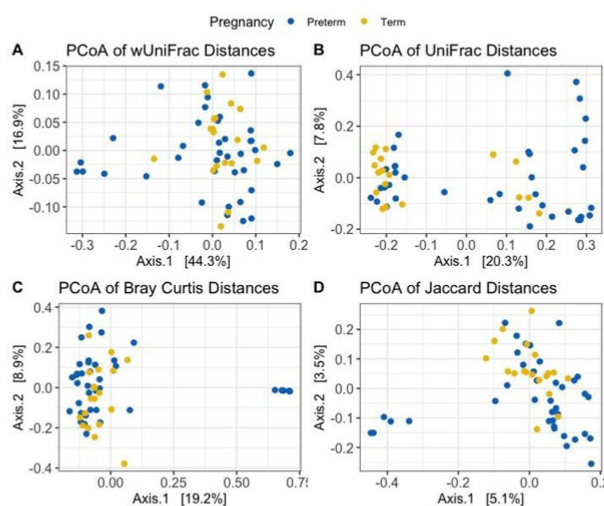


Figure 2. Beta diversity Principal Coordinate Analysis (PCoA) plots. **A.** weighted (w) UniFrac, **B.** unweighted UniFrac, **C.** Bray Curtis and, **D.** Jaccard Distances demonstrating the beta diversity of the placental microbial community for preterm birth (blue) and term birth (yellow). The values in parentheses show the percentages of total community variation explained.

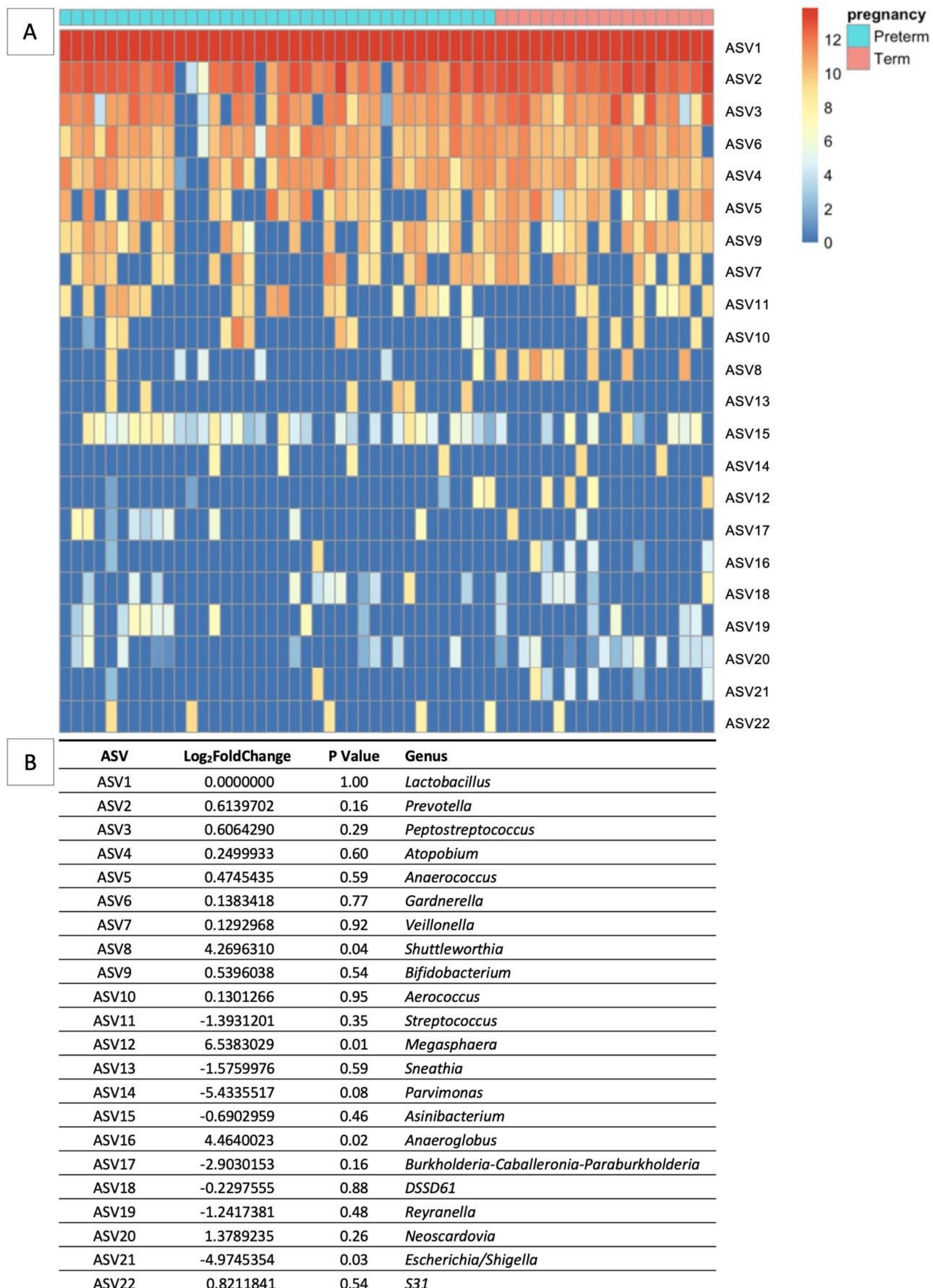
were seen between preterm and term samples in the Jaccard PCoA plot (PERMANOVA, P=0.03696) and unweighted UniFrac PCoA plot (PERMANOVA, P=0.003996) indicating that overall microbial community composition of placental samples was significantly different for preterm compared to term births at ASV level, but not for abundance (Bray Curtis PERMANOVA, P=0.1289 and W Unifrac PERMANOVA, P=0.1149).

Differential abundance altered for gestational age

A heat map (**Figure 3A**) of abundant genera between preterm and term pregnancies, with estimated fold change and P values (**Figure 3B**). Clustering of ASV's between preterm and term samples is visually difficult to interpret from the heat map generated, and therefore significance in differential abundance was determined with Log_2 fold change and associated P values. Statistical significance was observed for ASV 8 (*Shuttleworthia*, P=0.04), ASV 12 (*Megasphaera*, P=0.01), ASV 16 (*Anaeroglobus*, P=0.02) and ASV 21 (*Escherichia/Shigella*, P=0.03).

Based on the heat map generated (**Figure 3A**), the distribution of abundant genera by preterm and term identified that *Lactobacillus* were uniformly distributed in all preterm and term placentas (P=1.00). From visual inspection of the heat map and fold change values *Gardnerella, Prevotella, Peptostreptococcus, Atopobium, Anaerococcus* and *Bifidobacterium* were abundantly present in both preterm and term placentas. Non-bacterial sequences, unidentified bacteria, outliers, and taxa which were not prevalent in at least 1% of the samples were removed from subsequent analyses.

Bacterial genera significantly differentially expressed are represented in orange in the Volcano plot of the DESeq results



ASV	$Log_2FoldChange$	P Value	Genus
ASV1	0.0000000	1.00	*Lactobacillus*
ASV2	0.6139702	0.16	*Prevotella*
ASV3	0.6064290	0.29	*Peptostreptococcus*
ASV4	0.2499933	0.60	*Atopobium*
ASV5	0.4745435	0.59	*Anaerococcus*
ASV6	0.1383418	0.77	*Gardnerella*
ASV7	0.1292968	0.92	*Veillonella*
ASV8	4.2696310	0.04	*Shuttleworthia*
ASV9	0.5396038	0.54	*Bifidobacterium*
ASV10	0.1301266	0.95	*Aerococcus*
ASV11	-1.3931201	0.35	*Streptococcus*
ASV12	6.5383029	0.01	*Megasphaera*
ASV13	-1.5759976	0.59	*Sneathia*
ASV14	-5.4335517	0.08	*Parvimonas*
ASV15	-0.6902959	0.46	*Asinibacterium*
ASV16	4.4640023	0.02	*Anaeroglobus*
ASV17	-2.9030153	0.16	*Burkholderia-Caballeronia-Paraburkholderia*
ASV18	-0.2297555	0.88	*DSSD61*
ASV19	-1.2417381	0.48	*Reyranella*
ASV20	1.3789235	0.26	*Neoscardovia*
ASV21	-4.9745354	0.03	*Escherichia/Shigella*
ASV22	0.8211841	0.54	*S31*

Figure 3. Bacterial profile of placental samples. **A.** Heat map of mean abundance per genus illustrating differences in Log2 Fold Change of prominent amplicon sequence variants (ASVs) among placental samples of preterm (n=38) and term (n=19) births. **B.** Most prominent ASVs listed at genus level, with corresponding Log2 fold change and adjusted P values.

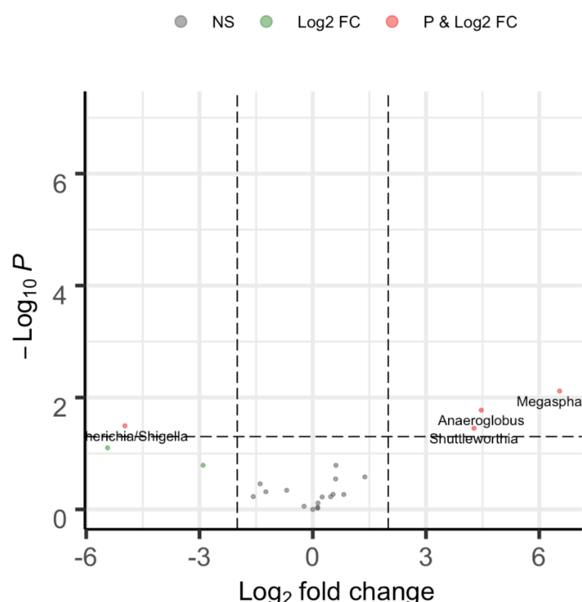


Figure 4. **Volcano plot of DESeq results indicating the positive Log2 fold change - Term of differentially expressed bacteria.** *Escherichia/Shigella, Anaeroglobus, Shuttleworthia,* and *Megasphaera* were considered statistically significant.

(**Figure 4**). Bacterial genera above the horizontal line remain significant after multiple testing correction (False Discovery Rate). Those on the left of the first vertical dotted line are more abundant in the preterm birth while those on the right of the second vertical line are less abundant in preterm birth. ASV 21 *Escherichia/Shigella* (P=0.03) were more abundant in preterm placentas, while ASV 8 *Shuttleworthia* (P=0.04), ASV12 *Megasphaera* (P=0.01) and ASV 16 *Anaeroglobus* (P=0.02) were less abundant in preterm placentas.

Discussion

This study uniquely characterizes the bacterial diversity in placentas from complicated pregnancies in South Africa using next generation 16S rRNA gene sequencing in conjunction with full placental histology. Next generation sequencing was performed on 16S rRNA gene amplicons derived from placentas from preterm and term births. A significantly higher alpha diversity was found in term than preterm placenta samples which correlated with the higher incidence of chorioamnionitis in term placentas and a lower alpha diversity in placentas from women who were HIV positive. There was also a significant difference in beta diversity between term and preterm placentas with *Escherichia/ Shigella, Shuttleworthia, Anaeroglobus* and *Megasphaera* differentially abundant.

It is well established that microbial infection of the amniotic cavity has been implicated in adverse pregnancy outcomes including preterm birth [39]. However, the origin and characterization of these microbes is less clear, as well as their clinical significance, in pregnancy outcomes.

The placental microbiome may vary with factors such as infant birthweight at term, maternal gestational weight gain, gestational diabetes, and preeclampsia [14,15,40,41]. Communities of bacteria were found to be spatially distinct within the placenta, specifically the fetal amniotic membranes, placental villi, and maternal basal plate [15]. Clinical vaginosis may be associated with changes in the placental microbiota [40]. Studies have shown that specific bacterial communities within the placental membranes were associated with preterm birth and chorioamnionitis and were independent of mode of delivery [6,42]. The growing body of evidence supporting the presence of a low biomass placental community or pathobiome in association with preterm birth risk may assist with clinical prediction of adverse pregnancy outcomes [43].

After quality filtering, 38 preterm and 19 term samples remained and were analyzed for alpha and beta diversity significance. Many published studies have used fewer samples for similar analyses; n=8 [5]; n=20 [43]; n=24 [14]; n=28 [16]; n=29 [17] and n=48 [11]. Together, these highlight the sufficiency of power within this study to detect differential abundance and to characterize the bacterial communities represented in the study groups. On the contrary, it highlights the need for larger study cohorts in this and similar settings to further validate these findings.

In this study, there was a significantly lower alpha diversity in preterm than term placenta samples as evidenced by the Shannon diversity index (Kruskal-Wallis, P=0.000075), which is in contrast with previous placental and vaginal microbiome studies [6,44]. This indicates that preterm placentas have fewer

microbial constituents within their microbiome. However, the higher alpha diversity in term placentas compared to preterm placentas, and higher alpha diversity in placentas with chorioamnionitis compared to those without CAM (P=0.0061) correlates with the higher incidence of CAM in term placentas compared to preterm placentas (P=0.002) [27]. Prince *et al*. also found that chorioamnionitis was associated with lower alpha diversity in the placental membrane microbiome, although associated with preterm birth in their study [6].

The effect of various histopathology, maternal characteristics and neonatal outcomes on alpha diversity was determined with the Shannon index and statistical significance assigned with the Wilcoxon rank sum test. There was a mean decrease in alpha diversity for patients who were HIV positive in comparison to HIV negative (P=0.0089). This agrees with previous gut microbiome studies which indicate that alpha diversity is decreased in association with recent HIV infection [45,46]. In microbiome studies of the placenta, vagina or amniotic fluid, HIV infection is often part of the exclusion criteria [44,40,51] or is not evaluated as a factor contributing to microbiome variances [17,18,49,50].

In a review by Gootenberg *et al*. which addresses changes in the enteric microbial community in association with HIV infection, two key points were made which may be applicable to this study [45]. The first is that there is HIV-driven destruction of gastrointestinal CD4+ T cells which may disturb the balance between the microbiota and mucosal immune system, leading to disruption of the stable gut microbiome and systemic inflammation. This same mechanism may occur in placental tissue as HIV is known to cross the placenta leading to infection of the neonate *in utero*, which may also disrupt the microbial balance. The second key point is that most HIV-enteric microbiome studies have occurred in developed countries, which may not be representative of countries with an increased HIV disease burden and potentially hindering the application of these outcomes to the populations of greatest need. The same can also be applied to microbiome studies of the amniotic cavity in developed countries [17,52]. A large-scale study by Doyle *et al.* reported HIV infection in 13.3% (n=1097) of included participants, compared to 37.5% HIV positive women in this study, however, they did not evaluate HIV infection as a factor contributing to the microbial diversity of the placenta [42]. In countries with a high HIV prevalence as well as high incidence of PTB, such as South Africa, it is of vital importance to further characterize the microbial diversity of the placenta in relation to HIV infection, PTB and associated outcomes. This study is the first to characterize the placental microbiome in South Africa, a country noted for its high incidence of HIV infection. In this study, Shannon diversity was significantly different between HIV positive versus negative groups in preterm birth, P=0.0008 and P=0.0118 respectively, implying that HIV may be a cofactor associated with decreased bacterial alpha diversity in placentas from preterm birth.

No significant difference in alpha diversity was seen for mode of delivery (Wilcoxon rank sum, P=0.75), suggesting that the diversity of microbes in the placenta is not associated with vaginal delivery and therefore not a consequence of contamination of the placenta by vaginal microbiota during birth. This is further confirmed by the presence of *Lactobacillus*, a common vaginal commensal, in placentas from both vaginal and caesarean delivery (P=1.00). Other studies also reported no significant difference in diversity by mode of delivery [6,11,42]. In contrast, some studies have found bacterial signals in association with a vaginal mode of delivery, which highlights the importance of sterile sample collection technique when assessing microbial diversity [19,51]. Studies investigating the vaginal microbiome in association with PTB often have disparate results, which emphasizes the need for further investigation of niche specific microbiomes during pregnancy and in PTB [52,53]. Previous studies on gut and oral microbiomes demonstrate that certain niche bacterial taxa may be implicated in intrauterine infection [53-58]. The hematogenous spread of common oral commensals, such as *Fusobacterium* spp. and *Streptococcus* spp., to the placenta in association with chorioamnionitis may account for the similarities observed between placental and oral or niche specific microbiomes [56].

In this study, there was a mean increase in alpha diversity for placentas with histological chorioamnionitis compared to those without chorioamnionitis (P=0.0061). In contrast, Prince *et al*. found a decrease in species diversity (Shannon index) in preterm subjects with severe chorioamnionitis and Doyle *et al*. reported a distinct difference in bacteria, a higher bacterial load and lower alpha diversity (species richness) in association with chorioamnionitis [6,57]. Various factors may have contributed to the different results obtained in this study, including differences in the maternal cohort. Alpha diversity has been reported as consistently higher in women of African ancestry than other ancestries in the vaginal microbiome, which may impact the placental microbiome if infection is due to ascending infection [50]. Although infection in the chorion and amnion is not always correlated with infection of the amniotic fluid, chorioamnionitis can be associated with hematogenous or ascending microbial infection of the amniotic cavity, which is often polymicrobial in nature and may influence species diversity in the preterm group [58]. In this study setting, patients in preterm labor are given prophylactic antibiotic treatment with erythromycin (Zithromax 500 mg daily) and the use of antibiotics for prophylaxis, suspected infection or clinically diagnosed chorioamnionitis may also have influenced the alpha diversity in this cohort of high-risk pregnancies. This is a limitation of the study as the information on duration of antibiotic prophylaxis administered to certain patients in preterm labor was not available. Prince *et al*. found that antibiotic use made no significant alteration to prevalent taxa at genus level in preterm and term placentas and suggested that variations in the placental membrane

microbiome may be associated with inflammation rather than infection [6].

The graphical representation of beta diversity in a PCoA plot indicates the similarity of microbiomes at the genus level between individual samples. Subjects can be characterized by categorical variables (e.g., preterm or term birth) and then visualized where each group clusters in relation to principal components (PC) on the axes. Most programs indicate the percentage of the difference between samples for each principal component. The higher the percentage, the more one can conclude that clusters are because of specific taxa, or lower percentages indicate that differences are based on multiple taxa which implies that no specific taxa are correlated with the outcome of preterm birth [15].

Upon analyzing beta diversity, a significant difference in clustering was found by PERMANOVA for the unweighted UniFrac distance (P=0.003996) and Jaccard distance (P=0.03696) on the PCoA plots, which indicate the richness and diversity of microbes significantly differs at the ASV level between preterm and term placentas. The large percentage for PC1 (44.3%) on the weighted UniFrac PCoA plot indicates that clustering may be a result of specific bacterial taxa shaping beta diversity between preterm and term placentas. These observations are consistent with previous evidence suggesting that the placental microbiome is altered in placentas from preterm birth [6,11,59].

Genera that were differentially abundant and were considered statistically significant were *Escherichia/ Shigella, Shuttleworthia, Anaeroglobus* and *Megasphaera*. *Escherichia* were found more abundantly in preterm placentas while *Shuttleworthia, Anaeroglobus* and *Megasphaera* were more abundant in term placentas. *Escherichia* spp. are considered as both human commensals and opportunistic pathogens and have been found in the urogenital and reproductive tracts. *Escherichia* was reported as one of the principal members of the placental microbiota and also in low abundance [6,11,13,14,17].

A study by Aagaard *et al.* also detected a higher abundance of *Escherichia* in the placenta and proposed that the presence of *Escherichia* in meconium, which is strongly associated with early onset neonatal sepsis, may be consistent with intrauterine seeding with the placenta acting as a reservoir [11]. They also suggest that the presence of oral bacteria in the amniotic cavity, such as *Fusobacterium nucleatum*, may facilitate hematogenous transmission of other bacteria during placentation by altering the mucosal permeability of the vascular endothelium [11,60]. *Anaeroglobus* is a relatively newly classified pathogen belonging to the family *Veillonellaceae* and is closely related to *Megasphaera* [60]. *Megasphaera* and *Shuttleworthia* have been strongly associated with bacterial vaginosis, which is linked to preterm birth [62-64]. The known association with bacterial vaginosis suggests that the presence of these bacteria within the placenta may arise because of ascending infection. However, in this study *Megasphaera* and *Shuttleworthia* were found more abundantly in term placentas from high-risk pregnancies which suggests their role may be associated with other adverse outcomes, though this association should be investigated further.

In bacterial vaginosis there is a shift in the *Lactobacillus* dominated microbiota to overgrowth of Gram negative or anaerobic bacteria such as *Megasphaera* spp., *Gardnerella vaginalis*, *Atopobium* spp., *Prevotella, Ureaplasma* spp., or *M. hominis*. *Megasphaera* has previously been associated with an increased risk of spontaneous preterm birth [63,65]. *Lactobacillus* are prevalent in the intestinal and vaginal flora, which may suggest that their presence in the placenta arises as a result of ascending transmission [15]. The ubiquitous presence of *Lactobacillus* in the placentas of preterm and term birth in this study may indicate that they are commensals, not associated with pathology of preterm birth, and are not influenced by mode of delivery as they were present in placentas delivered by caesarean section and therefore not exposed to vaginal flora during delivery. An overall increased bacterial diversity, together with a depletion of *Lactobacillus,* has been described as a universal marker for diagnosis of bacterial vaginosis [66].

This study used primers that targeted the V3-V4 region to amplify the 16S rRNA gene for next generation sequencing. Other studies have used V3–V4, as well as 16S regions V1–V3, and V6-8 [11,14,67]. Parnell *et al*. sequenced all nine variable regions of the 16S rRNA gene and compared results according to total amplification and amplification of negative controls [15]. They reported that targeting the areas flanking the V4 region successfully amplified DNA in most samples, had sufficient reads for analysis and the lowest incidence of reads in negative controls compared to other variable regions. Studies of samples with low microbial biomass, such as the placenta, are particularly vulnerable to contamination, either 'kitome' or 'splashome', during processing and should consider additional measures for absolute abundance of total 16S rRNA gene copies, i.e., qPCR, to validate sequence analysis and avoid reporting false positive signals [15,18,42]. This is a potential limitation of this study, as qPCR was not performed for placental samples and the two methods of PCR (conventional and next generation sequencing of 16S rRNA) already underscored discrepancies in bacterial detection. This may lead to under-reporting of bacterial species in studies where only the next generation sequencing of 16S rRNA genes is used for detection.

The following limitations should be acknowledged; the term control group was from high-risk pregnancies delivered at a tertiary hospital which specializes in complicated cases, and so does not represent a true 'healthy' term pregnancy cohort. The use of antibiotics was not routinely monitored by researchers, and therefore antibiotic use may have influenced

the microbiota present in the placenta, although Prince *et al*. report minimal influence of antibiotics on the placental microbiome in their study [6]. There was also a delay from sample collection to the NGS experiment of approximately 11 months and at least two freeze-thaw cycles before tissues were excised for DNA extraction, which may have influenced the integrity of the samples, although stored in RNAlater at -80°C. The concern of contamination is currently a hot topic in studies of low-biomass samples [18]. Contamination may occur during the automated DNA extraction process as well as during the sequence library build process if samples are not randomized for sequencing at a minimum. However, the results of this study show significant differences in alpha diversity between placentas from preterm vs term birth and in association with chorioamnionitis and maternal HIV status, as well as beta diversity with differentially expressed bacterial genera. The total study cohort is also relatively homogenous with regards to maternal race, therefore this factor could not be used for microbial diversity analysis. It is known that racioethnicity influences the vaginal microbiome during pregnancy [50] and as such, this study offers a unique cohort of predominantly African women who are considered 'high risk' for preterm birth and certainly adds to the body of knowledge on microbiomes of a specific cohort in the South African context.

A South African study by Lennard *et al*. found that the vaginal microbiome varies by geographical location between two cities within the same country [68]. They suggested that clinical diagnostic and therapeutic approaches be tailored accordingly as women from different cities but from similar low-income high population density communities, and otherwise analogous demographics, had significantly different vaginal microbiomes. This emphasizes the effect of cohort selection on microbial diversity analysis where very few studies from low to middle income countries have been reported.

Conclusion

This is the first South African study to characterize the bacterial diversity in placentas from complicated pregnancies using next generation 16S rRNA gene sequencing in conjunction with full placental histology. Microbial diversity differs between preterm and term placentas where HIV may act as a cofactor associated with decreased bacterial alpha diversity in placentas from preterm birth. Characterizing the bacteria in the placenta has contributed to further understanding of the pathology associated with preterm birth. This has the potential to predict clinical pathology in the fetus, thereby reducing the risk of preterm birth and its associated adverse outcomes.

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Author Contributions

KES collected and analyzed samples. SP, JES, and EW were involved in sample sequencing and data analysis. KES and SG drafted manuscript. SG edited the manuscript. SG and CAW designed and supervised the study which was part of the PhD thesis of KES, and all authors approved the final manuscript.

Consent to Participate

Written informed consent was obtained from patients prior to sample acquisition.

Availability of Data and Material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

None.

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