MATERNAL MICROBIOME IN HEALTH AND DISEASE: ADVANCES AND POSSIBLE OUTCOMES

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Table of Contents

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ORIGINAL RESEARCH
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Fecal Microbiota Changes in Patients With Postpartum Depressive Disorder

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Postpartum depressive disorder (PPD) is a unique subtype of major depressive disorder and a substantial contributor to maternal morbidity and mortality. However, the pathogenesis of PPD has still remained elusive, and it may associate with genetic and environmental factors. Gut microbiota has already been proved to be associated with depression; however, a limited number of studies have concentrated on PPD. The present study aimed to explore the potential correlations between gut microbiota and PPD. In this study, 57 participants were enrolled, in which fecal samples of 28 patients with PPD and 16 healthy controls (HCs) were collected and then analyzed by high-throughput sequencing of the 16S ribosomal RNA (rRNA) gene. The results showed that diversity and composition of gut microbial communities were partly different between PPD patients and HCs. The relative abundance of *Firmicutes* phyla was lower in PPD patients. The levels of several predominant genera were significantly different between PPD patients and HCs. More importantly, the PPD patients experienced reduced levels of *Faecalibacterium*, *Phascolarctobacterium*, *Butyricicoccus*, and *Lachnospiraceae*, as well as increased levels of *Enterobacteriaceae* family. In addition, a correlation was observed between levels of *Phascolarctobacterium*, *Lachnospiraceae*, *Faecalibacterium*, and *Tyzzerella.3* and the severity of depressive symptoms. Various kinds of bacteria, such as *Lachnospiraceae* and *Faecalibacterium*, were found to be associated with levels of sex hormones. This study indicated the correlation between gut microbiota and PPD, and gut microbiota-based biomarkers may be helpful for the diagnosis and treatment of PPD patients. However, further studies need to be conducted to clarify the cause–effect relationship between PPD patients and gut microbiota and to highlight the suitability of gut microbiome as a biomarker.

Keywords: postpartum depressive disorder, gut microbiota, 16SrRNA gene, gut-brain, sex hormone

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INTRODUCTION

Postpartum depressive disorder (PPD) is a type of mood disorder associated with childbirth, and it can affect both sexes, associating with the morbidity of 7.1% in the first three postnatal months (Gavin et al., 2005). The symptoms of PPD include extreme sadness, low energy, anxiety, crying episodes, irritability, and changes in sleeping or eating patterns, thereby resembling a major depressive disorder (MDD). PPD severely influences mothers' quality of life and daily activities, and it can also negatively affect the newborn child (Netsi et al., 2018; Weissman, 2018). Despite the fact that multiple antidepressants are recommended for alleviating symptoms of depression,

<50% of PPD patients have obvious responses to the existing drug therapies (Hansen et al., 2008). Hormonal abnormalities, neuroendocrine processes, or inflammation all play different roles in depression, although interactions between genetic and environmental factors have significantly attracted scholars' attention. In recent years, mounting evidence has supported the viewpoints that gut microbiome is closely associated with the function and behavior of the brain (Clemente et al., 2012).

The human microbiome, a complex assemblage of the microbes, inhabits, and interacts with human hosts, which are classified into beneficial or pathogenic bacteria. In the human body, great numbers of microbes can be found in the gastrointestinal tract, especially in the colon with the densest and most diverse microbial community (Tremaroli and Backhed, 2012). The gastrointestinal microbiota modulates host function from various aspects: apart from the decomposition of food residues and production of micronutrients, they could produce short-chain fatty acids (SCFAs) (Cummings et al., 1987) and neuroactive substance (Barrett et al., 2012), affect hypothalamic–pituitary–adrenal (HPA) axis (Ait-Belgnaoui et al., 2012) and gut barrier, and promote the balance within the immune system (Clemente et al., 2012).

A number of scholars have supported and characterized "microbiome–gut–brain (MGB) axis" and illuminated a possible role of gut microbiota dysbiosis in a variety of diseases, such as obesity (Torres-Fuentes et al., 2017), inflammatory bowel disease (Lahtinen et al., 2020), and hypertension (Li et al., 2020). Although previous studies have demonstrated that microbial communities may influence our health, further attention needs to be paid to the potential associations between maternal gut microbiome and PPD. However, several studies have suggested that alterations of gut microbiota may influence other depressive disorders. Compared with healthy subjects, the relative abundance of *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* has markedly increased in MDD patients, and it was unveiled that *Faecalibacterium* was negatively correlated with the severity of symptoms of depression (Jiang et al., 2015). Another study pointed out that the relative abundance of *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* has remarkably changed in MDD patients, and fecal microbiota transplantation of Germ-free (GF) mice with "depression microbiota" derived from MDD patients resulted in depression-like behaviors (Zheng et al., 2016). In those studies, the gut microbiota related to inflammatory status, oxidative stress, and disease severity could be further identified (Jiang et al., 2015). Additionally, animal studies showed that changes of gut microbiota were associated with depressive-like behaviors (Foster and McVey Neufeld, 2013), and probiotic supplementation could prevent and alleviate anxiety and depression in mice by regulating gut microbiota dysbiosis (Jang et al., 2019). Besides, a number of scholars have reported the characteristics of maternal microbiome during pregnancy or in the postpartum period. A study assessed the relationship between psychosocial stress and fecal microbiota in pregnant women, and their findings revealed a significant association between anxiety in late pregnancy and women's fecal microbiota composition at the genus level. More specifically, the fecal microbiota of mothers with lower anxiety could be characterized by higher abundance of the *Eubacterium* and *Oscillospira* compared with mothers with higher prenatal anxiety (Hechler et al., 2019). A randomized controlled trial (RCT) demonstrated that targeted supplementation with probiotics can correct PPD and postpartum anxiety-associated behavioral abnormalities (Slykerman et al., 2017). On the basis of these findings, we speculated that gut microbiota dysbiosis may be involved in the development of PPD.

The present study aimed to investigate whether the gut microbiota could be changed in PPD patients and identify the specific microbiota for PPD *via* high-throughput sequencing of the 16S ribosomal RNA (rRNA) gene. Additionally, the associations between gut microbiota and clinical patterns were explored.

MATERIALS AND METHODS

Ethics, Consent, and Permissions

This study was approved by the Ethics Committee of Shenzhen Traditional Chinese Medicine Hospital [Shenzhen, China; Registration No. (2018), 81]. All procedures were designed and conducted in accordance with the Declaration of Helsinki. Eligible participants were informed about all the procedures, benefits, as well as potential risks that they may encounter in this trial, and they could withdraw the study at any time without any specific reason. All the participants signed the written informed consent form prior to commencing the study.

Recruitment of Study Subjects

Healthy participants and patients with PPD were included in this study. All participants were recruited from the Shenzhen Traditional Chinese Medicine Hospital and Shenzhen Maternity & Child Healthcare Hospital (Shenzhen, China). The PPD was diagnosed according to the Fourth Edition of the Diagnostic and Statistical Manual of Mental Disorders (*DSM-IV*). Participants who gave birth within 1 year were recruited for further evaluation. People were enrolled in this study from June 2019 to October 2019.

The inclusion criteria for patients with PPD were as follows: (1) patients who are aged 20–49 years old; (2) patients who were diagnosed with PPD by a psychiatrist; (3) onset of disease within 12 months after delivery; (4) the scores of 17-item Hamilton depression rating scale (17-HAMD) ranging from 7 to 24; and (5) signing the written informed consent form. Patients with any one of the following items were excluded: (1) bipolar disorder (diagnostic criteria according to *DSM-IV*) or serious mental disorders, such as schizophrenia; (2) dysnoesia or having difficulty in understanding the content of the questionnaire due to brain diseases or other reasons, or incapable of effective interview; (3) pregnancy; (4) the score of "suicide" item in the 17-HAMD would be more than 2; (5) having committed suicide within 1 year; or (6) having anorexia nervosa.

The inclusion criteria for healthy participants were as follows: (1) participants who are aged 20–49 years old; (2) no obvious discomfort; (3) normal health examination after delivery (routine blood count, liver and kidney functions, and electrocardiogram); (4) 17-HAMD score would be <7; and (5) signing the written

informed consent form for voluntarily participation in this study. Participants with any of the following conditions were excluded: (1) with pregnancy; (2) having committed suicide in the last year; (3) the score of "suicide" item in 17-HAMD would be >2; or (4) participants who were involved in other clinical trials.

Evaluation of Clinical Scales

All participants completed the evaluation process of 17-HAMD and the Edinburgh Postnatal Depression Scale (EPDS). The 17-HAMD, a 17-item scale, was designed to measure the frequency and intensity of depressive symptoms in individuals with MDD (Hamilton, 1960). The 17-HAMD scores range from 0 to 52: "7 < scores ≤ 17" indicate clinically mild depression; "17 < scores ≤ 24" are indicative of moderate depression; "scores > 24" denote severe depression. The EPDS, a self-reporting scale, consists of 10 items with acceptable sensitivity, specificity, and positive predictive values (Cox et al., 1987), containing mood, fun, self-accusation, anxiety, fear, insomnia, coping ability, sadness, crying, and self-injury. Each item is divided into four grades: never (0 point), occasionally (1 point), often (2 points), and always (3 points). The total score of EPDS ranges from 0 to 30, and scores higher than 13 are indicative of clinically significant depression.

Measurement of Serum Levels of Sex Hormones

Blood samples were collected immediately after the 17-HAMD and EPDS assessment, then transferred to the laboratory, and stored in −20°C refrigerator for 15 min until preparation for further analysis. The serum levels of sex hormones, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactinemia (PRL), progesterone (PROG), estradiol (E2), and testosterone (TESTO), were detected using commercially available enzyme-linked immunosorbent assay (ELISA) kits (RayBiotech, Norcross, GA, USA).

Analysis of Clinical Characteristics

Demographic and clinical characteristics of healthy controls (HCs) and patients with PPD were compared using SPSS 22.0 software (IBM, Armonk, NY, USA). For continuous data, normally distributed data were analyzed by the Student's *t*-test and expressed as mean ± standard deviation, while abnormally distributed data were analyzed by the Mann–Whitney *U*-test and presented as median or interquartile range (IQR). For count data, the chi-square test was used. $P < 0.05$ was considered statistically significant.

Collection of Fecal Samples

Fecal samples of all participants were collected after being enrolled within 2 days after 17-HAMD and EPDS evaluation, which were put into a sterile plastic cup and immediately stored at −20°C after defecation, and further transported at −80°C storage to the laboratory of Shenzhen Traditional Chinese Medicine Hospital. Additionally, for hospitalized patients, the collected samples were stored in a −80°C refrigerator. The details of fecal sample collection have been previously described (Zhou et al., 2019).

DNA Extraction

DNA extraction was carried out using MOBIO PowerSoil® DNA Isolation kit, and DNA was stored at −80°C in Tris-EDTA buffer solution. To perform amplification of the V4 region of the 16S rRNA gene and add barcode sequences, unique fusion primers were designed based on the universal primer set, 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′), along with barcode sequences. PCR mixtures contained 1 μl of each forward and reverse primer (10 μM), 1 μl of template DNA, 4 μl of dNTPs (2.5 mM), 5 μl of 10× EasyPfu Buffer, 1 μl of Easy Pfu DNA Polymerase (2.5 U/μl), and 1 μl of double-distilled water in a 50-μl reaction volume. Thermal cycling consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 40 s, with a final extension step at 72°C for 4 min. Amplicons were run for each sample on an agarose gel. Expected band size for 515f-806r was ∼300–350 bp. Amplicons were quantified with Quant-iT PicoGreen dsDNA Assay Kit (P11496; Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. The amplicon library for high-throughput sequencing on the Illumina MiSeq V3 reagent PE150 (300 cycles) platform was combined to an equal amount and subsequently quantified using KAPA Library Quantification Kit (KK4824; Illumina, Inc., San Diego, CA, USA) according to manufacturer's protocols.

High-Throughput Sequencing of 16S Ribosomal RNA Gene

Using the Quantitative Insights Into Microbial Ecology (QIIME) 2.0, the raw sequences were processed to concatenate reads into tags according to the overlapping relationship; then, reads belonging to each sample were separated with barcodes, and low-quality reads were removed. The processed tags were clustered into the Amplicon Sequence Variants (ASVs) at the commonly used 97% similarity threshold. The ASVs were assigned to taxa by matching to the SILVA database. A phylogenetic tree of representative sequences was constructed. Evenness, observed species, Shannon, and Faith-PD indices were used to estimate the α-diversity. The measurement of β-diversity was undertaken using UniFrac that is a β-diversity measure that uses phylogenetic information, and the principal coordinate analysis (PCoA) was employed to calculate the distance matrixes. To further identify the specific bacteria as biomarkers at the genus level, linear discriminant analysis effect size (LEfSe) was applied through the Huttenhower Lab Galaxy Server (Segata et al., 2011) after taxa summaries were reformatted. In the setting of LEfSe, firstly, the Kruskal–Wallis test ($\alpha = 0.05$) was employed to detect taxa using differential abundance analysis; secondly, the Wilcoxon rank-sum test was used to investigate the biological consistency among subclasses; finally, the effect size of differentially abundant genera was estimated by linear discriminant analysis (LDA) (Segata et al., 2011), and the threshold on the logarithmic LDA score for discriminative features was 2. All the analyses were conducted using "vegan" package in R 3.4.1 software. Correlations between clinical variables and bacterial taxa were analyzed by using Spearman's Rho test.

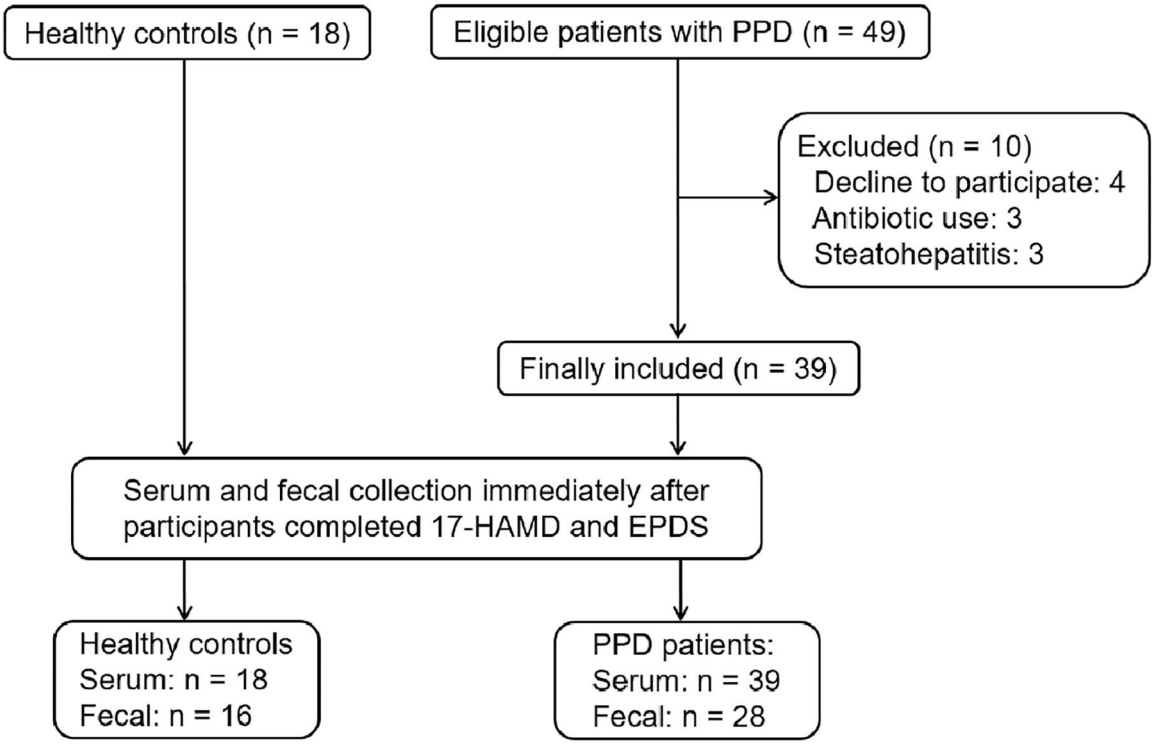

FIGURE 1 | The recruitment of participants and the process of sample collection.

TABLE 1 | Baseline characteristics and clinical symptoms in patients with PPD and HCs.

Parameter	HC group (n = 18)	PPD group (n = 39)	P-value
Sociodemographics			
Age, mean (SD), years	32.57 ± 3.98	33.64 ± 4.27	0.376
BMI, mean (SD),	20.90 ± 2.24	21.50 ± 2.87	0.434
High school or less, No. (%)	2 (11.11%)	7 (17.95%)	0.275
Duration of postpartum, mean (SD), days	142.94 ± 129.00	112.00 ± 91.84	0.372
Menstruating, No. (%)	11 (61.1%)	30 (76.0%)	0.217
Constipation, No. (%)	5 (27.8%)	16 (41.0%)	0.335
Severity of depressive symptoms			
HAMDs	3.83 ± 1.98	13.46 ± 3.51	<0.001*
EPDS	5.72 ± 3.72	15.33 ± 4.66	<0.001*

*HC, healthy control; PPD, postpartum depressive disorder; HAMDS, Hamilton's Depression Scale; EPDS, Edinburgh Postnatal Depression Scale. P < 0.05 is marked with *.*

RESULTS

Participants' Demographic and Clinical Data

From June 7, 2019 to October 15, 2019, 67 participants were recruited from Shenzhen Traditional Chinese Medicine Hospital and Shenzhen Maternity & Child Healthcare Hospital. Of the 67 participants, 10 patients withdrew from the study. Finally, 18 HCs and 39 patients with PPD were included in the study. All participants completed the 17-HAMD and EPDS evaluation. Among HCs, 18 participants completed the serum sample collection; however, two cases did not complete the fecal sample collection. Among patients with PPD, 39 patients completed the serum sample collection, and only 28 cases completed the fecal sample collection (**Figure 1**).

Demographic characteristics, such as age, body mass index (BMI), education background, duration of postpartum, etc., showed no significant difference between the HC group and PPD group (**Table 1**; $P > 0.05$). For the EPDS and 17-HAMD, the total score was significantly higher in the PPD group than that in the HC group (**Table 1**; $P < 0.001$). Other baseline information was also collected, including special diets, gastrointestinal disorders, metabolic disorders, and antibiotic/probiotic treatments. The surveys showed that all puerpera had no irritable bowel syndrome and bowel cancer; however, some of them had constipation (**Table 1**). None had hypertension or diabetes after childbirth, while three participants were excluded for steatohepatitis (**Figure 1**). Additionally, another three patients with antibiotic use were ruled out as well (**Figure 1**). Furthermore, all the participants were from Shenzhen (China), whose favorable diet was easy-to-digest foods and less consumption of greasy, Atsumi, and spicy food. All the eligible participants had a general appetite and nutritious food with balanced portion of vegetables and meat.

The serum levels of sex hormones in the PPD group were compared with those in the HC group (**Table 2**). No significant differences in the levels of FSH, LH, and PROG were observed between the two groups. However, the levels of E2 and TESTO in the PPD group were lower than those in the HC group ($P = 0.036$ and 0.012, respectively), and the PRL level in the PPD group was higher than that in the HC group ($P = 0.001$).

Collection of 16S Ribosomal RNA Sequences

Herein, 44 samples from participants who completed 16S rRNA sequences were collected, in which 1,765,950 qualified sequences from 1,852,840 raw sequences were filtered. Then, a total of 979 qualified ASVs were clustered for downstream analysis. The mean number of ASVs per sample was 92.70, and the standard deviation was 23.86. The sequencing results could be achieved from 28 PPD patients and 16 HCs.

Analysis of Microbial α- and β-Diversity

The indices of fecal bacterial α-diversity are shown in **Figure 2**. There were no significant differences between the PPD group and HC group, including observed species, Evenness, Shannon, and Faith-PD indices ($P = 0.669$, 0.526, 0.367, and 0.435, respectively) (**Figure 2**). However, the observed species seemed to be much higher in the HC group than those in the PPD group (**Figure 2D**).

The indices of fecal bacterial β-diversity are illustrated in **Figure 3**. The Wilcoxon rank-sum test and PCoA were performed to measure differences in β-diversity between the two groups using weighted UniFrac distance metrics (**Figures 3A,B**, respectively). The sample-based differences in the PPD group were significantly higher than those in the HC group ($P = 2e\text{-}14$) (**Figure 3A**). Additionally, as displayed in **Figure 3B**, the result of PCoA unveiled that there was a significant difference in bacterial communities between the PPD group and HC group ($P = 0.038$). The results disclosed that the indices of fecal bacterial β-diversity in the HC group were more centralized than those in the PPD group.

TABLE 2 | Sex hormone levels in patients with PPD and HCs.

Parameter	HCs group ($n = 18$)	PPD group ($n = 39$)	*P*-value
FSH, mean (SD), ng/ml	9.05 ± 5.74	8.59 ± 3.01	0.757
LH, mean (SD), ng/ml	14.73.90 ± 28.25	6.04 ± 5.26	0.212
E2, mean (SD), ng/ml	240.61 ± 331.17	109.42 ± 125.33	0.036*
PRL, mean (SD), ng/ml	346.43 ± 265.54	872.82 ± 860.03	0.001*
PROG, mean (SD), ng/ml	4.86 ± 13.49	2.005 ± 4.41	0.392
TESTO, mean (SD), ng/ml	1.27 ± 0.61	0.82 ± 0.612	0.012*

*HC, healthy control; PPD, postpartum depressive disorder; FSH, follicle-stimulating hormone; LH: luteinizing hormone; E2, estradiol; PRL, prolactinemia; PROG, progesterone; TESTO, testosterone. P < 0.05 is marked with *.*

Composition of Microbial Communities

The histograms of species were made for the two groups at the levels of phylum, class, order, family, and genus on the basis of the annotation results. The histograms showing the relative abundance of species uncovered the composition (species and corresponding proportion) of microbial communities in each group with higher relative abundance at different levels (**Figures 4A–F**). The analysis of the composition of the gut microbiota at the levels of phylum and genus reflected the entire structure of gut microbiota.

On the whole, there were significant differences in the composition of the gut microbiota at the phylum level (**Figure 4A**). Besides, 10 and 11 phyla were detected in the HC group and PPD group by sequence alignment analysis using the SILVA database, respectively. *p_Patescibacteria* was only found in the PPD group, although its relative abundance was low. In both groups, the proportion of *p_Firmicutes* was the highest among all phyla, which was higher in the HC group (88.91%) than that in PPD group (74.57%). *p_Actinobacteria* and *p_Bacteroidetes* ranked the second and third, while they were both relatively lower in the HC group than those in the PPD group.

Furthermore, we analyzed the characteristics and alterations in community structure of the gut microbiota in the two groups

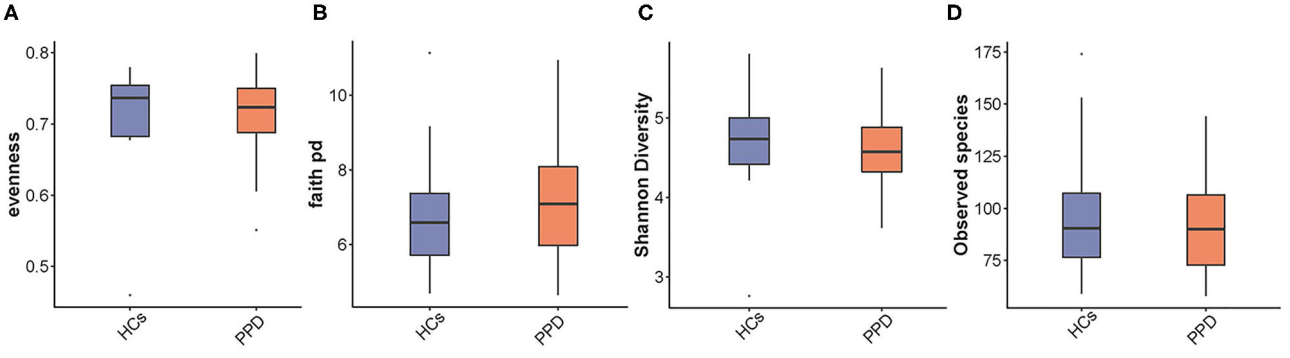

FIGURE 2 | An analysis of microbial α-diversity. Evenness index **(A)**; Faith pd index **(B)**; Shannon diversity **(C)**; Observed species **(D)**.

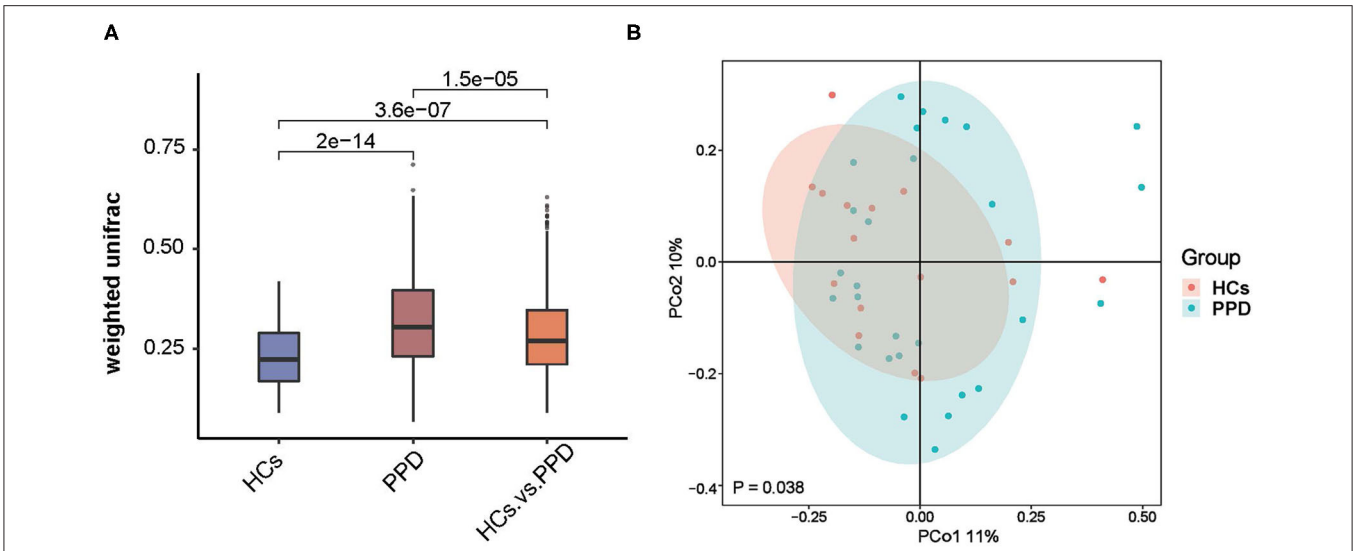

FIGURE 3 | An analysis of microbial β-diversity. The Wilcoxon rank-sum test analysis **(A)** and Principal coordinates analysis plots **(B)** of the fecal microbiome based on the weighted-UniFrac distance metric.

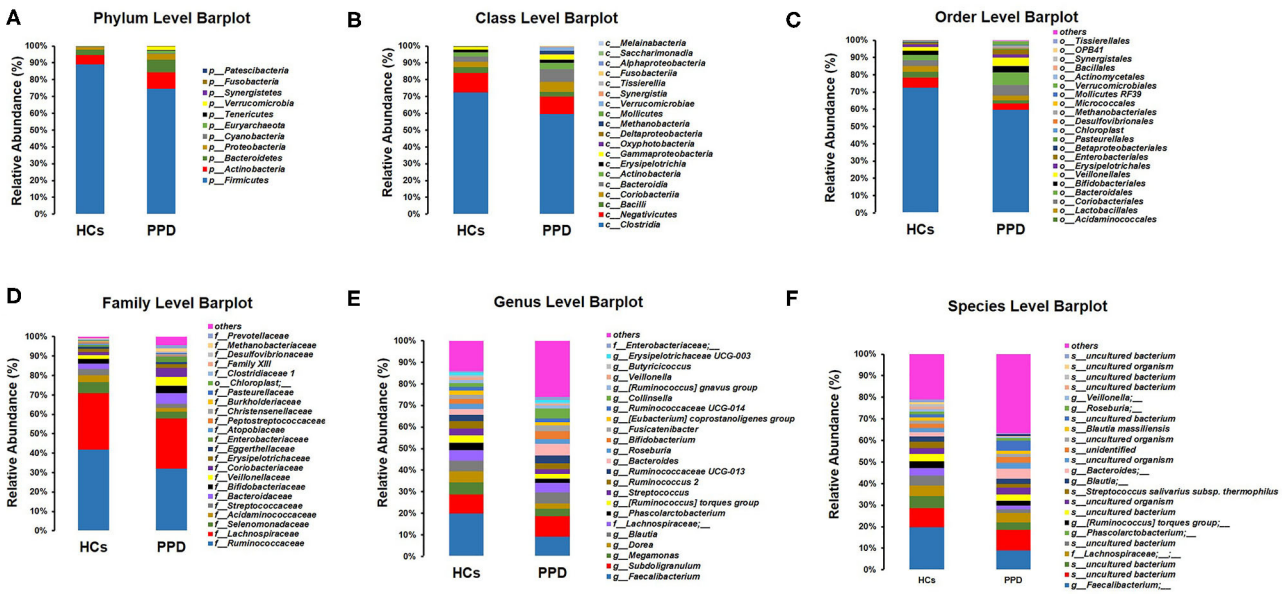

FIGURE 4 | Composition of microbial communities. Phylum level **(A)**; Class level **(B)**; Order level **(C)**; Family level **(D)**; Genus level **(E)**; Species level **(F)**.

at the genus level according to the relative abundance (**Figure 4E**, **Table 3**). Among all bacteria, *g_Faecalibacterium* was the most dominant one, accounting for 19.79% in HC group and 9.22% in the PPD group. The prominent changes of community structure of the gut microbiota at the genus level are related to the decrease of *g_Faecalibacterium* ($P = 0.003$), *g_Phascolarctobacterium* ($P = 0.022$), *g_Butyricicoccus* ($P = 0.024$), and *g_Megasphaera* ($P = 0.047$) in the PPD group compared to those in the HC group (**Table 3**).

Comparing Differences in Bacterial Genus Between the Healthy Control Group and Postpartum Depressive Disorder Group

To further investigate the differences in the abundance between the two groups and to explore the specific bacteria associated with PPD, LEfSe analysis was utilized ($P < 0.05$, LDA value > 2). The most significant difference among the two groups was that *Faecalibacterium*, *Phascolarctobacterium*, *Butyricicoccus*, *Lachnospiraceae*, *Acidaminococcaceae*,

TABLE 3 | Relative abundance of gut microbial communities at the genus level.

	Tax_name	HCs (%)	PPD (%)	*P*-value
1	*g_Faecalibacterium*	19.7913	9.2243	0.003
2	*g_Subdoligranulum*	8.9888	9.4907	0.778
3	*g_Megamonas*	5.6413	3.4550	0.366
4	*g_Dorea*	5.0988	2.3821	0.090
5	*g_Blautia*	4.9138	5.1350	0.826
6	*f_Lachnospiraceae;_;_*	4.7738	4.3886	0.534
7	*g_Phascolarctobacterium*	3.4688	1.9243	0.022
8	*g_[Ruminococcus] torques group*	3.3713	2.3286	0.163
9	*g_Streptococcus*	3.3200	2.0829	0.227
10	*g_Ruminococcus 2*	3.2888	2.7879	0.864
11	*g_Ruminococcaceae UCG-013*	2.9538	3.5471	0.788
12	*g_Bacteroides*	2.6638	5.5364	0.714
13	*g_Roseburia*	2.4225	2.0914	0.485
14	*g_Bifidobacterium*	2.3725	3.6564	0.288
15	*g_Fusicatenibacter*	1.9988	2.8257	0.668
16	*g_[Eubacterium] coprostanoligenes group*	1.8713	1.4979	0.837
17	*g_Ruminococcaceae UCG-014*	1.7888	1.6050	0.598
18	*g_Collinsella*	1.5913	4.5729	0.225
19	*g_[Ruminococcus] gnavus group*	1.5125	1.4343	0.882
20	*g_Veillonella*	1.1950	0.5864	0.193
21	*g_Butyricicoccus*	1.0888	0.6300	0.024
22	*g_Erysipelotrichaceae UCG-003*	1.0800	1.2386	0.892
23	*f_Enterobacteriaceae;_*	0.6763	1.5679	0.712
24	*g_Megasphaera*	0.6125	0.4636	0.047
25	*g_Anaerostipes*	0.6025	0.7200	0.345
26	*g_Tyzzerella 4*	0.5763	0.4671	0.516
27	*g_Adlercreutzia*	0.5600	0.2793	0.221
28	*g_Olsenella*	0.5200	0.1986	0.972
29	*Others*	11.2575	23.8821	

HC, healthy control; PPD, postpartum depressive disorder.

Eubacterium_xylanophilum, and *Megasphaera* were mainly enriched in the HC group, and *Enterocossus* and *Escherichia_Shigella* were mainly enriched in the PPD group (**Figures 5A,B**).

Associations of Gut Microbiota With Clinical Indicators and Sex Hormones

Associations of Gut Microbiota With Clinical Indicators

BMI was positively correlated with *g_Allisonella* but negatively correlated with *g_Holdemania*, *g_Coprobacillus*, and *g_Ruminococcaceae.UCG.014*. Age was positively correlated with *g_Allisonella*, *g_Raoultibacter*, and *g_Fournierella*, while that was negatively correlated with *g_Moryella* and *g_Megasphaera*.

EPDS scores were positively correlated with *g_Dialister*, *g_Clostridium.sensu.stricto.1*, *g_Senegalimassilia*, and *g_Lachnospiraceae.FCS020.group*, while those were negatively correlated with *g_Lachnospiraceae.UCG.004*, *g_Phascolarctobacterium*, *g_Lachnospiraceae.UCG.001*, *g_Lachnospiraceae.UCG.006*, and *g_Lachnospiraceae.ND3007.group*. The 17-HAMD scores were positively correlated with *g_Escherichia.Shigella*, *g_Dialister*, and *g_Enterococcus*, while those were negatively correlated with *g_Butyricicoccus*, *g_Lachnospiraceae.UCG.001*, *g_Lachnospiraceae.ND3007.group*, *g_Faecalibacterium*, and *g_Tyzzerella.3* (**Figure 6**).

Associations of Gut Microbiota With Levels of Sex Hormones

We also analyzed the associations between levels of sex hormones and gut microbiota. FSH level was positively correlated with *g_Raoultibacter*, *g_Fournierella*, *g_Shuttleworthia*, and *g_Family.XIII.AD3011.group*, while that was negatively correlated with *g_Veillonella*, *g_Lachnospiraceae.FCS020.group*, and *g_Lachnospira*. Moreover, PRL level was negatively correlated with *f_Erysipelotrichaceae_*, *g_Eubacterium..coprostanoligenes.group*, *g_Ruminococcus..torques.group*, *g_Lachnospiraceae.NK4A136.group*, *g_Pyramidobacter*, *g_Eubacterium..hallii.group*, *g_Lachnospiraceae.UCG.006*, *g_Megamonas*, and *g_Faecalibacterium*, while that was positively correlated with *g_Prevotella*, *g_Anaerostipes*, *g_Bifidobacterium*, and *g_Eggerthella*. PROG level was positively correlated with *g_Prevotella.2*, *f_Burkholderiaceae._*, *g_.Eubacterium..hallii.group*, *g_.Ruminococcus..torques.group*, *f_Lachnospiraceae._*, *g_Ruminococcus.1*, *g_Coprococcus.2*, *g_Lachnospiraceae.ND3007.group*, *g_Lachnospira*, and *g_Tyzzerella.3*, whereas that was negatively correlated with *g_Prevotella*, *g_Blautia*, *g_Absiella*, *g_Tyzzerella.4*, *g_.Ruminococcus..gnavus.group*, and *g_Peptoniphilus*. TESTO level was positively correlated with *f_Erysipelotrichaceae._*, *g_Alistipes*, *f_Burkholderiaceae._*, *g_Turicibacter*, *g_Faecalibacterium*, *g_.Eubacterium..xylanophilum.group*, and *g_Tyzzerella.3*, while it was negatively correlated with *g_Tyzzerella.4*, *g_Raoultibacter*, *g_Eggerthella*, and *g_Akkermansia*. LH level was negatively correlated with *g_Catenibacterium*, *g_Blautia*, *g_Collinsella*, *g_Parvibacter*, *g_Lachnospiraceae.UCG.006*, *g_Faecalibacterium*, and *g_Roseburia*, while that was positively correlated with *g_Escherichia.Shigella*, *g_.Eubacterium..eligens.group*, and *g_Parabacteroides*. E2 level was *g_Eisenbergiella*, *g_Parvibacter*, and *g_DTU089*, while that was positively correlated with *f_Burkholderiaceae._*, *g_Turicibacter*, g_.*Eubacterium..hallii.group*, *g_Coprococcus.2*, *g_Ruminiclostridium.9*, *g_Lachnospiraceae.ND3007.group*, and *g_Lachnospiraceae.UCG.006* (**Figure 6**).

DISCUSSION

In the current study, we characterized the gut microbiota of PPD and HC patients by high-throughput sequencing of the 16S rRNA gene. Moreover, associations of gut microbiota with clinical indicators and sex hormones were assessed. The results showed that diversity and composition of gut microbial communities were remarkably different between the PPD group and HC group. We found that some butyrate-producing genera were enriched in the HC group, such as *Faecalibacterium*,

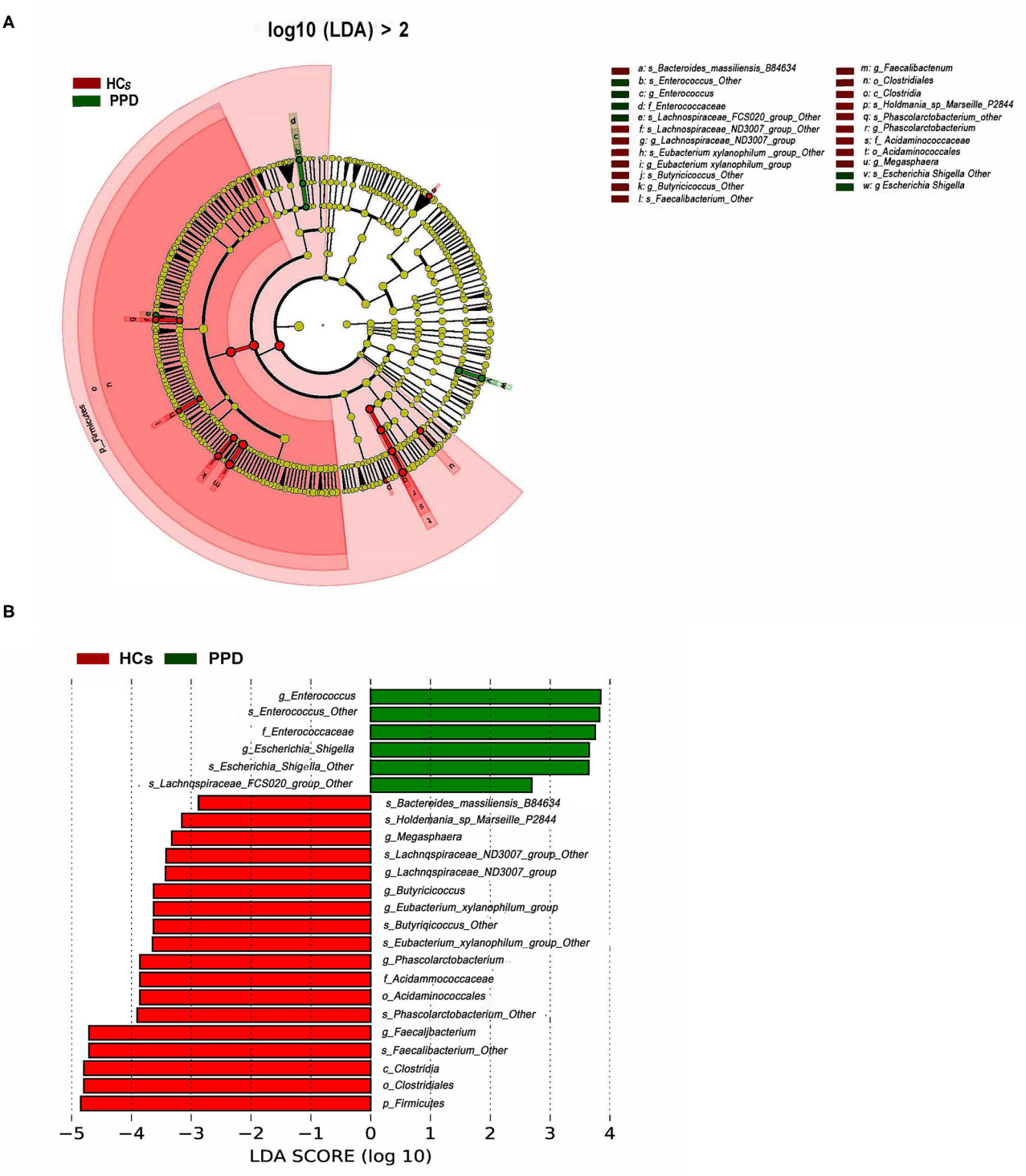

FIGURE 5 | Bacterial taxa differences between healthy control (HC) and postpartum depressive disorder (PPD) patient samples. Cladogram showing the most differentially abundant taxa identified by linear discriminant analysis effect size (LEfSe). Red indicates clades enriched in the HC group, whereas blue indicates clades enriched in the PPD group **(A)**. Comparisons of gut microbiota between the NC and PPD groups **(B)**. Only genera meeting a linear discriminant analysis score threshold >2 are shown.

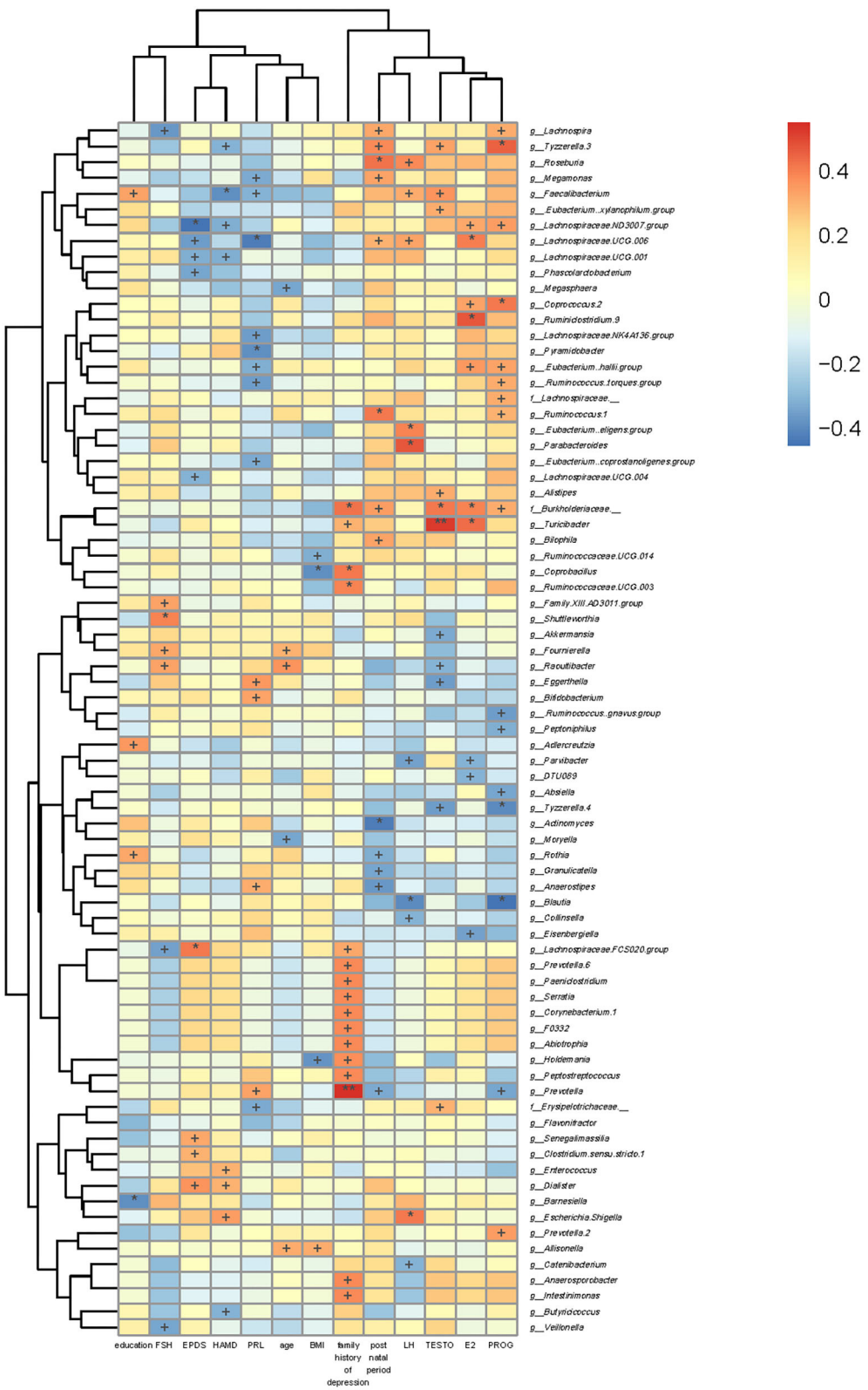

FIGURE 6 | Association of gut microbiota with clinical indicators. The heat map of Spearman's rank correlation coefficients between the gut microbiota and clinical indicators. $^{+}P < 0.05$; $^{*}P < 0.01$; $^{**}P < 0.001$.

Phascolarctobacterium, and *Butyricicoccus*. Additionally, the overgrowth of *Enterococcaceae* and *Escherichia_Shigella* was detected in the PPD group. Moreover, the present study revealed that the amounts of specific genera were correlated with clinical indicators and levels of sex hormones in the PPD group, which indicated that sex hormones might play a significant role in the gut microbiome of patients with PPD.

Additionally, no significant difference in α-diversity was noted between the PPD group and HC group. Similarly, a recent research revealed that there were no significant differences in the Shannon's index and Simpson's index between patients with MDD and non-depressed controls (Sanada et al., 2020). However, β-diversity of gut microbiota, referring to the sample-based differences, was markedly greater in the PPD group than those in the control group. Various factors may influence the diversity of gut microbiota, including age, diet, and health status, and a number of studies suggested that keeping a rich diversity of gut microbiota is highly beneficial for protecting from autoimmune and metabolic diseases (Manichanh et al., 2006; Claesson et al., 2012; Le Chatelier et al., 2013). However, other studies demonstrated that microbial diversity is reduced in patients with MDD (Dantzer et al., 2018; Winter et al., 2018). Hu et al. (2019) found that diversity of gut microbiota was noticeably different between patients with bipolar disorders and HCs. Another one studied the interaction between antidepression treatments and gut microbiota in a mouse model of depression and found an obvious increase of species richness in acupuncture-treated mice, which indicated that the diversity of the gut microbiota may be a factor influencing depression (Fu, 2019). Thus, due to controversial results about bacterial diversity, further research is warranted to explore the differences in bacterial α- and β-diversity between patients with PPD and HCs.

The present study demonstrated that the microbial composition in the PPD group was noticeably different from that in the HC group. Phylum *Firmicutes* ranked the first in both groups, and it was markedly lower in the PPD group than that in the HC group, which was consistent with findings of a previous research (Hu et al., 2019). Additionally, we found that *Escherichia_Shigella* was enriched in the PPD group. *Escherichia_Shigella*, belonging to the *Enterobacteriaceae* family, is Gram-negative bacteria and observed in normal gut flora. Overgrowth of *Enterbacteriaceae* could result in gut inflammation and increased permeability of the gut wall, which in turn favors bacterial translocation, promoting systemic inflammation (Winter and Bäumler, 2014). Clinical depression is accompanied by increased pro-inflammatory cytokine interleukin (IL), such as IL-1β and IL-6 (Berk et al., 2013; Wong et al., 2016). Some studies found an anti-inflammatory effect of *Faecalibacterium*, *Bifidobacterium*, and *Lactobacillus* on stress responses and depressive disorders (Jiang et al., 2015; Aizawa et al., 2016). Moreover, previous studies have also found that the increase of *Enterobacteriaceae* in the gastrointestinal tract can induce behavioral and psychological changes in animals and humans (Goehler et al., 2008; Löwe et al., 2014; Jiang et al., 2015; Borgo et al., 2017). More importantly, various bacteria with decreased abundance were found in the PPD group, including *Faecalibacterium*, *Phascolarctobacterium*, and *Butyricicoccus*. It was in accordance with some studies (Jiang et al., 2015; Zheng et al., 2016) that reported lower abundance of *Faecalibacterium* in depressive patients compared with non-depressed individuals, while the study by Chen et al. (2018) presented a contrary result. *Faecalibacterium* is a genus of bacteria, and *Faecalibacterium prausnitzii*, its only known species, produces butyric acid and other SCFAs (Louis and Flint, 2009). In addition, a recent study demonstrated that the intake of *Faecalibacterium prausnitzii* improves anxiety-related and depressive-like behaviors in the preclinical setting (Hao et al., 2019), and the study by Jiang et al. (2015) showed a negative correlation between the abundance of *Faecalibacterium* and the severity of depression symptoms. Besides, *Phascolarctobacterium*, affiliated with the *Acidaminococcaceae* at the family level, was significantly higher in the HC group, which is in line with a study that assessed and compared patients with MDD with non-depressed controls (Jiang et al., 2015). However, another study (Jeffery et al., 2012) concentrated on patients with both irritable bowel syndrome and depression and found that *Acidaminococcaceae* significantly overgrew using pyrosequencing fecal samples. Additionally, we also noted lower abundance of *Butyricicoccus* at the genus level in the PPD group. *Butyricicoccus* belongs to the *clostridial* cluster IV genus of the *Firmicutes* phylum, which is typically decreased in patients with inflammatory bowel disease (Eeckhaut et al., 2013). The inflammatory bowel conditions have been found to be correlated with high comorbidity with depression and anxiety (Bhandari et al., 2017). Moreover, we found that the abundance of *Butyricicoccus* was positively associated with EPDS scores. *Butyricicoccus pullicaecorum* is an anaerobic and butyrate-producing bacterium from the genus *Butyricicoccus*. Butyrate, one of the main products of colonic microbiota, has been found beneficial for a variety of diseases, such as insulin resistance and ischemic stroke (Canani et al., 2011). Furthermore, butyrate in the central nervous system can influence the function of the hippocampus and promote the expression of brain-derived neurotrophic factor (BDNF), which has been shown to have antidepressant-like effects in animal models (Yamawaki et al., 2012; Wei et al., 2014). Thus, decreased butyrate-producing bacteria in PPD patients may contribute to the disease pathology.

Furtherly, we noted that the gut microbial communities were relevant to confounding factors, including clinical indicators (age, BMI, etc.), disease severity, and levels of sex hormones. Among the PPD patients, BMI was found to be positively correlated with *g_Allisonella* but negatively correlated with *g_Holdemania*, *g_Coprobacillus*, and *g_Ruminococcaceae.UCG.014*. In another study that involved patients with bipolar disorders, *Holdemania* at the genus level was also found to be negatively correlated with BMI (Hu et al., 2019). Another study showed that the

abundance of *Ruminococcaceae* increased in obesity patients (Chávez-Carbajal et al., 2019), and *Ruminococcaceae* was associated with antidepressant effects in rats under chronic mild stress (Tung et al., 2019). Moreover, the increased levels of *Ruminococcaceae* would decrease after weight reduction (Kang et al., 2017). *Ruminococcaceae* is a dominant butyrate producer. Butyrate supports the energy for colonic mucosa and makes a different regulatory effect on gene expression and inflammation (Pajak et al., 2007; Hamer et al., 2008; Ivanov and Honda, 2012). These findings indicated that *Ruminococcaceae* may be related to the development of PPD through metabolic pathways. Besides, *Holdemania* at the genus level is involved in glucose metabolism and metabolic syndrome (Lippert et al., 2017).

Previous studies have reported some associations between the levels of several genera and depression severity in MDD (Naseribafrouei et al., 2014; Jiang et al., 2015). In the present study, EPDS scores were negatively associated with *Phascolarctobacterium* and *Lachnospiraceae* at the genus level, and 17-HAMD scores were positively associated with *Escherichia.Shigella*, *Dialister*, and *Enterococcus* and negatively associated with *Butyricicoccus*, *Lachnospiraceae*, *Faecalibacterium*, and *Tyzzerella.3* at the genus level. Hence, gut microbiota may play a pivotal role in the metabolic disturbance in PPD patients.

With respect to the causes of PPD, although it has been poorly understood, a number of human and animal studies supported the role of sex hormones in PPD (Schiller et al., 2015). In the current study, we also found that the serum levels of E2, PROG, and TESTO were noticeably different between the PPD group and HC group. Recently, various studies have reported that changes in the levels of sex hormones could be related to diversity and profiles of gut microbiota (Baker et al., 2017; Shin et al., 2019). The gut microbiota has been shown to be influenced by E2; meanwhile, the gut microbiota also could regulate E2 level through secreting β-glucuronidase (Flores et al., 2012; Huang et al., 2017). In the present research, our results also found that some bacteria genera were associated with serum sex hormone levels in PPD patients, such as *Faecalibacterium*, *Lachnospiraceae*, and *Megamonas*, which were significantly different from those in HCs. A recent study has demonstrated that the regulation of sex hormones–microbiota–inflammation axis could ameliorate polycystic ovary syndrome (PCOS), including *Faecalibacterium*, *Parabacteroides*, *Bifidobacterium*, and so on (Wang et al., 2020). Another study showed that prenatal androgen exposure causes hypertension and gut microbiota dysbiosis (Sherman et al., 2018). Moreover, a study found that small amounts of brain E2 and PROG could improve menopausal symptoms by decreasing serum FSH levels and maintaining the diversity of the gut microbiome in estrogen-deficient rats (Park et al., 2018). Our study used EPDS and 17-HAMD scores to assess symptoms of depression in PPD patients and found that *Faecalibacterium* and *Lachnospiraceae* were correlated with disease severity. Hence, the abovementioned findings showed that the interaction of sex hormones and gut microbiota may play a substantial role in PPD.

CONCLUSIONS

In summary, our findings may provide further evidence to support a number of previous reports that the gut microbial composition of PPD patients partially differs from that of HCs, and we explored new associations among gut microbiota, disease severity, and sex hormones. We also noted that *Faecalibacterium*, *Phascolarctobacterium*, and *Butyricicoccus* were significantly decreased in patients with PPD, which were all butyrate-producing, as well as *Enterobacteriaceae* family increased obviously. In addition, we demonstrated that *Phascolarctobacterium*, *Lachnospiraceae*, *Faecalibacterium*, and *Tyzzerella.3* were correlated with disease severity; besides, various kinds of bacteria, such as *Lachnospiraceae* and *Faecalibacterium*, were found to be associated with the levels of sex hormones. The abovementioned results may assist scholars to further explore the underlying pathogenesis of PPD. Moreover, the identified microbiota in this study could be a potential diagnostic biomarker of PPD. Therefore, our findings may provide significant clues for future researches.

LIMITATIONS

Serval limitations of this study should be pointed out. Firstly, this observational study may not contain reliable indicators of causal effects; thus, further longitudinal studies on patients with depression are required to clarify the cause–effect relationship between PPD and gut microbiota. Secondly, due to the small sample size, we could not adjust for multiple testing or ethnicity, and our results still need to be validated by further studies with larger sample sizes. Therefore, future studies may elucidate the temporal and causal relationships between gut microbiota and PPD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/, with accession number PRJNA637228.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Review committee of Shenzhen Traditional Chinese Medicine Hospital (Shenzhen, China; Approval No. [2018], 81). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YZ conceived and planned the experiments and wrote the manuscript. YZ and CC executed the experiments. HY analyzed

the data. ZY contributed to revise the final manuscript. All authors approved the submitted version.

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REFERENCES

Ait-Belgnaoui, A., Durand, H., Cartier, C., Chaumaz, G., Eutamene, H., Ferrier, L., et al. (2012). Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology* 37, 1885–1895. doi: 10.1016/j.psyneuen.2012.03.024

Aizawa, E., Tsuji, H., Asahara, T., Takahashi, T., Teraishi, T., Yoshida, S., et al. (2016). Possible association of Bifidobacterium and Lactobacillus in the gut microbiota of patients with major depressive disorder. *J. Affect. Disord.* 202, 254–257. doi: 10.1016/j.jad.2016.05.038

Baker, J. M., Al-Nakkash, L., and Herbst-Kralovetz, M. M. (2017). Estrogen-gut microbiome axis: physiological and clinical implications. *Maturitas* 103, 45–53. doi: 10.1016/j.maturitas.2017.06.025

Barrett, E., Ross, R. P., O'Toole, P. W., Fitzgerald, G. F., and Stanton, C. (2012). γ-aminobutyric acid production by culturable bacteria from the human intestine. *J. Appl. Microbiol.* 113, 411–417. doi: 10.1111/j.1365-2672.2012.05344.x

Berk, M., Williams, L. J., Jacka, F. N., O'Neil, A., Pasco, J. A., Moylan, S., et al. (2013). So depression is an inflammatory disease, but where does the inflammation come from? *BMC Med.* 11:200. doi: 10.1186/1741-7015-11-200

Bhandari, S., Larson, M. E., Kumar, N., and Stein, D. (2017). Association of inflammatory bowel disease (IBD) with depressive symptoms in the United States population and independent predictors of depressive symptoms in an IBD population: a NHANES study. *Gut Liver* 11, 512–519. doi: 10.5009/gnl16347

Borgo, F., Riva, A., Benetti, A., Casiraghi, M. C., Bertelli, S., Garbossa, S., et al. (2017). Microbiota in anorexia nervosa: the triangle between bacterial species, metabolites and psychological tests. *PLoS ONE* 12:e0179739. doi: 10.1371/journal.pone.0179739

Canani, R. B., Costanzo, M. D., Leone, L., Pedata, M., Meli, R., and Calignano, A. (2011). Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J. Gastroenterol.* 17, 1519–1528. doi: 10.3748/wjg.v17.i12.1519

Chávez-Carbajal, A., Nirmalkar, K., Pérez-Lizaur, A., Hernández-Quiroz, F., Ramírez-Del-Alto, S., García-Mena, J., et al. (2019). Gut microbiota and predicted metabolic pathways in a sample of mexican women affected by obesity and obesity plus metabolic syndrome. *Int. J. Mol. Sci.* 20:438. doi: 10.3390/ijms20020438

Chen, J. J., Zheng, P., Liu, Y. Y., Zhong, X. G., Wang, H. Y., Guo, Y. J., et al. (2018). Sex differences in gut microbiota in patients with major depressive disorder. *Neuropsychiatr. Dis. Treat.* 14, 647–655. doi: 10.2147/NDT.S159322

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack, S., et al. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature* 488, 178–184. doi: 10.1038/nature11319

Clemente, J. C., Ursell, L. K., Parfrey, L. W., and Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell* 148, 1258–1270. doi: 10.1016/j.cell.2012.01.035

Cox, J. L., Holden, J. M., and Sagovsky, R. (1987). Detection of postnatal depression. Development of the 10-item Edinburgh postnatal depression scale. *Br. J. Psychiatry* 150, 782–786. doi: 10.1192/bjp.150.6.782

Cummings, J. H., Pomare, E. W., Branch, W. J., Naylor, C. P., and Macfarlane, G. T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 28, 1221–1227. doi: 10.1136/gut.28.10.1221

Dantzer, R., Cohen, S., Russo, S. J., and Dinan, T. G. (2018). Resilience and immunity. *Brain Behav. Immun.* 74, 28–42. doi: 10.1016/j.bbi.2018.08.010

Eeckhaut, V., Machiels, K., Perrier, C., Romero, C., Maes, S., Flahou, B., et al. (2013). Butyricicoccus pullicaecorum in inflammatory bowel disease. *Gut* 62, 1745–1752. doi: 10.1136/gutjnl-2012-303611

Flores, R., Shi, J., Fuhrman, B., Xu, X., Veenstra, T. D., Gail, M. H., et al. (2012). Fecal microbial determinants of fecal and systemic estrogens and estrogen metabolites: a cross-sectional study. *J. Transl. Med.* 10:253. doi: 10.1186/1479-5876-10-253

Foster, J. A., and McVey Neufeld, K. A. (2013). Gut-brain axis: how the microbiome influences anxiety and depression. *Trends Neurosci.* 36, 305–312. doi: 10.1016/j.tins.2013.01.005

Fu, C. (2019). *Effects of Acupuncture Treatment on Intestinal Flora in Mice with Chronic Stress Anxiety Model*. Beijing: Beijing University of Chinese Medicine.

Gavin, N. I., Gaynes, B. N., Lohr, K. N., Meltzer-Brody, S., Gartlehner, G., and Swinson, T. (2005). Perinatal depression: a systematic review of prevalence and incidence. *Obstet. Gynecol.* 106(5 Pt 1), 1071–1083. doi: 10.1097/01.AOG.0000183597.31630.db

Goehler, L. E., Park, S. M., Opitz, N., Lyte, M., and Gaykema, R. P. (2008). *Campylobacter jejuni* infection increases anxiety-like behavior in the holeboard: possible anatomical substrates for viscerosensory modulation of exploratory behavior. *Brain Behav. Immun.* 22, 354–366. doi: 10.1016/j.bbi.2007.08.009

Hamer, H. M., Jonkers, D., Venema, K., vanhoutvin, S., Troost, F. J., and Brummer, R. J. (2008). Review article: the role of butyrate on colonic function. *Aliment. Pharmacol. Ther.* 27, 104–119. doi: 10.1111/j.1365-2036.2007.03562.x

Hamilton, M. (1960). A rating scale for depression. *J. Neurol. Neurosurg. Psychiatr.* 23, 56–62. doi: 10.1136/jnnp.23.1.56

Hansen, R., Gaynes, B., Thieda, P., Gartlehner, G., Deveaugh-Geiss, A., Krebs, E., et al. (2008). Meta-analysis of major depressive disorder relapse and recurrence with second-generation antidepressants. *Psychiatr. Serv.* 59, 1121–1130. doi: 10.1176/ps.2008.59.10.1121

Hao, Z., Wang, W., Guo, R., and Liu, H. (2019). Faecalibacterium prausnitzii (ATCC 27766) has preventive and therapeutic effects on chronic unpredictable mild stress-induced depression-like and anxiety-like behavior in rats. *Psychoneuroendocrinology* 104, 132–142. doi: 10.1016/j.psyneuen.2019.02.025

Hechler, C., Borewicz, K., Beijers, R., and Saccenti, E. (2019). Association between psychosocial stress and fecal microbiota in pregnant women. *Sci. Rep.* 9:4463. doi: 10.1038/s41598-019-40434-8

Hu, S., Li, A., Huang, T., Lai, J., Li, J., Sublette, M. E., et al. (2019). Gut microbiota changes in patients with bipolar depression. *Adv. Sci.* 6:1900752. doi: 10.1002/advs.201900752

Huang, G., Xu, J., Lefever, D. E., Glenn, T. C., Nagy, T., and Guo, T. L. (2017). Genistein prevention of hyperglycemia and improvement of glucose tolerance in adult non-obese diabetic mice are associated with alterations of gut microbiome and immune homeostasis. *Toxicol. Appl. Pharmacol.* 332, 138–148. doi: 10.1016/j.taap.2017.04.009

Ivanov, I. I., and Honda, K. (2012). Intestinal commensal microbes as immune modulators. *Cell Host Microbe* 12, 496–508. doi: 10.1016/j.chom.2012.09.009

Jang, H. M., Lee, K. E., and Kim, D. H. (2019). The preventive and curative effects of lactobacillus reuteri NK33 and bifidobacterium adolescentis NK98 on immobilization stress-induced anxiety/depression and colitis in mice. *Nutrients* 11:819. doi: 10.3390/nu11040819

Jeffery, I. B., O'Toole, P. W., Ohman, L., Claesson, M. J., Deane, J., Quigley, E. M., et al. (2012). An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut* 61, 997–1006. doi: 10.1136/gutjnl-2011-301501

Jiang, H., Ling, Z., Zhang, Y., Mao, H., Ma, Z., Yin, Y., et al. (2015). Altered fecal microbiota composition in patients with major depressive disorder. *Brain Behav. Immun.* 48, 186–194. doi: 10.1016/j.bbi.2015.03.016

Kang, C., Wang, B., Kaliannan, K., Wang, X., Lang, H., Hui, S., et al. (2017). Gut microbiota mediates the protective effects of dietary capsaicin against chronic low-grade inflammation and associated obesity induced by high-fat diet. *mBio* 8:e00470-17. doi: 10.1128/mBio.00470-17

Lahtinen, P., Jalanka, J., Hartikainen, A., Mattila, E., Hillilä, M., Punkkinen, J., et al. (2020). Randomised clinical trial: faecal microbiota transplantation versus autologous placebo administered via colonoscopy in irritable bowel syndrome. *Aliment. Pharmacol. Ther.* 51, 1321–1331. doi: 10.1111/apt.15740

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., et al. (2013). Richness of human gut microbiome correlates with metabolic markers. *Nature* 500, 541–546. doi: 10.1038/nature12506

Li, J., Yang, X., Zhou, X., and Cai, J. (2020). The role and mechanism of intestinal flora on blood pressure regulation and hypertension development. *Antioxid. Redox Signal*. doi: 10.1089/ars.2020.8104

Lippert, K., Kedenko, L., Antonielli, L., Kedenko, I., Gemeier, C., Leitner, M., et al. (2017). Gut microbiota dysbiosis associated with glucose metabolism disorders and the metabolic syndrome in older adults. *Benef. Microbes* 8, 545–556. doi: 10.3920/BM2016.0184

Louis, P., and Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol. Lett.* 294, 1–8. doi: 10.1111/j.1574-6968.2009.01514.x

Löwe, B., Andresen, V., Fraedrich, K., Gappmayer, K., Wegscheider, K., Treszl, A., et al. (2014). Psychological outcome, fatigue, and quality of life after infection with shiga toxin-producing *Escherichia coli* O104. *Clin. Gastroenterol. Hepatol.* 12, 1848–1855. doi: 10.1016/j.cgh.2014.02.035

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., et al. (2006). Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* 55, 205–211. doi: 10.1136/gut.2005.073817

Naseribafrouei, A., Hestad, K., Avershina, E., Sekelja, M., Linlokken, A., Wilson, R., et al. (2014). Correlation between the human fecal microbiota and depression. *Neurogastroenterol. Motil.* 26, 1155–1162. doi: 10.1111/nmo.12378

Netsi, E., Pearson, R. M., Murray, L., Cooper, P., Craske, M. G., and Stein, A. (2018). Association of persistent and severe postnatal depression with child outcomes. *JAMA Psychiatry* 75, 247–253. doi: 10.1001/jamapsychiatry.2017.4363

Pajak, B., Orzechowski, A., and Gajkowska, B. (2007). Molecular basis of sodium butyrate-dependent proapoptotic activity in cancer cells. *Adv. Med. Sci.* 52, 83–88.

Park, S., Kim, D. S., Kang, E. S., Kim, D. B., and Kang, S. (2018). Low-dose brain estrogen prevents menopausal syndrome while maintaining the diversity of the gut microbiomes in estrogen-deficient rats. *Am. J. Physiol. Endocrinol. Metab.* 315, E99–E109. doi: 10.1152/ajpendo.00005.2018

Sanada, K., Nakajima, S., Kurokawa, S., Barceló-Soler, A., Ikuse, D., Hirata, A., et al. (2020). Gut microbiota and major depressive disorder: a systematic review and meta-analysis. *J. Affect. Disord.* 266, 1–13. doi: 10.1016/j.jad.2020.01.102

Schiller, C. E., Meltzer-Brody, S., and Rubinow, D. R. (2015). The role of reproductive hormones in postpartum depression. *CNS Spectr.* 20, 48–59. doi: 10.1017/S1092852914000480

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Sherman, S. B., Sarsour, N., Salehi, M., Schroering, A., Mell, B., Joe, B., et al. (2018). Prenatal androgen exposure causes hypertension and gut microbiota dysbiosis. *Gut Microbes* 9, 400–421. doi: 10.1080/19490976.2018.1441664

Shin, J. H., Park, Y. H., Sim, M., Kim, S. A., Joung, H., and Shin, D. M. (2019). Serum level of sex steroid hormone is associated with diversity and profiles of human gut microbiome. *Res. Microbiol.* 170, 192–201. doi: 10.1016/j.resmic.2019.03.003

Slykerman, R. F., Hood, F., Wickens, K., Thompson, J. M. D., Barthow, C., Murphy, R., et al. (2017). Effect of Lactobacillus rhamnosus HN001 in pregnancy on postpartum symptoms of depression and anxiety: a randomised double-blind placebo-controlled trial. *EBioMedicine* 24, 159–165. doi: 10.1016/j.ebiom.2017.09.013

Torres-Fuentes, C., Schellekens, H., Dinan, T. G., and Cryan, J. F. (2017). The microbiota-gut-brain axis in obesity. *Lancet Gastroenterol. Hepatol.* 2, 747–756. doi: 10.1016/S2468-1253(17)30147-4

Tremaroli, V., and Backhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. *Nature* 489, 242–249. doi: 10.1038/nature11552

Tung, T. H., Tung, Y. T., Lin, I. H., Shih, C. K., Nguyen, N. T. K., Shabrina, A., et al. (2019). Fish oil, but not olive oil, ameliorates depressive-like behavior and gut microbiota dysbiosis in rats under chronic mild stress. *Biomolecules* 9:516. doi: 10.3390/biom9100516

Wang, T., Sha, L., Li, Y., Zhu, L., Wang, Z., Li, K., et al. (2020). Dietary α-linolenic acid-rich flaxseed oil exerts beneficial effects on polycystic ovary syndrome through sex steroid hormones-microbiota-inflammation axis in rats. *Front. Endocrinol.* 11:284. doi: 10.3389/fendo.2020.00284

Wei, Y., Melas, P. A., Wegener, G., Mathé, A. A., and Lavebratt, C. (2014). Antidepressant-like effect of sodium butyrate is associated with an increase in TET1 and in 5-hydroxymethylation levels in the Bdnf gene. *Int. J. Neuropsychopharmacol.* 18:pyu032. doi: 10.1093/ijnp/pyu032

Weissman, M. M. (2018). Postpartum depression and its long-term impact on children: many new questions. *JAMA Psychiatry* 75, 227–228. doi: 10.1001/jamapsychiatry.2017.4265

Winter, G., Hart, R. A., Charlesworth, R. P. G., and Sharpley, C. F. (2018). Gut microbiome and depression: what we know and what we need to know. *Rev. Neurosci.* 29, 629–643. doi: 10.1515/revneuro-2017-0072

Winter, S. E., and Bäumler, A. J. (2014). Dysbiosis in the inflamed intestine: chance favors the prepared microbe. *Gut Microbes* 5, 71–73. doi: 10.4161/gmic.27129

Wong, M. L., Inserra, A., Lewis, M. D., Mastronardi, C. A., Leong, L., Choo, J., et al. (2016). Inflammasome signaling affects anxiety- and depressive-like behavior and gut microbiome composition. *Mol. Psychiatry* 21, 797–805. doi: 10.1038/mp.2016.46

Yamawaki, Y., Fuchikami, M., Morinobu, S., Segawa, M., Matsumoto, T., and Yamawaki, S. (2012). Antidepressant-like effect of sodium butyrate (HDAC inhibitor) and its molecular mechanism of action in the rat hippocampus. *World J. Biol. Psychiatry* 13, 458–467. doi: 10.3109/15622975.2011.585663

Zheng, P., Zeng, B., Zhou, C., Liu, M., Fang, Z., Xu, X., et al. (2016). Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. *Mol. Psychiatry* 21, 786–796. doi: 10.1038/mp.2016.44

Zhou, Y., Yu, H., Guo, Y., Chen, C., Huang, X., Gou, Y., et al. (2019). Efficacy of acupuncture versus sham acupuncture for postpartum depression disorder: Study protocol for a randomized controlled trial. *Eur. J. Integr. Med.* 31:100982. doi: 10.1016/j.eujim.2019.100982

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A Human Microbiota-Associated Murine Model for Assessing the Impact of the Vaginal Microbiota on Pregnancy Outcomes

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Disease states are often linked to large scale changes in microbial community structure that obscure the contributions of individual microbes to disease. Establishing a mechanistic understanding of how microbial community structure contribute to certain diseases, however, remains elusive thereby limiting our ability to develop successful microbiome-based therapeutics. Human microbiota-associated (HMA) mice have emerged as a powerful approach for directly testing the influence of microbial communities on host health and disease, with the transfer of disease phenotypes from humans to germ-free recipient mice widely reported. We developed a HMA mouse model of the human vaginal microbiota to interrogate the effects of Bacterial Vaginosis (BV) on pregnancy outcomes. We collected vaginal swabs from 19 pregnant African American women with and without BV (diagnosed per Nugent score) to colonize female germ-free mice and measure its impact on birth outcomes. There was considerable variability in the microbes that colonized each mouse, with no association to the BV status of the microbiota donor. Although some of the women in the study had adverse birth outcomes, the vaginal microbiota was not predictive of adverse birth outcomes in mice. However, elevated levels of pro-inflammatory cytokines in the uterus of HMA mice were detected during pregnancy. Together, these data outline the potential uses and limitations of HMA mice to elucidate the influence of the vaginal microbiota on health and disease.

Keywords: vaginal microbiota, bacterial vaginosis (BV), humanization, pregnancy, inflammation

INTRODUCTION

The vagina houses a numerically immense and functionally consequential microbiota (Mendling, 2016; Kaminska and Gajecka, 2017). Distinct anatomic regions within the female reproductive tract house a dynamically changing microbial community of vastly different numbers and taxonomic composition. For example, it is well-known that the vagina maintains a numerically vast microbiota while the uterus (pregnant and non-pregnant) is normally colonized with very limited microbiota (Aagaard et al., 2014; Franasiak and Scott, 2017). Lactobacilli are the dominant taxa of the human vaginal microbiota and are one of the first bacteria to which neonates are exposed. The origin

and consequences of the unique lactobacilli-dominant community structure remains enigmatic, though it is generally accepted that lactate produced by these bacteria results in the characteristic acidic pH of the healthy vagina. This is a result of direct or syntrophic fermentation of the abundant glycogen found in apical squamous epithelia of the vagina (Charbonneau et al., 2016; Reid, 2016). This low pH is generally assumed to have intrinsic bacteriostatic effects and is a prime example of the innate defenses of the female reproductive tract. Thus, the female reproductive tract has a highly adapted microbiota with known beneficial effects including colonization resistance against pathogens (Sykiotis and Bohmann, 2008).

In support of this notion, a study that characterized the diversity of bacterial taxa within the vaginal microbiome of nearly 400 multi-ethnic reproductive age women discovered that the vaginal microbiota clustered into community state types (CSTs) (Ravel et al., 2011; Gajer et al., 2012). Indeed, about 25% of the women sampled clustered into a group (designated CST IV) where lactobacilli was not the dominant taxon. This group was associated with a less acidic pH and higher indices associated with bacterial vaginosis (BV). Intriguingly, African American and Hispanic women were overrepresented among CST IV. Additionally, African Americans are also at a higher risk for adverse pregnancy outcomes, including preterm birth (Kessel et al., 1988). Considering the association between BV and preterm birth (Goldenberg et al., 1998; Leitich et al., 2003), investigating the microbiota of the reproductive tract in this context may offer a path toward refined associations and molecular mechanisms underlying the development of adverse pregnancy outcomes in this population of women.

BV is a common clinical syndrome seen in gynecological practice. African American women are more commonly affected by BV, with prevalence estimates of 51.4% for African American women compared to 23.2% for US white women of reproductive age (Koumans et al., 2007). This condition is best conceptualized as an ecological disorder characterized by the displacement of a lactobacillus-dominant microbiota (and loss of acidic pH) by a variable mix of facultative organisms often including *Gardnerella vaginalis* (Nasioudis et al., 2017). Interestingly, BV can result in the formation of endometrial biofilms with *G. vaginalis*, observed in 50% of patients with BV, including both pregnant and non-pregnant patients (Swidsinski et al., 2013), indicating abnormal microbial community structure can result in ascending infection. Furthermore, a consequence of reduced lactobacilli abundance in the vaginal canal is associated with reduced implantation efficiency in humans (Moreno et al., 2016). These observations suggest that specific commensal taxa such as lactobacilli within the female reproductive tract mediate colonization resistance against pathogens and positively influence pregnancy outcomes.

Despite the known complications in patients with BV, animal models for human BV remain underdeveloped. To address this void, we aimed to develop a human microbiota-associated (HMA) mouse model of BV using germ-free mice. While the natural microbiota of mice differs greatly to that of humans (Ley et al., 2005), HMA mice allows microbial colonization of germ-free mice with the relevant human microbiota and later studied. Colonizing germ-free mice with bacteria from human donors aims to maintain a microbiota diversity and profile similar to the human donor and thus may be a faithful model to examine the role of the human vaginal microbiota in diseases of the reproductive tract and adverse pregnancy outcomes (Marcobal et al., 2013). Furthermore, housing of HMA mice in a hermetically sealed bio-exclusion cage system means that no further bacteria from the environment will enter and alter the diversity of the humanized mouse (Paik et al., 2015).

Herein, we describe the generation of HMA mice harboring the microbiota collected from the vaginal tract of pregnant women and describe the extent to which the human vaginal microbiota can colonize the vaginal tract of germ-free mice. We show that pregnant women with BV harbor a distinct microbiota and variable risk for adverse pregnancy outcomes. Further, we observed substantial variation in pregnancy outcomes and pro-inflammatory cytokine production among HMA mice, despite donor microbiota being a poor predictor of these properties. Together, these data outline the potential use and limitations of using HMA mice harboring the microbiota collected from the human vaginal tract to elucidate the impact of the vaginal microbiota on pregnancy outcomes.

RESULTS

Pregnant Women With BV Harbor a Distinct Microbiota Community Structure

To employ a rigorous experimental approach to generate HMA mice, it is essential to establish a well-characterized cohort of patients with well-characterized disease states as donors of human microbiota. To this end, 19 pregnant African American women were recruited and information pertaining to Nugent score of vaginal swab were collected at the same time as the microbiota sample (for gold standard diagnosis of BV), while patient demographics, health status, pregnancy complications and birth outcomes were recorded as well (**Table 1**). Specifically, seven women presented with a normal Nugent score between 0 and 3, four women presented with an intermediate Nugent score between 4 and 6, and eight women presented with a Nugent score of 7 or higher indicative of BV (**Table 1**). Seven women experienced a urogenital infection during gestation, with five of those women assigned intermediate or BV Nugent scores at the time of swab collection (**Table 2**). Of the five women diagnosed with a urogenital infection, four of them were given an antibiotic and/or antifungal prior to the swab collection (**Table 2**). In addition, eight women had white/gray vaginal discharge reported at the time of sampling, with all of those women assigned either intermediate or BV Nugent scores (**Table 2**). The vaginal and rectal microbiota community structures were characterized for each of the 19 patients. Healthy patients with a low Nugent score harbored a microbiota diversity typical of that previously detected in heathy women (Nunn and Forney, 2016) where the microbial community is dominated by lactobacilli (**Figure 1A**). However, patients with an intermediate or high Nugent score harbored a dysbiotic vaginal microbiota community structure, typified by the diminishment in the relative abundance of lactobacilli and an expansion in the relative

TABLE 1 | Clinical parameters of the 19 pregnant women used for HMA mouse generation.

Characteristics	Subjects ($n = 19$)
Age, years (mean ± sd)	25.1 ± 5.06,
Race/Ethnicity	
African American	19 (100%)
Educational level	
Less than high school	6 (31.6%)
High school or GED	6 (31.6%)
Some college	6 (31.6%)
College graduate	1 (5.3%)
Prenatal Insurance, *n* (%)	
Medicaid	17 (89.5%)
Private	2 (10.5%)
Nugent Score, *n* (%)	
Normal (0–3)	7 (36.8%)
Intermediate (4–6)	4 (21.1%)
BV (7+)	8 (42.1%)
Gestational Hypertension	1(5.3%)
Gestational Diabetes	1(5.3%)
Obstetrical history, *n* (%)	
Prior term birth	11 (57.9%)
Prior preterm birth	3 (15.8%)
Birth Outcome*, *n* (%)	
Full term	10 (52.6%)
Early term	6 (31.6%)
Preterm	1(5.3%)
Spontaneous abortion	1(5.3%)
Exposure to antibiotics during pregnancy, *n* (%)	
Yes	7 (36.8%)
No	12 (63.2%)

**Full term (39 weeks≥), Early term (39 weeks<x≤36 weeks), Preterm (36 weeks<).*

abundance of bacteria of genera Prevotella, Gardernella, and Shuttleworthia (**Figure 1A**). Furthermore, beta diversity analysis of the vaginal microbiota revealed distinct separation of patients with respect to their Nugent scores (**Figure 1C**). By contrast, characterization of the fecal microbiota diversity of these patients did not reveal any salient differences in either relative bacterial abundances (**Figure 1B**), nor in beta diversity, with no detectable separation of patients clustering with respect to their Nugent score (**Figure 1C**). The Shannon diversity index of the vaginal microbiota of each patient was also plotted with respect to the grouped Nugent score, and revealed that patients with a normal Nugent score between 0 and 3 had significantly lower microbiota diversity (Shannon diversity index) compared to patients with an intermediate Nugent score between 4 and 6, or compared to patients with a Nugent score of 7 or higher (**Figure 1D**). The gestational age and birthweight of each infant was also collected and revealed that while there was some variation among the infants, our total sample size of 19 did not demonstrate a statistically significant difference among infants carried by women with a normal, intermediate or high Nugent score (**Figures 1E,F**). Together, these data establish a cohort of normal and disease patients with defined and quantifiable disease activity for use in the generation of HMA mice.

Generation of Human Microbiota-Associated (HMA) Mice Harboring the Microbiota Collected From the Vaginal Tract of Pregnant Women With Bacterial Vaginosis

The patients' vaginal microbiota was swabbed at the hospital and the swabs were transported immediately to the Emory Gnotobiotic Animal Core (EGAC). The vaginal tract of female germ-free C57BL/6 mice was inoculated by physically wiping the vaginal swab on the mouse, concentrating the swab to the vaginal opening. This process was typically completed within 2 h of collecting the vaginal swab from the patient. Mice were then housed within bioexclusion husbandry cages for the vaginal microbiota to establish and colonize. After 2 weeks, a male germ-free mouse was introduced into the bioexclusion cages of each HMA female mouse, and conception date recorded by the appearance of a viscous copulatory plug. At 18.5 days post-coitum (18.5 dpc) and before delivery of litters, mice were sacrificed and the uterus, vagina, and fecal pellet collected under sterile conditions (**Figure 2A**). Characterization of the vaginal microbiota in HMA mice using 16S rDNA amplicon sequencing revealed that although each HMA mouse became colonized by microbes, the proportional abundance of those taxa in the HMA mice was considerably different to the donor sample (**Figures 1A**, **2B**). Importantly, the lactobacilli from the normal Nugent score patients did not efficiently colonize the mouse vaginal tract (**Figure 2B**). To compare the vaginal microbiota of the HMA mice to the vaginal microbiota of a pregnant conventionally raised mouse harboring an undisturbed microbiota, a female mouse housed in the adjacent specific pathogen-free murine vivarium was collected at 18.5 dpc. Microbiota analysis revealed the conventional mouse had a vaginal microbiota abundant in Sphingomonas and Corynebacterium (**Figure 2B**). During microbial DNA isolation and PCR amplification, a blank sample was included among our mouse vaginal samples to determine any environmental contaminants in our samples (denoted "kit blank"). While the kit blank contained some bacteria that were also found in our HMA mouse samples, the total number of reads obtained after PCR amplification was markedly less compared to those found in the HMA samples or the conventional sample (**Figure 2B**). Furthermore, there was high variability in the colonizing microbiota of each HMA mouse, and similarity between the microbiota of HMA mice was not driven by the BV status of the donor sample (**Figure 2C**). We also detected no significant differences in the fecal microbiota of the HMA mice and no separation based on patient Nugent score (**Figure 2C**). These results suggest that while the vaginal canal of germ-free mice provides a competition-free niche for bacteria in a donor sample, the vaginal microbiota of HMA mice generated using this method of inoculation does not closely resemble the community present in donor samples.

TABLE 2 | Urogenital infection and antibiotic/antifungal use among the 19 pregnant women during pregnancy.

Patient #	Nugent score	Vaginal discharge color	Infection during pregnancy	Antibiotic	Antifungal	GA at Antibiotic and/or Antifungal Use (weeks)
1	8	White/Gray				
2	1	NR				
3	1	Clear				
4	7	White/Gray	BV	Cleocin		12.2*
5	0	Clear				
6	7	White/Gray	Trichomoniasis	Flagyl		30
7	5	Clear	Chlamydia	Zithromax		12
8	6	White/Gray	Gonorrhea	Ceftriaxone		10*
9	0	Clear	GBS UTI	Cephalexin	Clotrimazole	6.5*
10	7	White/Gray				
11	8	White/Gray				
12	0	NR	BV	Flagyl		34.5
13	6	NR				
14	5	NR				
15	7	White/Gray				
16	0	Clear				
17	0	Clear				
18	8	White/Gray	Trichomoniasis	Flagyl		8*
19	10	NR				

NR, Not Recorded; GA, Gestational Age. Asterisk denotes antibiotic/antifungal use prior to vaginal swab collection.

Pregnancy Outcomes in Human Microbiota-Associated (HMA) Mice Harboring the Microbiota Collected From the Vaginal Tract of Pregnant Women With Bacterial Vaginosis

Despite no similarity between donor and HMA mouse vaginal microbiota, we recorded considerable variation in the number of pups *in utero* among the HMA mice and sought to determine whether the microbiota harbored in the vaginal canal correlated with litter size. We determined litter size in HMA mice by recording the number of pups within the uterine horns at 18.5 dpc. This was done before birth in order to obtain a faithful count of viable pups and to mitigate the prospect that the new moms' cannibalize their newborn offspring, which often occurs postpartum in C57BL/6 mice and could affect our data. We compared the litter size at 18.5 dpc with the Nugent score of the corresponding patient described in **Table 1**. We detected considerable variation in litter size, but no significant difference in litter size between HMA mice colonized with normal, intermediate or BV patient swabs (**Figure 3A**). We also compared the litter size of HMA mice with the Shannon diversity index of their vaginal microbiota and found that mice with smaller litter sizes did not have a significant difference in vaginal microbiota diversity (**Figure 3B**). Lastly, a PCA plot comparing the beta diversity of the mouse vaginal microbiota and the corresponding litter size (**Figure 3C**) shows that the vagina microbiota of the HMA mice did not have a significant influence on litter size.

Altered Pregnancy Outcomes in Human Microbiota-Associated (HMA) Mice Is Associated With Elevated Levels of Pro-inflammatory Cytokines in the Uterus of Mice During Pregnancy

Inflammation in the uterus, or endometritis is commonly associated with pregnancy complications. We examined the extent to which the colonizing microbes influenced levels of inflammation in the uterus of HMA mice. Although the uterus has considerably lower levels of bacteria compared to the vaginal tract, it may be possible that certain bacterial species in the vaginal tract have negative impacts on the physiology of the entire reproductive system. Linear regression analysis of cytokine concentrations in the uterus of HMA mice described in **Figure 2** revealed a significant negative correlation between IFNγ and IL-4 with litter size, whereas a significantly positive correlation was found with TNFα levels (**Figure 4A**). To determine the extent to which certain murine vaginal microbial communities were associated with altered uterine cytokine levels during pregnancy, we conducted PCA analysis comparing cytokine levels and the vaginal microbiota community. However, our analysis revealed that the HMA mouse vaginal microbiota did not group with any alterations in uterine cytokine levels and had no correlation with BV status of the patient (**Figure 4B**).

DISCUSSION

In order to better understand the effect of the vaginal microbiota on health, we assessed the utility of the HMA mouse approach,

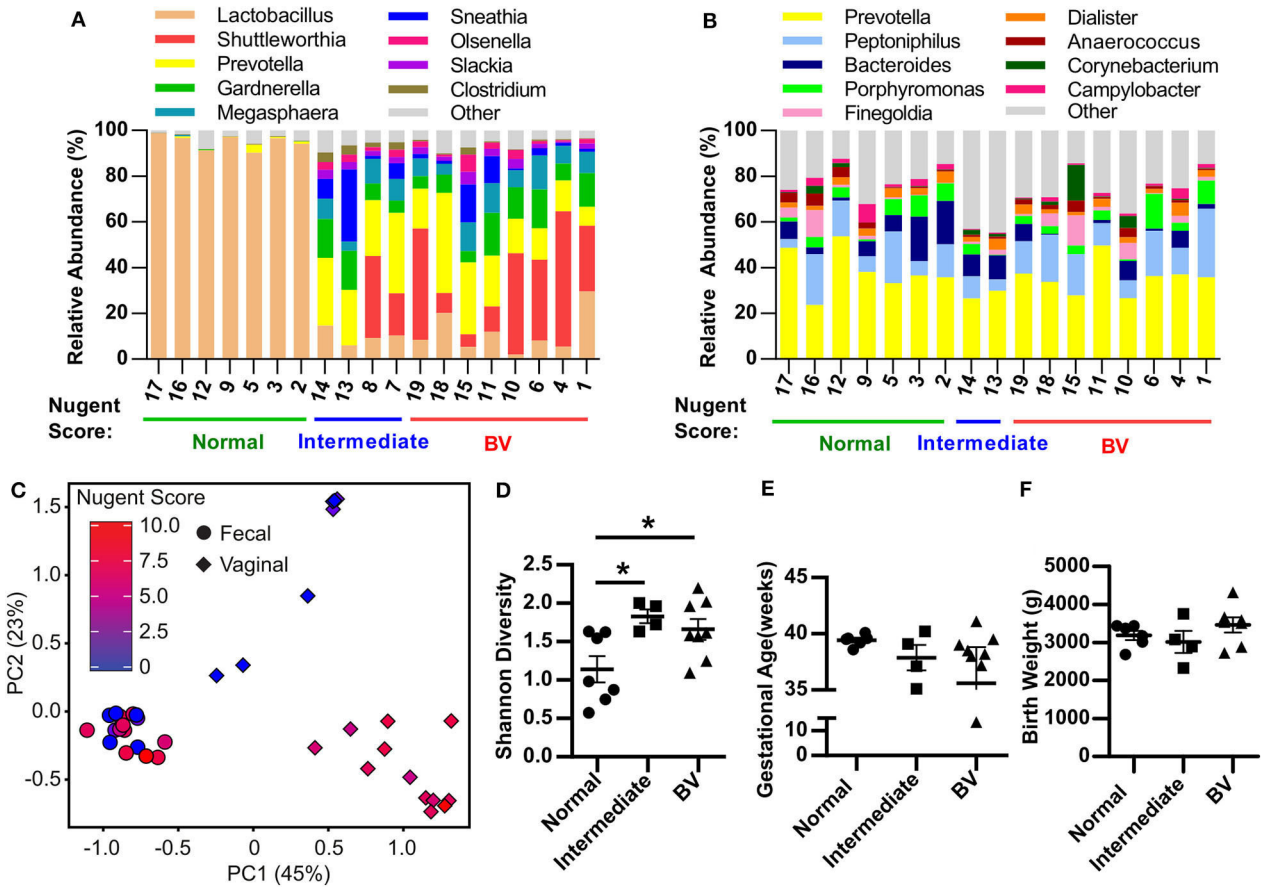

FIGURE 1 | Pregnant women with bacterial vaginosis harbor a distinct microbiota community structure. **(A,B)** Relative abundance of bacterial genera within the vaginal tract **(A)** and rectum **(B)** of pregnant women assigned as Normal, Intermediate, or BV by Nugent score described in **Table 1**. Data represents the top 10 most abundant bacterial genera detected. Each column represents one patient. **(C)** Principal Component Analysis (PCA) plot depicting the beta-diversity of the microbiota community structure within the vaginal tract and the rectum of pregnant patients described in **Table 1**. **(D)** Shannon diversity index of patient vaginal microbiota determined via 16S rRNA gene sequencing according to patient Nugent score. Data graphed as mean ± SEM. **(E)** Gestational age of delivery for pregnant patients described in **Table 1** with either a normal, intermediate or BV Nugent score. Data graphed as mean ± SEM. **(F)** Birth weight of infant delivered by patients described in **Table 1** with a normal, intermediate or BV Nugent score. Data graphed as mean ± SEM. Statistical significance determined via One-way ANOVA, Turkey's multiple comparison test **(D,F)** or Kruskal-Wallis test, Dunn's multiple comparisons test **(E)**. *$p < 0.05$. $n = 19$ patients.

which has been widely employed in the study of host cell and intestinal microbe interactions (Marcobal et al., 2013; Ridaura et al., 2013). Studies using HMA mice have not only attributed causality of specific microbial community structures in the development of many chronic diseases such as metabolic syndrome, but have also helped to unravel the mechanisms behind these associations- an approach that is unfeasible in human subjects (Chassaing et al., 2015). While mice with a humanized microbiota have offered an invaluable model for the study of the gastrointestinal microbiota and its role in human health, the same mouse model for the human vaginal microbiota has not been fully established (Bradshaw and Sobel, 2016). We first collected patient data on a cohort of pregnant women, including Nugent scores for the assessment of BV in patients. Using vaginal swabs from these patients, we generated HMA mice harboring the microbiota collected from the vaginal tract of pregnant women with BV. Comparison of the vaginal microbiota of BV patients and HMA mice revealed no immediately apparent similarity. In addition, there was no correlation detected between the number of pups within pregnant HMA mice and Nugent scores from BV patients. Significantly elevated levels of pro-inflammatory cytokines in the uterus of mice were detected during pregnancy, although there was no clear correlation between the murine vaginal microbiota, Nugent scores from BV patients and pro-inflammatory cytokine levels.

In both mice and humans, the uterus functions to nurture a fertilized egg until the fetus, or offspring, is ready to be delivered. However, the anatomy of the mouse female reproductive tract has clear differences compared to humans. The murine uterus is bicornuate, forming two uterine horns that help accommodate large litter sizes, with an average of 6.2 newborns per litter (Nagasawa et al., 1973). By comparison humans have a simplex uterus with a single cavity located between the bladder and

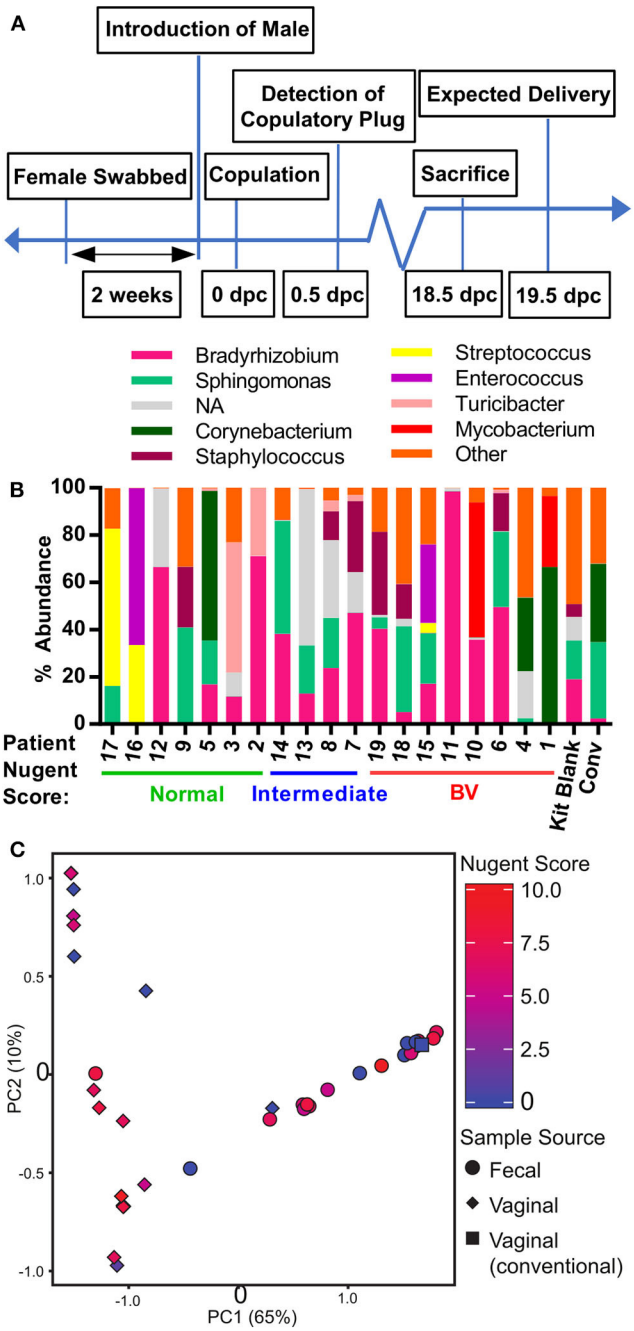

FIGURE 2 | Generation of human microbiota-associated (HMA) mice harboring the microbiota collected from the vaginal tract of pregnant women with bacterial vaginosis. **(A)** Graphical depiction of experimental approach to generate human microbiota-associated (HMA) mice harboring the microbiota collected from the vaginal tract of pregnant patients described in **Table 1**. Swabs were collected from the vaginal tract and immediately transported to the Emory Gnotobiotic Animal Core (EGAC). The vaginal tract of female germ-free C57BL/6 were inoculated by physically wiping the swab on the vaginal opening of the mouse. Mice were then housed in Tecniplast ISOcageP Bioexclusion cages for the microbiota to colonize. After 2 weeks, a male germ-free mouse was introduced to the HMA female mouse and conception monitored. On 18.5 dpc (days post-coitum), pregnant female mice were sacrificed under sterile conditions for sample collection and analysis.

(Continued)

FIGURE 2 | (B) Relative abundance of the bacterial genera detected via 16S analysis in the vaginal tract of HMA mice on 18.5 dpc. Data represents the top 10 bacterial genera detected and each stacked column represents one mouse. Data is separated by BV status of the corresponding human donor. The total read count for each sample is provided at the top of each bar. NA (not assigned) refers to sequences that were unclassifiable at this taxonomic level. **(C)** Principal Component Analysis (PCA) plot depicting the beta-diversity of the microbiota community structure within the vaginal tract and gastrointestinal tract of HMA mice on 18.5 dpc. Symbols are colored by the Nugent score of the corresponding human donor.

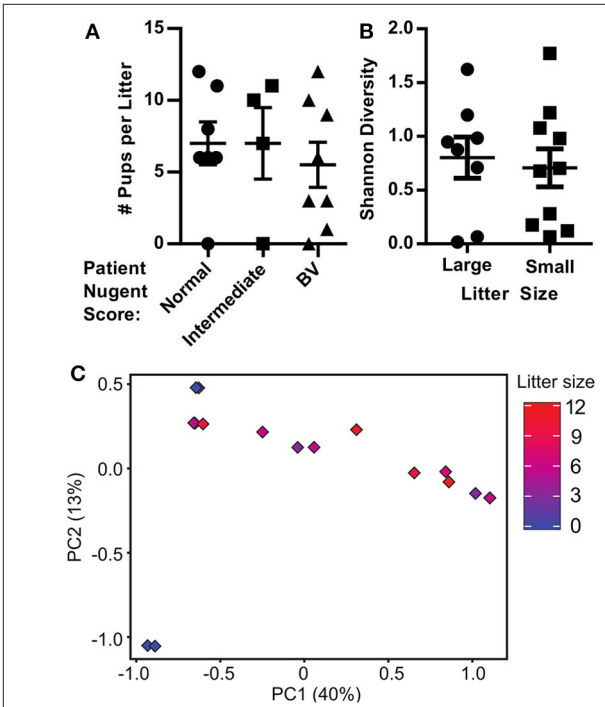

FIGURE 3 | Pregnancy outcomes in human microbiota-associated (HMA) mice harboring the microbiota collected from the vaginal tract of pregnant women with bacterial vaginosis. **(A)** The number of pups detected in the uterine horns of HMA mice on 18.5 dpc separated by the Nugent score of the corresponding donor. Data graphed as mean ± SEM. **(B)** Shannon diversity index of the mouse vaginal microbiota determined via 16S analysis on 18.5 dpc separated by litter size of the pregnant HMA mouse. Less than 7 pups on 18.5 dpc is considered a small litter, while 7 or more pups is considered a large litter. Data graphed as mean ± SEM. **(C)** Principal Component Analysis (PCA) plot depicting the beta-diversity of the microbiota community structure within the vaginal tract of HMA mice on 18.5 dpc. Symbols are colored by the litter size on 18.5 dpc.

the rectum, and typically harbors only one infant. Importantly, both humans and mice have a cervix which forms a tight physical barrier between the vagina and uterine cavity. The cervix plays a critical role in preventing vaginal bacteria from ascending into the uterus (Racicot et al., 2013; Pavlidis et al., 2020). As in humans, mice have a resident commensal vaginal microbiota and virtually undetectable numbers of microbes in the uterus (Han et al., 2004; Digiulio et al., 2010). As in humans, mice also undergo a hormonal cycle regulated by

steroid hormones, with their cycle being much shorter, about 4 days in total, compared to a human's 28 day cycle (Gonzalez, 2016). Interestingly, mice also do not undergo menstruation and instead only decidualize if fertilization occurs (Finn, 1998). The lack of menstruation may be a governing determinant in the establishment of the microbiota community structure within the mouse reproductive tract. Indeed, the composition of the vaginal microbiota differ greatly between mice and humans. In fact, humans have a distinct vaginal microbiota compared to most other mammalian species sampled including even non-human primates (Miller et al., 2017). Humans are the only species to have a vaginal microbiota dominated by lactobacilli, while all other mammals have a considerably more diverse vaginal microbe community structure (Swartz et al., 2014; Yildirim et al., 2014). The teleological explanation for the specific nature of the human vaginal microbiota diversity in terms of the purpose it serves remains enigmatic, with progress in our understanding perhaps hindered by the very fact that there is no suitable animal to model the human vaginal microbiota (Miller et al., 2016). Establishing an HMA animal model would greatly enhance our understanding of how the vaginal microbiota affects the development of several human reproductive diseases, many of which cause pre-term births, and may facilitate the development of novel therapies that are desperately needed for millions of women.

When generating the HMA mice using human vaginal swabs, ensuring viability of the bacteria present on the patient swab was critical and challenging. The vaginal swab was self-collected by the patients, stored in a sterile tube and immediately transferred to the facility housing the germ-free recipient female mice. Although we inoculated the mice without delay, about an hour would transpire between patient collection and mouse inoculation. During this time, it is unclear how many and what type of bacteria had lost viability by the time the germ-free mice were inoculated, since 16S microbiota analysis of the swabs also detects non-viable bacterial DNA. Because of this limitation, it is possible that germ-free mice are capable of harboring a vaginal microbiota similar to humans, but this particular method of inoculation using a patient swab may be not the optimal method. This challenge with viability may be a contributory factor explaining why the HMA mice did not harbor a vaginal microbiota similar to the human patient swabs. However, the total sequencing read counts of the HMA vaginal samples showed little variability, as 16/19 samples had at least 1.80×10^5 total reads and were comparable to the total read count of the conventional mouse vaginal sample from the SPF facility at 7.4×10^5 reads. In addition, antibiotic or antifungal use by the patient did not influence the bacterial abundance or total read count. Interestingly, many of the bacterial taxa detected in the HMA mouse vaginal samples were only present at low abundances in the patient swab. While the kit blank sample contained some of the bacterial taxa detected in the HMA mice, the kit blank had a very low read count compared to HMA mouse samples. Nevertheless, these observations do raise the possibility that environmental contaminants may be present in the HMA samples, albeit at very low levels. In addition, in future approaches, we propose that a blank microbiome sample should be generated by collecting samples from the vaginal tract of germ-free mice to detect any mouse-derived contaminants in the HMA samples. Nevertheless, for future investigations, colonization of the HMA mice may be more successful if the viability of microbes on the swab is ensured. Furthermore, determining the best method to store the patient swab during transport requires consideration given that conditions related to temperature and oxygen exposure would ultimately favor the viability of some bacteria over others. Therefore, due to the challenge of confirming the viability of all bacteria present on a patient swab, enhanced approaches to sample collection and inoculation should be considered.

As an alternative approach, and because the human vaginal community state types have been well-defined (Ravel et al., 2011), it may also be possible to isolate the major bacterial species that make up a particular vaginal community state type to generate a defined flora that may then be introduced to the germ-free mice via vaginal lavage. This approach would contribute to establishing the proof of principle that human vaginal microbiota isolates can indeed colonize the murine vaginal tract. This approach would be similar to the approach successfully implemented in the generation of mice with a standardized intestinal microbiota. For example, altered Schaedler flora (ASF) is a standardized cocktail of eight bacterial species that is employed to study gut-microbe interactions in mice (Wymore Brand et al., 2015; Lyte et al., 2019). In addition to a vaginal lavage of a relevant standardized cocktail of bacteria, the mice could also receive an oral gavage of the ASF cocktail to ensure the intestinal microbiota is comparable among all female mice. This would address any confounding effects the intestinal microbiota may have on pregnancy outcomes. Using a similar method to ASF may serve as a reliable approach to modeling the human vaginal microbiota in mice.

This study aimed to generate HMA mice to determine the effect of the vaginal microbiota on pregnancy outcomes. To measure reproductive fitness in the HMA mice, we enumerated the number of pups *in utero* at 18.5 dpc. Small litter sizes in mice have been correlated with pregnancy complications such as ascending uterine infections and dysregulation of protein expression critical to uterine remodeling and regeneration (Tuffrey et al., 1992; Zavan et al., 2016; Mccallum et al., 2018). Although we saw high variability in litter sizes among the HMA mice, smaller litter sizes did not correlate with the HMA mouse vaginal microbiota. In addition to litter size, we also measured the levels of 10 pro-inflammatory cytokines in order to detect any uterine inflammation. Chorioamnionitis, also known as intra-amniotic infection (IAI) is an inflammation of the fetal membranes due to a bacterial infection (Tita and Andrews, 2010). Up to 40% of preterm births are clinically associated with intrauterine infections (Agrawal and Hirsch, 2012). Elevated levels of pro-inflammatory cytokines such as TNFα, IL-1, IL-12, IL-6, and IL-2 have been linked to chorioamnionitis (Negishi et al., 1996; Holst et al., 2007; Berry et al., 2011; Revello et al., 2016) and therefore elevated levels of these cytokines serve to identify potential pregnancy complications.

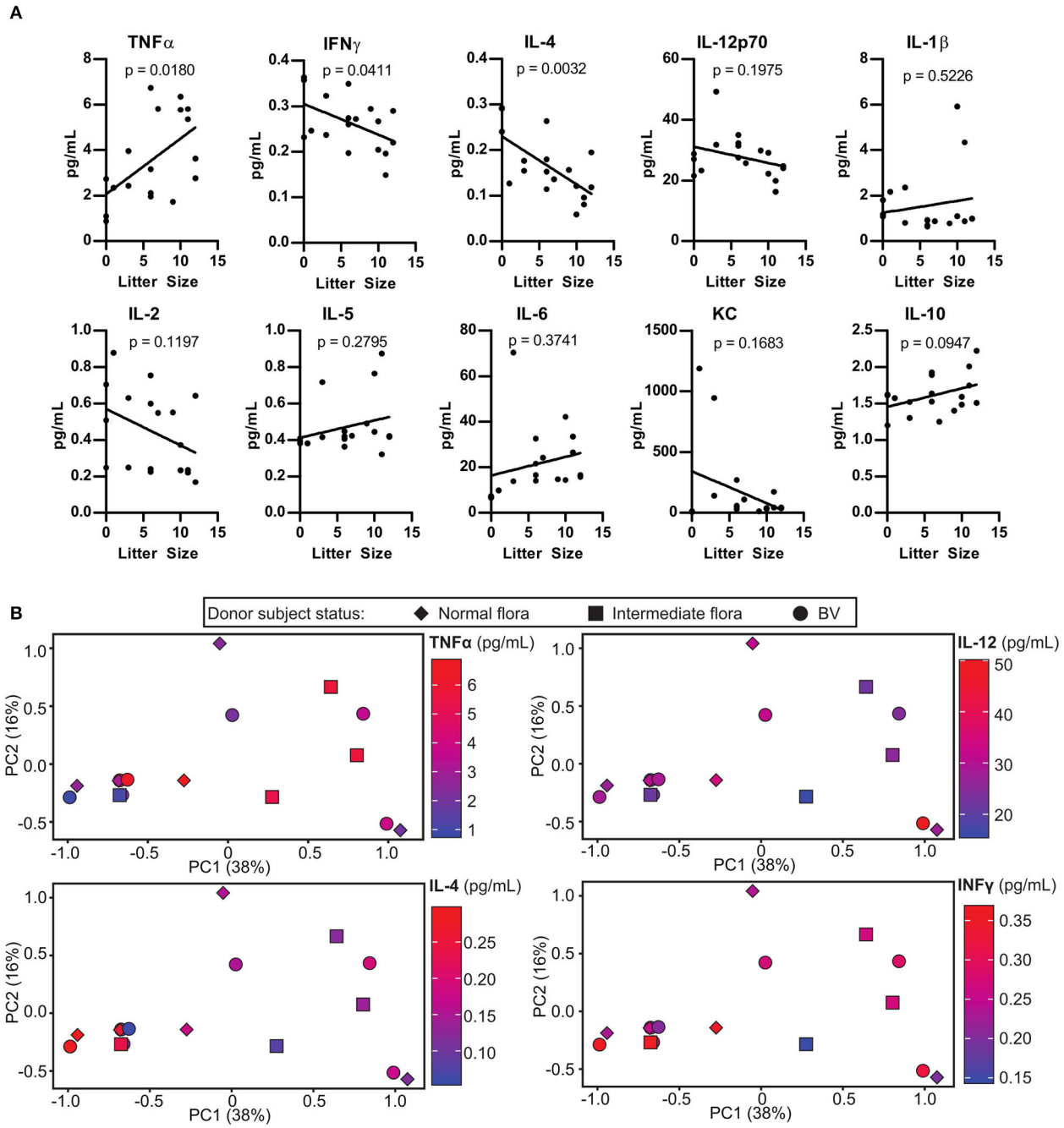

FIGURE 4 | Pregnancy outcome in human microbiota-associated (HMA) mice is associated with altered uterine cytokine levels during pregnancy. **(A)** Cytokine concentrations in the uterus of HMA mice on 18.5 dpc. Cytokine levels are plotted with respect to the litter size. Statistical significance determined using linear regression analysis, *p*-value is indicated on the figure for each cytokine. **(B)** Principal Component Analysis (PCA) analysis depicting the correlation between cytokines concentrations in the uterus that were significantly altered or trending toward significance in **(A)**, the HMA mouse vaginal microbiota community, and the Nugent score of the corresponding donor patient.

Interestingly, we saw a significant positive correlation between TNFα and litter size. While pro-inflammatory cytokines are associated with inflammation during chorioamnionitis, pro-inflammatory cytokines downstream of NF-κB activation, such as TNFα, play an important role in uterine homeostasis and initiating critical events during the estrous cycle and pregnancy (Evans and Salamonsen, 2012; Sierra-Mondragon et al., 2015). We hypothesize that the small litters during pregnancy could have altered the level of TNFα expression during pregnancy. While we did see a significant negative correlation between INFγ

and IL-4 with litter size, we did not detect a correlation between these cytokine levels and the HMA mouse vaginal microbiota. The number of reabsorptions could not be enumerated in this study, but may also have contributed to differences in cytokine levels. Given the variability seen in the HMA mouse vaginal microbiota, the failure to detect correlation between litter sizes, cytokine levels and the vaginal microbiota diversity is not surprising. Upon generation of a successful mouse model of the human vaginal microbiota, several additional metrics may potentially serve to compare pregnancy outcomes in mice which include, but are not limited to the presence of bacteria in the uterus during pregnancy, weight and survival of the pups after birth, time of gestation, and the number of subsequent viable litters produced by each female mouse.

The biological mechanisms by which the normal gravid uterus protects itself from ascending infection are not fully known. Recently, it has been established that the indigenous microbiota residing on host mucosal surfaces act in concert with the host to prevent pathogenic microbial colonization in a process called colonization resistance. The colonization resistance offered by the lactobacilli-rich microbiota combined with the physical barrier of the cervix is considered to be a major defense against ascending uterine infections. Furthermore, there is increasing literature describing the influence of microbial diversity within the female reproductive tract on uterine health and disease. Indeed, our approach did discover significant variation in uterine cytokine levels of HMA mice and large range of litter sizes, despite the colonizing communities bearing limited similarity to their donor sample. In that way, the approach we describe in this study could still be a valuable method for interrogating the mechanistic details through which microbes and microbial consortia influence reproductive health. Further, although we report limited similarity based on 16S rDNA amplicon sequencing, assessing microbial community composition using metagenomics and metatranscriptomics could reveal more meaningful similarities among the colonizing microbes in HMA mice. That is, it is possible that the HMA mice with more similar litter sizes and levels of inflammatory cytokines were disproportionately colonized by microbes enriched with similar metabolic capabilities. Ultimately, the HMA mouse model we describe here is a valuable step toward developing new methods that can provide mechanistic insight into how microbe-host interactions affect reproductive health.

METHODS

Patient Recruitment and Swab Collection

Women who participated in this study were part of the Emory University African American Vaginal, Oral, and Gut Microbiome in Pregnancy Cohort Study (Corwin et al., 2017). During the recruiting period for this study (7/31/2018 through 11/15/2018), women who were recruited for the parent study were offered the option of collecting an additional vaginal and rectal swab for participation in the present study. The study protocol was carried out in accordance with the review and approval of the Emory University Institutional Review Board and the Grady Research Oversight Committee. All women participating in this study provided written informed consent in accordance with the Declaration of Helsinki.

Pregnant women were recruited to participate in this study from the prenatal care clinics of two metropolitan hospitals in Atlanta, GA, affiliated with Emory University Woodruff Health Sciences Center: Grady Memorial Hospital, a county-supported hospital that serves as a safety net for low-income patients; and Emory University Hospital Midtown, a private hospital that serves patients from a wide economic range. Inclusion criteria were that each participant is: (1) African American by self-report; (2) Between 8 and 14 weeks' gestation (verified by clinical record) and expecting a singleton pregnancy; (3) Able to comprehend written and spoken English; (4) Between 18 and 40 years of age; (5) Experiencing no chronic medical condition or taking prescribed chronic medications (verified by prenatal record). For enrolled women, data collection consisted of completing a sociodemographic questionnaire as well as self-collecting vaginal and rectal swabs during the study visit and giving permission to complete a medical record abstraction at the end of the pregnancy. For the swab collection, participants were provided verbal and pictorial instructions directing them to obtain (in a private exam room) self-collected vaginal swabs (one for Gram staining according to Nugent's score, one for DNA extraction and 16S rRNA gene sequencing, and one for inoculation of the mouse model) and one rectal swab (for DNA extraction and 16S rRNA gene sequencing). The swabs for microbiota sequencing were Sterile Catch-All™ Sample Collection Swabs (Epicentre Biotechnologies, Madison WI) which were immediately plunged into MoBio bead tubes (MoBio Laboratories, Inc., Carlsbad, CA) and frozen upright on dry ice until transported to the lab, to be stored at −80°C until DNA extraction and preparation for vaginal microbiota measurement occurs. The swabs for vaginal Gram staining were dacron swabs that were stored in a sterile tube until transport to the Emory Clinical Microbiology Laboratory for Gram staining for Nugent criteria scoring for evaluation of BV (Nugent et al., 1991). Well-designed studies support that vaginal self-collection swabs sample the same microbial diversity as physician-collected swabs of the mid-vagina and have high overall morphotype-specific validity compared with provider-collected swabs based on microbiome analysis (Forney et al., 2010).

Maternal Medical Chart Abstraction was completed by the research team using a standardized chart abstraction tool to ascertain for the following: *Gestational age at birth*. All participants receive early pregnancy dating by last menstrual period (LMP) and/or early ultrasound, given enrollment criteria. Gestational age at birth is determined from the delivery record, based upon the date of delivery in relation to the estimated date of confinement established during the 8–14 week prenatal visit. *Complications/Type and Mode of Delivery*. Gestational diabetes, preeclampsia/eclampsia, etc., type and mode of delivery are ascertained from record review after delivery and defined according to standard clinical definitions of the American College of Obstetricians and Gynecologists. *Medication use*, including any antibiotic use, in the month prior to sampling was also ascertained.

Generation of Human Microbiota-Associated Mice

After collection of the human vaginal swabs by hospital staff the swabs were immediately transferred to research staff who inoculated 10 week-old germ-free female single-housed in a bioexclusion microisolator cage at the Emory Gnotobiotic Animal Core (EGAC). Germ-free status of mice was confirmed by bacterial 16S rDNA PCR assay paired with anaerobic culture testing, undertaken by IDEXX BioAnalytics (Columbia, MO). To handle the mice contained in the bioexclusion cages, the cage was saturated in disinfectant and placed in a sterile biosafety hood. Gloves and forceps for mouse handling were sterilized in disinfectant before opening the cage. Inoculation was achieved by physical wiping of the vaginal swab at the vaginal opening of the mouse for several seconds, ensuring adequate transfer of microbes from the swab onto the vaginal opening of the mouse. Inoculated females were housed for 2 weeks before introduction of a germ-free male. Evidence of copulation was monitored by inspection for a vaginal copulatory plug every morning after inoculation. Observance of a vaginal plug was designated as 0.5 days post-coitum (dpc). After identification of a vaginal plug the male mouse was removed to prevent the possibility of multiple copulations. The day before expected delivery (18.5 dpc) the female mouse was sacrificed and the uterine horns, vagina and fecal pellet were collected under strict sterile conditions. The number of developing pups in the uterus were enumerated and removed. The vaginal canal was immediately processed for microbial DNA isolation and the uterine horn was flash frozen for future cytokine analysis.

Microbial DNA Isolation and 16S Analysis

After sterile collection the vaginal canal from the HMA mouse was placed in a MagnaLyser tube (Sheikh et al., 1995) with 1 mL sterile PBS. The tube was vigorously vortexed three times for 10 seconds each to remove the mucosa and bacteria from the vaginal tissue. The 1 mL of PBS containing the vaginal mucosa and bacteria was collected and the microbial DNA was isolated using the QIAamp DNA microbiome kit (Qiagen, Hilden, Germany). Adequate and faithful amplification of the 16S rRNA V4 region required an initial PCR amplification of the near full length 16S rRNA genes using the 27F (5′- AGAGTTTGATCMTGGCTCAG−3′) and 1492R (5′- TACGGYTACCTTGTTACGACTT-3′) primer pair for 25 cycles. The resulting amplicon was then used as template for amplification of the V4 region using the 515F/806R primer pair (GTGCCAGCMGCCGCGGTAA and GGACTACHVGGGTWTCTAAT) for 30 cycles in triplicate. Each 806R primer had a unique 12 base golay barcode. The PCR products were quantified using a Qubit fluorimeter (Invitrogen, Carlsbad, CA) and ran on a bioanalyzer to confirm the presence of a single band at 350 bp and no band for the "kit-ome" control. DNA samples were pooled and sequenced using the Illumina MiSeq at the Emory Integrated Genomics Core. Sequencing data was processed using the analysis pipeline developed by Vaninsberghe et al. (2020). Briefly, primers were trimmed, allowing up to one mismatch and discarding all sequences without primers, and Dada2 was used to infer the Amplicon Sequence Variants (ASVs) (Callahan et al., 2016). Sequencing data is deposited at BioProject ID PRJNA655465 and can be retrieved at http://www.ncbi.nlm.nih.gov/bioproject/655465.

Measurement of Uterine Cytokine Levels

The uterus from HMA mice at 18.5 dpc was collected and the pups were carefully removed. The uterine wall was flash frozen with liquid nitrogen. To ensure adequate representation of the uterine tissue in subsequent cytokine analysis, the uterine tissue was ground while frozen with mortar and pestle. Fifty milligram of tissue was then added to homogenization buffer in a Magnalyser tube and homogenized twice for 30 s at 6,500 rpm using a Magnalyser (Roche, Basel, Switzerland). Protein concentrations were quantified using the Pierce BCA assay kit and the protein concentrations were diluted and normalized for cytokine quantification using the V-PLEX Proinflammatory Panel 1 Mouse Kit (Meso Scale Discovery, Rockville, Maryland) following manufacturer's protocol with the help of the Emory Multiplexed Immunoassay Core (Emory University, Atlanta, GA).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found in the NCBI short read archive (SRA) under the accession number PRJNA655465.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Emory University Institutional Review Board and the Grady Research Oversight Committee. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Emory University IACUC committee.

AUTHOR CONTRIBUTIONS

RJ and AD conceived and oversaw the project. AD and EC oversaw patient sample collections. AW and TS performed the experiments. DV, AW, and TS analyzed the data and produced the figures. RJ, AW, AD, and AN wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., and Versalovic, J. (2014). The placenta harbors a unique microbiome. *Sci. Transl. Med.* 6:237ra265. doi: 10.1126/scitranslmed.3008599

Agrawal, V., and Hirsch, E. (2012). Intrauterine infection and preterm labor. *Semin. Fetal Neonatal Med.* 17, 12–19. doi: 10.1016/j.siny.2011.09.001

Berry, C. A., Nitsos, I., Hillman, N. H., Pillow, J. J., Polglase, G. R., Kramer, B. W., et al. (2011). Interleukin-1 in lipopolysaccharide induced chorioamnionitis in the fetal sheep. *Reprod. Sci.* 18, 1092–1102. doi: 10.1177/1933719111404609

Bradshaw, C. S., and Sobel, J. D. (2016). Current treatment of bacterial vaginosis-limitations and need for innovation. *J. Infect. Dis.* 214(Suppl. 1), S14–S20. doi: 10.1093/infdis/jiw159

Callahan, B. J., Mcmurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Charbonneau, M. R., Blanton, L. V., Digiulio, D. B., Relman, D. A., Lebrilla, C. B., Mills, D. A., et al. (2016). A microbial perspective of human developmental biology. *Nature* 535, 48–55. doi: 10.1038/nature18845

Chassaing, B., Koren, O., Goodrich, J. K., Poole, A. C., Srinivasan, S., Ley, R. E., et al. (2015). Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature* 519, 92–96. doi: 10.1038/nature14232

Corwin, E. J., Hogue, C. J., Pearce, B., Hill, C. C., Read, T. D., Mulle, J., et al. (2017). Protocol for the emory university African American vaginal, oral, and gut microbiome in pregnancy cohort study. *BMC Pregn Childbirth* 17:161. doi: 10.1186/s12884-017-1550-y

Digiulio, D. B., Romero, R., Kusanovic, J. P., Gomez, R., Kim, C. J., Seok, K. S., et al. (2010). Prevalence and diversity of microbes in the amniotic fluid, the fetal inflammatory response, and pregnancy outcome in women with preterm pre-labor rupture of membranes. *Am. J. Reprod. Immunol.* 64, 38–57. doi: 10.1111/j.1600-0897.2010.00830.x

Evans, J., and Salamonsen, L. A. (2012). Inflammation, leukocytes and menstruation. *Rev. Endocr. Metab. Disord.* 13, 277–288. doi: 10.1007/s11154-012-9223-7

Finn, C. A. (1998). Menstruation: a nonadaptive consequence of uterine evolution. *Q. Rev. Biol.* 73, 163–173. doi: 10.1086/420183

Forney, L. J., Gajer, P., Williams, C. J., Schneider, G. M., Koenig, S. S., Mcculle, S. L., et al. (2010). Comparison of self-collected and physician-collected vaginal swabs for microbiome analysis. *J. Clin. Microbiol.* 48, 1741–1748. doi: 10.1128/JCM.01710-09

Franasiak, J. M., and Scott, R. T. (2017). Endometrial microbiome. *Curr. Opin. Obstet. Gynecol.* 29, 146–152. doi: 10.1097/GCO.0000000000000357

Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schutte, U. M., Zhong, X., et al. (2012). Temporal dynamics of the human vaginal microbiota. *Sci. Transl. Med.* 4:132ra152. doi: 10.1126/scitranslmed.3003605

Goldenberg, R. L., Iams, J. D., Mercer, B. M., Meis, P. J., Moawad, A. H., Copper, R. L., et al. (1998). The preterm prediction study: the value of new vs standard risk factors in predicting early and all spontaneous preterm births. NICHD MFMU Network. *Am. J. Public Health* 88, 233–238. doi: 10.2105/AJPH.88.2.233

Gonzalez, G. (2016). Determining the stage of the estrous cycle in female mice by vaginal smear. *Cold Spring Harb. Protoc.* 2016, 732–734. doi: 10.1101/pdb.prot094474

Han, Y. W., Redline, R. W., Li, M., Yin, L., Hill, G. B., and Mccormick, T. S. (2004). *Fusobacterium nucleatum* induces premature and term stillbirths in pregnant mice: implication of oral bacteria in preterm birth. *Infect. Immun.* 72, 2272–2279. doi: 10.1128/IAI.72.4.2272-2279.2004

Holst, R. M., Laurini, R., Jacobsson, B., Samuelsson, E., Savman, K., Doverhag, C., et al. (2007). Expression of cytokines and chemokines in cervical and amniotic fluid: relationship to histological chorioamnionitis. *J. Matern. Fetal Neonatal. Med.* 20, 885–893. doi: 10.1080/14767050701752601

Kaminska, D., and Gajecka, M. (2017). Is the role of human female reproductive tract microbiota underestimated? *Benef. Microbes* 8, 327–343. doi: 10.3920/BM2015.0174

Kessel, S. S., Kleinman, J. C., Koontz, A. M., Hogue, C. J., and Berendes, H. W. (1988). Racial differences in pregnancy outcomes. *Clin. Perinatol.* 15, 745–754. doi: 10.1016/S0095-5108(18)30671-7

Koumans, E. H., Sternberg, M., Bruce, C., Mcquillan, G., Kendrick, J., Sutton, M., et al. (2007). The prevalence of bacterial vaginosis in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. *Sex. Transm. Dis.* 34, 864–869. doi: 10.1097/OLQ.0b013e318074e565

Leitich, H., Bodner-Adler, B., Brunbauer, M., Kaider, A., Egarter, C., and Husslein, P. (2003). Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis. *Am. J. Obstet. Gynecol.* 189, 139–147. doi: 10.1067/mob.2003.339

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., and Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11070–11075. doi: 10.1073/pnas.0504978102

Lyte, J. M., Proctor, A., Phillips, G. J., Lyte, M., and Wannemuehler, M. (2019). Altered Schaedler flora mice: a defined microbiota animal model to study the microbiota-gut-brain axis. *Behav. Brain Res.* 356, 221–226. doi: 10.1016/j.bbr.2018.08.022

Marcobal, A., Kashyap, P. C., Nelson, T. A., Aronov, P. A., Donia, M. S., Spormann, A., et al. (2013). A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. *ISME J.* 7, 1933–1943. doi: 10.1038/ismej.2013.89

Mccallum, M. L., Pru, C. A., Smith, A. R., Kelp, N. C., Foretz, M., Viollet, B., et al. (2018). A functional role for AMPK in female fertility and endometrial regeneration. *Reproduction* 156, 501–513. doi: 10.1530/REP-18-0372

Mendling, W. (2016). Vaginal microbiota. *Adv. Exp. Med. Biol* 902, 83–93. doi: 10.1007/978-3-319-31248-4_6

Miller, E. A., Beasley, D. E., Dunn, R. R., and Archie, E. A. (2016). Lactobacilli dominance and vaginal pH: why is the human vaginal microbiome unique? *Front. Microbiol* 7:1936. doi: 10.3389/fmicb.2016.01936

Miller, E. A., Livermore, J. A., Alberts, S. C., Tung, J., and Archie, E. A. (2017). Ovarian cycling and reproductive state shape the vaginal microbiota in wild baboons. *Microbiome* 5:8. doi: 10.1186/s40168-017-0228-z

Moreno, I., Codoner, F. M., Vilella, F., Valbuena, D., Martinez-Blanch, J. F., Jimenez-Almazan, J., et al. (2016). Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am. J. Obstet. Gynecol.* 215, 684–703. doi: 10.1016/j.ajog.2016.09.075

Nagasawa, H., Miyamoto, M., and Fujimoto, M. (1973). [Reproductivity in inbred strains of mice and project for their efficient production (author's transl)]. *Jikken Dobutsu* 22, 119–126. doi: 10.1538/expanim1957.22.2_119

Nasioudis, D., Linhares, I. M., Ledger, W. J., and Witkin, S. S. (2017). Bacterial vaginosis: a critical analysis of current knowledge. *BJOG* 124, 61–69. doi: 10.1111/1471-0528.14209

Negishi, H., Yamada, H., Mikuni, M., Kishida, T., Okuyama, K., Sagawa, T., et al. (1996). Correlation between cytokine levels of amniotic fluid and

histological chorioamnionitis in preterm delivery. *J. Perinat. Med.* 24, 633–639. doi: 10.1515/jpme.1996.24.6.633

Nugent, R. P., Krohn, M. A., and Hillier, S. L. (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J. Clin. Microbiol.* 29, 297–301. doi: 10.1128/JCM.29.2.297-301.1991

Nunn, K. L., and Forney, L. J. (2016). Unraveling the dynamics of the human vaginal microbiome. *Yale J. Biol. Med.* 89, 331–337.

Paik, J., Pershutkina, O., Meeker, S., Yi, J. J., Dowling, S., Hsu, C., et al. (2015). Potential for using a hermetically-sealed, positive-pressured isocage system for studies involving germ-free mice outside a flexible-film isolator. *Gut Microbes* 6, 255–265. doi: 10.1080/19490976.2015.1064576

Pavlidis, I., Spiller, O. B., Sammut Demarco, G., Macpherson, H., Howie, S. E. M., Norman, J. E., et al. (2020). Cervical epithelial damage promotes *Ureaplasma parvum* ascending infection, intrauterine inflammation and preterm birth induction in mice. *Nat. Commun.* 11:199. doi: 10.1038/s41467-019-14089-y

Racicot, K., Cardenas, I., Wunsche, V., Aldo, P., Guller, S., Means, R. E., et al. (2013). Viral infection of the pregnant cervix predisposes to ascending bacterial infection. *J. Immunol.* 191, 934–941. doi: 10.4049/jimmunol.1300661

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., Mcculle, S. L., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U.S.A* 108(Suppl. 1), 4680–4687. doi: 10.1073/pnas.1002611107

Reid, G. (2016). Cervicovaginal microbiomes-threats and possibilities. *Trends Endocrinol. Metab.* 27, 446–454. doi: 10.1016/j.tem.2016.04.004

Revello, R., Alcaide, M. J., Dudzik, D., Abehsera, D., and Bartha, J. L. (2016). Differential amniotic fluid cytokine profile in women with chorioamnionitis with and without funisitis. *J. Matern. Fetal Neonatal. Med.* 29, 2161–2165. doi: 10.3109/14767058.2015.1077512

Ridaura, V. K., Faith, J. J., Rey, F. E., Cheng, J., Duncan, A. E., Kau, A. L., et al. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* 341:1241214. doi: 10.1126/science.1241214

Sheikh, M. S., Rochefort, H., and Garcia, M. (1995). Overexpression of p21WAF1/CIP1 induces growth arrest, giant cell formation and apoptosis in human breast carcinoma cell lines. *Oncogene* 11, 1899–1905.

Sierra-Mondragon, E., Gomez-Chavez, F., Murrieta-Coxca, M., Vazquez-Sanchez, E. A., Martinez-Torres, I., Cancino-Diaz, M. E., et al. (2015). Low expression of IL-6 and TNF-alpha correlates with the presence of the nuclear regulators of NF-kappaB, IkappaBNS and BCL-3, in the uterus of mice. *Mol. Immunol.* 68, 333–340. doi: 10.1016/j.molimm.2015.09.020

Swartz, J. D., Lachman, M., Westveer, K., O'neill, T., Geary, T., Kott, R. W., et al. (2014). Characterization of the vaginal microbiota of ewes and cows reveals a unique microbiota with low levels of lactobacilli and near-neutral pH. *Front. Vet. Sci.* 1:19. doi: 10.3389/fvets.2014.00019

Swidsinski, A., Verstraelen, H., Loening-Baucke, V., Swidsinski, S., Mendling, W., and Halwani, Z. (2013). Presence of a polymicrobial endometrial biofilm in patients with bacterial vaginosis. *PLoS ONE* 8:e53997. doi: 10.1371/journal.pone.0053997

Sykiotis, G. P., and Bohmann, D. (2008). Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*. *Dev. Cell.* 14, 76–85. doi: 10.1016/j.devcel.2007.12.002

Tita, A. T., and Andrews, W. W. (2010). Diagnosis and management of clinical chorioamnionitis. *Clin. Perinatol.* 37, 339–354. doi: 10.1016/j.clp.2010.02.003

Tuffrey, M., Alexander, F., Woods, C., and Taylor-Robinson, D. (1992). Genetic susceptibility to chlamydial salpingitis and subsequent infertility in mice. *J. Reprod. Fertil.* 95, 31–38. doi: 10.1530/jrf.0.0950031

Vaninsberghe, D., Elsherbini, J. A., Varian, B., Poutahidis, T., Erdman, S., and Polz, M. F. (2020). Diarrhoeal events can trigger long-term Clostridium difficile colonization with recurrent blooms. *Nat. Microbiol.* 5, 642–650. doi: 10.1038/s41564-020-0668-2

Wymore Brand, M., Wannemuehler, M. J., Phillips, G. J., Proctor, A., Overstreet, A. M., Jergens, A. E., et al. (2015). The altered schaedler flora: continued applications of a defined murine microbial community. *ILAR J.* 56, 169–178. doi: 10.1093/ilar/ilv012

Yildirim, S., Yeoman, C. J., Janga, S. C., Thomas, S. M., Ho, M., Leigh, S. R., et al. (2014). Primate vaginal microbiomes exhibit species specificity without universal *Lactobacillus* dominance. *ISME J* 8, 2431–2444. doi: 10.1038/ismej.2014.90

Zavan, B., de Almeida, E. M., Salles Eda, S., Do Amarante-Paffaro, A. M., and Paffaro, V. A. Jr. (2016). COX-2 plays a role in angiogenic DBA(+) uNK cell subsets activation and pregnancy protection in LPS-exposed mice. *Placenta* 44, 34–45. doi: 10.1016/j.placenta.2016.06.006

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Comparison of the Genital Microbiomes of Pregnant Aboriginal and Non-aboriginal Women

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The genital microbiomes of women varies with racial background. Preterm birth and early-onset neonatal sepsis are two outcomes associated with genital infections during pregnancy. The rate of preterm birth in Aboriginal Australian mothers is high, as is the rate of early-onset sepsis in their infants. To date, no studies have been conducted to investigate genital microbiome taxa associated infection in this group of women. A prospective cohort study to characterize the vaginal and placental microbiomes of a group of these women from the Pilbara region was conducted at the Hedland Health Campus in Western Australia. Included in the study were gravidae Aboriginal ($n = 23$) and Non-aboriginal ($n = 27$) women in labor or for planned lower uterine segment Caesarean section. Employing sterile swabs, vaginal samples were obtained under sterile conditions immediately prior to vaginal delivery or planned Caesarean section; and placental samples were obtained under the same conditions during labor. Taxa present in the samples were identified by 16S rRNA amplicon sequencing (V4 region, 515F-806R). Taxon identity and abundance were established from Operational Taxonomic Unit (OTU) counts. Statistical analyses combining clinical metadata and sequencing results were employed to determine associations of taxa with racial background. The findings of this work served to enhance the current understanding of microbiota associated with health and disease in Aboriginal and Non-Aboriginal women. Differences were found between the vaginal and placental microbiomes of Aboriginal and Non-aboriginal women during pregnancy, as well as notable differences between the abundance of specific taxa in each racial group. The relative abundances of specific taxa were significantly different between participants with clinical signs of infection and those with healthy pregnancies. This work will contribute to understanding the causes of differences in rates of infection-driven preterm birth in various racial populations.

Keywords: Australian aboriginal women, pregnancy, vaginal microbiome, placental microbiome, infection, preterm birth, neonatal sepsis

INTRODUCTION

Genital bacterial community profiles differ with racial background. Dominant *Lactobacillus* in the vaginal flora of pregnant women is more prevalent amongst Caucasian and Asian women, and anaerobic bacterial communities are more common amongst African American and Hispanic women (Ma et al., 2012; Romero et al., 2014; MacIntyre et al., 2015; Freitas et al., 2017; Fettweis et al., 2019). Studies of the vaginal microbiome also have found racial differences in the context of preterm birth (Callahan et al., 2017; Elovitz et al., 2019). The associations of intrauterine microbiota with racial background have been studied for placental samples of African, Caucasian and Chinese women (Zheng et al., 2015; Collado et al., 2016; Doyle et al., 2017; Gomez-Arango et al., 2017; Parnell et al., 2017; Zhou et al., 2018), but there are little data on the influence of racial background on other intra-amniotic bacterial populations. There have not been investigations of the genital microbiota of pregnant Australian Aboriginal women; this study aims at addressing this gap by providing data on their vaginal and placental microbiomes and comparing them to the genital microbiota of Non-aboriginal women from the same Pilbara region in Western Australia. Knowledge of bacterial taxa residing in the vagina and placenta in normal pregnancies provides a foundation for healthcare professionals to identify abnormal flora, enabling the possibility of targeted prevention of genital infections.

The genital microbiomes have an important role in maternal and neonatal health. At the beginning of pregnancy, microbial richness and diversity of bacterial populations are reduced. At the same time, in healthy pregnancies the prevalence of potential pathogens is reduced too (Goltsman et al., 2018). The taxonomic composition of the microbial community of the vagina remains stable during pregnancy with an increase of the microbial diversity before birth of a healthy infant at term (DiGiulio et al., 2015). The historical view that the amniotic cavity constitutes a sterile environment has been challenged by findings that the healthy maternofetal unit is colonized with microbes, and that this is a prerequisite for immune maturation as well as metabolic and hormonal homeostasis (Staude et al., 2018). However, a review of recent findings concluded that the evidence supporting the hypothesis of a uterine microbiome is extremely weak (Perez-Muñoz et al., 2017). In addition, a recent study with placental samples concluded that the human placenta does not have a microbiome (De Goffau et al., 2019). Nonetheless, there is evidence that the placenta, the amnion, and the fetus share large proportions of a common microbiome, and that the maternal microbiome drives the development of the fetal immune system (Collado et al., 2016; De Aguero et al., 2016) These data lead to the conclusion that the composition of the vaginal microbiota is tightly regulated during pregnancy and that the switch to the non-pregnant situation precedes and maybe even triggers birth (Romero et al., 2014; DiGiulio et al., 2015; Freitas et al., 2017).

Owing to the polymicrobial nature of the genital microbiota the definition of what is normal or abnormal genital tract microflora is very difficult; normal microflora is assumed to be present in the absence of disease (Lamont, 2015). Spontaneous preterm labor leading to preterm birth (PTB) is recognized as a syndrome caused by a number of pathological processes leading to activation of the common terminal pathway of parturition. Abundant evidence supports the view that local or systemic infection or inflammation is a major cause of early PTB (Lamont, 2015). Bacterial infections threaten pregnant women and the fetus by gaining access to gestational tissues, such as the decidua, placenta, and fetal membranes (Vinturache et al., 2016). There are correlations between the risk of PTB and genital bacterial populations in both the vaginal (Wen et al., 2014) and intrauterine (Mendz et al., 2013) microbiota. These correlations differ between racial groups; for example, a study observed significant associations of vaginal bacteria and PTB in African and Hispanic American women, but Caucasian Americans did not show significant associations between vaginal microbiota and birth outcome (Wen et al., 2014).

It is estimated that 25%-40% of PTB are attributable to intrauterine infection (Goldenberg et al., 2008), and the relationship between the presence of pathogens and spontaneous preterm birth is complex. These findings underline the need to consider women's race when evaluating the links of specific genital bacteria with preterm birth. Similarly, early-onset neonatal sepsis (EOS) is associated with pathogenic microorganism acquired from the mother during pregnancy or at birth (Schrag et al., 2016). Firm connections have been established between several taxa such as *E. coli* and *Streptococcus* B and EOS, but the association of other taxa such as *Enterococcus* and *Haemophilus* with this type of sepsis appears to depend on human populations (Simonsen et al., 2014; Singh et al., 2019).

The rate of preterm birth (PTB) of Aboriginal Australian mothers is 14% and of Non-aboriginal mothers is 8% (Australian Institute of Health and Welfare, 2016), the former is higher than those of many underdeveloped countries. Spontaneous preterm birth was the most commonly identified cause of perinatal death in infants of Aboriginal and/or Torres Strait Islander (ATSI) mothers: 26% compared with 19% for Non-indigenous mothers (Australian Institute of Health and Welfare, 2018). Historically, infants of ATSI mothers have been over-represented in EOS data, although recent studies suggest that this rate may be decreasing (Braye et al., 2019). The current study investigated the presence of bacterial taxa associated with clinical signs of infection and PTB or EOS in the participant Australian Aboriginal women with the aim of providing data that would serve to devise strategies to reduce adverse pregnancy outcomes in this population.

METHODS

Study Design and Participants

A prospective cohort study was performed at Hedland Health Campus (HHC), located in Port Hedland, Pilbara region, Western Australia. Aboriginal people account for 16.2% of the Pilbara's population. Inclusion criteria admitted pregnant women with no known fetal anomalies, 18 years of age or older, and ability to provide informed written consent. Racial background was determined by self-identity as Aboriginal or non-Aboriginal Australian. Included in the study were pregnant Aboriginal ($n = 23$) and non-Aboriginal ($n = 27$) women recruited upon

presenting to the birth suite in labor or for planned lower uterine segment Caesarean section (LUSCS). Demographic data were collected from maternal and infant medical records and de-identified. It included age, gravidity, parity, gestational age at birth, infection during pregnancy, and information on previous pregnancies. Vaginal and placental swabs from each woman were obtained in theater prior to vaginal delivery or planned Caesarean section and during labor, respectively.

Ethics Approval

Participants volunteered to be in the study and provided written informed consent. The study was undertaken following approvals from WA Country Health Service Human Research Ethics Committee (HREC #2015/40) and Western Australian Aboriginal Health Ethics Committee (HREC #686).

Study Protocol

Previous investigations found that during pregnancy bacterial communities are stable in the introitus, midpoint and posterior fornix of the vagina (Consortium HMP, 2012; Huang et al., 2015). In this study, vaginal samples were collected from the vaginal midpoint (3 cm from introitus) using a sterile plastic swab with a Dacron tip. Vaginal samples were collected under sterile conditions by a qualified midwife on arrival to birth suite for women in spontaneous labor, or in theater immediately prior to surgery in women booked for LUSCS. Placentas were collected under sterile conditions during labor and stored at 4°C; placental samples were obtained by swabbing between the amnion and chorion layers of the placenta using the same type of sterile plastic swab. This method reduced sampling potential microbial contaminants from the vagina during the passage of the placenta through it. Two samples were collected from the same placental site for each participant for quality assurance purposes. Swabs were obtained from the vagina and the placenta of each participant, labeled for their participant origin but de-identified from the participant, and immediately preserved frozen at 0°C in sterile tubes until DNA extraction.

DNA Extraction, Amplification, and Sequencing

DNA extraction and purification were performed using the QIAamp DNA Mini Kit according to the manufacturer's instructions (QIAGEN, Chadstone Center, VIC, Australia); the concentration and quality of the DNA was assessed using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies; Wilmington, DE, USA). To minimize environmental contamination, DNA was extracted following a recommend practice (Eisenhofer et al., 2019).

The composition of the microbial communities was determined at the Ramaciotti Centre for Genomics (University of NSW, Sydney, Australia) by amplicon sequencing using an Illumina MiSeq instrument (2 × 250 bp chemistry). The 16S rRNA gene was amplified using the KAPA HiFi HotStart ReadyMix (95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by a final step of 72°C for 5 min) and the Earth Microbiome primers (515F-806R). Indices and Illumina sequencing adapters were attached using the Nextera XT Index Kit according the manufacturer's instructions. The Ramaciotti Centre is an institution with a quality management system according to the ISO/IEC17025:2017 Standard, and it follows recommended best practice protocols (Eisenhofer et al., 2019).

All placental and vaginal samples were analyzed individually and keeping track of their paired origin. Raw reads were quality checked, trimmed and aligned using the MiSeq standard operating procedures implemented in Mothur v1.39.1 with the SILVA SEED 16S rRNA reference database and classification with RDP (read depth 81,681 ± 7,145 clean reads/per sample). DNA extraction procedures and microbiome analyses have been previously utilized and validated.

Reagents in the QIAGEN extraction kit were included in the analyses as negative controls. To address the potential contamination of the placental microbiome by taxa present only in the vagina, all placental and vaginal paired samples were analyzed individually, and the taxa present as well as their relative abundances were compared. The data provided evidence that a possible contamination of the placental microbiome by the vaginal microbiota at the time of delivery was negligible (**Supplementary Table 1**). All OTU with an abundance >1% in the negative controls ($n = 21$) were excluded from the analyses. Application of these quality control processes left a total of 1,667 OTU. A hierarchical cluster was further generated and consistency was found to be optimal between biological replicates.

Data Analyses

The de-identified demographic data collected from the clinical records of mother and baby were analyzed using the Statistical Package for the Social Sciences (SPSS), Version 2.2. One-way Analyses of Variance (ANOVA) were carried out to determine differences in the microbiomes of Aboriginal and Non-Aboriginal cohorts. Logistic regression modeling was conducted to control for potential confounding variables, such as previous PTB history, maternal age and drug and alcohol use during pregnancy.

Taxon relative abundance was determined from sequence counts and expressed in percent. Genital taxa sequence data were analyzed to determine species diversity and richness, and Shannon's Diversity Index for each participant and the entire cohort. To evaluate beta-diversity in this cohort, multivariate analyses including Distance Linear Model (distLM) analyses, non-metric multidimensional scaling (nMDS) and PERMDISP were performed on Bray-Curtis resemblance matrix of square-root transformed relative abundances using Primer-E v7 (Clarke, 1993).

Probability tests were used to combine the clinical metadata and sequencing data to generate *p*-values of the association of microbial taxa with genital infection. Multivariate Association with Linear Models analyses (MaAsLIN) were conducted to evaluate associations between clinical metadata and microbial community abundance. After removing confounders, Linear Discriminant Effect Size analyses (LEfSe) (Segata et al., 2011) were performed to compare taxa enrichment between vagina and placenta genital locations and Aboriginal and Non-aboriginal

women. These analyses were conducted using the Galaxy web application (Afgan et al., 2018). Taxon prevalence was calculated as the number of samples with a relative abundance > 1% expressed as a percentage of the total number samples. To focus on taxa with minimum frequencies in the populations rather than on those whose presence is circumscribed to a few individuals, the frequency with which taxa were found in the samples were included in the analyses; only taxa with frequencies equal or higher than 15% were reported.

RESULTS

Clinical Characteristics of the Study Population

Participants were grouped in two self-identified racial categories almost equally represented: Aboriginal ($n = 23$) and Non-Aboriginal ($n = 27$). The racial composition of the Non-aboriginal participants was 76% Caucasian, 16% Asian, 8% Middle East. Demographic, clinical and pregnancy outcome characteristics data of the two groups of women are summarized in **Table 1**. The maternal age was lower for the Aboriginal participants (25.6 years, range 18–34 y.o.) than for the Non-aboriginal participants (29.5 years, range 21–36 y.o.), with a significant difference between them on one-way ANOVA testing ($p = 0.01$). The mean gravidity and parity were significantly higher for the Aboriginal women (G3.1, P2.5), compared to the Non-aboriginal women (G1.8, P1.6).

None of the Non-aboriginal participants had a previous history of PTB, and eight Aboriginal women had a background of PTB. Antenatal smoking, alcohol consumption and illicit drug use were significantly higher in Aboriginal women. Antenatal urinary, respiratory, or sexually transmitted infections were found in 52.2% of Aboriginal and 14.8% of Non-Aboriginal participants. Apart from a history of antenatal infection, there were no significant differences in maternal complications at delivery between both groups of participants, only the PTB rate was higher in the group of Aboriginal women (**Table 1**). Neonatal sepsis was diagnosed by attending clinicians blinded to the study aims and outcomes using established clinical and microbiological criteria (Gerdes, 1991).

TABLE 1 | Demographic and clinical characteristics of Aboriginal ($n = 23$) and Non-aboriginal ($n = 27$) women.

Characteristics	Aboriginal	Non-aboriginal	*p*-values
Mothers			
Mean age	25.6 years	29.5 years	0.001
Mean gestational age	38.2 weeks	39.1 weeks	0.061
Mean gravidity	3.1	1.8	0.002
Mean parity	2.5	1.6	0.007
Preterm birth history	8 (34.8%)	0 (0%)	0.001
Alcohol consumption	8 (34.8%)	2 (7.4%)	0.015
Smoking consumption	14 (60.9%)	1 (3.7%)	0.000
Illicit drug use	8 (34.8%)	1 (3.7%)	0.004
Complications during pregnancy			
Infection	12 (52.2%)	4 (14.8%)	0.004
IUGR	4 (17.4%)	5 (18.5%)	0.920
Hypertension	4 (17.4%)	4 (14.8%)	0.809
Obesity	4 (17.4%)	4 (14.8%)	0.809
APH	2 (8.7%)	4 (14.8%)	0.517
Diabetes	3 (13%)	2 (7.4%)	0.518
None	9 (39.1%)	10 (37%)	0.882
GBS status			
Positive	9 (39.1%)	5 (18.5%)	0.369
Negative	7 (30.4%)	14 (51.9%)	0.023
Unknown	7 (30.4%)	8 (29.6%)	–
Birth mode			
SVD	17 (73.9%)	12 (44.4%)	–
AVD	1 (4.3%)	1 (3.7%)	–
Elective LUSC	3 (13%)	6 (22.2%)	–
Non-elective LUSC	2 (8.7%)	8 (29.6%)	–
Problems during labor			
Maternal fever	1 (4.3%)	3 (11.1%)	0.053
Mean blood loss	569.6 ml	440.7 ml	–
Neonates			
Preterm	6 (26.1%)	2 (7.4%)	–
Neonatal sepsis	4 (17.4%)	4 (14.8%)	–

**Data are presented as frequencies (proportion percentage in each cohort).*

Composition and Diversity of the Genital Microbiomes

Similar OTU, species richness, evenness, and Shannon diversity index were found in the microbiomes of Aboriginal and Non-aboriginal women participating in the study (**Table 2**). Placental samples, however, showed differences in their OTU and species richness between racial groups in the presence of infection (data not shown). The OTU, richness, and evenness were significantly different between placental and vaginal samples, but not Shannon diversity, and these differences remained after stratifying by racial background (**Table 2**).

The predominant Phylum in the vaginal microbiomes of all the participant women was Firmicutes, and Proteobacteria in their placental microbiomes. Actinobacteria were more abundant in the vaginal microbiomes of Aboriginal women compared to those of Non-aboriginal women. In the placental microbiomes, Actinobacteria were more abundant in Non-aboriginal than in Aboriginal women (**Figure 1**).

The compositions of Aboriginal and Non-aboriginal microbiomes appeared similar in non-metric multidimensional scaling 2D and 3D plots using a Bray-Curtis similarity resemblance matrix. However, distLM analyses showed significant differences between Aboriginal and Non-aboriginal microbiomes ($p = 0.002$) (**Table 3**). Further analyses stratifying by racial background and genital location, yielded an ordination plot with clustered placental samples of each group; but no significant differences were observed in dispersion of the data ($PERMDISP = 0.52$).

Placental and vaginal samples clustered separately (**Figure 2**), which was confirmed by a significant dispersion between them ($PERMDISP = 0.05$); also, distLM analyses showed significant differences ($p = 0.001$) (**Table 3**).

TABLE 2 | Number of OTU (S), species richness (d), evenness (J'), and Shannon diversity index (H'), and comparisons of population subgroups stratified by racial background, genital location, and presence of infection.

Group	Average				*p*-value			
	S	d	J'	H' (log_e)	S	d	J'	H' (log_e)
Aboriginal	36.85	4.06	0.46	1.44				
Non-aboriginal	41.46	4.57	0.44	1.41	0.930	0.940	0.269	0.780
Placental	35.36	3.89	0.46	1.43				
Vaginal	59.17	6.57	0.36	1.34	1.56 E-15	2.13 E-15	3.09 E-09	0.698
Placental (A)	35.72	3.93	0.46	1.43				
Vaginal (A)	58.78	6.53	0.36	1.34	1.37 E-08	1.47 E-08	0.003	0.768
Placental (N-a)	39.39	4.34	0.45	1.41				
Vaginal (N-a)	55.21	6.14	0.36	1.31	2.97 E-08	3.49 E-08	2.05 E-7	0.411
Non-infection	37.17	4.09	0.46	1.43				
Infection	34.91	3.84	0.44	1.37	0.153	0.149	0.025	0.003

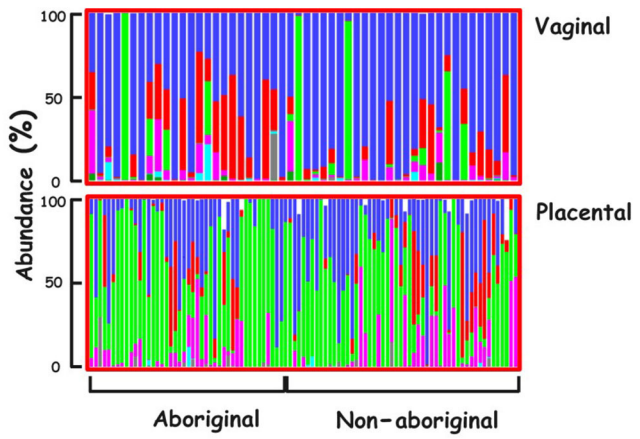

FIGURE 1 | Phyla relative abundances in the vaginal and placental microbiomes of the cohort. Firmicutes (blue), Actinobacteria (red), Proteobacteria (green), Bacteroidetes (magenta), Tenericutes (turquoise), Unclassified (black).

TABLE 3 | Composition of the microbiomes by racial background (Aboriginal vs. Non-aboriginal), genital location (vagina vs. placenta) and infection (yes vs. no) calculated by a Distance Linear model.

Variable	Pseudo-F	*p*-value	Residual difference
Racial background	2.842	0.002	147
Genital location	15.646	0.001	146
Infection	0.994	0.435	144

Comparison of Taxa Abundance in Genital Populations and Racial Backgrounds

In both populations of women at the taxon level, LEfSe analyses showed several taxa with enriched relative abundances at each genital location. The genera *Atopobium, Gardnerella, Lactobacillus, Prevotella* and *Staphylococcus* were more abundant in the vagina, and *Enterococcus* and *Flavobacterium* in the placenta (**Table 4**). In paired vaginal-placental samples of women who gave birth through the vagina, some taxa found in the vaginal microbiomes were present in negligible abundances in the placental microbiomes, and vice versa (**Supplementary Table 1**). Similar relationships were observed in Caesarean births. These and other instances rule out significant contamination of the placental samples during vaginal delivery. On the other hand, the cases that indicate greater relative abundances in the placental microbiomes suggest access to the placenta via routes other than ascending from the vagina, but do not rule out completely a possible migration of taxa to the amnion from the vagina during pregnancy.

Differences in taxa relative abundances were identified between the microbiomes of Aboriginal and Non-aboriginal women. **Table 5** shows genera with significantly different relative abundances and frequencies $> 15\%$ in each racial background. The taxon with highest relative abundance among the genital taxa associated with Aboriginal participants was *Lactobacillus iners* (OTU0002, 99% similarity), and associated with Non-aboriginal women was the taxon *Lactobacillus crispatus/acidophilus/helveticus* (OTU0001, all 99% similarity). These results were confirmed by MaAsLIN analysis ($p = 2.91 \times 10^{-4}$). The proportion of *Lactobacillus spp*. in vaginal samples followed a bimodal distribution with approximately 30% of samples with $< 1\%$ relative abundance, and 30% of samples with relative abundances 60–100%. Other genera found with higher relative abundance associated with Aboriginal women were: *Atopobium, Bifidobacterium, Prevotella, Pseudomonas,* and *Ureaplasma.* Enriched relative abundance genera associated with Non-aboriginal women were *Acinetobacter, Anaerococcus, Streptococcus,* and *Viellonella* (**Table 5**).

Significant differences between Aboriginal and Non-aboriginal women were identified by LEfSe analyses in the

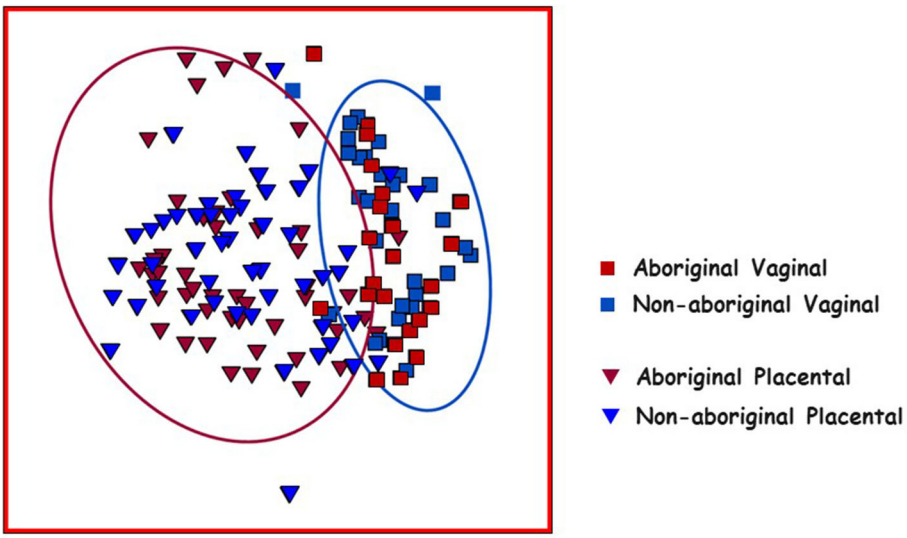

FIGURE 2 | Non-metric multidimensional scaling (nMDS) plot of Bray–Curtis resemblance generated from square root transformed OTU relative abundances. Red squares: Aboriginal participant vaginal microbiomes. Red triangles: Aboriginal participant placental microbiomes. Blue squares: Non-aboriginal participant vaginal microbiomes. Blue triangles: Non-aboriginal participant placental microbiomes. The oval outlines refer to the vaginal (blue oval) and placental (red oval) microbiomes.

TABLE 4 | Differences in the relative abundances between vaginal and placental taxa.

Taxon	Location	LDA Score	*p*-value
Atopobium	Vagina	3.792	<0.001
Gardenerella	Vagina	4.314	<0.001
Lactobacillus crispatus	Vagina	5.001	0.001
Lactobacillus iners	Vagina	4.525	0.003
Prevotella	Vagina	3.803	<0.001
Staphylococcus	Vagina	3.982	<0.001
Enterococcus	Placenta	3.950	0.011
Flavobacterium	Placenta	3.939	0.017

TABLE 5 | Taxa with significantly different relative abundances in Aboriginal and Non-aboriginal women.

Taxon	Racial group	LDA score	*p*-value
Atopobium	Aboriginal	3.622	0.012
Bifidobacterium	Aboriginal	2.898	<0.001
Lactobacillus iners	Aboriginal	4.651	0.001
Pseudomonas	Aboriginal	4.480	0.035
Prevotella	Aboriginal	3.197	0.025
Ureaplasma	Aboriginal	3.174	0.045
Acinetobacter	Non-aboriginal	2.918	0.038
Anaerococcus	Non-aboriginal	3.573	0.016
Lactobacillus crispatus	Non-aboriginal	4.697	<0.001
Streptococcus	Non-aboriginal	3.643	0.013
Veillonella	Non-aboriginal	3.710	0.010

TABLE 6 | Differences in the relative abundances of vaginal taxa between Aboriginal and Non-aboriginal women.

Taxon	Racial group	LDA score	*p*-value
Atopobium	Aboriginal	4.009	0.025
Corynebacterium	Aboriginal	3.137	0.009
Lactobacillus iners	Aboriginal	4.804	0.007
Megasphaera	Aboriginal	3.832	0.006
Ureaplasma	Aboriginal	3.281	0.013
Anaerococcus	Non-aboriginal	3.455	0.001
Escherichia/Shigella	Non-aboriginal	4.319	0.004
Enterococcus	Non-aboriginal	4.459	0.026
Prevotella	Non-aboriginal	3.318	0.031
Streptococcus	Non-aboriginal	3.989	0.017
Veillonella	Non-aboriginal	3.885	0.025

TABLE 7 | Differences in the relative abundances of placental taxa between Aboriginal and Non-aboriginal women.

Taxon	Racial Group	LDA Score	*p*-value
Pseudomonas	Aboriginal	4.742	0.019
Lactobacillus iners	Aboriginal	4.632	0.010
Paracoccus	Aboriginal	4.185	0.016
Lactobacillus crispatus	Non-aboriginal	4.577	<0.001
Streptococcus	Non-aboriginal	3.557	0.007
Veillonella	Non-aboriginal	3.565	0.037

relative abundances of several genera of vaginal taxa with frequencies > 15% (**Table 6**). The vaginal communities of Aboriginal women had a greater relative abundance of *Atopobium, Corynebacterium, L. iners, Megasphaera,* and *Ureaplasma*, The vaginal microbiota of Non-aboriginal women had higher relative abundance of the taxa *Anaerococcus, Enterococcus, Escherichia/Shigella*, *Prevotella, Streptococcus* and *Veillonella* (**Table 6**).

Several genera of placental taxa identified by LEfSe analyses and with frequencies > 15% showed significant differences in the relative abundances between Aboriginal and Non-aboriginal women (**Table 7**). The placental communities of Aboriginal women had greater relative abundances of the taxa *L. iners, Paracoccus,* and *Pseudomonas.* In Non-aboriginal women the taxa *L. crispatus, Streptococcus* and *Veillonella* had greater relative abundances (**Table 7**).

Genital Taxa Associated With Infection

The presence of infection did not affect the number of OTU or species richness in the genital microbiomes of both groups of women, but there were significant differences in their evenness and Shannon diversity index (**Table 2**).

The results of distLM multivariate analyses indicated that the presence of clinical signs of infection did not establish significant differences in the microbiomes of the studied women ($p = 0.44$) (**Table 5**).

Consistently, infection did not introduce significant differences in dispersion of the data were observed in the vaginal (*PERMDISP* = 0.94) or placental (*PERMDISP* = 0.10) microbiomes of Aboriginal women. Similarly, the presence of infection did not introduce significant differences between the vaginal microbiomes (*PERMDISP* = 0.10) of Non-aboriginal women, but did introduce significant dispersion differences (*PERMDISP* = 0.05) in their placental microbiomes.

Although no significant overall differences were found between the vaginal microbiomes of Aboriginal and Non-aboriginal women and those who experienced an infection, to ascertain whether there were taxa associated with the presence of infection, the relative abundance of each taxon in both racial groups and genital locations were compared employing LEfSe analyses. **Table 8** shows that the presence of infection enriched the relative abundance of the taxon *Atopobium* in the vagina of Aboriginal women, and of the taxa *Acinetobacter, Anaerococcus, Gardnerella, Megasphaera,* and *Prevotella* in the vagina of Non-aboriginal women. Notwithstanding that no significant overall differences were found between the placental microbiomes of healthy Aboriginal women and those who experienced an infection, LEfSe analyses showed that in this group of women the presence of infection resulted in enrichment of the relative abundances of *Pseudomonas* and a taxon of the order Sphingomonadales (**Table 8**). A significant difference was found between the placental microbiomes of healthy Non-aboriginal women and those who experienced an infection during pregnancy and LEfSe analyses showed that infection enriched the taxon *Corynebacterium* (**Table 8**).

Focusing on Aboriginal women with infection, **Table 9** summarizes microbiome data in the presence of *Pseudomonas* and *E. coli/Shigella* in the genital microbiomes of these women, and clinical data on *Streptococcus* B status, PTB and infant sepsis. *Pseudomonas* was detected in 25% of women in this group, and high rates of PTB and infant sepsis were noticed.

A number of taxa have been associated with PTB and sepsis. **Table 10** shows the relative abundance and the genital location of a dozen of these taxa with abundances > 1% and frequencies > 15% in the microbiomes of Aboriginal women who had signs of infection during pregnancy.

TABLE 8 | Taxa detected in higher relative abundances in the presence of infection in Aboriginal or Non-aboriginal women.

Taxon	Racial group	Location	LDA score	*p*-value
Atopobium	Aboriginal	Vagina	2.274	
Pseudomonas	Aboriginal	Placenta	4.854	0.009
Sphingomonadales	Aboriginal	Placenta	3.897	0.022
Acinetobacter	Non-aboriginal	Vagina	2.720	0.016
Anaerococcus	Non-aboriginal	Vagina	2.837	0.016
Gardnerella	Non-aboriginal	Vagina	2.820	0.011
Megasphaera	Non-aboriginal	Vagina	3.103	0.016
Prevotella	Non-aboriginal	Vagina	3.029	0.040
Corynebacterium	Non-aboriginal	Placenta	2.927	0.016

DISCUSSION

Investigations of the genital microbiomes of pregnant Aboriginal Australians were needed to understand their characteristics and identify taxa whose presence were associated with infection in this population. The study compared the genital microbiomes at the time of delivery of pregnant Aboriginal and Non-aboriginal women living in the Pilbara region employing high throughput 16S-rRNA sequencing. Some differences in demographic and clinical characteristics between both racial groups were statistically significant; however, the small size of each group in the cohort and the values of these differences made them in most cases not relevant to understanding the microbiological data. Nonetheless, the differences in infection and PTB rates between both groups of women helped to interpret the abundances of some taxa (**Table 1**).

The relative abundances of various Phyla showed differences between vaginal and placental microbiomes and between the microbiomes of Aboriginal and Non-aboriginal women (**Figure 1**), opening up the possibility for significant differences at lower taxonomic levels. The predominance of Firmicutes in the vagina was in agreement with the results of previous studies including Black, Asian, Caucasian and Hispanic American women (Aagaard et al., 2012; MacIntyre et al., 2015). The predominance of Proteobacteria in the placental microbiome agreed with results for Caucasian European (Collado et al., 2016) and Caucasian and Black American women (Parnell et al., 2017), but not with those of Chinese women in whom the predominant placental Phylum was Firmicutes (Zheng et al., 2015), indicating a dependence of placental bacterial populations on racial background.

The overall composition of the microbiomes of women in both racial groups showed significant differences in distLM calculations. However, comparison of the microbiomes of Aboriginal and Non-aboriginal women yielded similar OTU composition, species richness, evenness and Shannon diversity index for the vaginal and placental microbiomes (**Table 2**). A prospective study by Hyman et al. (2014), found that the vaginal microbiome species diversity was greatest among African American ($n = 8$) followed by Hispanic ($n = 13$) women compared with that of Asian ($n = 16$) and Caucasian ($n = 40$) women; in addition, statistically significant differences were

TABLE 9 | Clinical and microbiome sequencing data of Aboriginal women who presented signs of infection.

Woman	*Pseudomonas* (%)	GBS	*E. coli*/*Shigella* (%)	PTB	Sepsis	Weight (g)
A6003	N	?	2; P	N	Y	3,890
A6004	N	Y	6; P	N	Y	3,280
A6005	99; P, V	N	N	Y	Y	2,140
A6006	7; P	Y	1; P	Y	N	2,580
A6007	99; P	?	N	N	Y	2,440
A6009	93; P	?	N	N	N	4,190
A6010	N	Y	22; V	Y	Y	2,840
A6011	N	Y	N	N	N	2,410
A6014	N	Y	12; P	N	N	3,440
A6018	N	Y	6; P	N	N	4,130
A6021	N	N	N	Y	Y	3,170
A6023	N	Y	N	N	N	3,660

Taxa relative abundances are given as numbers. Genital location: P-placenta, V-vagina. GBS: Streptococcus Group B status; (?) unknown status. PTB: preterm birth. Sepsis: status. Weight at birth in g.

TABLE 10 | The relative abundances of a dozen taxa in the genital microbiomes of eight Aboriginal women who delivered preterm and/or the infant had sepsis.

Taxon	A6003	A6004	A6005	A6006	A6007	A6009	A6010	A6021
Acinetobacter	N	N	N	1; P	N	N	N	51; P
E. coli	2; P	6; P	N	1; P	N	N	22; V	N
Paracoccus	10; P	N	N	3; P	N	N	27; P	N
Pseudomonas	N	N	99; P, V	7; P	99; P	93; P	N	N
Ralstonia	29; P	N	N	N	N	N	N	N
Enterococcus	18; V	N	N	N	N	N	N	N
Staphylococcus	14; P	N	N	1; P	1; V	N	N	N
Streptococcus	?	Y	N	Y	?	?	Y	N
Atopobium	N	N	N	1; P	1; V	N	N	N
Gardnerella	N	N	N	17; P	11; V	N	2; P 5; V	N
Mycoplasma	N	N	N	N	N	N	N	N
Ureaplasma	10; V	N	N	N	N	N	N	N

Taxa relative abundances are given as percentage numbers. Genital location: P-placenta, V-vagina. Continuous dark borders indicate three women in whose placental microbiome Pseudomonas was the dominant taxon. The double line borders indicate the abundance of E. coli/Shigella and Streptococcus Group B. The discontinuous border indicates the abundance of Acinetobacter.

found between Asian and Caucasian women relative to African American or Hispanic women. This greater diversity in the vaginal microbiome of women of African descent was confirmed in an investigation comprising 300 pregnant women (Serrano et al., 2019). A study of the vaginal microbiome in the third trimester by Fettweis et al. (2019) that included women of African ancestry (71 delivered at term; 35 preterm) and European ancestry (13 delivered at term; 6 preterm) observed greater species diversity in women who delivered preterm. In contrast, Stout et al. (2017) reported that the vaginal microbiome of 24 non-African American women had lower species diversity than that of 53 African American women. It remains to be determined whether the absence of significant differences observed in the present work between Aboriginal and Non-aboriginal women will persist in analyses of larger numbers of samples. The presence of infection resulted in significant changes in evenness and diversity (**Table 2**) in agreement with the results of a study that observed greater species diversity in women who delivered preterm and included women of African ancestry ($n = 106$) and European ancestry ($n = 19$) (Fettweis et al., 2019).

Comparison of the vaginal and placental microbiomes indicated that they clustered separately in nMDS plots (**Figure 2**), and distLM calculations showed a significant difference between both genital locations in agreement with the results of previous studies (Aagaard et al., 2014). In healthy pregnant women, midpoint vaginal microbiomes are stable in time and are less rich and diverse than in healthy non-pregnant women (Aagaard et al., 2012; Walther-António et al., 2014). The numbers of

OTU, richness and evenness, but not the Shannon diversity, were different between vaginal and placental samples (**Table 2**). Analyses by location and racial background showed nMDS clustering of the placental microbiomes in these populations (**Figure 2**); but no significant differences were found in PERMDISP calculations. Nonetheless, the differences in OTU composition, richness and evenness remained after stratification by racial background (**Table 2**). The results of this work suggested that the microbiome in the normal placenta has significant lower abundance, richness and diversity than in the vagina in agreement with previous investigations (Aagaard et al., 2014; Taddei et al., 2018; Seferovic et al., 2019).

Differences in relative abundances of specific taxa with frequencies > 15% were identified between both genital microbiomes (**Table 4**). More taxa were enriched in the vagina than in the placenta reflecting the greater diversity and abundances found in the former genital location; exceptions were *Enterococcus* and *Flavobacterium*. The higher relative abundance of *Enterococcus* found in placental samples agreed with the results of a study comparing placental microbiomes of Chinese women who gave birth to normal or low weight infants (Zheng et al., 2015); also, in a study of 1,832 placental samples from Chinese women with healthy pregnancies, *Enterococcus* was recovered by cultivation in 24% of the placental samples second in abundance only to *E. coli*. (Zhou et al., 2018). Viable bacteria have been detected in 15 normal placentas from pregnancies delivered at term by Caesarean section with *Enterobacter* and *Escherichia/Shigella* as the most abundant taxa and smaller abundances of *Lactobacillus, Staphylococcus* and *Streptococus* taxa (Collado et al., 2016). *In situ* hybridization of the placentas of 52 women in term and preterm births without evidence of infection and independent of delivery mode yielded relative abundances greater than 5% for *Lactobacillus, Prevotella, Staphylococcus,* and *Streptococcus* taxa. (Seferovic et al., 2019).

Flavobacterium has been found as a common contaminant in reagents, but after applying the quality control with the reagents employed in the sequencing and in the analyses described in the Methods section, this taxon appeared at significant relative abundances in a number of placental microbiomes of both Aboriginal and Non-aboriginal women, and at over twice the abundance in the latter group of women. This higher relative abundance identified in the participating women, concurred with the results of a study of the endometrial microbiome of women undergoing assisted reproductive technology treatments (Franasiak et al., 2016).

The microbiomes of Aboriginal and Non-aboriginal women showed differences in relative abundances of taxa with frequencies > 15% (**Table 5**). The results were consistent with those of genital microbiome studies of healthy women from various racial backgrounds (Romero et al., 2014; MacIntyre et al., 2015). An unusually high relative abundance of *Pseudomonas* was present in genital microbiomes of Aboriginal women, three times more abundant than in the microbiomes of Non-aboriginal women. *Pseudomonas* has been detected in placental samples of overweight and obese Caucasian Australian women (Gomez-Arango et al., 2017); also, it has been identified at high relative abundance in samples from the endometrium of the healthy control group of a study of Chinese women with endometrial polyps (Fang et al., 2016), endometrial and Fallopian tube samples of a large cohort of reproductive age Chinese women operated for conditions not known to involve infection (Chen et al., 2017), and endometrial samples of women with chronic endometritis (Moreno and Franasiak, 2017). Considering that the distLM logistic regression confirmed that significant differences in microbiomes were due to racial background and genital location (**Table 3**), these results suggested that colonization with *Pseudomonas* is characteristic of the Aboriginal population of this study.

Comparison of the vaginal microbiomes of Aboriginal and Non-aboriginal women yielded significant differences in the relative abundances of a number of taxa (**Table 6**). In the vagina of Non-aboriginal women, *Corynebacterium* was found only in negligible or very small concentrations; also, these women had negligible or very low *Lactobacillus* spp. abundance. The highest relative abundances of *Corynebacterium* found in Aboriginal women with no clinical signs of infection suggested that the taxa present were non-pathogenic species. These species of corynebacteria produce organic acids and stimulate the production of anti-inflammatory cytokines that enhance the capacity of the vaginal mucosa to protect against opportunistic and pathogenic microorganisms (Gladysheva and Cherkasov, 2018); *Corynebacterium* may play a similar role in the vaginal microbiome of Aboriginal women.

Vaginal microbiomes abundant in *Atopobium* and low in *Lactobacillus* can reach up to 40% amongst Black and Hispanic women (Ma et al., 2012), and *Atopobium* and *Megasphaera* have been associated with vaginal infections (Hocevar et al., 2019). In the vaginal microbiota of Aboriginal women *Atopobium* was commonly found in microbiomes with low *Lactobacillus* abundance but it was not associated with clinical signs of disease. *Megaspahaera* also was not found in women with clinical signs of infection.

Differences in the community structures (CST) have been identified in the vaginal microbiomes of non-pregnant (Ravel et al., 2011) and of pregnant (Freitas et al., 2018) healthy women from various racial backgrounds. CST III is found at a greater frequency in women with Asian, Black and Hispanic racial backgrounds, and CST I is found in Asian and Caucasian women Ravel et al., 2011; Gajer et al., 2012; Freitas et al., 2018). In terms of population structures, the predominant species in the microbiomes of Aboriginal women was *L. iners* which is associated with vaginal community state type CST III, and in Non-aboriginal women the most abundant taxon was identified as *L. crispatus* which is associated with CST I. The study by Hyman et al. (2014) concluded that *L crispatus* had a higher relative abundance in the vaginal microbiome of Caucasian participants, and *L iners* in African-American and Hispanic participants, and both species had similar relative abundances in Asian women. An investigation of the vaginal microbiome in the third trimester found higher relative abundances of *L. iners* in women of African descent, and of *L. crispatus* in women of European descent (Fettweis et al., 2019). Serrano et al. (2019) studied a matched cohort of women of African ancestry ($n = 49$) and European ancestry ($n = 41$) who delivered at term

and found in the third trimester a predominance of *L. iners* in the former group and of *L. crispatus* in the latter. In contrast, Stout et al. (2017) determined that *L. iners* was significantly more abundant than *L. crispatus* in a study comprising both African Americans and non-African Americans. *Gardnerella* and *Atopobium* are dominant genera in the vaginal microbiome of CST IV, and present at lower abundances in the other CST (Freitas et al., 2018). In the study of women with predominantly African ancestry by Fettweis et al. (2019), the next most abundant taxa after *Lactobacillus* were *Gardnerella,* BVAB1, *Atopobium* and *Prevotella* cluster 2. No significant differences between the abundance of *Atopobium* and *Prevotella* were observed for women of African or European ancestry, but *Gardenerella* was more abundant in women of African ancestry. The work of Serrano et al. (2019) showed a linkage between *Gardnerell*a clade 3 and women of African ancestry, and no *Gardnerella* showed a significant association with women European ancestry. On the other hand, Stout et al. (2017) found no difference in *Gardnerella* abundance between were African Americans and non-African Americans. In Aboriginal women the most abundant vaginal taxon was *L. iners* followed by *L. crispatus*, *Gardnerella, Pseudomonas,* and *Enterococcus*. Considering that the genital microbiomes of these women were dominated by *L. iners,* and the abundance in healthy Aboriginal women of other taxa such as *Corynebacterium, Atopobium* and *Megaspahera* suggested in the context of the reduced diversity of the vaginal microbiomes of pregnant women, that there were some similarities with the vaginal microbiome of women of African descent, but overall the Aboriginal pregnant women showed an uncommon vaginal community structure.

Significant differences between the placental microbiomes of Aboriginal and Non-aboriginal women were the result of LEfSe analyses (**Table 7**). In addition to *L. crispatus*. The placental microbiomes of Non-aboriginal women were enriched with *Streptococcus* and *Veillonella.* The former taxon has been found commonly in placental samples of women of various racial backgrounds: Chinese (Zheng et al., 2015; Zhou et al., 2018), Caucasian European (Collado et al., 2016), Caucasian and African American (Parnell et al., 2017), Caucasian Australian (Gomez-Arango et al., 2017), and African Malawian women (Doyle et al., 2017), suggesting the colonization of a broad range of racial backgrounds. *Streptococcus* has been identified at high relative abundance also at the endometrium: in samples of Chinese women with endometrium polyps (Fang et al., 2016), of predominantly Caucasian American (Tao et al., 2017) and Caucasian Italian (Moreno and Simon, 2018) women undergoing IVF cycles. Similarly, *Veillonella* has been identified in the placenta of women from various racial backgrounds (Aagaard et al., 2014; Zheng et al., 2015; Doyle et al., 2017; Gomez-Arango et al., 2017; Moreno and Simon, 2019).

In Aboriginal women in addition to *L. iners,* the placental microbiomes were enriched with *Paracoccus* and *Pseudomonas* (**Table 7**). The former taxon has been identified in the placenta of women from only few racial backgrounds and at low relative abundances (Zheng et al., 2015; Theis et al., 2019), and has been considered a contaminant by others (Perez-Muñoz et al., 2017; Leiby et al., 2018). In the present study after elimination of OTU with an abundance <1% in the negative controls, *Paracoccus* was present at relative abundances > 1% and with a frequency higher than 15%, as were all the other taxa presented in tables.

Comparisons of the effects of infection on the genital microbiomes of Aboriginal women did not yield any overall significant differences; likewise, for the vaginal microbiomes of Non-aboriginal women. However, at the taxon level, LEfSe analyses showed important differences in the presence of infection (**Table 8**). Potential effects of colonization by *Atopobium, Corynebacterium, Gardnerella,* and *Megasphaera* and have been discussed. The presence of *Acinetobacter* has been associated with PTB and neonatal infections in American women (He et al., 2013), spontaneous preterm and chorioamnionitis in an Australian Caucasian woman (Quinlivan et al., 2014), and vaginal infection in Indian women (Gopalan et al., 2017). Genital infections of *Acinetobacte*r during pregnancy are associated with adverse outcomes in various racial backgrounds.

Post-delivery increases in the abundance of *Anaerococcus* and *Prevotella* with disturbance in the vaginal microbiota unrelated to gestational age at delivery were observed in a population of mostly Caucasian American women (DiGiulio et al., 2015). In contrast, significant *Anaerococus* vaginal colonization was detected in studies with mostly African American with normal pregnancies (Romero et al., 2014) and of Chinese women with uncomplicated pregnancies (Chen et al., 2019). *Anaerococcus* appeared to colonize the female genital tract of women of various racial backgrounds albeit with different pregnancy outcomes. In a study with mostly African American women, *Prevotella* was found with higher abundance in the vagina of women who delivered preterm (Subramaniam et al., 2016). Several *Prevotella* spp. were associated with both term and preterm in predominantly Caucasian American women. *Prevotella amnii* and *P. tannerae* had greater prevalence in the term cohort, whereas *P. timonensis, P. bivia, P. corpori*s, and *P. bucalis* were more prevalent in the preterm group (Freitas et al., 2018). *Prevoltella* was strongly associated with vaginal infection in a study of mostly Caucasian Brazilian women (Dobbler et al., 2019). These results suggest an overall association of *Prevotella* with vaginal infection in different racial backgrounds.

Amongst the 23 Aboriginal women in the study, 12 presented clinical signs of infection. The relative abundance of *Pseudomonas, E. coli/Shigella* and the prevalence of GBS in Aboriginal women with sign of infection is given in **Table 9**; included in the table are the metadata for PTB and sepsis. In the placental microbiome of 3 of these women the dominant taxon was *Pseudomonas* with relative abundances > 93%; this taxon was dominant also in the vaginal microbiome (99% relative abundance) of one of these women. *Pseudomonas* was identified at low relative abundance in another Aboriginal woman. This taxon has been found in the placenta of women from different racial backgrounds, e.g., Chinese women who delivered of low birth weight infants (Zheng et al., 2015), Chinese women with gestational diabetes mellitus (Zheng et al., 2017), overweight and obese Caucasian Australian women (Gomez-Arango et al., 2017), and African American women who delivered at term (Theis et al., 2019). Commonly, intrauterine presence of *Pseudomonas* has been associated with infection but not in all instances.

Many taxa have been associated with infection during pregnancy (DiGiulio et al., 2015; Freitas et al., 2018). **Table 10** shows the relative abundance of 12 of these taxa in eight of the Aboriginal women who had adverse pregnancy outcomes, either PTB and/or neonatal sepsis. In women A6005, A6007, and A6009 there is a correlation between these outcomes and the presence of *Pseudomonas* at high relative abundance in the absence of significant abundance of other potential pathogens. The presence of several potential pathogens at low and intermediate abundances in the genital microbiomes of woman A6003 would account for her infection. The Group B *Streptococcus* positive status of women A6004, A6006, and A6010 may explain the presence of infection in these participants. In addition, the low abundance of *Pseudomonas, Gardnerella* and other potentially pathogenic taxa in woman A6006, and the intermediate abundance of *E. coli* and *Paracoccus* in woman A6010 may have contributed to their respective infections. Participant A6021 did not show abundance of *E. coli,* GBS or other taxa that would explain satisfactorily that she delivered preterm a child with sepsis. However, *Acinetobacter* has been associated with preterm birth and was detected at relatively high abundance in the placenta of this woman.

Early-onset neonatal sepsis almost always is associated with pathogenic microorganisms acquired from the mother during pregnancy or at birth (Klinger et al., 2009); they are commonly GBS and *E. coli* (Schrag et al., 2016), but a significant proportion of EOS is due to other infections. *Pseudomonas* has been associated with PTB and late-onset sepsis (Harnaen et al., 2015), but in Australia and New Zealand also with EOS (Braye et al., 2019; Singh et al., 2019).

The abundance of *Pseudomonas* and *E. coli,* and the GBS status in the genital microbiomes of Aboriginal and Non-aboriginal women in this study are given in **Supplementary Table 2**, together with clinical data on PTB and sepsis. These data suggested the association of *Pseudomonas* with PTB and/or sepsis, particularly in Aboriginal women, also in the absence of other potential infections.

CONCLUSION

The study investigated the genital microbiomes at delivery in Aboriginal women of the Pilbara region of Western Australia by comparing them to Non-aboriginal women in the same region. The study is the first report on the genital microbiomes of pregnant Australian Aboriginal women using culture independent techniques. The results support previous findings that the most abundant vaginal Phyla during pregnancy are Firmicutes, Proteobacteria, and Actinobacteria, and that the vaginal microbiome of normal pregnancy commonly consolidates into communities dominated by *Lactobacillus*. At a global level distLM analyses yielded differences between the genital microbiomes of both groups of women, and at the taxon level significant differences were found between the vaginal or placental microbiomes of Aboriginal and Non-aboriginal women. *Pseudomonas* appeared to be associated with infection and adverse pregnancy outcomes, particularly in the group of Aboriginal women.

The study focused on a rural population with a high rate of spontaneous preterm birth and demonstrated differences in the microbiomes at the taxon level in women with infection. The findings served to enhance the current understanding of microbiota associated with health and disease in Aboriginal and Non-Aboriginal women, and will help the development of targeted prevention of chorioamnionitis. Further studies with larger cohorts will serve to verify the results of this work and the routes of invasion of pathogenic bacteria into the intra-uterine environment that would enable earlier detection of subclinical infection, and allow for further reduction of premature deliveries for both Aboriginal and Non-aboriginal women.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the European Nucleotide Archive under the accession number PRJEB39698.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by WA Country Health Service Human Research Ethics Committee (HREC #2015/40) and Western Australian Aboriginal Health Ethics Committee (HREC #686). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ND, JQ, and GM contributed to the design of the study. ND and JQ acquired the data. ND, NC-R, and GM helped with the analyses and interpretation of the data. ND, JQ, NC-R, and GM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.523764/full#supplementary-material

REFERENCES

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., and Versalovic, J. (2014). The placenta harbors a unique microbiome. *Sci. Transl. Med.* 6:237ra65. doi: 10.1126/scitranslmed.3008599

Aagaard, K., Riehle, K., Ma, J., Segata, N., Mistretta, T.-A., Coarfa, C., et al. (2012). A Metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. *PLoS ONE* 7:e36466. doi: 10.1371/journal.pone.0036466

Afgan, E., Baker, D., Batut, B., van den Beek, M., Bouvier, D., Ech, M., et al. (2018). The galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res.* 46, W537–W544. doi: 10.1093/nar/gky379

Australian Institute of Health and Welfare (2016) *Australia's Health Series no. 15. Catalogue No. AUS 199, 234-237*. Available online at: https://www.aihw.gov.au/getmedia/9844cefb-7745-4dd8-9ee2-f4d1c3d6a727/19787-AH16.pdf.aspx (accessed September 3, 2019).

Australian Institute of Health and Welfare (2018) *Perinatal deaths in Australia 2013-2014. Catalogue No. PER 94, 45-46*. https://www.aihw.gov.au/getmedia/78784f2e-2f61-47ea-9908-84b34441ae0a/~aihw-per-94.pdf.aspx?inline=true (accessed August 3, 2020).

Braye, K., Foureur, M., de Waal, K., Jones, M., Putt, E., and Ferguson, J. (2019). Epidemiology of neonatal early-onset sepsis in a geographically diverse Australian health district 2006-2016. *PLoS ONE* 14:e0214298. doi: 10.1371/journal.pone.0214298

Callahan, B. J., DiGiulio, D. B., Goltsman, D. S. A., Sun, C. L., Costello, E. K., Jeganathan, P., et al. (2017). Replication and refinement of a vaginal microbial signature of preterm birth in two racially distinct cohorts of US women. *Proc. Natl. Acad. Sci. U.S.A.* 114, 9966–9971. doi: 10.1073/pnas.1705899114

Chen, C., Song, X., Wei, W., Zhong, H., Dai, J., Lan, Z., et al. (2017). The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat. Commun.* 8:875. doi: 10.1038/s41467-017-00901-0

Chen, Y., Hong, Z., Wang, W., Gu, L., Gao, H., Qiu, L., et al. (2019). Association between the vaginal microbiome and high-risk human papillomavirus infection in pregnant Chinese women. *BMC Infect. Dis. (2019)* 19:677. doi: 10.1186/s12879-019-4279-6

Clarke, K. R. (1993). Non-parametric multivariate analyses of changes in community structure. *Austral. J. Ecol.* 18, 117–143. doi: 10.1111/j.1442-9993.1993.tb00438.x

Collado, M. C., Rautava, S., Aakko, J., Isolauri, E., and Salminen, S. (2016). Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Sci. Rep.* 6:23129. doi: 10.1038/srep23129

Consortium HMP. (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

De Aguero, M. G., Ganal-Vonarburg, S. C., and Fuhrer, T. (2016). The maternal microbiota drives early postnatal innate immune development. *Science* 351, 1296–1302. doi: 10.1126/science.aad2571

De Goffau, M. C., Lager, S., Sovio, U., Gaccioli, F., Cook, E., Peacock, S. J., et al. (2019). Human placenta has no microbiome but can contain potential pathogens. *Nature* 572, 329–334. doi: 10.1038/s41586-019-1451-5

DiGiulio, D. B., Callahan, B. J., McMurdiea, P. J., Costello, E. K., Lyell, D. J., Robaczewska, A., et al. (2015). Temporal and spatial variation of the human microbiota during pregnancy. *Proc. Nat. Acad. Sci. U.S.A.* 112, 11060–11065. doi: 10.1073/pnas.1502875112

Dobbler, P., Mai, V., Procianoy, R. S., Silveira, R. C., Corso, A. L., and Roesch, L. F. W. (2019). The vaginal microbial communities of healthy expectant Brazilian mothers and its correlation with the newborn's gut colonization. *World J. Mirobiol. Biotech.* 35:159. doi: 10.1007/s11274-019-2737-3

Doyle, R. M., Harris, K., Kamiza, S., Harjunmaa, U., Ashorn, U., Nkhoma, M., et al. (2017). Bacterial communities found in placental tissues are associated with severe chorioamnionitis and adverse birth outcomes. *PLoS ONE* 12:e0180167. doi: 10.1371/journal.pone.0180167

Eisenhofer, R., Minich, J. J., Marotz, C., Cooper, A., Knight, R., and Weyrich, L. S. (2019). Contamination in low microbial biomass microbiome studies: issues and recommendations. *Trends Microbiol.* 27, 105–116. doi: 10.1016/j.tim.2018.11.003

Elovitz, M. A., Gajer, P., Riis, V., Brown, A. G., Humphys,. M. S., Holm,. J. B., et al. (2019). Cervicovaginal microbiota and local immune response modulate the risk of spontaneous preterm delivery. *Nat. Commun.* 10:1305. doi: 10.1038/s41467-019-09285-9

Fang, R.-L., Chen, L.-X., Shu, W.-S., Yao, S.-Z., Wang, S.-W., and Chen, Y.-Q. (2016). Barcoded sequen- cing reveals diverse intrauterine microbiomes in patients suffering with endometrial polyps. *Am. J. Transl. Res.* 8, 1581–1592.

Fettweis, J. M., Serrano, M. G., Brooks, J. P., Edwards, D. J., Girerd, P. H., Parikh, H. I., et al. (2019). The vaginal microbiome and preterm birth. *Nat. Med.* 25, 1012–1021. doi: 10.1038/s41591-019-0450-2

Franasiak, J. M., Werner, M. D., Juneau, C. R., Tao, X., Landis, J., Zha, Y., et al. (2016). Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit. *J. Assist. Reprod. Genet.* 33, 129–136. doi: 10.1007/s10815-015-0614-z

Freitas, A. C., Bocking, A., Hill, J. E., Money, D. M., and VOGUE Research Group. (2018). Increased richness and diversity of the vaginal microbiota and spontaneous preterm birth. *Microbiome* 6:117. doi: 10.1186/s40168-018-0502-8

Freitas, A. C., Chaban, B., Bocking, A., Rocco, M., Yang, S., Hill, J. E., et al. (2017). The vaginal microbiome of pregnant women is less rich and diverse, with lower prevalence of mollicutes, compared to non-pregnant women. *Sci. Rep.* 7:9212. doi: 10.1038/s41598-017-07790-9

Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schütte, U. M. E., Zhong, X., et al. (2012). Temporal dynamics of the human vaginal microbiota. *Sci. Transl. Med.* 4:132ra52. doi: 10.1126/scitranslmed.3003605

Gerdes, J. S. (1991). Clinicopathologic approach to the diagnosis of neonatal sepsis. *Clin. Perinatol.* 18, 361–381. doi: 10.1016/S0095-5108(18)30528-1

Gladysheva, I., and Cherkasov, S. V. (2018). Corynebacterium species in the female genital tract - pathogens or potential probiotics. *Int. J. Pharma. Bio. Sci.* 9, 265–272. doi: 10.22376/ijpbs.2018.9.4.b265-272

Goldenberg, R. L., Culhane, J. F., Iams, J. D., and Romero, R. (2008). Epidemiology and causes of preterm birth. *Lancet* 371, 75–84. doi: 10.1016/S0140-6736(08)60074-4

Goltsman, D. S. A., Sun, C. L., Proctor, D. M., DiGiulio, D. B., Robaczewska, A., Thomas, B. C., et al. (2018). Metagenomic analysis with strain-level resolution reveals fine-scale variation in the human pregnancy microbiome. *Genome Res.* 28, 1467–1480. doi: 10.1101/gr.236000.118

Gomez-Arango, L. F., Barrett, H. L., McIntyre, H. D., Callaway, L. K., Morrison, M., and Nitert, M. D. (2017). Contributions of the maternal oral and gut microbiome to placental microbial colonization in overweight and obese pregnant women. *Sci. Rep.* 7:2860. doi: 10.1038/s41598-017-03066-4

Gopalan, U., Rajendiran, S., Jayakumar, K., and Karnaboopathy, R. (2017). Composition of vaginal microbiota and their antibiotic susceptibility in symptomatic women. *Int. J. Reprod. Contracept. Obstet. Gynecol.* 6, 427–432 doi: 10.18203/2320-1770.ijrcog20170190

Harnaen, E., Doctor, T. N., and Malhotra, A. (2015). Characteristics of *Pseudomonas aeruginosa* infection in a tertiary neonatal unit. *Int. J. Pediatr. Res.* 1:010. doi: 10.23937/2469-5769/1510010

He, M., Kostadinov, S., Gundogan, F., Struminsky, J., Pimar, H., and Sung, C. J. (2013). Pregnancy and perinatal outcomes associated with

Acinetobacter baumannii infection. *Am. J. Perinatol. Rep.* 3, 51–56. doi: 10.1055/s-0033-1334460

Hocevar, K., Maver, A., Simic, M. V., Hodzic, A., Halsberger, A., Sersen, T. P., et al. (2019). Vaginal microbiome signature is associated with spontaneous preterm delivery. *Front. Med.* 6:201. doi: 10.3389/fmed.2019.00201

Huang, Y. E., Wang, Y., He, Y., Ji, Y., Wang, L. P., Sheng, H. F., et al. (2015). Homogeneity of the vaginal microbiome at the cervix, posterior fornix, and vaginal canal in pregnant Chinese women. *Microb. Ecol.* 69, 407–414. doi: 10.1007/s00248-014-0487-1

Hyman, R. W., Fukushima, M., Jiang, H., Fung, E., Rand, L., Johnson, B., et al. (2014). Diversity of the vaginal microbiome correlates with preterm birth. *Reprod. Sci.* 21, 32–40. doi: 10.1177/1933719113488838

Klinger, G., Levy, I., Sirota, L., Boyko, V., Reichman, B., Lerner-Geva, L., et al. (2009). Epidemiology and risk factors for early onset sepsis among very-low-birthweight infants. *Am. J. Obstet. Gynecol.* 201, 38.e1–6. doi: 10.1016/j.ajog.2009.03.006

Lamont, R. F. (2015). Advances in the prevention of infection-related preterm birth. *Front. Immunol.* 6:566. doi: 10.3389/fimmu.2015.00566

Leiby, J. S., McCormick, K., Sherrill-Mix, S., Clarke, E. L., Kessler, L. R., Taylor, L. J., et al. (2018). Lack of detection of a human placenta microbiome in samples from preterm and term deliveries. *Microbiome* 6:196. doi: 10.1186/s40168-018-0575-4

Ma, B., Forney, L. J., and Ravel, J. (2012). Vaginal microbiome: rethinking health and disease. *Annu. Rev. Microbiol.* 66, 371–389. doi: 10.1146/annurev-micro-092611-150157

MacIntyre, D. A., Chandiramani, M., Lee, Y. S., Kindinger, L., Smith, A., Angelopoulos, N., et al. (2015). The vaginal microbiome during pregnancy and the postpartum period in a European population. *Sci. Rep.* 5:8988. doi: 10.1038/srep08988

Mendz, G. L., Kaakoush, N. O., and Quinlivan, J. A. (2013). Bacterial aetiological agents of intra-amniotic infections and preterm birth in pregnant women. *Front. Cell Infect. Microbiol.* 3:58. doi: 10.3389/fcimb.2013.00058

Moreno, I., and Franasiak, J. M. (2017). Endometrial microbiota-new player in town. *Fertil. Steril.* 108, 32–39. doi: 10.1016/j.fertnstert.2017.05.034

Moreno, I., and Simon, C. (2018). Relevance of assessing the uterine microbiota in infertility. *Fertil. Steril.* 110, 337–343. doi: 10.1016/j.fertnstert.2018.04.041

Moreno, I., and Simon, C. (2019). Deciphering the effect of reproductive tract microbiota on human reproduction. *Reprod. Med. Biol.* 18, 40–50. doi: 10.1002/rmb2.12249

Parnell, L. A., Briggs, C. M., Cao, B., Delannoy-Bruno, O., Schrieffer, A. E., and Mysorekar, I. U. (2017). Microbial communities in placentas from term normal pregnancy exhibit spatially variable profiles. *Sci. Rep.* 7:11200. doi: 10.1038/s41598-017-11514-4

Perez-Muñoz, M. E., Arrieta, M.-C., Ramer-Tait, A. E., and Walter, J. (2017). A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome. *Microbiome* 5:48. doi: 10.1186/s40168-017-0268-4

Quinlivan, J. A., Kaakoush, N. O., and Mendz, G. L. (2014). *Acinetobacter* species associated with spontaneous preterm birth and histological chorioamnionitis. *Br. J. Med. Res.* 4, 5293–5297. doi: 10.9734/BJMMR/2014/12004

Ravel, J., Gajer, P., Abdob, Z., Schneider, G. M., Koenig, S. S. K., McCulle, S. L., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4680–4687. doi: 10.1073/pnas.1002611107

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Nikita, L., et al. (2014). The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2:4. doi: 10.1186/2049-2618-2-10

Schrag, S. J., Farley, M. M., Petit, S., Reingold, A., Weston, E. J., Pondo, T., et al. (2016). Epidemiology of invasive early-onset neonatal sepsis, 2005 to 2014. *Pediatrics* 138:e20162013. doi: 10.1542/peds.2016-2013

Seferovic, M. D., Pace, R. M., Carroll, M., Belfort, B., Major, A. M., Chu, D. M., et al. (2019). Visualization of microbes by 16S in situ hybridization in term and preterm placentas without intraamniotic infection. *Am. J. Obstet. Gynecol.* 146, e1–e23. doi: 10.1016/j.ajog.2019.04.036

Segata, N, Izard, J., Waldron, L., Gevers, D., Miropolski L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Serrano, M. G., Parikh, H. I., Brooks, J. P., Edwards, D. J., Arodz, T. J., Edupuganti, L., et al. (2019). Racioethnic diversity in the dynamics of the vaginal microbiome during pregnancy. *Nat. Med.* 25, 1001–1011. doi: 10.1038/s41591-019-0465-8

Simonsen, K. A., Anderson-Berry, A. L., Delair, S. F., and Davies, H. D. (2014). Early-onset neonatal sepsis. *Clin. Microbiol. Rev.* 27, 21–47. doi: 10.1128/CMR.00031-13

Singh, T., Barnes, E. H., Isaacs, D., and Australian Group for Neonatal Infections. (2019). Early-onset neonatal infections in Australia and New Zealand, 2002-2012. *Arch. Dis. Child Fetal Neonatal. Ed.* 104, F248–F252. doi: 10.1136/archdischild-2017-314671

Staude, B., Oehmke, F., Lauer, T., Behnke, J., Gopel, W., Schloter, M., et al. (2018). The microbiome and preterm birth: a change in paradigm with profound implications for pathophysiologic concepts and novel therapeutic strategies. *Biomed. Res. Int.* 2018:7218187. doi: 10.1155/2018/7218187

Stout, M. J., Zhou, Y., Wylie, K.-M., Tarr, P. I., Macones, G. A., and Tuuli, M. G. (2017). Early pregnancy vaginal microbiome trends and preterm birth. *Am. J. Obstet. Gynecol.* 217, 356.e1–356.e18. doi: 10.1016/j.ajog.2017.05.030

Subramaniam, A., Kumar, R., Cliver, S. P., Zhi, D., Jeff, M., Szychowski1, J. M., et al. (2016). Vaginal microbiota in pregnancy: evaluation based on vaginal flora, birth outcome, and race. *Am. J. Perinatol.* 33, 401–408. doi: 10.1055/s-0035-1565919

Taddei, C. R., Cortez, R. V., Mattar, R., Torloni, M. R., and Daher, S. (2018). Microbiome in normal and pathological pregnancies: a literature overview. *Am. J. Reprod. Immunol.* 80:e12993. doi: 10.1111/aji.12993

Tao, X., Franasiak, J. M., Zhan, Y., Scott, R. T., Rajchel, J., Bedard, J., et al. (2017). Characterizing the endometrial microbiome by analyzing the ultra-low bacteria from embryo transfer catheter tips in IVF cycles: next gener- ation sequencing (NGS) analysis of the 16 S ribosomal gene. *Hum. Microbiome J.* 3, 15–21. doi: 10.1016/j.humic.2017.01.004

Theis, K. R., Romero, R., Winters, A. D., Greenberg, J. M., Gomez-Lopez, N., Alhousseini, A., et al. (2019). Does the human placenta delivered at term have a microbiota? Results of cultivation, quantitative real-time PCR, 16S rRNA gene sequencing, and metagenomics. *Am. J. Obstet. Gynecol.* 220, 267.e1–267.e39 doi: 10.1016/j.ajog.2018.10.018

Vinturache, A. E., Gyamfi-Bannerамn, C., Hwang, J., Mysorekar, I. U., Jacobsson, B., and Preterm Birth International Collaborative PREBIC. (2016). Maternal microbiome - A pathway to preterm birth semin. *Fetal Neonatal Med.* 21, 94–99. doi: 10.1016/j.siny.2016.02.004

Walther-António, M. R. S., Jeraldo, P., Miller, M. E. B., Yeoman, C. J., Nelson, K. E., Wilson, B. A., et al. (2014). Pregnancy's stronghold on the vaginal microbiome. *PLoS ONE* 9:e98514. doi: 10.1371/journal.pone.0098514

Wen, A., Srinivasan, U., Goldberg, D., Owen, J., Marrs, C. F., Misra, D., et al. (2014). Selected vaginal bacteria and risk of preterm birth: an ecological perspective. *J. Infect. Dis.* 209, 1087–1094. doi: 10.1093/infdis/jit632

Zheng, J., Xiao, X., Zhang, Q., Mao, L., Yu, M., and Xu, J. (2015). The placental microbiome varies in association with low birth weight in full-term neonates. *Nutrients* 7, 6924–6937. doi: 10.3390/nu7085315

Zheng, J., Xiao, X., Zhang, Q., Mao, L., Yu, M., and Xu, J., et al. (2017). The placental microbiota is altered among subjects with gestational diabetes mellitus: a pilot study. *Front. Physiol.* 8:675. doi: 10.3389/fphys.2017.00675

Zhou, L., Luo, F., Hu, W., Han, Y., Wang, Y., Zheng, H., et al. (2018). Bacterial communities in the womb during healthy pregnancy. *Front. Microbiol.* 9:2163. doi: 10.3389/fmicb.2018.02163

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REVIEW
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The Microbiota of the Human Mammary Ecosystem

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Human milk contains a dynamic and complex site-specific microbiome, which is not assembled in an aleatory way, formed by organized microbial consortia and networks. Presence of some genera, such as *Staphylococcus, Streptococcus, Corynebacterium, Cutibacterium* (formerly known as *Propionibacterium*), *Lactobacillus*, *Lactococcus* and *Bifidobacterium*, has been detected by both culture-dependent and culture-independent approaches. DNA from some gut-associated strict anaerobes has also been repeatedly found and some studies have revealed the presence of cells and/or nucleic acids from viruses, archaea, fungi and protozoa in human milk. Colostrum and milk microbes are transmitted to the infant and, therefore, they are among the first colonizers of the human gut. Still, the significance of human milk microbes in infant gut colonization remains an open question. Clinical studies trying to elucidate the question are confounded by the profound impact of non-microbial human milk components to intestinal microecology. Modifications in the microbiota of human milk may have biological consequences for infant colonization, metabolism, immune and neuroendocrine development, and for mammary health. However, the factors driving differences in the composition of the human milk microbiome remain poorly known. In addition to colostrum and milk, breast tissue in lactating and non-lactating women may also contain a microbiota, with implications in the pathogenesis of breast cancer and in some of the adverse outcomes associated with breast implants. This and other open issues, such as the origin of the human milk microbiome, and the current limitations and future prospects are addressed in this review.

Keywords: human milk, microbiota, microbiome, vertical transfer, biological functions, mastitis, breast tissue, breast cancer

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INTRODUCTION

Historically human milk was considered sterile under physiological conditions and, therefore, the presence of microbes was considered either as an infection or as a contamination. The mammary glands are made up of a moist intra-mammary mucosal ecosystem which, during late pregnancy and throughout the lactation period, becomes an ideal environment for bacterial growth due to the availability of a wide range of nutrients, and the optimum temperature for many microbes. In addition, the extremely complex duct system may favor the growth and spreading of biofilm-forming

bacteria, a property that is common among *Staphylococcus* strains isolated from human milk (Delgado et al., 2009; Delgado et al., 2011; Begović et al., 2013). In addition, the mammary ecosystem is exposed to the external environment (via the ducts/nipple) and, also, to the internal environment since tight junctions remain open for a few days after birth. Since the first culture-dependent and -independent reports of the human milk microbiome (Heikkilä and Saris, 2003; Martin et al., 2003), all published studies have provided evidence of the presence of bacteria or bacterial DNA in milk collected from healthy women under hygienic conditions. In contrast, no modern study has found evidence of their absence.

THE MICROBIOTA OF HUMAN MILK: HISTORICAL PERSPECTIVE

Knowledge of the bacterial content of fresh mammalian milk is as old as Pasteur's "germ" theory (**Figure 1**). Numerous studies, dating back more than a century ago, demonstrated that bacteria are common in milk of healthy ruminants. However, milk microbes were traditionally viewed from three perspectives: (a) as a potential cause of mastitis, leading to economic losses in farms (Macfadyen, 1891; French, 1903; Jones, 1918); (b) as a potential cause of milk spoilage (Hall and Trout, 1968); or (c) as a potential threat to human health because of the potential transfer of pathogenic microbes, including those with a zoonotic origin and those arising from a non-hygienic handling (Anonymous, 1889; Park, 1901). Many milk-borne diseases, including tuberculosis, brucellosis, typhoid fever, diphtheria or scarlet fever, had been recognized before 1900 (Holsinger et al., 1997), and that it is estimated that approximately 65,000 people died of milk-borne tuberculosis in England alone between 1912 and 1937 (Wilson, 1943). More than half a century later (mid-1950s), the first studies dealing with the bacteriological composition of human milk were published (Lindemann and Rupp, 1954; Novel and Pongratz, 1954; Lindemann, 1955; Mocquot, 1955; Meyer and Potel, 1956; Sager, 1956; Mossel and Weijers, 1957; Lindemann, 1958). Most of them reported an unexpected fact: the abundant and widespread bacterial "contamination" of milk donated to human milk banks. Since these institutions were rapidly spreading in Western countries, the number of studies proposing either bacterial criteria for acceptance of donor milk,

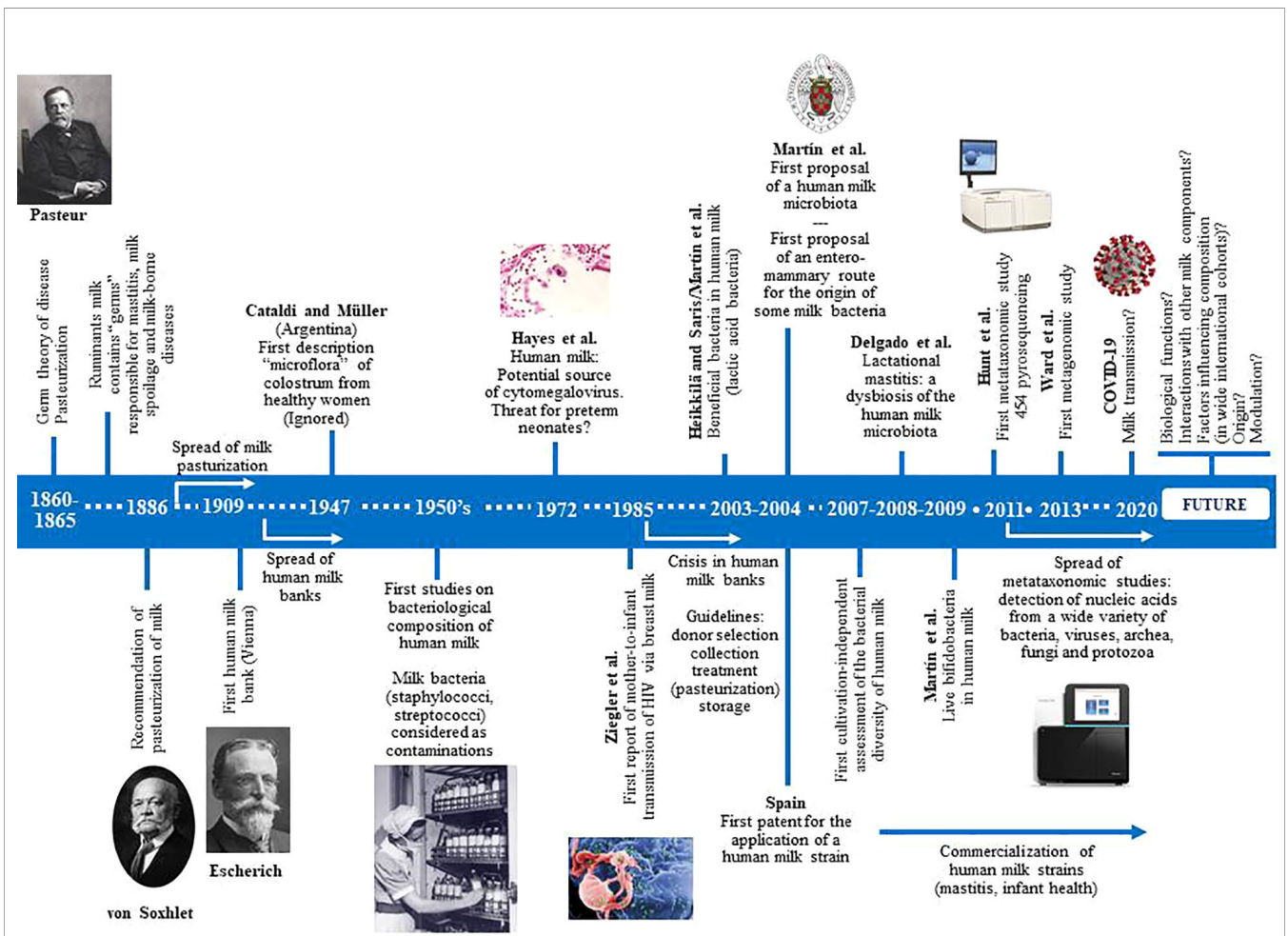

FIGURE 1 | Historical perspectives of the main milestones in research focused in human milk microbes.

or collection, storage and processing procedures to reduce the milk bacterial load, also increased rapidly from the 1950s to the early 1980s (Bachmann and Pulverer, 1962; Krieg, 1966; Liebhaber et al., 1978; Carroll et al., 1979; Davidson et al., 1979; Eidelman and Szilagyi, 1979; Jones et al., 1979; Lucas and Roberts, 1979; West et al., 1979; Björkstén et al., 1980; Carroll et al., 1980).

In parallel, a number of studies warned about the presence of some potential pathogenic bacteria among such "contaminants", which posed a risk for infant health. They included mainly *Staphylococcus aureus* but, also, *Streptococcus pyogenes*, *Streptococcus agalactiae* and some enterobacteria (*Salmonella* spp., *Klebsiella pneumoniae*) (Dubois, 1954; Cayla et al., 1955; Rantasalo and Kauppinen, 1959; Foster and Harris, 1960; Ottenheimer et al., 1961; Dluzniewska, 1966; Burbianka and Dluzniewska, 1971; Fleischrocker et al., 1972; Kenny, 1977; Ryder et al., 1977; Schreiner et al., 1977; Lucas and Roberts, 1978; Williamson et al., 1978; Donowitz et al., 1981; Cooke et al., 1987; Lemoine, 1987). Finally, a few studies were focused on the relationship between milk bacteria and mastitis (Dorr and Sittel, 1953; Cherkasskaia et al., 1980; Thomsen, 1982).

In 1985, the first report of a case of presumed mother-to-infant transmission of human immunodeficiency virus (HIV) *via* milk was published (Ziegler et al., 1985). In the following years, fear of HIV transmission led human milk banks to a strong crisis and to the closing of many of them because of the financial burden of serological testing of donors (Haiden and Ziegler, 2016). In addition, a few years earlier, human milk had also been recognized as a source of cytomegalovirus (CMV) (Hayes et al., 1972). This worsened the situation since most banked milk was intended for preterm neonates, a population where CMV may be particularly harmful, and no routine procedure for CMV screening was available at that time. As a result, most microbial studies of human milk published in the second half of the 80's and throughout the 90's were related to its role as a vehicle of HIV and/or CMV, and to the search for the best methods for donors screening and for viral inactivation (Stagno et al., 1980; Friis and Andersen, 1982; Cheeseman and McGraw, 1983; Dworsky et al., 1983; Boyes, 1987; Anonymous, 1988). At this stage, presence of any kind of microbe in milk was generally seen as a potential threat for infant health.

This disease-centric view of milk changed in 2003 following the publication of two articles that described the presence of lactic acid bacteria in human milk (Heikkilä and Saris, 2003; Martin et al., 2003). Such bacteria were generally recognized as safe and beneficial for infant health. One year later, it was proposed that human milk contains its own site-specific microbiota (Martín et al., 2004). Soon, it was found that some strains isolated from human milk displayed a wide array of probiotic traits (Beasley and Saris, 2004; Martín et al., 2005; Martín et al., 2006; Olivares et al., 2006a). In fact, the first description of the presence of lactobacilli (*Lactobacillus acidophilus*) and bifidobacteria (then the so-called *Lactobacillus bifidus*) in colostrum from healthy women was made in 1947 (Cataldi and Müller, 1947); however, such work was (and still is) ignored by the medical and scientific community, likely due to the fact that it was published in an Argentine journal with no diffusion outside of Spanish-talking countries.

Presence of microorganisms in human milk is no longer valid as a predictor of the risk of infant infection (Boer et al., 1981; Schanler et al., 2011; Zimmermann et al., 2017), despite the fact that additional cases of human milk-related infant infections have been reported (Qutaishat et al., 2003; Kayıran et al., 2014; Weems et al., 2015).

THE COMPOSITION OF THE HUMAN MILK MICROBIOTA

From 2003, the study of the human milk microbiota has attracted the interest of many research groups worldwide (Fernandez et al., 2013; Jost et al., 2015; Ojo-Okunola et al., 2018), enabling the detection of approximately 200 different bacterial, archeal and fungal species from more than 50 different genera (Fernandez et al., 2013), including new species (Martín et al., 2011), and new genera, including *Lactomassilus*, *Lactimicrobium*, *Anaerolactibacter*, *Galactobacillus*, and *Acidipropionibacterium* (Togo et al., 2017; Togo et al., 2019a).

Culture-based methods have revealed that some species of the genera *Staphylococcus* (*Staphylococcus epidermidis* and other coagulase-negative species [CNS]), *Streptococcus* (*S. salivarius*, *S. mitis* and other species of the mitis group), *Corynebacterium*, *Cutibacterium* and other taxonomically-related Gram-positive bacteria are usually the dominant cultivable bacteria in samples of milk from healthy women (Jiménez et al., 2008a; Jiménez et al., 2008b; Solís et al., 2010; Schanler et al., 2011; Ding et al., 2019). Less frequently, lactic acid bacteria (*Lactococcus*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, and *Weissella*) and bifidobacteria are isolated from this biological fluid (Martin et al., 2003; Martín et al., 2006; Abrahamsson et al., 2009; Martín et al., 2009; Solís et al., 2010; Arboleya et al., 2011; Makino et al., 2015; Murphy et al., 2017). Some *Lactobacillus* (*L. salivarius*, *L. reuteri, L. gasseri*, *L. fermentum*) and *Bifidobacterium* (*B. breve* and *B. longum*) species have received particular interest because of the potential of the strains belonging to such species to be employed as probiotics. It must be highlighted that the different species of the genera *Lactobacillus* and *Leuconostoc* have been recently reclassified into 25 different genera (Zheng et al., 2020).

Under physiological conditions, milk bacterial concentrations may range from <1 to 4 $\log_{10}$ colony-forming units (cfu)/mL if the samples are obtained by either manual expression or through sterile pumps following hygienic practices (Espinosa-Martos et al., 2016). In contrast, bacterial concentrations can rise up to 6 $\log_{10}$ cfu/mL, or even higher, in mastitis cases (Fernández et al., 2014) or if milk is collected through the use of non-sterile pumps (Boo et al., 2001; Brown et al., 2005; Marín et al., 2009; Jiménez et al., 2017). At present, cell count methods, from classic counting chambers to flow cytometry, are the best methods for an accurate quantification of (live) bacterial cells in milk while quantitative PCR methods usually lead to an overestimation due to the presence of dead bacterial cells, exosomes and/or free

bacterial DNA but, also, to mispriming with human DNA (Walker et al., 2020), and copy number bias (Větrovský and Baldrian, 2013; Louca et al., 2018).

Introduction of new media, supplements and incubation conditions have allowed the isolation of bacteria that were previously unnoticed. However, recent developments in culturomics have revealed that many bacteria previously regarded as non-cultivable can now be isolated from complex ecosystems when a proper combination of culture conditions is provided (Lagier et al., 2012; Lagier et al., 2015; Lau et al., 2016; Lagier et al., 2018; Schwab et al., 2019). These new methodologies have served as critical tests to validate data derived from metagenomic studies regarding the gut ecosystem (Lau et al., 2016). Culture-based techniques enable the isolation, preservation and characterization of strains (Bäckhed et al., 2005; Lara-Villoslada et al., 2007; Jiménez et al., 2008b; Delgado et al., 2009; Jiménez et al., 2010a; Jiménez et al., 2010b; Arboleya et al., 2011; Delgado et al., 2011; Gueimonde et al., 2012; Jiménez et al., 2012; Langa et al., 2012; Martín et al., 2012a; Martín et al., 2013; Cárdenas et al., 2014; Cárdenas et al., 2015).

In relation to the milk microbiome, the use of first generation of culture-independent techniques, including PCR, combined or not with creation of bacterial gene libraries, denaturing gradient gel electrophoresis (DGGE), and temperature gradient gel electrophoresis (TGGE), allowed a better knowledge of the milk bacterial populations (Gueimonde et al., 2007; Martín et al., 2007a; Martín et al., 2007b; Delgado et al., 2008; Collado et al., 2009). Nowadays, they have been replaced by high-throughput Next Generation Sequencing (NGS) techniques, including metataxonomics (16S rRNA amplicon analysis) and metagenomics (total DNA sequencing). Sequencing of bacterial 16S rRNA genes has revealed that milk contains DNA of diverse microbial groups that were previously undetected with conventional culture-based techniques (Hunt et al., 2011; Cabrera-Rubio et al., 2012; Jost et al., 2013; Jost et al., 2014; Cabrera-Rubio et al., 2016; Boix-Amorós et al., 2017; Pannaraj et al., 2017). Although results from such studies do not provide evidence for viability, they have been useful to highlight the complexity of the milk microbiota and its role in modulating mammary homeostasis and gut colonization during early life. Shotgun sequencing of milk microbial DNA has been rarely performed to date (Ward et al., 2013; Jiménez et al., 2015), despite the fact that it can reveal the presence and potential functions of neglected members of this microbiota (archaea, viruses, fungi, protozoa). In addition, although DNA-based studies cannot demonstrate function, they can imply functional capacity based on the genes present in the samples. Comprehensive metagenomic, metatranscriptomic and metabolomic investigations are required for a holistic understanding of genetic diversity and functionality within the milk ecosystem (**Figure 2**).

Human milk microbiota is not aleatory assembled and contains organized bacterial consortia and networks (Ma et al., 2015; Drago et al., 2017). The milk microbiota of a woman is very stable throughout the lactation period, particularly in relation to the most abundant genera (Hunt et al., 2011; Williams et al., 2017a). Culture-independent studies have shown a wide diversity of bacterial signatures belonging to more than 800 different bacterial species, mainly from four major phyla (*Firmicutes*,

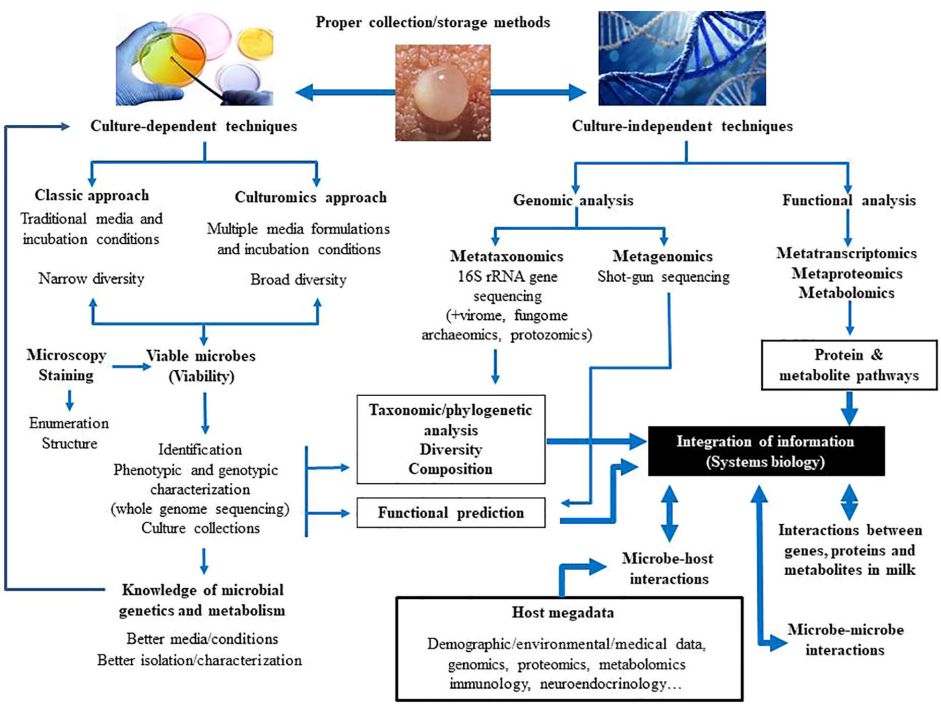

FIGURE 2 | Current and future approaches to study the composition and functions of the human milk microbiota.

Actinobacteria, *Bacteroidetes*, and *Proteobacteria*) (Togo et al., 2019b), including all the cultivable species (Gueimonde et al., 2007; Martín et al., 2007a; Martín et al., 2007b; Delgado et al., 2008; Collado et al., 2009; Hunt et al., 2011; Cabrera-Rubio et al., 2012; Jost et al., 2013; Ward et al., 2013; Jost et al., 2014; Jiménez et al., 2015; Boix-Amorós et al., 2016; Cabrera-Rubio et al., 2016; Sakwinska et al., 2016; Chen et al., 2018; Simpson et al., 2018; Tuominen et al., 2018; Ding et al., 2019; Lackey et al., 2019). Additionally, DNA from some gut-associated strict anaerobes (*Faecalibacterium, Bacteroides*, *Clostridium*, *Blautia, Coprococcus*, *Ruminococcus, Roseburia*, *Eubacterium, Veillonella*, and others) has also been repeatedly detected (Cabrera-Rubio et al., 2012; Jost et al., 2013; Jost et al., 2014; Jiménez et al., 2015; Gómez-Gallego et al., 2016).

Different studies have also reported the frequent detection of DNA sequences from soil- and water-related bacteria, including *Bradyrhizobium*, *Novosphingobium*, *Methylobacterium*, *Pseudomonas*, *Sphingobium*, *Sphingopyxis*, *Stenotrophomonas*, *Sphingomonas* or *Xanthomonas*, in human milk (Hunt et al., 2011; Cabrera-Rubio et al., 2012; Urbaniak et al., 2014a; Urbaniak et al., 2014b; Li et al., 2017). It is possible that a high proportion of such sequences are the result of technical artifacts. DNA from the bacterial genera cited above is generally present in molecular biology reagents, solutions and kits (Grahn et al., 2003; Mühl et al., 2010; Salter et al., 2014; Lauder et al., 2016). This represents a major challenge assessing the composition of microbiotas characterized by a low microbial biomass, as is the case of the human milk microbiota in healthy women. Upon amplification, contaminating DNA may overcome the low amount of starting material in the biological sample and lead to incorrect results (Laurence et al., 2014). Sequences belonging to such genera (e.g., *Pseudomonas*) may be so abundant that they can be wrongly included into the milk core microbiome. There are several measures that can be implemented to minimize this problem, such as sequencing of negative (blank) controls and contaminant removal procedures (Salter et al., 2014; Hornung et al., 2019; Karstens et al., 2019; Stinson et al., 2019), while providing microbial DNA-free sampling containers and molecular reagents is still a pending challenge for companies working in this field.

Differences in the techniques and procedures employed in different studies may account, at least partly, for conflicting results regarding the frequency and abundance of sequences belonging to *Lactobacillus*, *Bifidobacterium* and strict anaerobes in human milk (Lagier et al., 2012). These kind of controversies have also happened in relation to the microbiota of the infant gut (Palmer et al., 2007; Turroni et al., 2012). Other limitations and biases of molecular techniques include the lack of discrimination between live or dead organisms when techniques compatible with viability assessments are not selected (Emerson et al., 2017), and the over- or underestimation of some microbial groups because of the composition of their plasmatic membranes, outer membranes or cell walls, methods used for extraction of nucleic acids, copy number of the target gene, the specific 16S rRNA region(s) targeted by the selected primers, and the pipelines used for the bioinformatic analysis (McGuire and McGuire, 2015; Gómez-Gallego et al., 2016; McGuire and McGuire, 2017).

Some studies have revealed the presence of cells, DNA and/or RNA from viruses, archea, fungi and protozoa in human milk (Jiménez et al., 2015; Pannaraj et al., 2018; Boix-Amorós et al., 2019). Although some pathogenic viruses, such as HIV, CMV, Ebola and Zika viruses may be found in human milk (Kourtis et al., 2003; Van de Perre et al., 2012; Bardanzellu et al., 2019; Sampieri and Montero, 2019; Ververs and Arya, 2019), other viruses, including bacteriophages, are also present (Mohandas and Pannaraj, 2020). The human milk virome is distinct from other body anatomical sites and includes eukaryotic viruses, bacteriophages, and viral elements integrated in the host chromosomes (Duranti et al., 2017; Pannaraj et al., 2018). The most abundant eukaryotic viruses belong to the families *Herpesviridae, Poxviridae, Mimiviridae* and *Iridoviridae*. Bacteriophages comprise 95% of the human milk viruses and have the ability of modulating the bacterial ecology by killing specific bacteria or by supplying them with additional gene functions (Pannaraj et al., 2018). Human endogenous retroviruses, accounting for 0.06 to 3.63% of all reads, have also been identified in milk samples (Jiménez et al., 2015). Both pathogenic and non-pathogenic viruses can be vertically transferred from mother to infant (Kuehn et al., 2013; Lugli et al., 2016; Duranti et al., 2017; Blohm et al., 2018; Pannaraj et al., 2018).

Some studies have identified archaeal sequences in human milk (Ward et al., 2013; Jiménez et al., 2015; Togo et al., 2019c). In addition, Togo et al. (2019b) were able to isolate *Methanobrevibacter smithii* from 3 colostrum and 5 milk samples out of a total of 20 samples while *Methanobrevibacter oralis* was cultured from one milk sample. Methanogenic archaea have remained largely underestimated in human microbiome studies due to technical difficulties in their assessment (Bang and Schmitz, 2015). However, they are particularly adapted to the human gut, participating actively in metabolism and health through methanogenesis (Samuel et al., 2007; Dridi et al., 2009). Presence of *M. smithii* in the human gut is a feature of healthy lean adults while there is a depletion of this species in obese adults (Le Chatelier et al., 2013; Goodrich et al., 2014). In this context, *M. smithii* was less frequently detected by either culture or PCR in the milk samples obtained from obese mothers in the study of Togo et al. (2019c). The human gastrointestinal tract is colonized by *M. smithii* early in life (Grine et al., 2017), and colostrum and milk may represent relevant sources of methanogenic archaea.

In relation to fungi, a metagenomic analysis detected fungal-related sequences in 17 out of the 20 milk samples included in the study (Jiménez et al., 2015). More specifically, the reads belonged to the phyla *Basidiomycota* and *Ascomycota*, and to the species *Calocera cornea*, *Candida dubliniensis*, *Guepiniopsis buccina*, *Malassezia globosa*, *Malassezia restricta*, *Podospora anserina*, *Sordaria macrospora*, *Talaromyces stipitatus*, and *Yarrowia lipolytica*, with *M. globosa* being the most widespread species (Jiménez et al., 2015). Boix-Amorós et al. (2017) could visualize and isolate yeasts from 17 out of 41 milk samples from healthy women, and most of the isolates belonged to the species *Candida parapsilosis* and *Rhodotorula mucilaginosa*. Later, the same group analyzed 80 milk samples from women of 4 different countries by sequencing of the ITS1 region of the fungal rDNA gene, and found that *Malassezia* and *Davidiella* were the most prevalent genera independenty of the country, while delivery mode and geographic location were associated with shifts in the milk mycobiome

composition (Boix-Amorós et al., 2017). Sequencing of the ITS2 region allowed the identification of *Candida* and *Saccharomyces* sequences in 6 milk samples from women whose infant stayed in a neonatal intensive care unit (NICU) althouh sequences of the same genera were also identified in samples from the NICU environment (Heisel et al., 2019). Overall, these studied suggest that milk may be a source of fungi for the infant gut, thus contributing to the acquisition and development of the gut mycobiota. However, more studies are required to confirm this role, and to elucidate the potential interactions with other microbes. The significance of the protozoa (*Toxoplasma gondii*, *Giardia intestinalis*) detected in milk from some mothers remains unclear (Jiménez et al., 2015).

FACTORS AFFECTING THE COMPOSITION OF THE HUMAN MILK MICROBIOTA

The milk microbiome is characterized by a certain degree of interindividual variability (Martín et al., 2007a; Martín et al., 2007b; Hunt et al., 2011; Boix-Amorós et al., 2016; Cabrera-Rubio et al., 2016; Avershina et al., 2018; Chen et al., 2018). Modifications in its composition may have biological implications for infant colonization, metabolism, immune and neuroendocrine development and for mammary health. However, the current knowledge about the impact of a wide variety of factors (genetic background, ethnicity, milk sampling, geographical location, circadian rhythm, maternal age, diet and body mass index [BMI], delivery mode, gestational age, therapies and food supplements, infant and maternal health status, and others) on human milk microbial communities is very limited (Fernández et al., 2014; Gómez-Gallego et al., 2016) (**Figure 3**).

Although some studies have tried to elucidate the influence of some of these factors, most of them involved a low number of samples/women and/or have relied on short amplicon sequencing, a technology which poor resolution at the lower taxonomical levels may mask differences or overinflate them; these limitations together with the fact that many factors with a potential impact on the composition of the milk microbiome may interact, makes it difficult to evaluate their true impact on the milk microbiome (LeMay-Nedjelski et al., 2018).

Sample Collection

When milk is collected through the use of domestic (non-single use) milk pumps, a high concentration of yeasts and Gram-negative

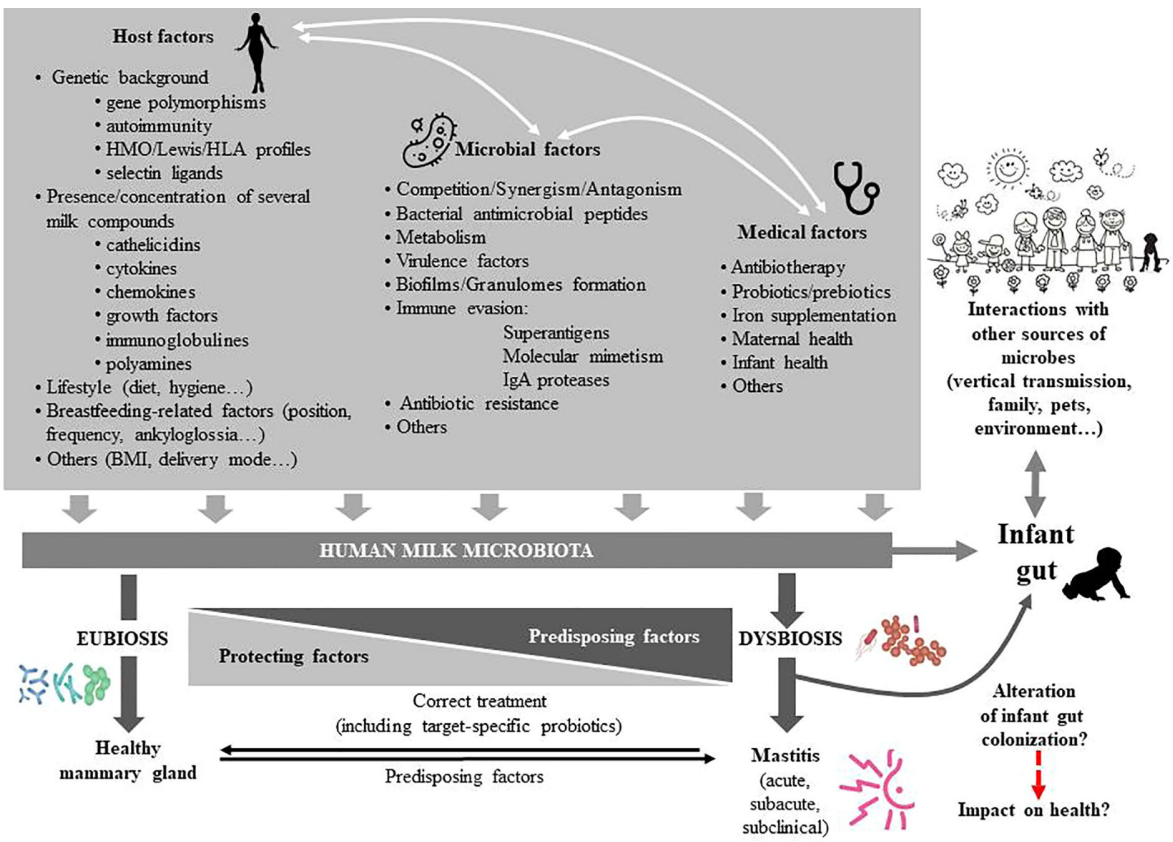

FIGURE 3 | Factors affecting the composition of the human milk microbiota. HMO, human milk oligosaccharides; HLA, human leukocyte antigen; BMI, body mass index; IgA, immunoglobulin A.

bacteria (*Enterobacteriaceae*, *Stenotrophomonas*, *Pseudomonas*) may arise from the water used to rinse the devices or as a consequence of unhygienic practices (Knoop et al., 1985; Moloney et al., 1987; Thompson et al., 1997; Boo et al., 2001; Brown et al., 2005; Marín et al., 2009; Jiménez et al., 2017); in fact, some cases of neonatal and infant sepsis and infections initially associated with human milk were the result of contamination of breast pumps (Gransden et al., 1986). A recent study comparing milk samples from mothers who regularly used pumps versus those who never used pumps showed that pumped milk was consistently related to an enrichment of potential pathogens compared to hand expressed milk (Moossavi et al., 2019a). This observation suggests that regular pump use may alter the milk microbiome over time. In fact, it has been shown that when sterile single-use pumps are used, no difference is detected between hand-expressed and pump-expressed samples, while the use of the mother's own multi-use pump does result in a significant change in the apparent milk microbiome (Rodríguez-Cruz et al., 2020).

Human Milk Oligosaccharides

Human milk oligosaccharides (HMOs) exert prebiotic effects on some bacteria that are frequently detected in human milk, including *Bifidobacterium* spp. and *Staphylococcus epidermidis* (Hunt et al., 2012; Thongaram et al., 2017). Milk oligosaccharides and microbes confer a highly personalized "symbiotic" complex which may be crucial for the development of the infant gut microbiota (Jost et al., 2015; Lewis et al., 2015; Wang et al., 2015; Williams et al., 2017a; Moossavi et al., 2019b). Positive correlations between HMO concentration and the abundance of *Staphylococcus* (Williams et al., 2017b), between *B. breve* and sialylated HMOs, between *B. longum* group and non-fucosylated/non-sialylated HMOs, between fucosylated HMOs and *Akkermansia muciniphila*, and between fucosylated/sialylated HMOs and *S. aureus* have been described (Aakko et al., 2017). A study found that the maternal secretor status was associated with the composition of the milk microbiota for the first 4 weeks after parturition (Cabrera-Rubio et al., 2019). Although no differences on diversity and richness were detected, *Lactobacillus* spp., *Enterococcus* spp., *Streptococcus* spp., and *Bifidobacterium* spp. were lower or less prevalent in non-secretor samples than in secretor samples (Cabrera-Rubio et al., 2019).

There are several mechanisms by which HMOs may modulate the human milk microbiota, including prebiotic, antimicrobial, or immunomodulatory properties (Ackerman et al., 2017; Ackerman et al., 2018; Triantis et al., 2018; Bode, 2020). The ability to assimilate, metabolize or use HMOs is conserved among the *Bifidobacterium* species that are most frequently detected in the infant gut and in human milk (Sela and Mills, 2010; Underwood et al., 2015), although the pathways vary among species and strains (Sakanaka et al., 2019). However, a recent study has shown that there is not a strict correlation between bifidobacterial populations in human milk and their ability to metabolize HMOs (Lugli et al., 2020). The exact growth-promoting effect of HMOs on *Staphylococcus* isolates from human milk remains unknown, since these bacteria do not metabolize these compounds (Hunt et al., 2012). Recently, novel pathways enabling the growth of *Roseburia* and *Eubacterium* (two butyrate-producing genera that have been repeteadly detected in human milk) on HMOs have been published (Pichler et al., 2020). More studies are required to elucidate how HMOs can contribute to shape the microbiota of milk.

Other Human Milk Metabolites

The complexity and dynamic chemical composition of human milk represents a challenge for researchers (Ojo-Okunola et al., 2020). In addition to HMOs, human milk contains a plethora of metabolites produced by either human or bacterial cells that are relevant for mammary and infant health (Gay et al., 2018). Some of them have the potential to shape the composition of the human milk and/or the infant gut microbiotas because of their roles in changing environmental conditions that are relevant for bacterial growth, such as the redox potential or by other mechanisms that promote or inhibit the growth of certain species or strains. Up to 68 metabolites were identified in one study assessing the metabolic and microbiota profiles of milk from 79 women from Finland, Spain, South Africa, and China. Bacilli, Actinobacteria. and Proteobacteria were the bacterial groups displaying more positive or negative associations with milk metabolites (Gómez-Gallego et al., 2018).

Short chain fatty acids (SCFA), including butyrate, acetate, and propionate, are the result of bacterial metabolism in the maternal gut and may reach the mammary gland through the bloodstream. These metabolites are key players in human homeostasis because of their interactions with the microbiota, the immune system, and the neuroendocrine system, contributing to the immunological, metabolic, and neurological programming of the host (Stinson et al., 2020). In this context, the concentrations of acetate and butyrate in milk samples from atopic mothers are significantly lower than in samples from non-atopic mothers (Stinson et al., 2020). Levels of butyrate in milk have also been negatively associated with infant BMI and this fact may program healthy adiposity outcomes later in life (Prentice et al., 2019). Interestingly, administration of some *Lactobacillus* strains isolated from human milk (*Lactobacillus salivarius* CECT 5713, *Lactobacillus fermentum* CECT 5716) to infants has shown ability for increasing the fecal concentration of butyrate (Maldonado et al., 2010; Gil-Campos et al., 2012). Variations in the concentrations of some hormones present in human milk (leptin, insulin) have also been associated with changes in the composition of the infant gut microbiota, SCFA concentrations, and gut permeability (Lemas et al., 2016). Current knowledge on the interactions between the metabolome and the microbiota of human milk is scarce despite the fact that they may have a paramount relevance for infant and mammary outcomes. Integrative studies are required to address these complex interactions.

Maternal Diet and Body Mass Index

Maternal diet is associated with the abundance of some bacterial genera (Williams et al., 2017a; Williams et al., 2017b). Protein intake is positively correlated with the abundance of *Gemella* while consumption of monounsaturated and saturated fatty acids is negatively related with the abundance of *Corynebacterium*; similarly, a negative correlation was found between Firmicutes

and total carbohydrates, disaccharides, and lactose ingestion (Williams et al., 2017a). A previous study had revealed that monounsaturated fatty acids were positively associated with *Lactobacillus* genus, while the contrary was observed for Proteobacteria (Kumar et al., 2016).

An association between vitamin C intake during pregnancy and the bacterial diversity of milk has also been reported (Padilha et al., 2019a). The same study found a positive relationship between ingestion of some fatty acids (linoleic and polyunsaturated fatty acids) during lactation and the level of *Bifidobacterium* in milk.

In relation to BMI, prepregnancy BMI has been associated with a higher milk microbial diversity and a lower abundance of *Streptococcus* (Davé et al., 2016). Another study found that the abundance of *Granulicatella* in milk from overweight and obese mothers was higher than in milk from normoweight women (Williams et al., 2017a). A recent study showed that the milk of mothers with a high postpartum BMI contained more *Staphylococcus* and less *Lactobacillus* and *Streptococcus* sequences than milk from normoweight mothers (Ding et al., 2019).

Immune Cells

The lactating mammary gland is a component of the mucosal-associated immune system that displays unique features when compared with other mucosal sites. For example, it contains a low biomass microbiota in contrast to the high bacterial concentration that characterizes the intestinal or the upper respiratory tracts (Mestecky, 2020). In addition, the mammary gland is considered a relevant component of the infant immune system since this link enables maternal-infant immune dialogue (Brandtzaeg, 2010; Hassiotou and Geddes, 2015). It is long known that the gut microbiota is essential for programming the infant immune system and vice versa (Milani et al., 2017a). However, while the development of the immune system and the development of the microbiota are coordinated in the gut, they remain independently regulated in the breast (Niimi et al., 2018). Interactions between milk microbiota and the mammary immune system are poorly known despite the fact that maternal mononuclear cells can transport gut-derived bacteria and bacterial components to the breast during pregnancy and lactation (Perez et al., 2007). It has been speculated that this process facilitates the discrimination between pathogens and commensal microbes by the neonatal immune system (Perez et al., 2007).

Studies dealing with potential associations between the milk concentrations of bacteria and immune cells are scarce and have provided conflicting results from no correlation (Boix-Amorós et al., 2016) to a negative association between the relative abundance of *Serratia* and both the somatic cell count and the neutrophil concentration (Williams et al., 2017b). As in the case of HMOs and other milk metabolites, there is a need for integrative approaches to clarify the interactions between the immune system and the microbiota in the mammary ecosystem. Information on the potential roles of specific bacterial strains isolated from human milk on the infant immune system is provided in *Moving From Composition to Function*.

Gestational Age, Mode of Delivery, and Postpartum Period

Controversial results have been obtained from studies comparing the human milk microbiome composition among women delivering preterm or term neonates. So, while Urbaniak et al. (2016a) did not detect differences in bacterial profiles between preterm and term births, Soeorg et al. (2017a) reported that milk of mothers of preterm infants had higher staphylococcal counts but lower species diversity compared with term mothers. The same authors found that the *S. epidermidis* strains that colonized the gastrointestinal tract and skin of preterm neonates were different to those found in milk while the opposite was observed in term neonates (Soeorg et al., 2017b). However, the gut of breast-fed preterm infants was gradually enriched with strains present in the milk of their mothers.

Concerning the delivery mode, Urbaniak et al. (2016a) found no significant differences in bacterial profiles between Cesarean section (either elective or non-elective) and vaginal deliveries, which is in contrast with the data provided by other authors (Khodayar-Pardo et al., 2014; Cabrera-Rubio et al., 2016; Kumar et al., 2016; Ding et al., 2019). Toscano et al. (2017) described many differences in the microbiome of colostrum depending on the mode of delivery. Compared to Cesarean section, vaginal delivery was associated with a lower abundance of *Staphylococcus*, *Pseudomonas*, and *Prevotella*. In addition, colostrum from women delivering by Cesarean section was associated with a higher number of bacterial hubs and was richer in environmental bacteria. In practice, it is difficult to separate the influence of the mode of delivery with other factors since, as an example, antibiotherapy administered per protocol to women delivering by Cesarean section may be responsable for some of the differences in the microbial population. A recent work reported that the effect of intrapartum antibiotic exposure was less decisive than that of delivery mode on the milk microbiome assessed one month after delivery (Hermansson et al., 2019).

In relation to the postpartum period, Hoashi et al. (2016) found changes in bacterial abundance and glycosylation patterns associated with the amount of time passed since delivery but only in milk from women who delivered vaginally. Drago et al. (2017) detected differences between the microbiomes of colostrum and milk since abundance of anaerobic intestinal bacteria was lower in colostrum than in mature milk. The permeability of the tight junctions in the mammary epithelium is greater in the first days after parturition and this fact determines major differences in the biochemical and immunological composition of colostrum with respect to mature milk. Such differences may be responsible for differences in the microbial composition of colostrum and mature milk. In addition, increasing exposures of the breast to the infant microbiota may also determine microbial changes over time.

Another study showed that the relative abundance of *Staphylococcus* in samples collected at 3 months postpartum was lower in comparison to samples collected at 10 days postpartum which, in turn, had a lower diversity of operational taxonomic units (OTUs) from the genera *Rothia*, *Veillonella* and *Granulicatella* (Simpson et al., 2018). Biagi et al. (2018) reported that the composition of the milk microbiota changes after the first infant

latching, becoming more diverse and being dominated by oral microbes (*Rothia* and *Streptococcus*). In contrast, Li et al. (2017) found that neither the lactation stage nor the maternal BMI influenced the milk microbiota of Taiwanese and Chinese women.

Geographical Location

Geographical location of the mother seems to exert a strong impact on the microbiota composition of human milk. A study including samples from mothers of Spain, Finland, South Africa and China found location-related differences in the relative abundance of Bacteroidetes, Actinobacteria and Proteobacteria (Kumar et al., 2016). Geographical differences in the microbiota of samples obtained from 133 mothers in seven regions of China and Taiwan have also been described (Li et al., 2017). Similarly, differences in the alpha diversity and *Lactobacillus* occurrence in milk were also observed depending on the region of China where the mothers were recruited (Ding et al., 2019). A study involving a higher number of samples from mothers living in Ethiopia, Gambia, US, Ghana, Kenya, Peru, Spain, and Sweden provided evidence of substantial variability within and across cohorts (Lackey et al., 2019).

There are several reasons that may explain why geographical location, even within the same country, might influence the bacterial composition of human milk. Ethnicity, genetic background, diet and climate vary across regions, but people with different ethnicity, genetic background, age, diet, housing, or contact with other people and animals usually coexist in the same village or town. The environmental microbiome (air, surfaces, water, plants, animals, food, waste, etc.) reflects the influences existing in a given place and might drive differences in the composition of the microbiome in any body site. Experiments to elucidate the impact of the geographical location on the milk microbiome are very challenging because of the high number of factors that may bias the interpretation of the data. They should have to take into account the factors cited above but also use identical protocols from sample collection, storage and shipping to data analysis, which is very difficult in practice.

Maternal Treatments (Antibiotics, Probiotics, Prebiotics, and Chemotherapy) and Infections

Antibiotic administration is one of the main drivers of dysbiosis in mucosal surfaces and the lactating mammary gland does not seem to be an exception. Lactobacilli and bifidobacteria are more abundant in milk from women who are not treated with antibiotics during pregnancy, delivery or lactation (Soto et al., 2014). Similarly, the *Bifidobacterium* load in milk obtained in the first week after delivery was lower among women receiving antibiotic prophylaxis in comparison to the control group (Padilha et al., 2019b). It is interesting to note that significantly lower amounts of bifidobacteria have been found in milk of allergic mothers compared with non-allergic ones (Grönlund et al., 2007).

Soeorg et al. (2017a) found that the probability of finding *mecA*-positive CNS in milk increases if the mother is hospitalized during the first month after delivery or if the neonate received antibiotherapy or needed an arterial catheter. These authors suggested that the presence in milk of pathogenic *Staphylococcus* could be reduced by limiting the exposition of women to the hospital environment. Intrapartum antibiotic prophylaxis is associated with a higher presence of transmissible genes conferring resistance to antibiotics in milk, which are subsequently shared with their infants (Pärnänen et al., 2018)

In relation to probiotics, oral administration of the probiotic VSL#3 (a mix of 8 strains belonging to the species *B. breve*, *B. longum*, *L. acidophillus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Streptococcus thermophilus*) to pregnant and lactating women led to higher concentrations of *Lactobacillus* and *Bifidobacterium* in colostrum and milk of those women who ingested the product when compared to the placebo group (Mastromarino et al., 2015). However, this effect was only seen among women with vaginal deliveries. In contrast, ingestion of milk containing *Bifidobacterium animalis* ssp. *lactis* Bb-12, *L. acidophilus* La-5 and *Lactobacillus rhamnosus* GG during late pregnancy and early lactation, did not modified significantly the composition of the milk microbiota (Simpson et al., 2018). Recently, Padilha et al. (2020) reported that the intake of prebiotics (fructooligosaccharides) affects the composition of the milk microbiota and found that this effect was influenced by the age of the mother.

Two studies studied the impact of chemotherapy employed during the treatment of Hodgkin's lymphoma in the milk microbiome. The first study included milk samples obtained regularly during four months from a single treated woman and from 8 healthy lactating women (Urbaniak et al., 2014b). Chemotherapy led to significant changes in the milk bacterial composition, including an increase of *Xanthomonadaceae*, *Acinetobacter* and *Stenotrophomonas*, and a drastic reduction in the presence of *Eubacterium*, *Bifidobacterium*, *Cloacibacterium* and *Staphylococcus*. Changes in the metabolic profile of milk, characterized by a decrease of inositol and docosahexaenoic, were also observed (Urbaniak et al., 2014b). The second study provided contradictory results since no negative effect of chemotherapy on community diversity was found (Ma et al., 2016).

Maternal and infant infections exert a strong influence on the immunological composition of milk (Bryan et al., 2007; Riskin et al., 2012; Hassiotou et al., 2013) and, therefore, they likely impact on its microbiological composition. However, there is an almost complete absence of studies addresing this question. Human papilloma virus (HPV) infection was not correlated with a modification of the bacterial composition of milk (Tuominen et al., 2018) although the authors stated that this result might be due to the low number of HPV positive milk samples (3 out of 35) in the analyzed population.

TRANSFER OF MILK BACTERIA TO THE INFANT GUT

The bacteria present in colostrum and milk are among the first microbes to enter the neonatal gastrointestinal tract and, as a

consequence, they may have a paramount relevance as drivers in the acquisition and development of a healthy microbiota. The role of milk as a source of bacteria, including strict anaerobes, to the infant gut has been repeteadly reported at the species and/or the strain level, by classical culture techniques (Martin et al., 2003; Martín et al., 2006; Martín et al., 2009; Solís et al., 2010; Albesharat et al., 2011; Makino et al., 2011; Martín et al., 2012b; Kozak et al., 2015; Makino et al., 2015; Murphy et al., 2017), and by culture-independent approaches (Milani et al., 2015; Asnicar et al., 2017; Duranti et al., 2017; Milani et al., 2017b). In addition, mother-to-infant transfer of *Bifidobacterium* phages through human milk has also been described (Duranti et al., 2017). However, the significance and contribution to infant gut colonization of milk bacteria remains an open question. Clinical studies trying to elucidate the question are confounded by the profound impact of non-microbial human milk components to intestinal microecology.

The microbiota of healthy breastfed infants is related to that present in the milk of their respective mothers. Pannaraj et al. (2017) suggested that approximately a quarter of the bacteria detected in infant feces during early life may derive from milk while Murphy et al. (2017) reported that the genera responsible for most of the bacterial abundance (>70%) in infant feces are shared with human milk. However, these two studies achieved genus level resolution, and only strain level analysis is valid for identifying shared taxa. Asnicar et al. (2017) described the vertical milk transmission of microbial strains, including some belonging to strict anaerobic species, and characterized their transcriptional activity once in the infant gut. Bacterial-host networks are different when breast-fed infants are compared to formula-fed infants (Martín et al., 2016), a fact that is reflected in the host transcriptome (Praveen et al., 2015). Some studies have also found long lasting differences in the fecal microbiota when comparing exclusively breastfed and non-exclusively breastfed infants (Pannaraj et al., 2017; Ho et al., 2018; Moossavi et al., 2019a). Overall, bacterial diversity and microbial pathways implied in the metabolism of carbohydrates are higher in non-exclusively breastfed infants while the contrary is observed for those pathways involved in the metabolism of vitamins, lipids and xenobiotic compounds (Ho et al., 2018).

Although the introduction of solid food during weaning was once thought to be associated with a sharp increase in the diversity of the gut microbiota in breastfed infants (Favier et al., 2002), the fecal microbiota of infants are dominated by milk bacteria, independently of the introduction or not of other foods, as long as they are breastfed (Bäckhed et al., 2015).

MOVING FROM COMPOSITION TO FUNCTION

Human milk is a source of a wide spectrum of beneficial microorganisms that might play a role in priming the development and function of many infant systems (Ojo-Okunola et al., 2018). However, studies dealing with the functions of such microbiota/microbiome are very scarce and, as stated by Theobald Smith, (1904), "*it is what bacteria do rather than what they are that commands attention, since our interest centers in the host rather than in the parasite*". Human colostrum and milk contain a complex array of bioactive molecules and cells, including the microbiota, which may act synergistically to preserve infant and maternal health through a wide variety of mechanisms (Morrow and Rangel, 2004).

The human milk microbiota may contribute to, at least, some of the functional properties and health benefits that epidemiological studies have associated with breastfeeding (Renfrew et al., 2012), including protection against infections, metabolic programming, immunomodulation and neuromodulation. Human milk bacteria may provide a certain degree of protection against infections caused by viruses, bacteria or fungi through a variety of mechanisms: (a) biosynthesis of compounds with antimicrobial activity, including organic acids (lactic acid, acetic acid, ethanol), bacteriocins, reuterin or hydrogen peroxide (Heikkilä and Saris, 2003; Beasley and Saris, 2004; Martín et al., 2005; Martín et al., 2006; Cárdenas et al., 2016; Cárdenas et al., 2019; Angelopoulou et al., 2020; García-Gutierrez et al., 2020); (b) coaggregation with pathobionts, impeding their access to the gut epithelial cells (Cárdenas et al., 2019); (c) competitive exclusion with pathobionts for nutrients or host receptors (Olivares et al., 2006a; Martín et al., 2010; Langa et al., 2012); (d) reinforcement of the infant gut barrier by preserving and decreasing intestinal permeability and increasing mucin biosynthesis (Olivares et al., 2006a; Vanhaecke et al., 2017; Liu et al., 2020); and (e) inmmunomodulation (Liu et al., 2020).

The analysis of the genome of some strains isolated from human milk provides some clues that may explain their anti-infectious properties. As an example, *L. salivarius* CECT 5713 is able to inhibit HIV-1 infectivity *in vitro* and its genome contains a gene encoding a protein containing a recognition motif of the high mannose N-linked oligosaccharides displayed by many pathogen antigens, such as gp120, which is essential for HIV pathogenesis (Langa et al., 2012). In developing countries, the World Health Organization (WHO) recommends exclusive breastfeeding among HIV-infected women during the first six months after birth "*unless replacement feeding is acceptable, feasible, affordable, sustainable, and safe for them and their infants*", and to continue breastfeeding thereafter, with gradual introduction of solid foods (WHO, 2016). In these settings, the advantages of breastfeeding for mother and infant health compensate the potential risk of viral transmission (Barthel et al., 2013). Unfortunately, the contribution of the human milk microbiota in protecting from infant infections caused by this or other life-threatening viruses which are relatively frequent in developing (dengue, Ebola, and zika) or developed countries (CMV) remains largely unexplored. Interestingly, the outcome of neonatal rotavirus infections is influenced by the complex interplay between HMOs, the milk microbiome and the infant gut microbiome (Ramani et al., 2018). In the context of the ongoing pandemic caused by the SARS-CoV-2 virus, a recent study did not detect the virus in human milk (Chambers et al., 2020), and COVID-19-positive women are recommended to continue breastfeeding by the WHO and the United Nations

Children's Fund (UNICEF) (United Nations Children's Fund, 2020).

Although most of the information about potential antimicrobial properties of human milk bacteria has arisen from *in vitro* studies and animal models, the beneficial effects of some strains have been confirmed in human clinical trials. Daily intake of a formula containing *L. fermentum* CECT5716 by 6-month-old children significantly decreased the rates of upper respiratory tract infections, gastrointestinal infections and total infections in the following 6 months (Maldonado et al., 2012). *L. salivarius* PS7, a human milk strain with a strong antimicrobial activity against several otopathogens, has been shown to be efficient in preventing recurrent acute otitis media in children (Cárdenas et al., 2019).

Studies addressing the potential role of the human milk microbiota on infant metabolism are scarce but promising. Bacterial diversity in milk is positively correlated with metabolites that are known to exert beneficial effects, including docosahexaenoic acid (DHA), as assessed by the construction of diversity-metabolites networks (Ma et al., 2016). Additionally, some species seem to be critical to regulate the concentrations of relevant milk metabolites, including inositol, DHA or butanal (Ma et al., 2016). Previous studies revealed that some strains originally isolated from human milk display metabolic activivity once in the human gut and are able to participate in the biosynthesis of functional metabolites, such as butyrate, leading to better bowel habits (Olivares et al., 2006c). More recently, administration of *L. fermentum* CECT5716 to pregnanat and lactating rats induced beneficial changes in the fatty acid composition of milk by increasing total polyunsaturated fatty acids including linoleic and α-linolenic acids and decreasing the proportion of palmitic acid (Azagra-Boronat et al., 2020).

Human milk bacteria may also be involved in immune programming of infants through different mechanisms, impacting both innate and acquired immunity (Díaz-Ropero et al., 2006; Olivares et al., 2006b; Pérez-Cano et al., 2010; Azagra-Boronat et al., 2020). Such mechanisms seem to be complementary and to exhibit a high degree of flexibility depending on factors related to the gut environment, such as the exposition to lipopolysaccharide (Díaz-Ropero et al., 2006). *L. fermentum* CECT5716 and *L. salivarius* CECT5713 behave as activators of NK, CD4+, CD8+, and regulatory T cells, and their immunomodulatory effects are different to those displayed by other strains of the same species but isolated from other sources (Pérez-Cano et al., 2010). In fact, *L. fermentum* CECT5716 is able to significantly ameliorate the inflammatory response and the intestinal damage in an animal model of intestinal inflammation (Peran et al., 2005; Peran et al., 2006; Peran et al., 2007).

As cited above, immunomodulation is involved in the anti-infectious protection conferred by some human milk bacteria or in the restoration of the damage caused by infections. *In vivo* assays have shown that *L. rhamnosus* SHA113 inhibits the expression of TNF-α and IL-6 caused by a multi-drug resistant *S. aureus* strain, restoring the concentration of leukocytes in blood (Liu et al., 2020).

Bacterial strains originally isolated from milk of healthy women are appealing as probiotic candidates because of their origin, which implies a history of safe intake by infants (Lara-Villoslada et al., 2009; Maldonado et al., 2010; Gil-Campos et al., 2012; Maldonado-Lobón et al., 2015a), and complex symbiotic interactions from the time we are born (Lara-Villoslada et al., 2007; Fernández et al., 2013; Jeurink et al., 2013). Among them, those species belonging to the genera *Bifidobacterium* and *Lactobacillus* (*B. longum*, *B. breve, L. salivarius*, *L. fermentum*, *L. reuteri*, *L. gasseri*, *L. plantarum*, and *L. rhamnosus*), have received special attention since many of them enjoy the GRAS (Generally Recognised As Safe) status (Food and Drug Administration, USA) and the QPS (Qualified Presumption of Safety) of the European Food Safety Authority (EFSA). In contrast, *S. mitis* and other mitis streptococci, *S. salivarius* and CNS have received marginal attention because of theoretical safety concerns despite of the fact that they are among the dominant bacteria both in this biological fluid (Jiménez et al., 2008a; Hunt et al., 2012; Martín et al., 2012b; Cacho et al., 2017) and in the feces of breast-fed infants (Jiménez et al., 2008a). However, they may provide relevant probiotic functions in practice (Kirjavainen et al., 2001; Uehara et al., 2001; Otto, 2009; Iwase et al., 2010; Park et al., 2011), and therefore, their potential beneficial roles in infants deserve future studies.

Desciphering the genomes of representative collections of human milk isolates and analysis of the human milk metagenome will allow the expansion of our knowledge on the functions that a "healthy" human milk microbiome should provide to the mother-infant dyad. The design of human milk bacterial consortia specifically tailored to meet early life requeriments is an attractive strategy for those infants that are devoid of the benefits of breastfeeding (Fernández et al., 2018).

THE ORIGINS OF THE HUMAN MILK MICROBIOTA

While suckling, some oral bacteria from the mouth or nasopharynx of the infant may seed milk (Ramsey et al., 2004; Moossavi et al., 2019a); however, pre-colostrum expressed during late pregnancy contain some of the bacteria usually detected in human milk (Martín et al., 2004). In fact, oral-associated bacteria have also been isolated in precolostrum collected at the end of the first pregnancy and, therefore, before any contact with the newborn (Ruiz et al., 2014).

The origin of the microbes that constitute the oral microbiome remains widely unknown (Zaura et al., 2014) but streptococci are already present in edentulous infants (Li et al., 1997; Caufield et al., 2000; Bearfield et al., 2002; Cephas et al., 2011). The fact that these bacteria are also frequently detected in human milk (Jiménez et al., 2008a; Jiménez et al., 2008b; Hunt et al., 2011; Martín et al., 2015), might suggest a role in the acquisition or modulation of the oral bacterial communities. A recent study investigating potential relationships among bacterial communities in samples of milk, maternal and infant feces, and maternal and infant oral swabs from some mother-infant pairs found strong associations among these three complex microbial communities and *Streptococcus* was the

most abundant genus not only in infant and maternal oral samples but also in milk (Williams et al., 2019). Similar results had been previously obtained by Davé et al. (2016). Biagi et al. (2017) showed that only a limited number of OTUs were shared between milk and the mouth of the infants, but they included specific *Streptococcus* and *Staphylococcus* OTUs.

Some species and genera commonly detected from human milk, such as *Corynebacterium C. acnes* and, especially, *S. epidermidis*, are also inhabitants of the human skin (Oh et al., 2014). Therefore, breast skin, nipples, and mammary areolas may be a source of such bacteria for human breast and milk (**Figure 4**). However, no studies on the specific transfer of skin bacterial strains to human milk or breast tissue have been performed yet. CNS, *Cutibacterium* and *Corynebacterium* are also present in all the human mucosal surfaces, including that of the digestive tract, which is, very likely, a relevant source of these bacteria for the mammary environment (**Figure 4**).

Although milk, breast skin and the infant oral cavity may share some phylotypes, there are major differences between their respective microbial communities (Hunt et al., 2011; Cabrera-Rubio et al., 2012; Jost et al., 2013; Jiménez et al., 2015). A study that compared the bacteriome of areolar skin, milk, and feces of 107 mother-infant dyads found differences in diversity and composition among the bacterial communities of the three ecosystems (Pannaraj et al., 2017).

The mucosal surfaces of the maternal digestive tract (oral cavity, gut) may be a source of bacteria for the mammary ecosystem from late pregnancy to the end of lactation (Martín et al., 2004; Rodríguez, 2014; Mira and Rodríguez, 2017; Fernández and Rodríguez, 2020). These oral- and entero-mammary routes imply a highly regulated cross-talk between bacterial cells, immune cells (dendritic cells [DCs] and macrophages) and epithelial cells. These complex cross-talks would drive the physiological translocation of certain bacteria without compromising the integrity of the gut epithelium (Vazquez-Torres et al., 1999; Rescigno et al., 2001; Macpherson and Uhr, 2004). Upon translocation, the mammary gland would exert a homing effect on the immune cells that act as bacterial carriers (Perez et al., 2007).

Low level bacterial translocation from the digestive tract to extra-digestive locations is a relatively common process (Ouwehand et al., 2001; Vankerckhoven et al., 2004; Begier et al., 2005; Dasanayake et al., 2005), while an increase in the rates of bacterial translocation from the gastrointestinal tract to the mammary environment has been observed in pregnant and lactating rodents without compromising host health (Perez et al., 2007; Treven et al., 2015; de Andrés et al., 2017; Azagra-Boronat et al., 2020). In addition, the existence of an entero-mammary traffic of immune cells during late pregnancy and lactation has long been known (Bertotto et al., 1991; Roitt, 2001; Newburg, 2005). Such efflux is responsible for the integration of the lactating breast into the mucosal immune system and its transformation in a formidable organ from the immunological point of view (Pabst, 1987; Brandtzaeg, 2010). It has also been reported that some bacterial strains isolated from human milk can cross Caco-2 monolayer by using a mechanism that involves interactions with DCs while this ability is lost in the absence of DC-like cells (Langa, 2006; Langa et al., 2012). Carrying of bacterial cells, including streptococci, lactobacilli, and bifidobacteria, by blood and milk maternal mononuclear cells has already been reported (Perez et al., 2007).

In fact, presence in milk of specific lactic acid bacteria after their *per os* administration has been reported in lactating rodents (Treven et al., 2015; de Andrés et al., 2017; Azagra-Boronat et al.,

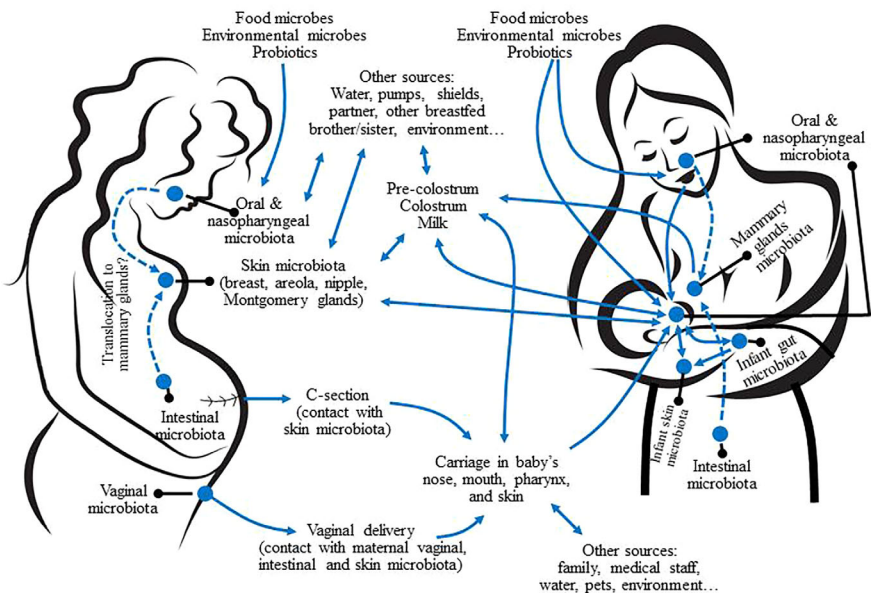

FIGURE 4 | Potential sources of the microbes present in human milk and interactions with other mother-infant microbiotas. Dashed arrows represent potential translocation through an endogenous pathway.

2020), lactating women (Jiménez et al., 2008c; Abrahamsson et al., 2009; Arroyo et al., 2010), or pregnant women (Fernández et al., 2016). Similarly, mothers who had ingested *L. rhamnosus* GG, *L. acidophilus* La-5, and *B. animalis* ssp. *lactis* Bb-12 can transfer such strains to their infants through breastfeeding (Avershina et al., 2018; Simpson et al., 2018).

Previously, it has been shown a *Lactobacillus* strain ingested during pregnancy could be detected in the feces of the breastfed infants, including those who were born by Caesarean section (Schultz et al., 2004). However, the authors did not investigate if the strain was present in the milk of the mothers. More recently, a *B. breve* strain detected in a rectal sample and in the milk of a woman could be also detected in the feces of her infant who was delivered *via* Caesarean section, suggesting a direct mother-to-infant transmission and supporting the possibility of a microbial translocation through an entero-mammary pathway (Kordy et al., 2020). In addition, the possibility of a shared environmental source, including the indoor microbiome, must not be ruled out.

Many transient anatomical and physiological changes may increase the translocation rate from the digestive system to the mammary glands in pregnant and lactating women (Rodríguez, 2014; Mira and Rodríguez, 2017; Fernández and Rodríguez, 2020). Such a homing effect may involve a physiological immunodepression to tolerate the fetus, a formidable angiogenesis process in the breast and the filling of the mammary duct system with pre-colostrum during late pregnancy, which would be an excellent source of nutrients for bacteria.

Although more studies are necessary to elucidate the existence of oral- and entero-mammary pathways, if this was confirmed, it would provide new approaches to manipulate the microbiota of mothers and infants inorder to improve infant health and development (Rautava et al., 2002; Martín et al., 2004).

MAMMARY DYSBIOSIS AND LACTATIONAL MASTITIS

Mastitis constitutes a common feeding problem for most mammalian species and humans are not an exception (Contreras and Rodríguez, 2011). This condition is the major cause of undesired weaning from the medical point of view and represents a relevant public health issue since such premature weaning prevents the health benefits that breastfeeding provides to infants and mothers (U.S. Department of Health and Human Services, 2011; American Academy of Pediatrics, 2012; Renfrew et al., 2012).

A wide variety of bacteria may inhabit the mammary gland ecosystem during a healthy lactation period, including potential mastitis-causing species; however, if there is a disturbance of this balanced state, milk dysbiosis may occur, eventually leading to mastitis (Delgado et al., 2008; Fernández et al., 2014; Mediano et al., 2017) (**Figure 3**). Patel et al. (2017) analyzed milk samples from women suffering either subacute or acute mastitis and, also, from healthy controls. In comparison to controls, the microbiome of samples from acute and subacute cases were distinct: their diversity was drastically reduced, and they were significantly enriched in some aerotolerant bacteria, including *Staphylococcus*, while depleted in obligate anaerobes, such as *Ruminococcus*, *Faecalibacterium*, or *Eubacterium*. Similar alterations in the milk microbiome have also been found in cases of mastitis involving other mammalian species (Kuehn et al., 2013; Oikonomou et al., 2014; Derakhshani et al., 2018).

The etiology and pathogenesis of acute and subacute lactational mastitis have been reviewed by Fernández et al. (2014) and Rodríguez and Fernández (2017). Empiric use of antibiotics has been, and still is, the most common approach to treat mastitis. However, many cases do not respond to such therapy since mastitis agents are becoming increasingly resistant to antimicrobials through different mechanisms, including intrinsic resistances, presence of transmissible antibiotic resistance genes and/or the formation of biofilms (Marín et al., 2017). The high rates of antimicrobial resistance among mastitis-causing bacteria have clinical relevance in relation to treatment options. In addition, wide-spectrum antibiotics may alter the bacterial composition of milk, impairing vertical transmission of microbes through breastfeeding (Soto et al., 2014). Therefore, new strategies for the management of mastitis are needed, and in this context, those based in the modulation of the mammary bacterial communities through the selection and application of probiotic strains that were originally isolated from human milk seem particularly suited for this target (Fernández et al., 2014).

Several human trials have shown that oral administration of some human milk strains (*L. salivarius* CECT5713, *L. salivarius* PS2, *Lactobacillus gasseri* CECT5714, *L. fermentum* CECT5716) provoke relevant changes in a variety of milk microbiological, biochemical and immunological parameters, including a significant decrease in the concentration of mastitis-causing agents (Jiménez et al., 2008c; Maldonado-Lobón et al., 2015b; Espinosa-Martos et al., 2016). In fact, such an approach has been found to be more efficient than empiric antibiotherapy for the treatment of this condition (Arroyo et al., 2010). Metabolomic studies have revealed that the impact of the probiotic treatment can be also observed in the urine of the treated women (Vazquez-Fresno et al., 2014). As an example, lactose was present in urine samples before the treatment but it was no longer detected after the probiotic treatment, indicating a restoration of the integrity of the mammary epithelium. Assessment of transcriptomic changes in milk somatic cells associated with the intake of *L. salivarius* PS2 by women with mastitis has also provided valuable information about the potential mechanisms responsible for the efficacy of specific probiotics in treating mastitis (de Andrés et al., 2018). Finally, a few strains (*L. salivarius* PS2 and *L. fermentum* CECT5716) have been successfully applied as a preventive strategy compared to a placebo, when administered either during late pregnancy (Fernández et al., 2016) or during lactation (Hurtado et al., 2017) to women with a history of mastitis after previous pregnancies.

Significant increases in the milk concentrations of TGF-β2 and IgA have been observed after intake of other probiotics during pregnancy or lactation (Rautava et al., 2002; Prescott et al., 2008; Nikniaz et al., 2013), suggesting that the probiotic approach may control the growth of mastitis-causing bacteria in the mammary gland preventing potential damage to the mammary epithelium.

MICROBIOTA OF THE BREAST TISSUE OF NON-LACTATING WOMEN

Most microbial studies addressing the human mammary ecosystem have been focused on the lactation period and limited to the analysis of colostrum and milk samples. However, the number of studies dealing with the microbiota of breast tissue in non-lactating women is rapidly increasing, particularly in the frame of breast cancer research. Breast cancer ranks as the most common malignancy in women. Some studies have observed a correlation between dysbiosis of the gut microbiota and breast cancer (Goedert et al., 2015; Goedert et al., 2018; Laborda-Illanes et al., 2020). In addition, changes in the microbiota of breast tissue have also been linked to breast cancer.

In a pioneering study, samples of breast tissue from 81 women with and without cancer were analyzed by sequencing of the V6 region of the 16S rRNA gene (Urbaniak et al., 2014a). Sequences corresponding to *Staphylococcus*, *Cutibacterium*, *Acinetobacter*, *Enterobacteriaceae*, *Bacillus*, *Pseudomonas*, or *Prevotella* were detected in a high percentage of the samples, including some obtained from women without a previous history of lactation. Cultures confirmed the presence of viable bacteria in some of the samples. In parallel, Xuan et al. (2014) employed 454 pyrosequencing of the V4 region of 16S rRNA gene to analyze breast tissue samples from 20 patients with breast cancer, each one providing tumor tissue and normal adjacent tissue. These authors found that *Methylobacterium radiotolerans* was enriched in tumor tissue, while *Sphingomonas yanoikuyae* was enriched in normal tissue. However, some doubts arise from their results since this technique does not discriminate at the species level, the DNA of the two genera that discriminated between both groups are typically found in DNA extraction reagents and kits, and no blank controls were included in the study. Later, Urbaniak et al. (2016b) reported that the bacteriome of breast tissue adjacent to breast cancer differs from that found in breast tissue from healthy controls undergoing cosmetic surgery. Compared to healthy controls, the relative abundances of *Bacillus*, *Enterobacteriaceae* and *Staphylococcus* were higher in samples from women with breast cancer while the abundance of lactic acid bacteria was lower (Urbaniak et al., 2016b).

Hieken et al. (2016) found different bacterial communities in breast tissue from women with breast cancer in comparison to women suffering from a benign breast disease. Malignancy was associated with an increase in some taxa that were present at a low abundance level, including the genera *Atopobium*, *Fusobacterium*, *Gluconacetobacter* and *Hydrogenophaga*. Chan et al. (2016) investigated the presence of bacteria in the nipple aspirate fluid obtained from women with a previous history of breast cancer and from healthy women and found a higher incidence of the genus *Alistipes* in the first group. In addition, the bacteria associated with breast cancer shared β-glucuronidase activity and, therefore, the authors suggested that this enzymatic activity may promote breast cancer. Recently, two studies described racial differences in the microbiome of breast tumors (Smith et al., 2019; Thyagarajan et al., 2020) while another study identified differences in diversity and composition not only between tumor and normal tissue but also among women and between the breasts of the same woman (Klann et al., 2020).

The microbiota may promote or inhibit tumorigenesis by several mechanisms, including the alteration of immune responses or the effect of bacterial-derived enzymes (e.g., β-glucuronidase and β-glucosidase activities) and metabolites, such as short-chain fatty acids, lipopolysaccharides, secondary bile acids, estrogens, or genotoxins (Kovács et al., 2020). In addition, the microbiota and its metabolites may exert a strong influence on the efficacy of chemotherapy and radiation therapies (Kovács et al., 2020). Overall, the results of previous studies warrant further research to elucidate the relationships between breast microbiota and breast cancer.

The breast microbiome may also play a key role in the outcomes of breast plastic or cosmetic surgery, including breast reconstruction, breast reduction or breast augmentation. Breast augmentation is one of the most frequent cosmetic surgical procedures practiced worldwide and can lead to several complications, capsular contracture being the most common one (Rieger et al., 2013; Cook et al., 2020). Bacterial growth, and subsequent biofilm formation, is one of the main risk factors for capsular contracture (Dancey et al., 2012; Ajdic et al., 2016; Walker et al., 2019). It has been suggested that chronic biofilm infection of breast implants may be implicated in the development of breast implant-associated lymphoma (Hu et al., 2015; Hu et al., 2016). Bacteria that are often associated with human milk, breast tissue and breast skin (*S. epidermidis* and other CNS species; *C. acnes*) have been repeatedly isolated within or surrounding breast implants from patients with capsular contracture by using classic culture-based approaches (Virden et al., 1992; Dobke et al., 1995; Ahn et al., 1996; Pajkos et al., 2003; Galdiero et al., 2018) and culture-independent techniques (Cook et al., 2020). At present, the origin of the bacteria detected in the explants (breast tissue and skin contamination) remains unclear (Bachour et al., 2019), and more studies are required to elucidate the role of mammary-related bacteria in the tolerance toward these devices or in the adverse outcomes that are relatively frequently associated with their implantation.

FUTURE TRENDS AND CONCLUSIONS

Conflicting results when trying to analyze the factors that may play a role in shaping the composition of the human milk microbiota can be explained, at least partially, by the low number of samples/women analyzed in most of the studies carried out so far and, also, by many host, environmental, perinatal, and technical factors (LeMay-Nedjelski et al., 2018). International and collaborative studies, involving a high number of participants and performed under identical conditions, are required in order to elucidate the impact of these factors and their interactions.

The origin of the microbes found in the mammary ecosystem remains a largely open question. Some of the bacteria detected in milk most likely originate from the maternal skin and areola. During lactation, shared features between the microecology in the infant mouth and milk suggest interactions but the precise directions and significance of which are yet to be determined. Perhaps most intriguingly, experimental and human data indicates that some bacteria in milk may originate in the maternal digestive

tract and that an enteromammary pathway for microbes may exist. Further assessment of this hypothesis demands sophisticated experimental and clinical studies and state-of-the-art methods to ensure accurate strain-level identification of specific bacteria in not only the intestine and milk but also in the bloodstream and within the immune cells thought to be responsible for the transfer.

The emerging data indicating that various maternal characteristics and exposures, including BMI, antibiotic exposure, gestational age or delivery mode, are associated with the composition of the milk microbiota suggest that the microbes in milk are linked to health and disease in the mother and perhaps, also, in the infant. As of present, however, the biological role and clinical significance of the bacteria in human milk remain poorly characterized and several fundamental questions require elucidation. Detailed metagenomic, metatranscriptomic and metabolomic studies are paramount for understanding the functionality of the milk microbiota. It is also important to appreciate the fact that most published studies describing the bacterial communities of human milk are based on culture-independent molecular methods, such as qPCR and sequencing of either of the 16S rRNA gene or the whole bacterial genome. While these tools are highly sensitive, detection of bacterial DNA does not entail the presence of viable or even intact bacteria. Furthermore, there are published data indicating that at least some bacteria in milk may actually be found on and inside immune cells (Perez et al., 2007). Distinguishing between intact and viable free or intracellular bacteria and the mere presence of bacterial fragments is a crucial step in understanding their biological function. The presence of bacterial components such as DNA in milk may be sufficient to induce immune responses in the mammary epithelium, immune cells, or the neonatal gut. Milk immune cells interacting with bacteria, on the other hand, may mediate immune responses specific to these bacteria. Given the role of human milk in establishing immune tolerance towards antigens in the maternal diet (Verhasselt et al., 2008), it is conceivable that milk may serve as a vehicle for inducing tolerance in the newborn to colonizing microbes from the mother. Currently, no direct evidence to corroborate or refute this hypothesis exists.

Live bacteria in human milk serve biological purposes in the mother. The relationships between the bacterial composition of milk and the risk of mastitis suggest that the indigenous bacteria may be necessary for mammary gland health. Mammary bacteria may also play some roles in the non-lactating mammary gland and the development of breast carcinoma, which is a subject of substantial clinical significance and an area of active research.

In addition to implications to maternal health, the human milk microbiota may be transferred to the infant, potentially acting as a driver in early oral and gut colonization. As reviewed above, shared bacterial taxa detected in maternal stool, human milk and the infant gut suggest that milk may be a vehicle for early colonization. This has to be interpreted with caution since there is the possibility of other routes of bacterial transfer and taxonomic discrimination should be performed at the strain level to confirm identity. As in the case of the origin of the milk bacteria, experimental studies in animal models offer a more reductionist means of dissecting the role of milk microbes for gut colonization. A translational approach complementing clinical studies with basic science is therefore needed.

So far, most studies dealing with the human milk microbiome have been focused on the taxonomical composition and only a few of them have dealt with its potential functions for the infant-mother dyad. Breastfeeding has been associated with reduced risk of chronic conditions such as obesity (Victora et al., 2016), which has also been linked to aberrant early gut colonization. The extent to which the beneficial health effects of breastfeeding are mediated by modulation of the developing gut microbiota remains an open question. Even less clear is the impact of human milk bacteria on child health. Indeed, studying the roles of the human milk microbiota in health and disease is a difficult task since there are synergistic activities among different milk molecules and cells, and several non-microbial components in human milk have the potential to modify the infant gut microbiota. However, we have a rapidly increasing variety of powerful *in vitro* and *in vivo* tools, techniques, and procedures to address such a question from cell biology to well-designed clinical trials, from human breast organoids to human milk microbiota-associated mouse models (Wang et al., 2017) and from true metagenome (integrating data from microbiome, microbial genomes and human genome projects) to systems biology approaches.

AUTHOR CONTRIBUTIONS

All the authors contributed equally to the review of the literature and to the writing and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Aakko, J., Kumar, H., Rautava, S., Wise, A., Autran, C., Bode, L., et al. (2017). Human milk oligosaccharide categories define the microbiota composition in human colostrum. *Benef. Microbes* 8, 563–567. doi: 10.3920/BM2016.0185

Abrahamsson, T. R., Sinkiewicz, G., Jakobsson, T., Fredrikson, M., and Björkstén, B. (2009). Probiotic lactobacilli in breast milk and infant stool in relation to oral intake during the first year of life. *J. Pediatr. Gastroenterol. Nutr.* 49, 349–354. doi: 10.1097/MPG.0b013e31818f091b

Ackerman, D. L., Doster, R. S., Weitkamp, J. H., Aronoff, D. M., Gaddy, J. A., and Townsend, S. D. (2017). Human milk oligosaccharides exhibit antimicrobial and antibiofilm properties against group B *Streptococcus*. *ACS Infect. Dis.* 3, 595–605. doi: 10.1021/acsinfecdis.7b00064

Ackerman, D. L., Craft, K. M., Doster, R. S., Weitkamp, J. H., Aronoff, D. M., Gaddy, J. A., et al. (2018). Antimicrobial and antibiofilm activity of human milk oligosaccharides against *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Acinetobacter baumannii*. *ACS Infect. Dis.* 4, 315–324. doi: 10.1021/acsinfecdis.7b00183

Ahn, C. Y., Ko, C. Y., Wagar, E. A., Wong, R. S., and Shaw, W. W. (1996). Microbial evaluation: 139 implants removed from symptomatic patients. *Plast. Reconstr. Surg.* 98, 1225–1229. doi: 10.1097/00006534-199612000-00016

Ajdic, D., Zoghbi, Y., Gerth, D., Panthaki, Z. J., and Thaller, S. (2016). The relationship of bacterial biofilms and capsular contracture in breast implants. *Aesthet. Surg. J.* 36 (3), 297–309. doi: 10.1093/asj/sjv177

Albesharat, R., Ehrmann, M. A., Korakli, M., Yazaji, S., and Vogel, R. F. (2011). Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. *Syst. Appl. Microbiol.* 34, 148–155. doi: 10.1016/j.syapm.2010.12.001

American Academy of Pediatrics (2012). Breastfeeding and the use of human milk. *Pediatrics* 129, e827–e841. doi: 10.1542/peds.2011-3552

Angelopoulou, A., Warda, A. K., O'Connor, P. M., Stockdale, S. R., Shkoporov, A. N., Field, D., et al. (2020). Diverse bacteriocins produced by strains from the human milk microbiota. *Front. Microbiol.* 11, 788. doi: 10.3389/fmicb.2020.00788

Anonymous (1889). Bacteria in milk and its products. *Science* 14, 116–118. doi: 10.1126/science.ns-14.341.116

Anonymous (1988). Breast-feeding/breast milk and human immunodeficiency virus (HIV). *Bull. Pan. Am. Health Organ.* 22, 87–88.

Arboleya, S., Ruas-Madiedo, P., Margolles, A., Solís, G., Salminen, S., de Los Reyes-Gavilán, C. G., et al. (2011). Characterization and in vitro properties of potentially probiotic *Bifidobacterium* strains isolated from breast-milk. *Int. J. Food Microbiol.* 149, 28–36. doi: 10.1016/j.ijfoodmicro.2010.10.036

Arroyo, R., Martin, V., Maldonado, A., Jiménez, E., Fernández, L., and Rodríguez, J. M. (2010). Treatment of infectious mastitis during lactation: antibiotics versus oral administration of lactobacilli isolated from breast milk. *Clin. Infect. Dis.* 50, 1551–1558. doi: 10.1086/652763

Asnicar, F., Manara, S., Zolfo, M., Truong, D. T., Scholz, M., Armaninni, F., et al. (2017). Studying vertical microbiome transmission from mothers to infants by strain-level metagenomics profiling. *mSystems* 2, e00164. doi: 10.1128/mSystems.00164-16

Avershina, E., Angell, I. L., Simpson, M., Storrø, O., Øien, T., Johnsen, R., et al. (2018). Low maternal microbiota sharing across gut, breast milk and vagina, as revealed by 16S rRNA gene and reduced metagenomic sequencing. *Genes* 9, E231. doi: 10.3390/genes9050231

Azagra-Boronat, I., Tres, A., Massot-Cladera, M., Franch, À., Castell, M., Guardiola, F., et al. (2020). *Lactobacillus fermentum* CECT5716 supplementation in rats during pregnancy and lactation affects mammary milk composition. *J. Dairy Sci.* 103, 2982–2992. doi: 10.3168/jds.2019-17384

Bachmann, K. D., and Pulverer, G. (1962). [Studies on the microorganism content of human milk. With a contribution to the problem of preservation by freeze drying]. *Z. Kinderheilkd.* 87, 52–59. doi: 10.1007/BF00456354

Bachour, Y., Poort, L., Verweij, S. P., van Selms, G., Winters, H., Ritt, M., et al. (2019). PCR characterization of microbiota on contracted and non-contracted breast capsules. *Aesthetic Plast. Surg.* 43, 918–926. doi: 10.1007/s00266-019-01383-9

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I (2005). Host-bacterial mutualism in the human intestine. *Science* 307, 1915–1920. doi: 10.1126/science.1104816

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* 17, 690–703. doi: 10.1016/j.chom.2015.04.004

Bang, C., and Schmitz, R. A. (2015). Archaea associated with human surfaces: not to be underestimated. *FEMS Microbiol. Rev.* 39, 631–648. doi: 10.1093/femsre/fuv010

Bardanzellu, F., Fanos, V., and Reali, A. (2019). Human breast milk-acquired cytomegalovirus infection: Certainties, Doubts and Perspectives. *Curr. Pediatr. Rev.* 15, 30–41. doi: 10.2174/1573396315666181126105812

Barthel, A., Gourinat, A. C., Cazorla, C., Joubert, C., Dupont-Rouzeyrol, M., and Descloux, E. (2013). Breast milk as a possible route of vertical transmission of dengue virus? *Clin. Infect. Dis.* 57, 415–417. doi: 10.1093/cid/cit227

Bearfield, C., Davenport, E. S., Sivapathasundaram, V., and Allaker, R. P. (2002). Possible association between amniotic fluid micro-organism infection and microflora in the mouth. *Br. J. Obstet. Gynaecol.* 109, 527–533. doi: 10.1111/j.1471-0528.2002.01349.x

Beasley, S. S., and Saris, P. E. J. (2004). Nisin-producing *Lactococcus lactis* strains isolated from human milk. *Appl. Environ. Microbiol.* 70, 5051–5053. doi: 10.1128/AEM.70.8.5051-5053.2004

Begier, E. M., Barrett, N. L., Mshar, P. A., Johnson, D. G., Hadler, J. L. Connecticut Bioterrorism Field Epidemiology Response Team (2005). Gram-positive rod surveillance for early anthrax detection. *Emerg. Infect. Dis.* 11, 1483–1486. doi: 10.3201/eid1109.041013

Begović, J., Jovčić, B., Papić-Obradović, M., Veljović, K., Lukić, J., Kojić, M., et al. (2013). Genotypic diversity and virulent factors of *Staphylococcus epidermidis* isolated from human breast milk. *Microbiol. Res.* 168, 77–83. doi: 10.1016/j.micres.2012.09.004

Bertotto, A., Gerli, R., Castellucci, G., Scalise, F., and Vaccaro, R. (1991). Human milk lymphocytes bearing the gamma/delta T-cell receptor are mostly delta TCS1-positive cells. *Immunology* 74, 360–361.

Biagi, E., Quercia, S., Aceti, A., Beghetti, I., Rampelli, S., Turroni, S., et al. (2017). The bacterial ecosystem of mother's milk and infant's mouth and gut. *Front. Microbiol.* 8, 1214. doi: 10.3389/fmicb.2017.01214

Biagi, E., Aceti, A., Quercia, S., Beghetti, I., Rampelli, S., Turroni, S., et al. (2018). Microbial community dynamics in mother's milk and infant's mouth and gut in moderately preterm infants. *Front. Microbiol.* 9, 2512. doi: 10.3389/fmicb.2018.02512

Björkstén, B., Burman, L. G., De Château, P., Fredrikzon, B., Gothefors, L., Hernell, O., et al. (1980). Collecting and banking human milk: to heat or not to heat? *Br. Med. J.* 281, 765–769. doi: 10.1136/bmj.281.6243.765

Blohm, G. M., Lednicky, J. A., Márquez, M., White, S. K., Loeb, J. C., Pacheco, C. A., et al. (2018). Evidence for mother-to-child transmission of Zika virus through breast milk. *Clin. Infect. Dis.* 66, 1120–1121. doi: 10.1093/cid/cix968

Bode, L. (2020). Human milk oligosaccharides: structure and functions. *Nestle Nutr. Inst. Workshop Ser.* 94, 115–123. doi: 10.1159/000505339

Boer, H. R., Anido, G., and Macdonald, N. (1981). Bacterial colonization of human milk. *South Med. J.* 74, 716–718. doi: 10.1097/00007611-198106000-00022

Boix-Amorós, A., Collado, M. C., and Mira, A. (2016). Relationship between milk microbiota, bacterial load, macronutrients, and human cells during lactation. *Front. Microbiol.* 7, 492. doi: 10.3389/fmicb.2016.00492

Boix-Amorós, A., Martinez-Costa, C., Querol, A., Collado, M. C., and Mira, A. (2017). Multiple approaches detect the presence of fungi in human breastmilk samples from healthy mothers. *Sci. Rep.* 7, 13016. doi: 10.1038/s41598-017-13270-x

Boix-Amorós, A., Puente-Sánchez, F., du Toit, E., Linderborg, K. M., Zhang, Y., Yang, B., et al. (2019). Mycobiome profiles in breast milk from healthy women depend on mode of delivery, geographic location, and interaction with bacteria. *Appl. Environ. Microbiol.* 85, e02994–e02918. doi: 10.1128/AEM.02994-18

Boo, N. Y., Nordiah, A. J., Alfizah, H., Nor–Rohaini, A. H., and Lim, V. K. (2001). Contamination of breast milk obtained by manual expression and breast pumps in mothers of very low birth weight infants. *J. Hosp. Infect.* 49, 274–281. doi: 10.1053/jhin.2001.1117

Boyes, S. M. (1987). AIDS virus in breast milk: a new threat to neonates and donor breast milk banks. *Neonatal Netw.* 5, 37–39.

Brandtzaeg, P. (2010). The mucosal immune system and its integration with the mammary glands. *J. Pediatr.* 156 (2 Suppl), S8–S15. doi: 10.1016/j.jpeds.2009.11.014

Brown, S. L., Bright, R. A., Dwyer, D. E., and Foxman, B. (2005). Breast pump adverse events: reports to the Food and Drug Administration. *J. Hum. Lact.* 21, 169–174. doi: 10.1177/0890334405275445

Bryan, D. L., Hart, P. H., Forsyth, K. D., and Gibson, R. A. (2007). Immunomodulatory constituents of human milk change in response to infant bronchiolitis. *Pediatr. Allergy Immunol.* 18, 495–502. doi: 10.1111/j.1399-3038.2007.00565.x

Burbianka, M., and Dluzniewska, I. (1971). [Enterotoxic staphylococci and enterotoxins in human milk]. *Rocz. Panstw. Zakl. Hig.* 22, 537–544.

Cabrera-Rubio, R., Collado, M. C., Laitinen, K., Salminen, S., Isolauri, E., and Mira, A. (2012). The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am. J. Clin. Nutr.* 96, 544–551. doi: 10.3945/ajcn.112.037382

Cabrera-Rubio, R., Mira-Pascual, L., Mira, A., and Collado, M. C. (2016). Impact of mode of delivery on the milk microbiota composition of healthy women. *J. Dev. Orig. Health Dis.* 7, 54–60. doi: 10.1017/S2040174415001397

Cabrera-Rubio, R., Kunz, C., Rudloff, S., García-Mantrana, I., Crehuá-Gaudiza, E., Martínez-Costa, C., et al. (2019). Association of maternal secretor status and human milk oligosaccharides with milk microbiota: an observational pilot study. *J. Pediatr. Gastroenterol. Nutr.* 68, 256–263. doi: 10.1097/MPG.0000000000002216

Cacho, N. T., Harrison, N. A., Parker, L. A., Padgett, K. A., Lemas, D. J., Marcial, G. E., et al. (2017). Personalization of the microbiota of donor human milk with mother's own milk. *Front. Microbiol.* 8, 1470. doi: 10.3389/fmicb.2017.01470

Cárdenas, N., Martín, V., Delgado, S., Rodríguez, J. M., and Fernández, L. (2014). Characterization of Lactobacillus gastricus strains isolated from human milk. *Int. Dairy J.* 39, 167–177. doi: 10.1016/j.idairyj.2014.06.008

Cárdenas, N., Laiño, J. E., Delgado, S., Jiménez, E., Juárez del Valle, M., Savoy de Giori, G., et al. (2015). Relationships between the genome and some phenotypical properties of *Lactobacillus fermentum* CECT 5716, a probiotic strain isolated from human milk. *Appl. Microbiol. Biotechnol.* 99, 4343–4353. doi: 10.1007/s00253-015-6429-0

Cárdenas, N., Arroyo, R., Calzada, J., Peirotén, Á., Medina, M., Rodríguez, J. M., et al. (2016). Evaluation of technological properties of *Enterococcus faecium* CECT 8849, a strain isolated from human milk, for the dairy industry. *Appl. Microbiol. Biotechnol.* 100, 7665–7677. doi: 10.1007/s00253-016-7616-3

Cárdenas, N., Martín, V., Arroyo, R., López, M., Carrera, M., Badiola, C., et al. (2019). Prevention of recurrent acute otitis media in children through the use of *Lactobacillus salivarius* PS7, a target-specific probiotic strain. *Nutrients* 11, 376. doi: 10.3390/nu11020376

Carroll, L., Osman, M., Davies, D. P., and McNeish, A. S. (1979). Bacteriology of raw breast milk. *Lancet* 2, 1186. doi: 10.1016/s0140-6736(79)92409-7

Carroll, L., Osman, M., and Davies, D. P. (1980). Does discarding the first few millilitres of breast milk improve the bacteriological quality of bank breast milk? *Arch. Dis. Child.* 55, 898–899. doi: 10.1136/adc.55.11.898

Cataldi, M. S., and Müller, A. (1947). Microflora del calostro y de la superficie cutánea del seno materno en relación con la del tubo digestivo del recién nacido. *El Día Médico 7 abril* 1945, 473–475.

Caufield, P. W., Dasanayake, A. P., Li, Y., Pan, Y., Hsu, J., and Hardin, J. M. (2000). Natural history of *Streptococcus sanguinis* in the oral cavity of infants: evidence for a discrete window of infectivity. *Infect. Immun.* 68, 4018–4023. doi: 10.1128/IAI.68.7.4018-4023.2000

Cayla, A., Perrot, R., and Bamberger, J. (1955). [Etiological role of *Staphylococcus aureus* in erythroderma desquamativum in infants (Leiner-Moussous disease); the presence of *Staphylococcus aureus* in the breast milk]. *Arch. Fr. Pediatr.* 12, 410–413.

Cephas, K. D., Kim, J., Mathai, R. A., Barry, K. A., Dowd, S. E., Meline, B. S., et al. (2011). Comparative analysis of salivary bacterial microbiome diversity in edentulous infants and their mothers or primary care givers using pyrosequencing. *PloS One* 6, e23503. doi: 10.1371/journal.pone.0023503

Chambers, C., Krogstad, P., Bertrand, K., Contreras, D., Tobin, N. H., Bode, L., et al. (2020). Evaluation for SARS-CoV-2 in breast milk from 18 infected women. *JAMA*, 324, 1347–1348. doi: 10.1001/jama.2020.15580

Chan, A. A., Bashir, M., Rivas, M. N., Duvall, K., Sieling, P. A., Pieber, T. R., et al. (2016). Characterization of the microbiome of nipple aspirate fluid of breast cancer survivors. *Sci. Rep.* 6, 28061. doi: 10.1038/srep28061

Cheeseman, S., and McGraw, B. R. (1983). Studies on cytomegalovirus in human milk. *J. Infect. Dis.* 148, 615–616. doi: 10.1093/infdis/148.3.615a

Chen, P.-W., Lin, Y.-L., and Huang, M.-S. (2018). Profiles of commensal and opportunistic bacteria in human milk from healthy donors in Taiwan. *J. Food Drug Anal.* 26, 1235–1244. doi: 10.1016/j.jfda.2018.03.004

Cherkasskaia, R. S., Dodonov, V. N., Darbeeva, O. S., Severov, A. M., and Khodoc, V. G. (1980). [Microbiologic characteristics of postpartum lactation mastitis]. *Zh. Mikrobiol. Epidemiol. Immunobiol.* 3, 100–105.

Collado, M. C., Delgado, S., Maldonado, A., and Rodríguez, J. M. (2009). Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. *Lett. Appl. Microbiol.* 48, 523–528. doi: 10.1111/j.1472-765X.2009.02567.x

Contreras, G. A., and Rodríguez, J. M. (2011). Mastitis: comparative etiology and epidemiology. *J. Mammary Gland Biol. Neoplasia* 16, 339–356. doi: 10.1007/s10911-011-9234-0

Cook, J., Holmes, C. J., Wixtrom, R., Newman, M. I., and Pozner, J. (2020). Characterizing the microbiome of the contracted breast capsule using next generation sequencing. *Aesthet. Surg. J.* sjaa097. Epub ahead of print. doi: 10.1093/asj/sjaa097

Cooke, R. P., Devlin, J., and Robinson, M. J. (1987). Breast milk and methicillin-resistant *Staphylococcus aureus*. *J. Hosp. Infect.* 10, 312. doi: 10.1016/0195-6701(87)90016-8

Dancey, A., Nassimizadeh, A., and Levick, P. (2012). Capsular contracture - what are the risk factors? A 14 year series of 1400 consecutive augmentations. *J. Plast. Reconstr. Aesthetic Surg.* 65, 213–218. doi: 10.1016/j.bjps.2011.09.011

Dasanayake, A. P., Li, Y., Wiener, H., Ruby, J. D., and Lee, M. J. (2005). Salivary *Actinomyces naeslundii* genospecies 2 and *Lactobacillus casei* levels predict pregnancy outcomes. *J. Periodontol.* 76, 171–177. doi: 10.1902/jop.2005.76.2.171

Davé, V., Street, K., Francis, S., Bradman, A., Riley, L., Eskenazi, B., et al. (2016). Bacterial microbiome of breast milk and child saliva from low-income Mexican-American women and children. *Pediatr. Res.* 79, 846–854. doi: 10.1038/pr.2016.9

Davidson, D. C., Poll, R. A., and Roberts, C. (1979). Bacteriological monitoring of unheated human milk. *Arch. Dis. Child.* 54, 760–764. doi: 10.1136/adc.54.10.760

de Andrés, J., Jiménez, E., Chico-Calero, I., Fresno, M., Fernández, L., and Rodríguez, J. M. (2017). Physiological translocation of lactic acid bacteria during pregnancy contributes to the composition of the milk microbiota in mice. *Nutrients* 10, E14. doi: 10.3390/nu10010014

de Andrés, J., Jiménez, E., Espinosa-Martos, I., Rodríguez, J. M., and García-Conesa, M. T. (2018). An exploratory search for potential molecular targets responsive to the probiotic *Lactobacillus salivarius* PS2 in women with mastitis: gene expression profiling vs. interindividual variability. *Front. Microbiol.* 9, 2166. doi: 10.3389/fmicb.2018.02166

Delgado, S., Arroyo, R., Martín, R., and Rodríguez, J. M. (2008). PCR-DGGE assessment of the bacterial diversity of breast milk in women with lactational infectious mastitis. *BMC Infect. Dis.* 8, 51. doi: 10.1186/1471-2334-8-51

Delgado, S., Arroyo, R., Jiménez, E., Marín, M. L., del Campo, R., Fernández, L., et al. (2009). *Staphylococcus epidermidis* strains isolated from breast milk of women suffering infectious mastitis: potential virulence traits and resistance to antibiotics. *BMC Microbiol.* 9, 82. doi: 10.1186/1471-2180-9-82

Delgado, S., García, P., Fernández, L., Jiménez, E., Rodríguez-Baños, M., del Campo, R., et al. (2011). Characterizationn of *Staphylococcus aureus* strains involved in human and bovine mastitis. *FEMS Immunol. Med. Microbiol.* 62, 225–235. doi: 10.1111/j.1574-695X.2011.00806.x

Derakhshani, H., Fehr, K. B., Sepehri, S., Francoz, D., De Buck, J., Barkema, H. W., et al. (2018). Invited review: Microbiota of the bovine udder: Contributing factors and potential implications for udder health and mastitis susceptibility. *J. Dairy Sci.* 101, 10605–10625. doi: 10.3168/jds.2018-14860

Díaz-Ropero, M. P., Martín, R., Sierra, S., Lara-Villoslada, F., Rodríguez, J. M., Xaus, J., et al. (2006). Two *Lactobacillus* strains, isolated from breast milk, differently modulate the immune response. *J. Appl. Microbiol.* 102, 337–343. doi: 10.1111/j.1365-2672.2006.03102.x

Ding, M., Qi, C., Yang, Z., Jiang, S., Bi, Y., Lai, J., et al. (2019). Geographical location specific composition of cultured microbiota and *Lactobacillus* occurrence in human breast milk in China. *Food Funct.* 10 (2), 554–564. doi: 10.1039/C8FO02182A

Dluzniewska, I. (1966). [Pathogenic staphylococci in milk]. *Ginekol. Pol.* 37, 745–751.

Dobke, M. K., Svahn, J. K., Vastine, V. L., Landon, B. N., Stein, P. C., and Parsons, C. L. (1995). Characterization of microbial presence at the surface of silicone mammary implants. *Ann. Plast. Surg.* 34, 563–569. doi: 10.1097/00000637-199506000-00001

Donowitz, L. G., Marsik, F. J., Fisher, K. A., and Wenzel, R. P. (1981). Contaminated breast milk: A source of *Klebsiella* bacteremia in a newborn intensive care unit. *Rev. Infect. Dis.* 3, 716–720. doi: 10.1093/clinids/3.4.716

Dorr, H., and Sittel, I. (1953). [Bacteriological examination of human milk and its relation to mastitis]. *Zentralbl. Gynakol.* 75, 1833–1835.

Drago, L., Toscano, M., De Grandi, R., Grossi, E., Padovani, E. M., and Peroni, D. G. (2017). Microbiota network and mathematic microbe mutualism in colostrum and mature milk collected in two different geographic areas: Italy versus Burundi. *ISME J.* 11, 875–884. doi: 10.1038/ismej.2016.183

Dridi, B., Henry, M., El Khéchine, A., Raoult, D., and Drancourt, M. (2009). High prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. *PloS One* 4, e7063. doi: 10.1371/journal.pone.0007063

Dubois, M. (1954). Role of contamination by breast milk in staphylococcus infection of newborn]. *Rev. Prat.* 4, 493–495.

Duranti, S., Lugli, G. A., Mancabelli, L., Armanini, F., Turroni, F., James, K., et al. (2017). Maternal inheritance of bifidobacterial communities and bifidophages in infants through vertical transmission. *Microbiome* 5, 66. doi: 10.1186/s40168-017-0282-6

Dworsky, M., Yow, M., Stagno, S., Pass, R. F., and Alford, C. (1983). Cytomegalovirus infection of breast milk and transmission in infancy. *Pediatrics* 72, 295–299.

Eidelman, A.II, and Szilagyi, G. (1979). Patterns of bacterial colonization of human milk. *Obstet. Gynecol.* 53, 550–552.

Emerson, J. B., Adams, R.II, Román, C., Brooks, B., Coil, D. A., Dahlhausen, K., et al. (2017). Schrödinger's microbes: Tools for distinguishing the living from the dead in microbial ecosystems. *Microbiome* 5, 86. doi: 10.1186/s40168-017-0285-3

Espinosa-Martos, I., Jiménez, E., de Andrés, J., Rodríguez-Alcala, L. M., Tavarez, S., Manzano, S., et al. (2016). Milk and blood biomarkers associated to the clinical efficacy of a probiotic for the treatment of infectious mastitis. *Benef. Microbes* 7, 305–318. doi: 10.3920/BM2015.0134

Favier, C. F., Vaughan, E. E., de Vos, W. M., and Akkermans, A. D. L. (2002). Molecular monitoring of succession of bacterial communities in human neonates. *Appl. Environ. Microbiol.* 68, 219–226. doi: 10.1128/AEM.68.1.219-226.2002

Fernández, L., and Rodríguez, J. M. (2020). Human milk microbiota: origin and potential uses. *Nestle Nutr. Inst. Workshop Ser.* 94, 75–85. doi: 10.1159/000505031

Fernández, L., Langa, S., Martin, V., Maldonado, A., Jiménez, E., Martin, R., et al. (2013). The Human milk microbiota: origin and potential roles in health and disease. *Pharmacol. Res.* 69, 1–10. doi: 10.1016/j.phrs.2012.09.001

Fernández, L., Arroyo, R., Espinosa, I., Marín, M., Jiménez, E., and Rodríguez, J. M. (2014). Probiotics for human lactational mastitis. *Benef. Microbes* 5, 169–183. doi: 10.3920/BM2013.0036

Fernández, L., Cardenas, N., Arroyo, R., Manzano, S., Jiménez, E., Martin, V., et al. (2016). Prevention of infectious mastitis by oral administration of *Lactobacillus salivarius* PS2 during late pregnancy. *Clin. Infect. Dis.* 62, 568–573. doi: 10.1093/cid/civ974

Fernández, L., Ruiz, L., Jara, J., Orgaz, B., and Rodríguez, J. M. (2018). Strategies for the preservation, restoration and modulation of the human milk microbiota. Implications for human milk banks and neonatal intensive care units. *Front. Microbiol.* 9, 2676. doi: 10.3389/fmicb.2018.02676

Fleischrocker, G., Vutuc, C., and Werner, H. P. (1972). [Infection of a newborn infant by breast milk containing *Salmonella typhimurium*]. *Wien Klin. Wochenschr.* 84, 394–395.

Foster, W. D., and Harris, R. E. (1960). The incidence of *Staphylococcus pyogenes* in normal human breast milk. *J. Obstet. Gynaecol. Br. Emp.* 67, 463–464. doi: 10.1111/j.1471-0528.1960.tb07027.x

French, R. (1903). Epidemic sore throat and suppurative mammitis in cows. *Br. Med. J.* 2, 1492–1493. doi: 10.1136/bmj.2.2240.1492

Friis, H., and Andersen, H. K. (1982). Rate of inactivation of cytomegalovirus in raw banked milk during storage at -20 degrees C and pasteurisation. *Br. Med. J. (Clin. Res. Ed.)* 285, 1604–1605. doi: 10.1136/bmj.285.6355.1604

Galdiero, M., Larocca, F., Iovene, M. R., Martora, F., Pieretti, G., D'Oriano, V., et al. (2018). Microbial evaluation in capsular contracture of breast implants. *Plast. Reconstr. Surg.* 141 (1), 23–30. doi: 10.1097/PRS.0000000000003915

García-Gutierrez, E., O'Connor, P. M., Colquhoun, I. J., Vior, N. M., Rodríguez, J. M., Mayer, M. J., et al. (2020). Production of multiple bacteriocins, including the novel bacteriocin gassericin M, by *Lactobacillus gasseri* LM19, a strain isolated from human milk. *Appl. Microbiol. Biotechnol.* 104, 3869–3884. doi: 10.1007/s00253-020-10493-3

Gay, M., Koleva, P. T., Slupsky, C. M., Toit, E. D., Eggesbo, M., Johnson, C. C., et al. (2018). Worldwide variation in human milk metabolome: indicators of breast physiology and maternal lifestyle? *Nutrients* 10, 1151. doi: 10.3390/nu10091151

Gil-Campos, M., López, M. A., Rodríguez-Benítez, M. V., Romero, J., Roncero, I., Linares, M. D., et al. (2012). *Lactobacillus fermentum* CECT 5716 is safe and well tolerated in infants of 1-6 months of age: a randomized controlled trial. *Pharmacol. Res.* 65, 231–238. doi: 10.1016/j.phrs.2011.11.016

Goedert, J. J., Jones, G., Hua, X., Xu, X., Yu, G., Flores, R., et al. (2015). Investigation of the association between the fecal microbiota and breast cancer in postmenopausal women: a population-based case-control pilot study. *J. Natl. Cancer Inst.* 107, djv147. doi: 10.1093/jnci/djv147

Goedert, J. J., Hua, X., Bielecka, A., Okayasu, I., Milne, G. L., Jones, G. S., et al. (2018). Postmenopausal breast cancer and oestrogen associations with the IgA-coated and IgA-noncoated faecal microbiota. *Br. J. Cancer.* 118, 471–479. doi: 10.1038/bjc.2017.435

Gómez-Gallego, C., Garcia-Mantrana, I., Salminen, S., and Collado, M. C. (2016). The human milk microbiome and factors influencing its composition and activity. *Semin. Fetal Neonatal Med.* 21, 400–405. doi: 10.1016/j.siny.2016.05.003

Gómez-Gallego, C., Morales, J., Monleón, D., du Toit, E., Kumar, H., Linderborg, K., et al. (2018). Human breast milk NMR metabolomic profile across specific geographical locations and its association with the milk microbiota. *Nutrients* 10, 1355. doi: 10.3390/nu10101355

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., et al. (2014). Human genetics shape the gut microbiome. *Cell* 159, 789–799. doi: 10.1016/j.cell.2014.09.053

Grahn, N., Olofsson, M., Ellnebo-Svedlund, K., Monstein, H. J., and Jonasson, J. (2003). Identification of mixed bacterial DNA contamination in broad-range PCR amplification of 16S rDNA V1 and V3 variable regions by pyrosequencing of cloned amplicons. *FEMS Microbiol. Lett.* 219, 87–91. doi: 10.1016/S0378-1097(02)01190-4

Gransden, W. R., Webster, M., French, G. L., and Phillips, I. (1986). An outbreak of *Serratia marcescens* transmitted by contaminated breast pumps in a special care baby unit. *J. Hosp. Infect.* 7, 149–154. doi: 10.1016/0195-6701(86)90057-5

Grine, G., Boualam, M. A., and Drancourt, M. (2017). Methanobrevibacter smithii, a methanogen consistently colonising the newborn stomach. *Eur. J. Clin. Microbiol. Infect. Dis.* 36, 2449–2455. doi: 10.1007/s10096-017-3084-7

Grönlund, M. M., Gueimonde, M., Laitinen, K., Kociubinski, G., Grönroos, T., Salminen, S., et al. (2007). Maternal breast-milk and intestinal bifidobacteria guide the compositional development of the *Bifidobacterium* microbiota in infants at risk of allergic disease. *Clin. Exp. Allergy* 37, 1764–1772. doi: 10.1111/j.1365-2222.2007.02849.x

Gueimonde, M., Laitinen, K., Salminen, S., and Isolauri, E. (2007). Breast milk: A source of bifidobacteria for infant gut development and maturation? *Neonatology* 92, 64–66. doi: 10.1159/000100088

Gueimonde, M., Bottacini, F., van Sinderen, D., Ventura, M., Margolles, A., and Sánchez, B. (2012). Genome sequence of *Parascardovia denticolens* IPLA 20019, isolated from human breast milk. *J. Bacteriol.* 194, 4776–4777. doi: 10.1128/JB.01035-12

Haiden, N., and Ziegler, E. E. (2016). Human Milk Banking. *Ann. Nutr. Metab.* 69 (2), 8–15. doi: 10.1159/000452821

Hall, C. W., and Trout, G. M. (1968). *Milk pasteurization* (Westport, Connecticut: AVI Publishing Co.).

Hassiotou, F., and Geddes, D. T. (2015). Immune cell-mediated protection of the mammary gland and the infant during breastfeeding. *Adv. Nutr.* 6, 267–275. doi: 10.3945/an.114.007377

Hassiotou, F., Hepworth, A. R., Metzger, P., Lai, C.-T., Trengove, N., Hartmann, P. E., et al. (2013). Maternal and infant infections stimulate a rapid leukocyte response in breastmilk. *Clin. Transl. Immunol.* 2, e3. doi: 10.1038/cti.2013.1

Hayes, K., Danks, D. M., Gibas, H., and Jack, I. (1972). Cytomegalovirus in human milk. *N. Engl. J. Med.* 287, 177–178. doi: 10.1056/NEJM197207272870407

Heikkilä, M. P., and Saris, P. E. J. (2003). Inhibition of *Staphylococcus aureus* by the commensal bacteria of human milk. *J. Appl. Microbiol.* 95, 471–478. doi: 10.1046/j.1365-2672.2003.02002.x

Heisel, T., Nyaribo, L., Sadowsky, M. J., and Gale, C. A. (2019). Breastmilk and NICU surfaces are potential sources of fungi for infant mycobiomes. *Fungal Gen. Biol.* 128, 29–35. doi: 10.1016/j.fgb.2019.03.008

Hermansson, H., Kumar, H., Collado, M. C., Salminen, S., Isolauri, E., and Rautava, S. (2019). Breast milk microbiota is shaped by mode of delivery and intrapartum antibiotic exposure. *Front. Nutr.* 6, 4. doi: 10.3389/fnut.2019.00004

Hieken, T. J., Chen, J., Hoskin, T. L., Walther-Antonio, M., Johnson, S., Ramaker, S., et al. (2016). The microbiome of aseptically collected human breast tissue in benign and malignant disease. *Sci. Rep.* 6, 30751. doi: 10.1038/srep30751

Ho, N. T., Li, F., Lee-Sarwar, K. A., Tun, H. M., Brown, B. P., Pannaraj, P. S., et al. (2018). Meta-analysis of effects of exclusive breastfeeding on infant gut microbiota across populations. *Nat. Comm* 9, 4169. doi: 10.1038/s41467-018-06473-x

Hoashi, M., Meche, L., Mahal, L. K., Bakacs, E., Nardella, D., Naftolin, F., et al. (2016). Human milk bacterial and glycosylation patterns differ by delivery mode. *Reprod. Sci.* 23, 902–907. doi: 10.1177/1933719115623645

Holsinger, V. H., Rajkowski, K. T., and Stabel, J. R. (1997). Milk pasteurisation and safety: a brief history and update. *Rev. Sci. Tech.* 16, 441–451. doi: 10.20506/rst.16.2.1037

Hornung, B. V. H., Zwittink, R. D., and Kuijper, E. J. (2019). Issues and current standards of controls in microbiome research. *FEMS Microbiol. Ecol.* 95, pii: fiz045. doi: 10.1093/femsec/fiz045

Hu, H., Jacombs, A., Vickery, K., Merten, S. L., Pennington, D. G., and Deva, A. K. (2015). Chronic biofilm infection in breast implants is associated with an

increased T-cell lymphocytic infiltrate: implications for breast implant-associated lymphoma. *Plast. Reconstr. Surg.* 135, 319–329. doi: 10.1097/PRS.0000000000000886

Hu, H., Johani, K., Almatroudi, A., Vickery, K., Van Natta, B., Kadin, M. E., et al. (2016). Bacterial biofilm infection detected in breast implant-associated anaplastic large-cell lymphoma. *Plast. Reconstr. Surg.* 137, 1659–1669. doi: 10.1097/PRS.0000000000002010

Hunt, K. M., Foster, J. A., Forney, L. J., Schütte, U. M. E., Beck, D. L., Abdo, Z., et al. (2011). Characterization of the diversity and temporal stability of bacterial communities in human milk. *PloS One* 6, e21313. doi: 10.1371/journal.pone.0021313

Hunt, K. M., Preuss, J., Nissan, C., Davlin, C. A., Williams, J. E., Shafii, B., et al. (2012). Human milk oligosaccharides promote the growth of staphylococci. *Appl. Environ. Microbiol.* 78, 4763–4770. doi: 10.1128/AEM.00477-12

Hurtado, J. A., Maldonado-Lobón, J. A., Díaz-Ropero, M. P., Flores-Rojas, K., Uberos, J., Leante, J. L., et al. (2017). Oral administration to nursing women of *Lactobacillus fermentum* CECT5716 Prevents lactational mastitis development: a randomized controlled trial. *Breastfeed. Med.* 12, 202–209. doi: 10.1089/bfm.2016.0173

Iwase, T., Uehara, Y., Shinji, H., Tajima, A., Seo, H., Takada, K., et al. (2010). *Staphylococcus epidermidis* Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization. *Nature* 465, 346–349. doi: 10.1038/nature09074

Jeurink, P. V., van Bergenhenegouwen, J., Jimenez, E., Knippels, L. M., Fernandez, L., Garssen, J., et al. (2013). Human milk: a source of more life than we imagine. *Benef. Microbes* 4, 17–30. doi: 10.3920/BM2012.0040

Jiménez, E., Delgado, S., Maldonado, A., Arroyo, R., Albujar, M., García, N., et al. (2008a). *Staphylococcus epidermidis*: A differential trait of the fecal microbiota of breast-fed infants. *BMC Microbiol.* 8, 143. doi: 10.1186/1471-2180-8-143

Jiménez, E., Delgado, S., Fernández, L., García, N., Albújar, M., Gómez, A., et al. (2008b). Assessment of the bacterial diversity of human colostrum and screening of staphylococcal and enterococcal populations for potential virulence factors. *Res. Microbiol.* 159, 595–601. doi: 10.1016/j.resmic.2008.09.001

Jiménez, E., Fernández, L., Maldonado, A., Martin, R., Olivares, M., Xaus, J., et al. (2008c). Oral administration of lactobacillus strains isolated from breast milk as an alternative for the treatment of infectious mastitis during lactation. *Appl. Environ. Microbiol.* 74, 4650–4655. doi: 10.1128/AEM.02599-07

Jiménez, E., Langa, S., Martín, V., Arroyo, R., Martín, R., Fernández, L., et al. (2010a). Complete genome sequence of *Lactobacillus fermentum* CECT 5716, a probiotic strain isolated from human milk. *J. Bacteriol.* 192, 4800. doi: 10.1128/JB.00702-10

Jiménez, E., Martín, R., Maldonado, A., Martín, V., Gomez de Segura, A., Fernández, L., et al. (2010b). Complete genome sequence of *Lactobacillus salivarius* CECT5713, a probiotic strain isolated from human milk and infant feces. *J. Bacteriol.* 192, 5266–5267. doi: 10.1128/JB.00703-10

Jiménez, E., Villar-Tajadura, M. A., Marín, M., Fontecha, J., Requena, T., Arroyo, R., et al. (2012). Complete genome sequence of *Bifidobacterium breve* CECT 7263, a strain isolated from human milk. *J. Bacteriol.* 194, 3762–3763. doi: 10.1128/JB.00691-12

Jiménez, E., de Andrés, J., Manrique, M., Pareja-Tobes, P., Tobes, R., Martínez-Blanch, J. F., et al. (2015). Metagenomic Analysis of milk of healthy and mastitis-suffering women. *J. Hum. Lact.* 31, 406–415. doi: 10.1177/0890334415585078

Jiménez, E., Arroyo, R., Cárdenas, N., Marín, M., Serrano, P., Fernández, L., et al. (2017). Mammary candidiasis, a medical condition without scientific evidence? *PloS One* 12, e0181071. doi: 10.1371/journal.pone.0181071

Jones, C. L., Jennison, R. F., and D'Souza, S. W. (1979). Bacterial contamination of expressed breast milk. *Br. Med. J.* 2, 1320–1322. doi: 10.1136/bmj.2.6201.1320

Jones, F. S. (1918). Studies in bovine mastitis: II. The relation of hemolytic streptococci to udder infections. *J. Exp. Med.* 28, 253–267. doi: 10.1084/jem.28.3.253

Jost, T., Lacroix, C., Braegger, C., and Chassard, C. (2013). Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. *Br. J. Nutr.* 110, 1253–1262. doi: 10.1017/S0007114513000597

Jost, T., Lacroix, C., Braegger, C. P., Rochat, F., and Chassard, C. (2014). Vertical mother-neonate transfer of maternal gut bacteria via breastfeeding. *Environ. Microbiol.* 16, 2891–2904. doi: 10.1111/1462-2920.12238

Jost, T., Lacroix, C., Braegger, C., and Chassard, C. (2015). Impact of human milk bacteria and oligosaccharides on neonatal gut microbiota establishment and gut health. *Nutr. Rev.* 73, 426–437. doi: 10.1093/nutrit/nuu016

Karstens, L., Asquith, M., Davin, S., Fair, D., Gregory, W. T., Wolfe, A. J., et al. (2019). Controlling for contaminants in low-biomass 16S rRNA gene sequencing experiments. *mSystems* 4, e00290–e00219. doi: 10.1128/mSystems.00290-19

Kayıran, P. G., Can, F., Kayıran, S. M., Ergonul, O., and Gürakan, B. (2014). Transmission of methicillin-sensitive *Staphylococcus aureus* to a preterm infant through breast milk. *J. Matern. Fetal Neonatal Med.* 27, 527–529. doi: 10.3109/14767058.2013.819332

Kenny, J. F. (1977). Recurrent group B streptococcal disease in an infant associated with the ingestion of infected mother's milk. *J. Pediatr.* 91, 158–159. doi: 10.1016/S0022-3476(77)80473-3

Khodayar-Pardo, P., Mira-Pascual, L., Collado, M. C., and Martínez-Costa, C. (2014). Impact of lactation stage, gestational age and mode of delivery on breast milk microbiota. *J. Perinatol.* 34, 599–605. doi: 10.1038/jp.2014.47

Kirjavainen, P. V., Apostolou, E., Arvola, T., Salminen, S. J., Gibson, G. R., and Isolauri, E. (2001). Characterizing the composition of intestinal microflora as a prospective treatment target in infant allergic disease. *FEMS Immunol. Med. Microbiol.* 32, 1–7. doi: 10.1111/j.1574-695X.2001.tb00526.x

Klann, E., Williamson, J. M., Tagliamonte, M. S., Ukhanova, M., Asirvatham, J. R., Chim, H., et al. (2020). Microbiota composition in bilateral healthy breast tissue and breast tumors. *Cancer Causes Control.* 31, 1027–1038. doi: 10.1007/s10552-020-01338-5

Knoop, U., Matheis, G., and Schütt-Gerowitt, H. (1985). [Bacterial contamination of pump-collected breast milk]. *Monatsschr Kinderheilkd.* 133, 537–541.

Kordy, K., Gaufin, T., Mwangi, M., Li, F., Cerini, C., Lee, D. J., et al. (2020). Contributions to human breast milk microbiome and enteromammary transfer of *Bifidobacterium breve*. *PloS One* 15, e0219633. doi: 10.1371/journal.pone.0219633

Kourtis, A. P., Butera, S., Ibegbu, C., Belec, L., and Duerr, A. (2003). Breast milk and HIV-1: vector of transmission or vehicle of protection? *Lancet Infect. Dis.* 3, 786–793. doi: 10.1016/s1473-3099(03)00832-6

Kovács, T., Mikó, E., Ujlaki, G., Sári, Z., and Bai, P. (2020). The microbiome as a component of the tumor microenvironment. *Adv. Exp. Med. Biol.* 1225, 137–153. doi: 10.1007/978-3-030-35727-6_10

Kozak, K., Charbonneau, D., Sanozky-Dawes, R., and Klaenhammer, T. (2015). Characterization of bacterial isolates from the microbiota of mothers' breast milk and their infants. *Gut Microbes* 6, 341. doi: 10.1080/19490976.2015.1103425

Krieg, H. (1966). The bacterial content of raw and freeze-dried women's milk. *Med. Klin.* 61, 953–956.

Kuehn, J., Kuhn, L., Kim, H. Y., Walter, J., Thea, D. M., Sinkala, M., et al. (2013). HIV-1 concentrations in human breast milk before and after weaning. *Sci. Trans. Med.* 5, 181ra51. doi: 10.1126/scitranslmed.3005113

Kumar, H., du Toit, E., Kulkarni, A., Aakko, J., Linderborg, K. M., Zhang, Y., et al. (2016). Distinct patterns in human milk microbiota and fatty acid profiles across specific geographic locations. *Front. Microbiol.* 7, 1619. doi: 10.3389/fmicb.2016.01619

Laborda-Illanes, A., Sanchez-Alcoholado, L., Dominguez-Recio, M. E., Jimenez-Rodriguez, B., Lavado, R., Comino-Méndez, I., et al. (2020). Breast and gut microbiota action mechanisms in breast cancer pathogenesis and treatment. *Cancers* 12, E2465. doi: 10.3390/cancers12092465

Lackey, K. A., Williams, J. E., Meehan, C. L., Zachek, J. A., Benda, E. D., Price, W. J., et al. (2019). What's Normal? Microbiomes in human milk and infant feces are related to each other but vary geographically: The INSPIRE study. *Front. Nutr.* 6, 45. doi: 10.3389/fnut.2019.00045

Lagier, J. C., Million, M., Hugon, P., Armougom, F., and Raoult, D. (2012). Human gut microbiota: repertoire and variations. *Front. Cell Infect. Microbiol.* 2, 136. doi: 10.3389/fcimb.2012.00136

Lagier, J. C., Hugon, P., Khelaifia, S., Fournier, P. E., La Scola, B., and Raoult, D. (2015). The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. *Clin. Microbiol. Rev.* 28, 237–264. doi: 10.1128/CMR.00014-14

Lagier, J. C., Dubourg, G., Million, M., Cadoret, F., Bilen, M., Fenollar, F., et al. (2018). Culturing the human microbiota and culturomics. *Nat. Rev. Microbiol.* 1, 540–550. doi: 10.1038/s41579-018-0041-0

Langa, S., Maldonado-Barragán, A., Delgado, S., Martín, R., Martín, V., Jiménez, E., et al. (2012). Characterization of *Lactobacillus salivarius* CECT 5713, a strain isolated from human milk: from genotype to phenotype. *Appl. Microbiol. Biotechnol.* 94, 1279–1287. doi: 10.1007/s00253-012-4032-1

Langa, S. (2006). *Interactions between lactic acid bacteria, intestinal epithelial cells and immune cells. Development of in vitro models. PhD Thesis* (Madrid: Complutense University of Madrid).

Lara-Villoslada, F., Sierra, S., Díaz-Ropero, M. P., Olivares, M., and Xaus, J. (2007). Safety assessment of the human milk-isolated probiotic *Lactobacillus salivarius* CECT5713. *J. Dairy Sci.* 90, 3583–3589. doi: 10.3168/jds.2006-685

Lara-Villoslada, F., Sierra, S., Díaz-Ropero, M. P., Rodríguez, J. M., Xaus, J., and Olivares, M. (2009). Safety assessment of *Lactobacillus fermentum* CECT5716, a probiotic strain isolated from human milk. *J. Dairy Res.* 76, 216–221. doi: 10.1017/S0022029909004014

Lau, J. T., Whelan, F. J., Herath, I., Lee, C. H., Collins, S. M., Bercik, P., et al. (2016). Capturing the diversity of the human gut microbiota through culture-enriched molecular profiling. *Genome Med.* 8, 72. doi: 10.1186/s13073-016-0327-7

Lauder, A. P., Roche, A. M., Sherrill-Mix, S., Bailey, A., Laughlin, A. L., Bittinger, K., et al. (2016). Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. *Microbiome* 4, 29. doi: 10.1186/s40168-016-0172-3

Laurence, M., Hatzis, C., and Brash, D. E. (2014). Common contaminants in next-generation sequencing that hinder discovery of low-abundance microbes. *PloS One* 9, e97876. doi: 10.1371/journal.pone.0097876

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., et al. (2013). Richness of human gut microbiome correlates with metabolic markers. *Nature* 500, 541–546. doi: 10.1038/nature12506

Lemas, D. J., Young, B. E., Baker, P. R., Tomczik, A. C., Soderborg, T. K., Hernandez, T. L., et al. (2016). Alterations in human milk leptin and insulin are associated with early changes in the infant intestinal microbiome. *Am. J. Clin. Nutr.* 103, 1291–1300. doi: 10.3945/ajcn.115.126375

LeMay-Nedjelski, L., Copeland, J., Wang, P. W., Butcher, J., Unger, S., Stintzi, A., et al. (2018). Methods and strategies to examine the human breastmilk microbiome. *Methods Mol. Biol.* 1849, 63–86. doi: 10.1007/978-1-4939-8728-3_5

Lemoine, L. (1987). Possible transmission of methicillin-resistant *Staphylococcus aureus* by expressed human breast milk. *J. Hosp. Infect.* 9, 93–94. doi: 10.1016/0195-6701(87)90105-8

Lewis, Z. T., Totten, S. M., Smilowitz, J. T., Popovic, M., Parker, E., Lemay, D. G., et al. (2015). Maternal fucosyl transferase 2 status affects the gut bifidobacterial communities of breastfed infants. *Microbiome* 3, 13. doi: 10.1186/s40168-015-0071-z

Li, Y., Teague, E., Zhuang, Z., and Caufield, P. W. (1997). Screening for the *spaP* gene of *Streptococcus mutans* in predentate infants. *J. Dental Res.* 76, 102.

Li, S.-W., Watanabe, K., Hsu, C.-C., Chao, S.-H., Yang, Z.-H., Lin, Y.-J., et al. (2017). Bacterial composition and diversity in breast milk samples from mothers living in Taiwan and mainland China. *Front. Microbiol.* 8, 965. doi: 10.3389/fmicb.2017.00965

Liebhaber, M., Lewiston, N. J., Asquith, M. T., and Sunshine, P. (1978). Comparison of bacterial contamination with two methods of human milk collection. *J. Pediatr.* 92, 236–237. doi: 10.1016/S0022-3476(78)80014-6

Lindemann, G., and Rupp, H. (1954). [Bacteriological control test in human milk collection center]. *Zentralbl. Gynakol.* 76, 1167–1176.

Lindemann, G. (1955). [Possibilities of improving the bacteriologic quality of human milk]. *Zentralbl. Gynakol.* 77, 1383–1389.

Lindemann, G. (1958). [Studies on samples from human milk banks]. *Monatsschr. Kinderheilkd.* 106, 52–55.

Liu, G., Pang, B., Li, N., Jin, H., Li, J., Wu, W., et al. (2020). Therapeutic effect of *Lactobacillus rhamnosus* SHA113 on intestinal infection by multi-drug-resistant *Staphylococcus aureus* and its underlying mechanisms. *Food Funct.* 11, 6226–6239. doi: 10.1039/d0fo00969e

Louca, S., Doebeli, M., and Parfrey, L. W. (2018). Correcting for 16S rRNA gene copy numbers in microbiome surveys remains an unsolved problem. *Microbiome* 6, 41. doi: 10.1186/s40168-018-0420-9

Lucas, A., and Roberts, C. D. (1978). Group B streptococci in pooled human milk. *Br. Med. J.* 1 (6117), 919–920. doi: 10.1136/bmj.1.6117.919-b

Lucas, A., and Roberts, C. D. (1979). Bacteriological quality control in human milk-banking. *Br. Med. J.* 1 (6156), 80–82. doi: 10.1136/bmj.1.6156.80

Lugli, G. A., Milani, C., Turroni, F., Tremblay, D., Ferrario, C., Mancabelli, L., et al. (2016). Prophages of the genus *Bifidobacterium* as modulating agents of the infant gut microbiota. *Environ. Microbial.* 18, 2196–2213. doi: 10.1111/1462-2920.13154

Lugli, G. A., Duranti, S., Milani, C., Mancabelli, L., Turroni, F., Alessandri, G., et al. (2020). Investigating bifidobacteria and human milk oligosaccharide composition of lactating mothers. *FEMS Microbiol. Ecol.* 96, fiaa049. doi: 10.1093/femsec/fiaa049

Ma, Z. S., Guan, Q., Ye, C., Zhang, C., Foster, J. A., and Forney, L. J. (2015). Network analysis suggests a potentially 'evil' alliance of opportunistic pathogens inhibited by a cooperative network in human milk bacterial communities. *Sci. Rep.* 5, 8275. doi: 10.1038/srep08275

Ma, Z. S., Li, L., Li, W., Li, J., and Chen, H. (2016). Integrated network-diversity analyses suggest suppressive effect of Hodgkin's lymphoma and slightly relieving effect of chemotherapy on human milk microbiome. *Sci. Rep.* 6, 28048. doi: 10.1038/srep28048

Macfadyen, A. (1891). Observations on a Mastitis *Bacillus*. *J. Anat. Physiol.* 25, 571–577.

Macpherson, A. J., and Uhr, T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303, 1662–1665. doi: 10.1126/science.1091334

Makino, H., Kushiro, A., Ishikawa, E., Muylaert, D., Kubota, H., Sakai, T., et al. (2011). Transmission of intestinal *Bifidobacterium longum* subsp. *longum* strains from mother to infant, determined by multilocus sequencing typing and amplified fragment length polymorphism. *Appl. Environ. Microbiol.* 77, 6788–6793. doi: 10.1128/AEM.05346-11

Makino, H., Martin, R., Ishikawa, E., Gawad, A., Kubota, H., Sakai, T., et al. (2015). Multilocus sequence typing of bifidobacterial strains from infant's faeces and human milk: are bifidobacteria being sustainably shared during breastfeeding? *Benef. Microbes* 6, 563–572. doi: 10.3920/BM2014.0082

Maldonado, J., Lara-Villoslada, F., Sierra, S., Sempere, L., Gómez, M., Rodríguez, J. M., et al. (2010). Safety and tolerance of the human milk probiotic strain *Lactobacillus salivarius* CECT5713 in 6-month-old children. *Nutrition* 26, 1082–1087. doi: 10.1016/j.nut.2009.08.023

Maldonado, J., Cañabate, F., Sempere, L., Vela, F., Sánchez, A. R., Narbona, E., et al. (2012). Human milk probiotic *Lactobacillus fermentum* CECT5716 reduces the incidence of gastrointestinal and upper respiratory tract infections in infants. *J. Pediatr. Gastroenterol. Nutr.* 54, 55–61. doi: 10.1097/MPG.0b013e3182333f18

Maldonado-Lobón, J. A., Gil-Campos, M., Maldonado, J., López-Huertas, E., Flores-Rojas, K., Valero, A. D., et al. (2015a). Long-term safety of early consumption of *Lactobacillus fermentum* CECT5716: A 3-year follow-up of a randomized controlled trial. *Pharmacol. Res.*, 95–96, 12-9. doi: 10.1016/j.phrs.2015.01.006

Maldonado-Lobón, J. A., Díaz-López, M. A., Carputo, R., Duarte, P., Díaz-Ropero, M. P., Valero, A. D., et al. (2015b). *Lactobacillus fermentum* CECT 5716 reduces *Staphylococcus* load in the breastmilk of lactating mothers suffering breast pain: a randomized controlled trial. *Breastfeed. Med.* 10, 425–432. doi: 10.1089/bfm.2015.0070

Marín, M. L., Arroyo, R., Jiménez, E., Gómez, A., Fernández, L., and Rodríguez, J. M. (2009). Cold storage of human milk, effect on its bacterial composition. *J. Ped. Gastroenterol. Nutr.* 49, 343–348. doi: 10.1097/MPG.0b013e31818cf53d

Marín, M., Arroyo, R., Espinosa-Martos, I., Fernández, L., and Rodríguez, J. M. (2017). Identification of emerging human mastitis pathogens by MALDI-TOF and assessment of their antibiotic resistance patterns. *Front. Microbiol.* 8, 1258. doi: 10.3389/fmicb.2017.01258

Martin, R., Langa, S., Reviriego, C., Jiminez, E., Marin, M. L., Xaus, J., et al. (2003). Human milk is a source of lactic acid bacteria for the infant gut. *J. Pediatr.* 143, 754–758. doi: 10.1016/j.jpeds.2003.09.028

Martín, R., Langa, S., Reviriego, C., Jiménez, E., Marín, M., Olivares, M., et al. (2004). The commensal microflora of human milk: new perspectives for food bacteriotherapy and probiotics. *Trends Food Sci. Technol.* 15, 121–127. doi: 10.1016/j.tifs.2003.09.010

Martín, R., Olivares, M., Marín, M. L., Fernández, L., Xaus, J., and Rodríguez, J. M. (2005). Probiotic potential of 3 lactobacilli strains isolated from breast milk. *J. Hum. Lact.* 21, 8–17. doi: 10.1177/0890334404272393

Martín, R., Jiménez, E., Olivares, M., Marín, M. L., Fernández, L., Xaus, J., et al. (2006). *Lactobacillus salivarius* CECT 5713, a potential probiotic strain isolated from infant feces and breast milk of a mother-child pair. *Int. J. Food Microbiol.* 112, 35–43. doi: 10.1016/j.ijfoodmicro.2006.06.011

Martín, R., Heilig, G. H. J., Zoetendal, E. G., Jiménez, E., Fernández, L., Smidt, H., et al. (2007a). Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. *Res. Microbiol;* 158, 31–37. doi: 10.1016/j.resmic.2006.11.004

Martín, R., Heilig, G. H. J., Zoetendal, E. G., Smidt, H., and Rodríguez, J. M. (2007b). Diversity of the *Lactobacillus* group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. *J. Appl. Microbiol.* 103, 2638–2644. doi: 10.1111/j.1365-2672.2007.03497.x

Martín, R., Jiménez, E., Heilig, H., Fernandez, L., Marin, M. L., Zoetendal, E. G., et al. (2009). Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-DGGE and qRTi-PCR. *Appl. Environ. Microbiol.* 75, 965–969. doi: 10.1128/AEM.02063-08

Martín, V., Maldonado, A., Fernández, L., Rodríguez, J. M., and Connor, R. I. (2010). Inhibition of human immunodeficiency virus type 1 by lactic acid bacteria from human breast milk. *Breastfeed. Med.* 5, 153–158. doi: 10.1089/bfm.2010.0001

Martín, V., Mañés-Lázaro, R., Rodríguez, J. M., and Maldonado, A. (2011). *Streptococcus lactarius* sp. nov., isolated from breast milk of healthy women. *Int. J. Syst. Evol. Microbiol.* 61, 1048–1052. doi: 10.1099/ijs.0.021642-0

Martín, V., Maldonado, A., Jiménez, E., Ruas-Madiedo, P., Fernández, L., and Rodríguez, J. M. (2012a). Complete genome sequence of *Streptococcus salivarius* PS4, a strain isolated from human milk. *J. Bacteriol.* 194, 4466–4467. doi: 10.1128/JB.00896-12

Martín, V., Maldonado, A., Moles, L., Rodríguez-Baños, M., Del Campo, R., Fernández, L., et al. (2012b). Sharing of bacterial strains between breast milk and infant feces. *J. Hum. Lact.* 28, 36–44. doi: 10.1177/0890334411424729

Martín, V., Cárdenas, N., Jiménez, E., Maldonado, A., Rodríguez, J. M., and Fernández, L. (2013). Genome sequence of *Lactobacillus gastricus* PS3, a strain isolated from human milk. *Genome Announc.* 1, e00489–e00413. doi: 10.1128/genomeA.00489-13

Martín, V., Mediano, P., Del Campo, R., Rodríguez, J. M., and Marín, M. (2015). Streptococcal diversity of human milk and comparison of different methods for the taxonomic identification of streptococci. *J. Hum. Lact.* 32, NP84–NP94. doi: 10.1177/0890334415597901

Martín, R., Makino, H., Cetinyurek Yavuz, A., Ben-Amor, K., Roelofs, M., Ishikawa, E., et al. (2016). Early-life events, including mode of delivery and type of feeding, siblings and gender, shape the developing gut microbiota. *PloS One* 11, e0158498. doi: 10.1371/journal.pone.0158498

Mastromarino, P., Capobianco, D., Miccheli, A., Praticò, G., Campagna, G., Laforgia, N., et al. (2015). Administration of a multistrain probiotic product (VSL#3) to women in the perinatal period differentially affects breast milk beneficial microbiota in relation to mode of delivery. *Pharmacol. Res.* 95-96, 63–70. doi: 10.1016/j.phrs.2015.03.013

McGuire, M. K., and McGuire, M. A. (2015). Human milk: mother nature's prototypical probiotic food? *Adv. Nutr.* 6, 112–123. doi: 10.3945/an.114.007435

McGuire, M. K., and McGuire, M. A. (2017). Got bacteria? The astounding, yet not-so-surprising, microbiome of human milk. *Curr. Opin. Biotechnol.* 44, 63–68. doi: 10.1016/j.copbio.2016.11.013

Mediano, P., Fernández, L., Jiménez, E., Arroyo, R., Espinosa-Martos, I., Rodríguez, J. M., et al. (2017). Microbial diversity in milk of women with mastitis: potential role of coagulase-negative staphylococci, viridans group streptococci, and corynebacteria. *J. Hum. Lact.* 33, 309–318. doi: 10.1177/0890334417692968

Mestecky, J. (2020). The mammary gland as an integral component of the common mucosal immune system. *Nestle Nutr. Inst. Workshop Ser.* 94, 27–37. doi: 10.1159/000505336

Meyer, J., and Potel, J. (1956). [Qualitative improvement of human milk by bacteriological studies]. *Dtsch. Gesundheitsw.* 11, 1254–1256.

Milani, C., Mancabelli, L., Lugli, G. A., Duranti, S., Turroni, F., Ferrario, C., et al. (2015). Exploring vertical transmission of bifidobacteria from mother to child. *Appl. Environ. Microbiol.* 81, 7078–7087. doi: 10.1128/AEM.02037-15

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., et al. (2017a). The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiol. Mol. Biol. Rev.* 81, e00036–e00017. doi: 10.1128/MMBR.00036-17

Milani, C., Mangifesta, M., Mancabelli, L., Lugli, G. A., James, K., Duranti, S., et al. (2017b). Unveiling bifidobacterial biogeography across the mammalian branch of the tree of life. *ISME J.* 11, 2834–2847. doi: 10.1038/ismej.2017.138

Mira, A., and Rodriguez, J. (2017). "The origin of human milk bacteria," in *Prebiotics and Probiotics in Human Milk: Origins and Functions of Milk-Borne Oligosaccharides and Bacteria*. Eds. M. McGuire, M. McGuire and L. Bode (London: Academic Press), 349–364.

Mocquot, G. (1955). Technical and hygienic aspects of the problem of milk destined for human consumption]. *Ann. Nutr. Aliment.* 9, 251–270.

Mohandas, S., and Pannaraj, P. S. (2020). Beyond the bacterial microbiome: virome of human milk and effects on the developing Infant. *Nestle Nutr. Inst. Workshop Ser.* 94, 86–93. doi: 10.1159/000504997

Moloney, A. C., Quoraishi, A. H., Parry, P., and Hall, V. (1987). A bacteriological examination of breast pumps. *J. Hosp. Infect.* 9, 169–174. doi: 10.1016/0195-6701(87)90056-9

Moossavi, S., Sepehri, S., Robertson, B., Bode, L., Goruk, S., Field, C. J., et al. (2019a). Composition and variation of the human milk microbiota are influenced by maternal and early-life factors. *Cell Host Microbe* 25, 324–335.e4. doi: 10.1016/j.chom.2019.01.011

Moossavi, S., Atakora, F., Miliku, K., Sepehri, S., Robertson, B., Duan, Q. L., et al. (2019b). Integrated analysis of human milk microbiota with oligosaccharides and fatty acids in the CHILD Cohort. *Front. Nutr.* 6, 58. doi: 10.3389/fnut.2019.00058

Morrow, A. L., and Rangel, J. M. (2004). Human milk protection against infectious diarrhea: implications for prevention and clinical care. *Semin. Pediatr. Infect. Dis.* 15, 221–228. doi: 10.1053/j.spid.2004.07.002

Mossel, D. A., and Weijers, H. A. (1957). [Results of bacteriological analysis of human milk & its significance for pediatric practice]. *Maandschr. Kindergeneeskd.* 25, 37–63.

Mühl, H., Kochem, A.-J., and Disqué, C. S. S. (2010). Activity and DNA contamination of commercial polymerase chain reaction reagents for the universal 16SrDNA real-time polymerase chain reaction detection of bacterial pathogens in blood. *Diagn. Microbiol. Infect. Dis.* 66, 41–49. doi: 10.1016/j.diagmicrobio.2008.07.011

Murphy, K., Curley, D., O'Callaghan, T. F., O'Shea, C. A., Dempsey, E. M., O'Toole, P. W., et al. (2017). The composition of human milk and infant fecal microbiota over the first three months of life: A pilot study. *Sci. Rep.* 7, 40597. doi: 10.1038/srep40597

Newburg, D. S. (2005). Innate immunity and human milk. *J. Nutr.* 135, 1308–1312. doi: 10.1093/jn/135.5.1308

Niimi, K., Usami, K., Fujita, Y., Abe, M., Furukawa, M., Suyama, Y., et al. (2018). Development of immune and microbial environments is independently regulated in the mammary gland. *Mucosal Immunol.* 11, 643–653. doi: 10.1038/mi.2017.90

Nikniaz, L., Ostadrahimi, A., Mahdavi, R., Hejazi, M. A., and Hosseini Salekdeh, G. (2013). Effects of synbiotic supplementation on breast milk levels of IgA, TGF-β1, and TGF-β2. *J. Hum. Lact.* 29, 591–596. doi: 10.1177/0890334413490833

Novel, E., and Pongratz, E. (1954). [Three years of bacteriological analysis of the human milk]. *Mitt. Geb. Lebensmittelunters Hyg.* 45, 33–37.

Oh, J., Byrd, A. L., Deming, C., Conlan, S., NISC Comparative Sequencing Program, Kong, H. H., et al. (2014). Biogeography and individuality shape function in the human skin metagenome. *Nature* 514, 59–64. doi: 10.1038/nature13786

Oikonomou, G., Bicalho, M. L., Meira, E., Rossi, R. E., Foditsch, C., Machado, V. S., et al. (2014). Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. *PloS One* 9, e85904. doi: 10.1371/journal.pone.0085904

Ojo-Okunola, A., Nicol, M., and du Toit, E. (2018). Human breast milk bacteriome in health and disease. *Nutrients* 10, 1643. doi: 10.3390/nu10111643

Ojo-Okunola, A., Cacciatore, S., Nicol, M. P., and du Toit, E. (2020). The determinants of the human milk metabolome and its role in infant health. *Metabolites* 10, 77. doi: 10.3390/metabo10020077

Olivares, M., Díaz-Ropero, M. P., Martín, R., Rodríguez, J. M., and Xaus, J. (2006a). Antimicrobial potential of four *Lactobacillus* strains isolated from breast milk. *J. Appl. Microbiol.* 101, 72–79. doi: 10.1111/j.1365-2672.2006.02981.x

Olivares, M., Díaz-Ropero, M. P., Gómez, N., Lara-Villoslada, F., Sierra, S., Maldonado, J. A., et al. (2006b). The consumption of two new probiotic strains, *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711, boost the immune system of healthy adults. *Int. Microbiol.* 9, 47–52. doi: 10.1016/j.ijfoodmicro.2005.08.019

Olivares, M., Díaz-Ropero, M. P., Gómez, N., Lara-Villoslada, F., Sierra, S., Maldonado, J. A., et al. (2006c). Oral administration of two probiotic strains, *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711, enhances the intestinal function of healthy adults. *Int. J. Food Microbiol.* 107, 104–111. doi: 10.1016/j.ijfoodmicro.2005.08.019

Ottenheimer, E. J. Jr, Minchew, B. H., Cohen, L. S., and Cluff, L. E. (1961). Studies of the epidemiology of staphylococcal infection. II. Isolation of staphylococci from the milkof nursing mothers under non-epidemic conditions. *Bull. Johns Hopkins Hosp.* 109, 114–123.

Otto, M. (2009). *Staphylococcus epidermidis*, the 'accidental' pathogen. *Nat. Rev. Microbiol.* 7, 555–567. doi: 10.1038/nrmicro2182

Ouwehand, A. C., Isolauri, E., He, F., Hashimoto, H., Benno, Y., and Salminen, S. (2001). Differences in *Bifidobacterium* flora composition in allergic and healthy infants. *J. Allergy Clin. Immunol.* 108, 144–145. doi: 10.1067/mai.2001.115754

Pabst, R. (1987). The anatomical basis for the immune function of the gut. *Anat. Embryol. (Berl)* 176, 135–144. doi: 10.1007/BF00310046

Padilha, M., Danneskiold-Samsøe, N. B., Brejnrod, A., Hoffmann, C., Cabral, V. P., Iaucci, J. M., et al. (2019a). The human milk microbiota is modulated by maternal diet. *Microorganisms* 7, 502. doi: 10.3390/microorganisms7110502

Padilha, M., Iaucci, J. M., Cabral, V. P., Diniz, E. M. A., Taddei, C. R., and Saad, S. M. I. (2019b). Maternal antibiotic prophylaxis affects *Bifidobacterium* spp. counts in the human milk, during the first week after delivery. *Benef. Microbes* 10, 155–163. doi: 10.3920/BM2018.0046

Padilha, M., Brejnrod, A., Danneskiold-Samsøe, N. B., Hoffmann, C., de Melo Iaucci, J., Cabral, V. P., et al. (2020). Response of the human milk microbiota to a maternal prebiotic intervention is individual and influenced by maternal age. *Nutrients* 12, 1081. doi: 10.3390/nu12041081

Pajkos, A., Deva, A. K., Vickery, K., Cope, C., Chang, L., and Cossart, Y. E. (2003). Detection of subclinical infection in significant breast implant capsules. *Plast. Reconstr. Surg.* 111 (5), 1605–1611. doi: 10.1097/01.PRS.0000054768.14922.44

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A., and Brown, P. O. (2007). Development of the human infant intestinal microbiota. *PloS Biol.* 5, e177. doi: 10.1371/journal.pbio.0050177

Pannaraj, P. S., Li, F., Cerini, C., Bender, J. M., Yang, S., Rollie, A., et al. (2017). Association between breast milk bacterial communities and establishment and development of the infnat gut microbiome. *JAMA Pediatr.* 171, 647–654. doi: 10.1001/jamapediatrics.2017.0378

Pannaraj, P. S., Ly, M., Cerini, C., Saavedra, M., Aldrovandi, G. M., Saboory, A. A., et al. (2018). Shared and distinct features of human milk and infant stool viromes. *Front. Microbiol.* 9, 1162. doi: 10.3389/fmicb.2018.01162

Park, B., Iwase, T., and Liu, G. Y. (2011). Intranasal application of *S. epidermidis* prevents colonization by methicillin-resistant *Staphylococcus aureus* in mice. *PloS One* 6, e25880. doi: 10.1371/journal.pone.0025880

Park, W. H. (1901). The bacterial condition of city milk and the need of health authorities to prevent the sale of milk containing excessive numbers of bacteria. *J. Boston Soc Med. Sci.* 5, 370–371.

Pärnänen, K., Karkman, A., Hultman, J., Lyra, C., Bengtsson-Palme, J., Larsson, D., et al. (2018). Maternal gut and breast milk microbiota affect infant gut antibiotic resistome and mobile genetic elements. *Nat. Commun.* 9, 3891. doi: 10.1038/s41467-018-06393-w

Patel, S. H., Vaidya, Y. H., Patel, R. J., Pandit, R. J., Joshi, C. G., and Kunjadiya, A. P. (2017). Culture independent assessment of human milk microbial community in lactational mastitis. *Sci. Rep.* 7, 7804. doi: 10.1038/s41598-017-08451-7

Peran, L., Camuesco, D., Comalada, M., Nieto, A., Concha, A., Diaz-Ropero, M. P., et al. (2005). Preventative effects of a probiotic, *Lactobacillus salivarius* ssp. *salivarius*, in the TNBS model of rat colitis. *World J. Gastroenterol.* 11, 5185–5192. doi: 10.3748/wjg.v11.i33.5185

Peran, L., Camuesco, D., Comalada, M., Nieto, A., Concha, A., Adrio, J. L., et al. (2006). *Lactobacillus fermentum*, a probiotic capable to release glutathione, prevents colonic inflammation in the TNBS model of rat colitis. *Int. J. Colorectal Dis.* 21, 737–746. doi: 10.1007/s00384-005-0773-y

Peran, L., Sierra, S., Comalada, M., Lara-Villoslada, F., Bailón, E., Nieto, A., et al. (2007). A comparative study of the preventative effects exerted by two probiotics, *Lactobacillus reuteri* and *Lactobacillus fermentum*, in the trinitrobenzenesulfonic acid model of rat colitis. *Br. J. Nutr.* 97, 96–103. doi: 10.1017/S0007114507257770

Perez, P. F., Doré, J., Leclerc, M., Levenez, F., Benyacoub, J., Serrant, P., et al. (2007). Bacterial imprinting of the neonatal immune system: lessons from maternal cells? *Pediatrics* 119, e724–e732. doi: 10.1542/peds.2006-1649

Pérez-Cano, F. J., Dong, H., and Yaqoob, P. (2010). *In vitro* immunomodulatory activity of *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713: two probiotic strains isolated from human breast milk. *Inmunobiology* 12, 996–1004. doi: 10.1016/j.imbio.2010.01.004

Pichler, M. J., Yamada, C., Shuoker, B., Alvarez-Silva, C., Gotoh, A., Leth, M. L., et al. (2020). Butyrate producing colonic Clostridiales metabolise human milk oligosaccharides and cross feed on mucin via conserved pathways. *Nat. Commun.* 11, 3285. doi: 10.1038/s41467-020-17075-x

Praveen, P., Jordan, F., Priami, C., and Morine, M. J. (2015). The role of breast-feeding in infant immune sytem: a systems perspective on the intestinal microbiome. *Microbiome* 3, 41. doi: 10.1186/s40168-015-0104-7

Prentice, P. M., Schoemaker, M. H., Vervoort, J., Hettinga, K., Lambers, T. T., van Tol, E., et al. (2019). Human milk short-chain fatty acid composition is associated with adiposity outcomes in infants. *J. Nutr.* 149, 716–722. doi: 10.1093/jn/nxy320

Prescott, S. L., Wickens, K., Westcott, L., Jung, W., Currie, H., Black, P. N., et al. (2008). Supplementation with *Lactobacillus rhamnosus* or *Bifidobacterium lactis* probiotics in pregnancy increases cord blood interferon-gamma and breast milk transforming growth factor-beta and immunoglobin A detection. *Clin. Exp. Aller.* 38, 1606–1614. doi: 10.1111/j.1365-2222.2008.03061.x

Qutaishat, S. S., Stemper, M. E., Spencer, S. K., Borchardt, M. A., Opitz, J. C., Monson, T. A., et al. (2003). Transmission of *Salmonella enterica* serotype *typhimurium* DT104 to infants through mother's breast milk. *Pediatrics* 111, 1442–1446. doi: 10.1542/peds.111.6.1442

Ramani, S., Stewart, C. J., Laucirica, D. R., Ajami, N. J., Robertson, B., Autran, C. A., et al. (2018). Human milk oligosaccharides, milk microbiome and infant gut microbiome modulate neonatal rotavirus infection. *Nat. Commun.* 9, 5010. doi: 10.1038/s41467-018-07476-4

Ramsey, D. T., Kent, J. C., Owens, R. A., and Hartmann, P. E. (2004). Ultrasound imaging of milk ejection in the breast of lactating women. *Pediatrics* 113, 361–367. doi: 10.1542/peds.113.2.361

Rantasalo, I., and Kauppinen, M. A. (1959). The occurrence of *Staphylococcus aureus* in mother's milk. *Ann. Chir. Gynaecol. Fenn.* 48, 246–258.

Rautava, S., Kalliomäki, M., and Isolauri, E. (2002). Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. *J. Allergy Clin. Immunol.* 109, 119–121. doi: 10.1067/mai.2002.120273

Renfrew, M. J., Pokhrel, S., Quigley, M., McCormick, F., Fox-Rushby, J., Dodds, R., et al. (2012). *Preventing disease and saving resources: the potential contribution of increasing breastfeeding rates in the UK* (London: UNICEF UK).

Rescigno, M., Urbano, M., Valzasina, B., Francolín, M., Rotta, G., Bonasio, R., et al. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* 2, 361–367. doi: 10.1038/86373

Rieger, U. M., Mesina, J., Kalbermatten, D. F., Haug, M., Frey, H. P., Pico, R., et al. (2013). Bacterial biofilms and capsular contracture in patients with breast implants. *Br. J. Surg.* 100, 768–774. doi: 10.1002/bjs.9084

Riskin, A., Almog, M., Peri, R., Halasz, K., Srugo, I., and Kessel, A. (2012). Changes in immunomodulatory constituents of human milk in response to active infection in the nursing infant. *Pediatr. Res.* 71, 220–225. doi: 10.1038/pr.2011.34

Rodríguez, J. M., and Fernández, L. (2017). "Infectious mastitis during lactation: a mammary dysbiosis model," in *Prebiotics and Probiotics in Human Milk*. Eds. M. McGuire, M. McGuire and L. Bode (London: Academic Press), 401–428.

Rodríguez, J. M. (2014). The origin of human milk bacteria: is there a bacterial entero-mammary pathway during late pregnancy and lactation? *Adv. Nutr.* 5, 779–784. doi: 10.3945/an.114.007229

Rodríguez-Cruz, M., Alba, C., Aparicio, M., Checa, M. Á., Fernández, L., and Rodríguez, J. M. (2020). Effect of sample collection (manual expression vs. pumping) and skimming on the microbial profile of human milk using culture techniques and metataxonomic analysis. *Microorganisms* 8, E1278. doi: 10.3390/microorganisms8091278

Roitt, I. (2001). *Essential Immunology* (Oxford: Blackwell Scientific Publications).

Ruiz, L., Bacigalupe, R., García-Carral, C., Boix-Amoros, A., Argüello, H., Silva, C. B., et al. (2019). Microbiota of human precolostrum and its potential role as a source of bacteria to the infant mouth. *Sci. Rep.* 9, 8435. doi: 10.1038/s41598-019-42514-1

Ryder, R. W., Crosby-Ritchie, A., McDonough, B., and Hall, W. J. 3. (1977). Human milk contaminated with *Salmonella kottbus*. A cause of nosocomial illness in infants. *JAMA* 238, 1533–1534. doi: 10.1001/jama.1977.03280150103039

Sager, C. A. (1956). Biology of human milk. II. Decrease of germ content in human milk after freeze-drying. *Monatsschr. Kinderheilkd.* 104, 223–228.

Sakanaka, M., Gotoh, A., Yoshida, K., Odamaki, T., Koguchi, H., Xiao, J. Z., et al. (2019). Varied pathways of infant gut-associated *Bifidobacterium* to assimilate human milk oligosaccharides: prevalence of the gene set and its correlation with bifidobacteria-rich microbiota formation. *Nutrients* 12, 71. doi: 10.3390/nu12010071

Sakwinska, O., Moine, D., Delley, M., Combremont, S., Rezzonico, E., Descombes, P., et al. (2016). Microbiota in breast milk of chinese lactating mothers. *PloS One* 11, e0160856. doi: 10.1371/journal.pone.0160856

Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., et al. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol.* 12, 87. doi: 10.1186/s12915-014-0087-z

Sampieri, C. L., and Montero, H. (2019). Breastfeeding in the time of Zika: a systematic literature review. *Peer J.* 7, e6452. doi: 10.7717/peerj.6452

Samuel, B. S., Hansen, E. E., Manchester, J. K., Coutinho, P. M., Henrissat, B., Fulton, R., et al. (2007). Genomic and metabolic adaptations of *Methanobrevibacter smithii* to the human gut. *Proc. Natl. Acad. Sci. U. S. A.* 104, 10643–10648. doi: 10.1073/pnas.0704189104

Schanler, R. J., Fraley, J. K., Lau, C., Hurst, N. M., Hovarth, L., and Rossmann, S. N. (2011). Breastmilk cultures and infection in extremely premature infants. *J. Perinatol.* 31, 335–338. doi: 10.1038/jp.2011.13

Schreiner, R. L., Coates, T., and Shackelford, P. G. (1977). Possible breast milk transmission of group B streptococcal infection. *J. Pediatr.* 91, 159. doi: 10.1016/S0022-3476(77)80474-5

Schultz, M., Göttl, C., Young, R. J., Iwen, P., and Vanderhoof, J. A. (2004). Administration of oral probiotic bacteria to pregnant women causes temporary infantile colonization. *J. Ped. Gastroenterol. Nutr.* 38, 293–297. doi: 10.1097/00005176-200403000-00012

Schwab, C., Voney, E., Ramirez Garcia, A., Vischer, M., and Lacroix, C. (2019). Characterization of the cultivable microbiota in fresh and stored mature human breast milk. *Front. Microbiol.* 10, 2666. doi: 10.3389/fmicb.2019.02666

Sela, D. A., and Mills, D. A. (2010). Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides. *Trends Microbiol.* 18, 298–307. doi: 10.1016/j.tim.2010.03.008

Simpson, M. R., Avershina, E., Storrø, O., Johnsen, R., Rudi, K., and øien, T. (2018). Breastfeeding-associated microbiota in human milk following supplementation with *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* La-5, and *Bifidobacterium animalis* ssp. *lactis* Bb-12. *J. Dairy Sci.* 101, 889–899. doi: 10.3168/jds.2017-13411

Smith, A., Pierre, J. F., Makowski, L., Tolley, E., Lyn-Cook, B., Lu, L., et al. (2019). Distinct microbial communities that differ by race, stage, or breast-tumor subtype in breast tissues of non-Hispanic Black and non-Hispanic White women. *Sci. Rep.* 9, 11940. doi: 10.1038/s41598-019-48348-1

Smith, T. (1904). Some problems in the life-history of pathogenic microörganisms. *Amer. Med.* viii, 711–718.

Soeorg, H., Metsvaht, T., Eelmäe, I., Metsvaht, H. K., Treumuth, S., Merila, M., et al. (2017a). Coagulase-negative staphylococci in human milk from mothers of preterm compared with term neonates. *J. Hum. Lact.* 33, 329–340. doi: 10.1177/0890334417691505

Soeorg, H., Metsvaht, T., Eelmäe, I., Merila, M., Treumuth, S., Huik, K., et al. (2017b). The role of breast milk in the colonization of neonatal gut and skin with coagulase-negative staphylococci. *Pediatr. Res.* 82, 759–767. doi: 10.1038/pr.2017.150

Solís, G., de Los Reyes-Gavilan, C. G., Fernández, N., Margolles, A., and Gueimonde, M. (2010). Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. *Anaerobe* 16, 307–310. doi: 10.1016/j.anaerobe.2010.02.004

Soto, A., Martín, V., Jiménez, E., Mader, I., Rodríguez, J. M., and Fernández, L. (2014). Lactobacilli and bifidobacteria in human breast milk: influence of antibiotherapy and other host and clinical factors. *J. Pediatr. Gastroenterol. Nutr.* 59, 78–88. doi: 10.1097/MPG.0000000000000347

Stagno, S., Reynolds, D. W., Pass, R. F., and Alford, C. A. (1980). Breast milk and the risk of cytomegalovirus infection. *N. Engl. J. Med.* 302, 1073–1076. doi: 10.1056/NEJM198005083021908

Stinson, L. F., Keelan, J. A., and Payne, M. S. (2019). Identification and removal of contaminating microbial DNA from PCR reagents: impact on low-biomass microbiome analyses. *Lette. Appl. Microbiol.* 68, 2–8. doi: 10.1111/lam.13091

Stinson, L. F., Gay, M., Koleva, P. T., Eggesbø, M., Johnson, C. C., Wegienka, G., et al. (2020). Human milk from atopic mothers has lower levels of short chain fatty acids. *Front. Immunol.* 11, 1427. doi: 10.3389/fimmu.2020.01427

Thompson, N., Pickler, R. H., Munro, C., and Shotwell, J. (1997). Contamination in expressed breast milk following breast cleansing. *J. Hum. Lact.* 13, 127–130. doi: 10.1177/089033449701300213

Thomsen, A. C. (1982). Infectious mastitis and occurrence of antibody-coated bacteria in milk. *Am. J. Obstet. Gynecol.* 144, 350–351. doi: 10.1016/0002-9378(82)90590-7

Thongaram, T., Hoeflinger, J. L., Chow, J., and Miller, M. J. (2017). Human milk oligosaccharide consumption by probiotic and human-associated bifidobacteria and lactobacilli. *J. Dairy Sci.* 100, 7825–7833. doi: 10.3168/jds.2017-12753

Thyagarajan, S., Zhang, Y., Thapa, S., Allen, M. S., Phillips, N., Chaudhary, P., et al. (2020). Comparative analysis of racial differences in breast tumor microbiome. *Sci. Rep.* 10, 14116. doi: 10.1038/s41598-020-71102-x

Togo, A. H., Camara, A., Konaté, S., Doumbo, O. K., Raoult, D., and Million, M. (2017). '*Lactomassilus timonensis*,' a new anaerobic bacterial species isolated from the milk of a healthy African mother. *New Microbes New Infect.* 21, 122–124. doi: 10.1016/j.nmni.2017.12.004

Togo, A. H., Diop, A., Camara, A., Kuete, E., Konate, S., Brevaut, V., et al. (2019a). *Lactimicrobium massiliense* gen. nov., sp. nov.; *Anaerolactibacter massiliensis* gen. nov., sp. nov.; *Galactobacillus timonensis* gen. nov., sp. nov. and *Acidipropionibacterium timonense* sp. nov. isolated from breast milk from healthy breastfeeding African women. *New Microbes New Infect.* 29, 100537. doi: 10.1016/j.nmni.2019.100537

Togo, A., Dufour, J. C., Lagier, J. C., Dubourg, G., Raoult, D., and Million, M. (2019b). Repertoire of human breast and milk microbiota: a systematic review. *Future Microbiol.* 14, 623–641. doi: 10.2217/fmb-2018-0317

Togo, A. H., Grine, G., Khelaifia, S., des Robert, C., Brevaut, V., Caputo, A., et al. (2019c). Culture of methanogenic archaea from human colostrum and milk. *Sci. Rep.* 9, 18653. doi: 10.1038/s41598-019-54759-x

Toscano, M., De Grandi, R., Peroni, D. G., Grossi, E., Facchin, V., Comberiati, P., et al. (2017). Impact of delivery mode on the colostrum microbiota composition. *BMC Microbiol.* 17, 205. doi. 10.1186/s12866-017-1109-0

Treven, P., Mrak, V., Bogovič Matijašić, B., Horvat, S., and Rogelj, I. (2015). Administration of probiotics *Lactobacillus rhamnosus* GG and *Lactobacillus gasseri* K7 during pregnancy and lactation changes mouse mesenteric lymph nodes and mammary gland microbiota. *J. Dairy Sci.* 98, 2114–2128. doi: 10.3168/jds.2014-8519

Triantis, V., Bode, L., and van Neerven, R. (2018). Immunological effects of human milk oligosaccharides. *Front. Pediatr.* 6, 190. doi: 10.3389/fped.2018.00190

Tuominen, H., Rautava, S., Collado, M. C., Syrjänen, S., and Rautava, J. (2018). HPV infection and bacterial microbiota in breast milk and infant oral mucosa. *PloS One* 13, e0207016. doi: 10.1371/journal.pone.0207016

Turroni, F., Peano, C., Pass, D. A., Foroni, E., Severgnini, M., Claesson, M. J., et al. (2012). Diversity of bifidobacteria within the infant gut microbiota. *PloS One* 7, e36957. doi: 10.1371/journal.pone.0036957

U.S. Department of Health and Human Services (2011). *The Surgeon General's Call to Action to Support Breastfeeding* (Washington, DC: U.S. Department of Health and Human Services, Office of the Surgeon General).

Uehara, Y., Kikuchi, K., Nakamura, T., Nakama, H., Agematsu, K., Kawakami, Y., et al. (2001). H_2O_2 produced by viridans group streptococci may contribute to inhibition of methicillin-resistant *Staphylococcus aureus* colonization of oral cavities in newborns. *Clin. Infect. Dis.* 32, 1408–1413. doi: 10.1086/320179

Underwood, M. A., German, J. B., Lebrilla, C. B., and Mills, D. A. (2015). *Bifidobacterium longum* subspecies. *infantis: champion colonizer infant gut. Pediatr. Res.* 77, 229–235. doi: 10.1038/pr.2014.156

United Nations Children's Fund (UNICEF) (2020). *Coronavirus disease (COVID-19): What parents should 177 know*. (New York: United Nations Children's Fund, UNICEF). Available at: https://www.unicef.org/stories/novel-coronavirus-outbreak178what-parents-should-know.

Urbaniak, C., Cummins, J., Brackstone, M., Macklaim, J. M., Gloor, G. B., Baban, C. K., et al. (2014a). Microbiota of human breast tissue. *Appl. Environ. Microbiol.* 80, 3007–3014. doi: 10.1128/AEM.00242-14

Urbaniak, C., McMillan, A., Angelini, M., Gloor, G. B., Sumarah, M., Burton, J. P., et al. (2014b). Effect of chemotherapy on the microbiota and metabolome of human milk, a case report. *Microbiome* 2, 24. doi: 10.1186/2049-2618-2-24

Urbaniak, C., Angelini, M., Gloor, G. B., and Reid, G. (2016a). Human milk microbiota profiles in relation to birthing method, gestation and infant gender. *Microbiome* 4, 1. doi: 10.1186/s40168-015-0145-y

Urbaniak, C., Gloor, G. B., Brackstone, M., Scott, L., Tangney, M., and Reid, G. (2016b). The microbiota of breast tissue and its association with breast cancer. *Appl. Environ. Microbiol.* 82, 5039–5048. doi: 10.1128/AEM.01235-16

Van de Perre, P., Rubbo, P. A., Viljoen, J., Nagot, N., Tylleskär, T., Lepage, P., et al. (2012). HIV-1 reservoirs in breast milk and challenges to elimination of breast-feeding transmission of HIV-1. *Sci. Transl. Med.* 4, 143sr3. doi: 10.1126/scitranslmed.3003327

Vanhaecke, T., Aubert, P., Grohard, P. A., Durand, T., Hulin, P., Paul-Gilloteaux, P., et al. (2017). *L. fermentum* CECT5716 prevents stress-induced intestinal barrier dysfunction in newborn rats. *Neurogastroenterol. Motil.* 29, e13069. doi: 10.1111/nmo.13069

Vankerckhoven, V. V., Autgaerden, T. A., Huys, G., Vancanneyt, M., Swings, J., and Goossens, H. (2004). Establishment of the PROSAFE collection of probiotic and human lactic acid bacteria. *Microb. Ecol. Health Dis.* 16, 131–136. doi: 10.1080/08910600410032349

Vazquez-Fresno, R., Llorach, R., Marinic, J., Tulipani, S., Garcia-Aloy, M., Espinosa-Martos, I., et al. (2014). Urinary metabolomic fingerprinting after consumption of a probiotic strain in women with mastitis. *Pharmacol. Res.* 87, 160–165. doi: 10.1016/j.phrs.2014.05.010

Vazquez-Torres, A., Jones-Carson, J., Baumler, A. J., Falkow, S., Valdivia, R., Brown, W., et al. (1999). Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. *Nature* 401, 804–808. doi: 10.1038/44593

Verhasselt, V., Milcent, V., Cazareth, J., Kanda, A., Fleury, S., Dombrowicz, D., et al. (2008). Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. . *Nat. Med.* 14, 170–175. doi: 10.1038/nm1718

Ververs, M., and Arya, A. (2019). Ebola virus disease and breastfeeding: time for attention. *Lancet* 394, 825. doi: 10.1016/S0140-6736(19)32005-7

Větrovský, T., and Baldrian, P. (2013). The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PloS One* 8, e57923. doi: 10.1371/journal.pone.0057923

Victora, C. G., Bahl, R., Barros, A. J., França, G. V., Horton, S., Krasevec, J., et al. (2016). Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. *Lancet* 387, 475–490. doi: 10.1016/S0140-6736(15)01024-7

Virden, C. P., Dobke, M. K., Stein, P., Parsons, C. L., and Frank, D. H. (1992). Subclinical infection of the silicone breast implant surface as a possible cause of capsular contracture. *Aesthetic Plast. Surg.* 16, 173–179. doi: 10.1007/BF00450610

Walker, J. N., Pinkner, C. L., Pinkner, J. S., Hultgren, S. J., and Myckatyn, T. M. (2019). The detection of bacteria and matrix proteins on clinically benign and pathologic implants. *Plast. Reconstr. Surg. Glob. Open* 7, e2037. doi: 10.1097/GOX.0000000000002037

Walker, S. P., Barrett, M., Hogan, G., Flores Bueso, Y., Claesson, M. J., and Tangney, M. (2020). Non-specific amplification of human DNA is a major challenge for 16S rRNA gene sequence analysis. *Sci. Rep.* 10, 16356. doi: 10.1038/s41598-020-73403-7

Wang, M., Li, M., Wu, S., Lebrilla, C. B., Chapkin, R. S., Ivanov, I., et al. (2015). Fecal microbiota composition of breast-fed infants is correlated with human milk oligosaccharides consumed. *J. Pediatr. Gastroenterol. Nutr.* 60, 825–833. doi: 10.1097/MPG.0000000000000752

Wang, X., Lu, H., Feng, Z., Cao, J., Fang, C., Xu, X., et al. (2017). Development of human breast milk microbiota-associated mice as a method to identify breast milk bacteria capable of colonizing gut. *Front. Microbiol.* 8, 1242. doi: 10.3389/fmicb.2017.01242

Ward, T. L., Hosid, S., Ioshikhes, I., and Altosaar, I. (2013). Human milk metagenome: a functional capacity analysis. *BMC Microbiol.* 13, 116. doi: 10.1186/1471-2180-13-116

Weems, M. F., Dereddy, N. R., and Arnold, S. R. (2015). Mother's milk as a source of *Enterobacter cloacae* sepsis in a preterm infant. *Breastfeed. Med.* 10, 503–504. doi: 10.1089/bfm.2015.0146

West, P. A., Hewitt, J. H., and Murphy, O. M. (1979). Influence of methods of collection and storage on the bacteriology of human milk. *J. Appl. Bacteriol.* 46, 269–277. doi: 10.1111/j.1365-2672.1979.tb00820.x

WHO (2016). *Guideline: updates on HIV and infant feeding: the duration of breastfeeding, and support from health services to improve feeding practices among mothers living with HIV* (Geneva: World Health Organization).

Williams, J. E., Carrothers, J. M., Lackey, K. A., Beatty, N. F., York, M. A., Brooker, S. L., et al. (2017a). Human milk microbial community structure is relatively stable and related to variations in macronutrient and micronutrient intakes in healthy lactating women. *J. Nutr.* 147, 1739–1748. doi. 10.3945/jn.117.248864

Williams, J. E., Price, W. J., Shafii, B., Yahvah, K. M., Bode, L., McGuire, M. A., et al. (2017b). Relationships among microbial communities, maternal cells, oligosaccharides, and macronutrients in human milk. *J. Hum. Lact.* 33, 540–551. doi: 10.1177/0890334417709433

Williams, J. E., Carrothers, J. M., Lackey, K. A., Beatty, N. F., Brooker, S. L., Peterson, H. K., et al. (2019). Strong multivariate relations exist among milk, oral, and fecal microbiomes in mother-infant dyads during the first six months postpartum. *J. Nutr.* 149, 902–914. doi: 10.1093/jn/nxy299

Williamson, S., Finucare, E., Gamsu, H. R., and Hewitt, J. H. (1978). *Staphylococcus aureus* in raw milk for neonates. *Br. Med. J.* 1 (6120), 1146. doi: 10.1136/bmj.1.6120.1146

Wilson, G. S. (1943). The Pasteurization of Milk. *Br. Med. J.* 1 (4286), 261–262. doi: 10.1136/bmj.1.4286.261

Xuan, C., Shamonki, J. M., Chung, A., Dinome, M. L., Chung, M., Sieling, P. A., et al. (2014). Microbial dysbiosis is associated with human breast cancer. *PloS One* 9, e83744. doi: 10.1371/journal.pone.0083744

Zaura, E., Nicu, E. A., Krom, B. P., and Keijser, B. J. (2014). Acquiring and maintaining a normal oral microbiome: current perspective. *Front. Cell Infect. Microbiol.* 2485. doi: 10.3389/fcimb.2014.00085

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M. A. P., Harris, H. M. B., Mattarelli, P., et al. (2020). A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *Int. J. Syst. Evol. Microbiol.* 70, 2782–2858. doi: 10.1099/ijsem.0.004107

Ziegler, J. B., Cooper, D. A., Johnson, R. O., and Gold, J. (1985). Postnatal transmission of AIDS-associated retrovirus from mother to infant. *Lancet* 1 (8434), 896–898. doi: 10.1016/S0140-6736(85)91673-3

Zimmermann, P., Gwee, A., and Curtis, N. (2017). The controversial role of breast milk in GBS late-onset disease. *J. Infect.* 74 (1), S34–S40. doi: 10.1016/S0163-4453(17)30189-5

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REVIEW
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The Interaction Between Microorganisms, Metabolites, and Immune System in the Female Genital Tract Microenvironment

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The female reproductive tract microenvironment includes microorganisms, metabolites, and immune components, and the balance of the interactions among them plays an important role in maintaining female reproductive tract homeostasis and health. When any one of the reproductive tract microorganisms, metabolites, or immunity is out of balance, it will affect the other two, leading to the occurrence and development of diseases and the appearance of corresponding symptoms and signs, such as infertility, miscarriage, premature delivery, and gynecological tumors caused by infectious diseases of the reproductive tract. Nutrients in the female reproductive tract provide symbiotic and pathogenic microorganisms with a source of nutrients for their own reproduction and utilization. At the same time, this interaction with the host forms a variety of metabolites. Changes in metabolites in the host reproductive tract are related not only to the interaction between the host and microbiota under dysbiosis but also to changes in host immunity or the environment, all of which will participate in the pathogenesis of diseases and lead to disease-related phenotypes. Microorganisms and their metabolites can also interact with host immunity, activate host immunity, and change the host immune status and are closely related to persistent genital pathogen infections, aggravation of infectious diseases, severe pregnancy outcomes, and even gynecological cancers. Therefore, studying the interaction between microorganisms, metabolites, and immunity in the reproductive tract cannot only reveal the pathogenic mechanisms that lead to inflammation of the reproductive tract, adverse pregnancy outcomes and tumorigenesis but also provide a basis for further research on the diagnosis and treatment of targets.

Keywords: microenvironment, female genital tract, immunity, metabolites, microbiota

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INTRODUCTION

Different from the high diversity of the gastrointestinal tract, the female genital tract microbiome has low diversity, and it changes dynamically through the female menstrual cycle (Consortium, 2012; Chen et al., 2017). Most microbes have a symbiotic relationship with the host. Accounting for 90–95% of the total bacterial biomass, *Lactobacillus* spp. represents a healthy female genital tract microbiota

that produces lactic acid to maintain an acidic microenvironment. It can also inhibit pathogens through competition, adhesion prevention, and the secretion of antibacterial and immunomodulatory substances (Anahtar et al., 2018; Van der Veer et al., 2019). Vaginitis, cervicitis, and pelvic inflammatory disease (PID) will occur if pathogenic bacteria surpass lactobacilli in the female genital tract and can cause uncomfortable symptoms such as increased vulvovaginal discharge, itching, odor, and lower abdominal pain (Workowski and Bolan, 2015). However, there are differences in the microbes between subjects and in the ability of the host to resist dysbiosis that may be related to race, diet, age, living habits, immunity, disease susceptibility, and genetic polymorphism (Consortium, 2012; Anahtar et al., 2018; Chu et al., 2018; Serrano et al., 2019). Furthermore, the dominance of different microflora is not necessarily related to symptoms because partial non-*Lactobacillus*-dominant women do not experience uncomfortable symptoms of vulvovaginitis; hence, we cannot define disease by the number of bacteria alone, and we cannot define dysbiosis without the internal milieu of the host and disease environment (Anahtar et al., 2018; Scott et al., 2019).

Metabolites in the reproductive tract play an important role in female genital tract inflammation, pregnancy and tumors and can be considered biomarkers of disease severity, diagnosis, and prognosis (Ghartey et al., 2015; McMillan et al., 2015; Ilhan et al., 2019; Song et al., 2019). Metabolites in the female reproductive tract are the substrates, intermediates and byproducts of biochemical reactions caused by the interaction of human nutrients and bacteria, reflecting downstream events of gene expression (Altmäe et al., 2014; McMillan et al., 2015; Turkoglu et al., 2016; Watson and Reid, 2018) (**Figure 1**). These metabolites are better than genome, transcriptome, and proteome substances at predicting the disease phenotype (Altmäe et al., 2014). Genital infections, adverse pregnancy outcomes, and cancers possess different metabolic signatures that are often accompanied by dysbiosis of the female genital tract (Ghartey et al., 2015; Ceccarani et al., 2019; Ilhan et al., 2019). The metabolic pathways affected by these metabolites mainly include amino acids, carbohydrates, and lipid metabolism, which are closely related to life activities (Srinivasan et al., 2015). These activities further affect host cell function, immunity, and disease susceptibility and help maintain the balance of the host's reproductive tract microenvironment.

The host's innate and adaptive immune systems perform complex interactions with microorganisms and metabolites (Agostinis et al., 2019; Delgado-Diaz et al., 2019). Microbial ligands bind to host receptors to produce inflammatory factors, chemokines and antimicrobial products to regulate the immune response of the reproductive tract (Hooper et al., 2012).Vaginal dysbiosis cannot only directly cause vaginal epithelial injury through pathogens (Tao et al., 2019), but also indirectly cause vaginal epithelial injury through immune components, which in turn release metabolites into the microenvironment (Olive and Sassetti, 2016; Serrano et al., 2019). This metabolite may be ingested by the vaginal microbiota, leading to increased microbial metabolism, which is beneficial to the growth and reproduction of the microbiota (Serrano et al., 2019). The local competition between the host, pathogen and different immune cells for metabolic precursors will also affect the ability of immune cells to respond effectively to infection, affecting the growth and immunogenicity of the pathogen and further affecting the host response (Hooper et al., 2012; Olive and Sassetti, 2016; Postler and Ghosh, 2017). Therefore, the interaction between microorganisms, metabolites, and immunity in the host reproductive tract microenvironment plays an important role in maintaining the balance of the reproductive tract (Pruski et al., 2018). An imbalance in any part will result in host phenotype changes, disease, and even serious complications. Therefore, this article intended to review the relationship and importance of

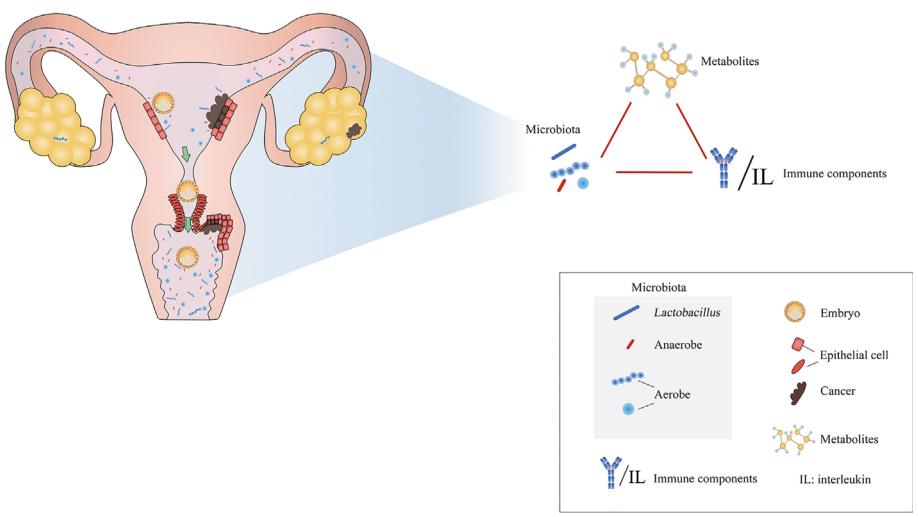

FIGURE 1 | Microenvironmental disorders of the female reproductive tract are closely related to inflammations, adverse pregnancy outcomes, and tumors. Modified from Paweł Łaniewski et al. (2020).

reproductive tract microorganisms, metabolites, and immunity to obtain a deeper understanding of the reproductive tract microenvironment, reproductive tract diseases and adverse reproductive tract outcomes.

NORMAL VAGINAL MICROENVIRONMENT

The vaginal microbiota community state types (CSTs) of women of childbearing age are divided into five categories (Ravel et al., 2011). CST I is dominated by *Lactobacillus crispatus*; CST II by *L. gasseri*; CST V by *L. jensenii*; and CST III by *L. iners*. CST IV belongs to the *Lactobacillus*-deficient type, which is dominated by anaerobic bacteria (classified by bacterial vaginosis, BV), partial aerobic bacteria (classified by aerobic vaginitis, AV) or a modest proportion of *Lactobacillus* spp. (Gajer et al., 2012). The vaginal microbiota is dynamic and occasionally transitions to an intermediate state or a disease state in most normal non-pregnant women. A high Nugent score does not indicate the disease status or microecological disorders (Gajer et al., 2012). The internal milieu of the host will make the microbiota return to a stable state. Factors that cause vaginal microbiota changes are primarily related to menstruation. Others include sexual intercourse, hormonal contraception, antimicrobial agents, use of lubricants, and vaginal douching, but they have less impact than menstrual periods and cause changes in the vaginal microbiota for a shorter duration (Gajer et al., 2012; Mitchell et al., 2012). In addition, in a few women, the vaginal CST does not change with the menstrual cycle and hormonal contraception, but further study by future researchers is needed to determine whether it is related to the metabolic function of bacteria (Gajer et al., 2012; Song et al., 2020).

Lactobacillus abundance in the female genital tract is strongly positively correlated with lactate and 4-hydroxyphenylacetate and correlated to a lesser extent with isoleucine, leucine, tryptophan, phenylalanine, aspartate, dimethylamine, sarcosine and pi-methylhistidine, all of which are typically associated with vaginal health (Srinivasan et al., 2015; Ceccarani et al., 2019) (**Table 1**). *L. crispatus* and *L. jensenii* have similar metabolic patterns (Srinivasan et al., 2015), while, *L. crispatus* and *L. iners* have different metabolic characteristics (Pruski et al., 2018). For example, studies have found that the genome of *L. crispatus* is almost twice that of *L. iners* (France et al., 2016). However, the carbon metabolism of *L. iners* is fermented by fewer compounds than *L. crispatus* (Pruski et al., 2018). When the dominant bacteria are *L. crispatus* and/or *L. jensenii*, most of the metabolites in the vagina are amino acids and dipeptide, such as higher levels of ornithine, lysine, glycylproline, phenylalanine (Srinivasan et al., 2015). Similar to BV-related flora, *L. iners* are correlated with amino acid catabolites, such as higher levels of

TABLE 1 | Current existing articles analyzing the correlation between the genital tract flora and metabolites in normal non-pregnant women of reproductive age.

Year	Author	Population	Sample	CST	Metabolites
2019	(Ceccarani et al., 2019)	Healthy women (n = 21), women with BV (n = 20) and women with Chlamydia trachomatis infection (n = 20)	Vaginal swabs	Contains all *Lactobacillus* spp. without distinguishing CST	High levels of lactate, 4-hydroxyphenylacetate, isoleucine, leucine, tryptophan, phenylalanine, aspartate, dimethylamine, sarcosine and pi-methylhistidine
2018	(Parolin et al., 2018)	Healthy women (n = 22), women with Chlamydia trachomatis infection (n = 20), and women with BV (n = 19)	Vaginal swabs	Contains all *Lactobacillus* spp. without distinguishing CST	Higher levels of lactate, 4-hydroxyphenylacetate, diverse amino acids (phenylalanine, glutamate, leucine, threonine, tryptophan, aspartate) and amino acid derivatives(sarcosine)
2015	(Srinivasan et al., 2015)	Women with BV (n = 40) and women without BV (n = 20)	Vaginal fluid	CST-I	1) Higher levels of sugars (maltose, maltotriose, and maltohexose), lipid metabolism biochemicals (such as arachidonate and carnitine), amino acids (ornithine, lysine), dipeptide (glycylproline, phenylalanine) as well as lactate, and urea 2) Lower levels of *N*-acetylneuraminate, succinate, the carnitine precursor deoxycarnitine, the eicosanoid 12-hydroxyeicosatetraenoic acid, the fatty acid 13-hydroxyoctadecadienoic acid, the nucleobase uracil, and glutathione
				CST-III	1) Higher levels of proline, threonine, aspartate, serine, and valinylglutamate 2) Lower levels of glutamate and glycylleucine
				CST-V	Same as CST-I: 1) Higher levels of sugars (maltose, maltotriose, and maltohexose), lipid metabolism biochemicals (such as arachidonate and carnitine), amino acids (ornithine, lysine), dipeptide (glycylproline, phenylalanine) as well as lactate, and urea 2) Lower levels of *N*-acetylneuraminate, succinate, the carnitine precursor deoxycarnitine, the eicosanoid 12-hydroxyeicosatetraenoic acid, the fatty acid 13-hydroxyoctadecadienoic acid, the nucleobase uracil, and glutathione
2015	(McMillan et al., 2015)	Pregnant women (n = 67) and non-pregnant women (n = 64)	Vaginal fluid	CST-I	Higher levels of succinate
2012	(Gajer et al., 2012)	Reproductive age women (n = 32)	Vaginal swabs	CST-III	1) Higher levels of lactate 2) Lower levels of succinate and acetate

CST, Community state types; BV, bacterial vaginosis.

proline, threonine, aspartate, serine, and valinylglutamate (Srinivasan et al., 2015). In addition, *L. iners* often has a symbiotic relationship with *G. vaginalis*, and both produce similar levels of cholesterol-dependent cytolysin (Macklaim et al., 2013). Therefore, the metabolic characteristics of *L. crispatus* and/or *L. jensenii* dominance can be defined as a healthy vaginal microenvironment. However, whether the metabolic characteristics of the non-*Lactobacillus* abundance of some asymptomatic women are similar to those of *L. crispatus* and/or *L. jensenii* dominance needs to be further explored.

The mucous layer on the surface of female genital tract epithelial cells plays an important role as the first line of defense against microbial invasion (Mirmonsef et al., 2011; Aldunate et al., 2015). When microorganisms break through the line of defense, epithelial cells use pattern recognition receptors (PPRs) to identify microorganisms to produce inflammatory factors and recruit inflammatory cells to resist microbial invasion and colonization. The dominance of *Lactobacillus* in the genital tract is essential, as it inhibits pathogens and maintains immune equilibrium (Smith and Ravel, 2017). Studies have found that the concentration of inflammatory factors in the vagina is very low when *L. crispatus* and *L. jensenii* are dominant (Kyongo et al., 2012). Lactic acid, as a metabolite derived primarily from *Lactobacillus* spp., is also related to reproductive tract immunity (Delgado-Diaz et al., 2019). L-lactic acid produced by *Lactobacillus* spp. can cause an anti-inflammatory response and inhibit the production of proinflammatory cytokines and chemokines induced by toll-like receptor (TLR) in cervical and vaginal epithelial cells at low pH (Delgado-Diaz et al., 2019). In addition, lactic acid can induce the secretion of the anti-inflammatory cytokine interleukin (IL)-10, reduce the production of the proinflammatory cytokine IL-12 in dendritic cells (DCs), and reduce the cytotoxicity of natural killer cells (Ilhan et al., 2019). The anti-inflammatory activity of lactic acid also requires the presence of organic acids produced by microorganisms to maintain vaginal health, mainly by increasing the production of the anti-inflammatory cytokine IL-1RA, inhibiting the proinflammatory signal of the IL-1 cytokine, and slightly reducing the production of the proinflammatory cytokines IL-6 and macrophage inflammatory protein 3 alpha (MIP-3α) (Delgado-Diaz et al., 2019). Therefore, the interaction between the flora, metabolites, and immunity in the healthy reproductive tract is very important for maintaining the health of the reproductive tract. When any one party is imbalanced, it will affect the balance of the reproductive tract.

COMMON REPRODUCTIVE TRACT INFECTIONS

Female genital tract infections mainly include vaginitis, cervicitis, and PID (Sherrard et al., 2018). The main cause is exogenous pathogen interference or endogenous dysbiosis (Workowski and Bolan, 2015; Song et al., 2020). At present, the relevant research on infectious diseases caused by the interaction between reproductive tract microorganisms, metabolites and the host is mainly focused on BV, *Chlamydia trachomatis* (*C. trachomatis*), and AV (Ceccarani et al., 2019). In addition, inflammation of the reproductive tract caused by bacterial flora disorders involving BV, AV, and *C. trachomatis* infection is closely related to adverse pregnancy outcomes and tumors (Sherrard et al., 2018). Other diseases, such as trichomoniasis (*Trichomonas vaginalis*), vulvovaginal candidiasis, and gonorrhea, have received less relevant research in this area and may become future research directions. Therefore, this section mainly discusses the interaction between the microorganisms, metabolites, and host immunity of three common RTIs: BV, *C. trachomatis* infection, and AV.

BV

BV is the most common vaginal microbial disorder of women of childbearing age, and can lead to adverse obstetrics and gynecological outcomes such as infertility, miscarriage, premature rupture of membranes, and premature delivery (Workowski and Bolan, 2015; Baqui et al., 2019; Peebles et al., 2019). It also increases the risk of sexually transmitted infections (Shipitsyna et al., 2020). BV is characterized by an increase in the diversity of vaginal microbiota, a decrease in *Lactobacillus* spp. in the vagina, and an increase in BV-related anaerobic and microaerobes (Srinivasan et al., 2015). BV-related bacteria mainly include *Gardnerella*, *Atopobium*, *Mycoplasma*, *Megasphaera*, *Mobiluncus*, *Roseburia*, *Dialister.*, *Sneathia* and *Prevotella* spp. (McMillan et al., 2015; Ceccarani et al., 2019). However, flora analysis alone cannot distinguish between a normal vaginal environment and BV because *Atopobium* spp., *Prevotella* spp. and *Mycoplasma hominis* can also be detected in healthy people, therefore, the vaginal microenvironment needs to be analyzed in combination with metabolomics (Vitali et al., 2015).

BV is closely related to metabolites in the genital tract (Spiegel et al., 1980; Wolrath et al., 2002) (**Table 2**). The metabolites of amines, organic acids, short chain fatty acids (SCFAs), amino acids, nitrogenous bases and monosaccharides of BV patients are significantly different from those of healthy individuals (Vitali et al., 2015). Current studies suggest that metabolites better reflect the disease phenotype than microorganisms. Before disease symptoms appear, the appearance or disappearance of certain metabolites in the vagina has a positive or negative correlation with the metabolic function of certain microorganisms (Yeoman et al., 2013). Changes in maltose, kynurenine, nicotinate, malonate, acetate and nicotinamide adenine dinucleotide (NAD^+) represent the occurrence of BV and can be used as metabolic biomarkers to distinguish BV from a healthy vagina (Vitali et al., 2015). When BV is cured, the metabolites associated with BV decrease significantly (Stanek et al., 1992; Srinivasan et al., 2015). In addition, genital tract metabolic analysis plays a prominent role in the diagnosis of BV (Watson and Reid, 2018). In 2015, McMillan et al. (2015) found that an increase in 2-hydroxyisovalerate and γ-hydroxybutyrate and a decrease in lactic acid and tyrosine in the vagina are the most sensitive

TABLE 2 | Current existing articles analyzing the correlation between the genital tract flora and metabolites in women with vaginitis.

Year	Author	Population	Sample	CST	Metabolites
BV					
2019	(Ceccarani et al., 2019)	Healthy women (n = 21), women with BV (n = 20) and women with Chlamydia trachomatis infection (n = 20)	Vaginal swabs	CST-IV	1) Higher levels of organic acids (*i.e.*: formate, pyruvate, propionate, acetate, 2-hydroxyisovalerate), amines (*i.e.*: trimethylamine, putrescine), amino acids (*i.e.*: proline and alanine) and 5-aminopentanoate 2) Lower levels of lactate, 4-hydroxyphenylacetate, phenylalanine, pi-methylhistidine, glycine, isoleucine, leucine, tryptophan, aspartate, dimethylamine, and sarcosine
2018	(Parolin et al., 2018)	Healthy women (n = 22), women with Chlamydia trachomatis infection (n = 20), and women with BV (n = 19)	Vaginal swabs	CST-IV	Higher levels of biogenic amines (methylamine, putrescine, trimethylamine, tyramine, desaminotyrosine), organic acids (succinate, malonate, 2-hydroxyisovalerate, and short-chain fatty acids) and alanine
2015	(McMillan et al., 2015)	Pregnant women (n = 67) and non-pregnant women (n = 64)	Vaginal fluid	CST-IV	1) Organic acid: higher levels of 2-hydroxyisovalerate, γ -hydroxybutyrate, 2-hydroxyglutarate and 2-hydroxyisocaproate; lower levels of lactate 2) Amines: higher levels of tyramine, putrescine, and cadaverine 3) Amino acids: lower levels of tyrosine
2015	(Srinivasan et al., 2015)	Women with BV (n = 40) and women with non-BV (n = 20)	Vaginal fluid	CST-IV	1) Amino acid: higher levels of cadaverine, pipecolate, tyramine, 4-hydroxyphenylacetate, 3- (4-hydroxyphenyl) propionate, tryptamine, citrulline and putrescine; lower concentrations of arginine, ornithine, spermine and dipeptides 2) Carbohydrates: higher levels of N-acetylneuraminate, galactose, threitol and succinate; lower levels of glucosamine, maltotriose, maltotetraose, maltopentaose, maltohexaose, lactate, fructose, and mannitol 3) NAD: lower levels of nicotinamide; higher levels of nicotinate 4) Lipids: higher levels of 12-hydroxyeicosatetraenoic acid, deoxycarnitine, 4-hydroxybutyrate and 13-hydroxyoctadecadienoic acid; lower levels of arachidonate, carnitine, ascorbic acid, acetylcarnitine, propionylcarnitine, butyrylcarnitine, glycerol and glycerol-3-phosphate
2015	(Vitali et al., 2015)	BV-affected patients (n = 43) and healthy controls (n = 37)	Vaginal fluid	CST-IV	1) Amines: higher levels of tyramine, ethanolamine, trimethylamine, methylamine, cadaverine 2) Organic acids: higher levels of formate, malonate, succinate, pyruvate, acetate 3) Short-chain fatty acids: higher levels of propionate, butyrate, 2-hydroxyisovalerate 4) Amino acids: higher levels of proline; lower levels of tryptophan, phenylalanine, tyrosine, glutamate, isoleucine, leucine 5) Nitrogenous bases: higher levels of nicotinate, uracil; lower levels of NAD+, inosine 6) Sugars: higher levels of glucose; lower levels of maltose 7) Others: higher levels of urocanate, 2-aminoadipate, 3-methyl-2-oxovalerate; lower levels of kynurenine, sn-glycero-3-phosphocholine, sarcosine
2013	(Yeoman et al., 2013)	Pre-menopausal women of reproductive age (n = 36)	Vaginal lavage fluid	CST-IV	1) Higher levels of putrescine, cadaverine, 2-methyl-2-hydroxybutanoic acid, hydroxylamine, glycolic acid, tetradecanoic acid, and butyrolactone 2) Lower levels of 2,3-hydroxypropyl-2-aminoethyl phosphate, cis-11-octadecanoic acid, and ribose-5-phosphate
2012	(Gajer et al., 2012)	Reproductive age women (n = 32)	Vaginal swabs	CST-IV	1) Higher levels of succinate and acetate 2) Lower concentrations of lactate

(Continued)

TABLE 2 | Continued

Year	Author	Population	Sample	CST	Metabolites
2002	(Wolrath et al., 2002)	Women of childbearing age with various lower genital tract disorders (n = 61)	Vaginal fluid	CST-IV	Higher levels of trimethylamine
1980	(Spiegel et al., 1980)	Women with non-specific vaginitis (n = 53)	Vaginal fluid	CST-IV	1) Higher levels of succinate, acetate, butyrate, and propionate 2) Lower levels of lactate
Chlamydia trachomatis					
2018	(Parolin et al., 2018)	Women with Chlamydia trachomatis infection (n = 20), healthy women (n = 22), and women with BV (n = 19)	Vaginal swabs	CST-III	Lower levels of tyramine, dimethylamine, cadaverine, succinate, valine, isoleucine, glycine, sarcosine, creatinine, 4-aminobutyrate
2019	(Ceccarani et al., 2019)	Healthy women (n = 21), women with BV (n = 20) and women with Chlamydia trachomatis infection (n = 20)	Vaginal swabs	CST-IV	Lower levels of lactate, certain amino acids and biogenic amines
AV					
2012	(Gajer et al., 2012)	Reproductive age women (n = 32)	Vaginal swabs	CST-IV	Higher levels of acetate and lactate

CST, Community state types; BV, bacterial vaginosis; AV, aerobic vaginitis.

and specific indicators for the diagnosis of BV. Therefore, not only the microbiota but also metabolites can be used as effective reference indicators for clinical diagnosis.

The vaginal microbiota and metabolites of BV patients are also closely related to the clinical symptoms and signs of the host. The odor of vaginal secretions in patients with BV is related to the increase in tyramine, trimethylamine, cadaverine, and putrescine and the decrease in the aromatic substances 2 (5H)-furanone and 2-ethyl-4-methyl-1,3-dioxolane (Yeoman et al., 2013; Srinivasan et al., 2015; Vitali et al., 2015). Odor is also closely related to *Dialister* spp. (Yeoman et al., 2013; McMillan et al., 2015; Srinivasan et al., 2015). Thin and homogeneous secretions are positively related to cadaverine, and cadaverine is related to *Streptococcus* spp. and *Mycoplasma* spp. (Yeoman et al., 2013; Srinivasan et al., 2015). Clue cells are positively correlated with deoxycarnitine and pipecolate, while deoxycarnitine is positively correlated with BV-associated bacterium 1 (BVAB1), *Megasphaera* sp. type 2, and several *Prevotella* species (Srinivasan et al., 2015). Vaginal discharge is related to 2-methyl-2-hydroxybutanoic acid and *Mobiluncus* spp. (Yeoman et al., 2013). In addition, the metabolic pathways of amino acids, carbohydrates, NAD, and lipids in the vaginal flora of BV patients are active and are closely related to cellular life activities (Srinivasan et al., 2015; Ceccarani et al., 2019).Therefore, understanding the interaction between the flora and metabolites of BV patients provides a basis for understanding the molecular mechanisms of microbe-microbe and microbe-host interactions (Ilhan et al., 2019).

BV-related bacteria can activate the host's genital tract immune response, but they do not cause obvious inflammatory symptoms such as redness, swelling, heat and pain (Smith and Ravel, 2017). The reason may be related to the influence of BV-related microorganisms and their metabolites on immunity. In 2019, Delgado-Diaz et al. (2019) found that the sustained action of organic acids, metabolites of the vaginal microbiota associated with BV, led to dysregulation of the immune response of cervical and vaginal epithelial cells *in vitro*. SCFAs can recruit and activate female reproductive tract innate immune cells, such as neutrophils and monocytes (Vitali et al., 2015). However, SCFAs can also inhibit the production of proinflammatory cytokines and affect the migration and phagocytic response of immune cells to regulate the immune response (Al-Mushrif et al., 2000). In addition, succinic acid produced by *Prevotella* spp. and *Mobiluncus* spp. in the genital tract can also inhibit leukocyte chemotaxis and regulate the immune response (Al-Mushrif et al., 2000; McMillan et al., 2015; Vitali et al., 2015). In 1985, Rotstein et al. (1985) demonstrated that succinic acid has the strongest chemotaxis inhibitory effect at pH 5.5 and at concentrations of 20–30 mM. Therefore, the current research has proven that the interaction between BV flora, metabolites and immunity is of great significance for understanding clinical symptoms and signs. However, more research on the interaction mechanism between immunity and metabolites in BV patients is needed to confirm the influence of metabolites on flora and immunity.

Chlamydia trachomatis

In 2016, the World Health Organization announced the newest global *C. trachomatis* prevalence rate of 1.5–7% for women aged 15–49 years and an estimated 127 million new cases women worldwide that year (Organization, 2020). Most women infected with *C. trachomatis* are asymptomatic (Ceccarani et al., 2019). Approximately 10% of *C. trachomatis* infections will progress to PID without timely treatment, which will cause severe ectopic pregnancy, reproductive dysfunction and cancer (Workowski and Bolan, 2015; Idahl et al., 2020). Lactic acid is an important inhibitor of *C. trachomatis* infection (Gong et al., 2014). However, *L. iners* produces less lactic acid, so the microbiota dominated by *L. iners* increases the risk of *C. trachomatis* infection (Van Houdt et al., 2018). Similarly, BV also increases the risk of *C. trachomatis* infection due to a reduction in the lactate concentration (Shipitsyna et al., 2020). Therefore, *C. trachomatis* infection is greatly affected by lactic acid in the reproductive tract microenvironment.

The interactions among microorganisms, *C. trachomatis* and metabolites in the reproductive tract are closely related (**Table 2**). In 2016, Ceccarani et al. (2019) performed a combined metagenomic and metabolomics analysis on the vaginal secretions of non-pregnant Caucasians of childbearing age with risk factors for *C. trachomatis* infection. The study showed that *C. trachomatis* infection was dominated by *Lactobacillus* in most

people, and *L. iners* was increased, and some patients had anaerobic bacteria as the dominant bacteria. Compared with healthy controls, women infected with *C. trachomatis* showed only slight changes in vaginal metabolites that were mainly manifested as a reduction in certain amino acids and biogenic amines (Ceccarani et al., 2019). In 2018, Parolin et al. (2018) studied the characteristics of vaginal microbes and metabolites in the case of *C. trachomatis* infection and found that vaginal valine, isoleucine, tyramine, cadaverine, and succinate in patients with *C. trachomatis* infection were significantly decreased compared with those in healthy controls, indicating that *C. trachomatis* may use nitrogen as the first nutrient source or that *C. trachomatis* may affect the nitrogen metabolism of infected host cells. There is a correlation between the vaginal microbiome, metabolites, and genital symptoms of *C. trachomatis* infection (Parolin et al., 2018). More than half of *C. trachomatis*-infected patients are completely asymptomatic, while symptomatic patients mainly manifest with abnormal vaginal discharge, dyspareunia, dysuria, and abnormal bleeding. The concentration of 4-aminobutyrate is significantly different between asymptomatic and symptomatic women with *C. trachomatis* infection. However, all asymptomatic women have *L. crispatus* as the dominant bacteria in the vagina, and only half of symptomatic women have *L. crispatus* as the dominant bacteria (Parolin et al., 2018). Therefore, *C. trachomatis* infection is related to the genital tract flora, metabolites and clinical symptoms. However, the effect of 4-aminobutyrate on host immunity against *C. trachomatis* infection needs further study.

There are complicated interactions between *C. trachomatis*, microorganisms, genital tract metabolites and immunity (Ziklo et al., 2016b). Epithelial cells and immune cells infected by *C. trachomatis* can secrete several proinflammatory cytokines and chemokines to eliminate pathogen infection (Rasmussen et al., 1997; Johnson, 2004; Brunham and Rey-Ladino, 2005). Interferon (IFN)-γ is an important factor that inhibits the reproduction of *C. trachomatis* (Shemer and Sarov, 1985). The ability to synthesize tryptophan in the IFNγ-rich infection microenvironment is an important virulence factor of the genital *C. trachomatis* serovars (Aiyar et al., 2014). IFN-γ mediates the activation of host indoleamine 2,3-dioxgenase (IDO), leading to the consumption of tryptophan necessary for the growth of *C. trachomatis* and inhibiting the growth of *C. trachomatis* (Beatty et al., 1994; Aiyar et al., 2014; Olive and Sassetti, 2016; Molenaar et al., 2018). The tryptophan needed for the growth of *C. trachomatis* is reduced, and *C. trachomatis* forms a static state (Byrne et al., 1989). It is known that BV infection increases the risk of *C. trachomatis* infection. BV-related bacteria, such as partial *Prevotella species*, can produce indole (Romanik et al., 2007; Sasaki-Imamura et al., 2011), which is increased in the vaginal discharge of BV patients (Lewis et al., 2014). *C. trachomatis* in the genital tract can use indole produced by microorganisms as a substrate to activate alternative tryptophan synthesis pathways- the *trpA*, *trpB* and *trpR* genes, synthesize tryptophan, and make *C. trachomatis* evade the clearance of IFN-γ in the genital tract (Fehlner-Gardiner et al., 2002; Wood et al., 2003; Ziklo et al., 2016b). However, in patients who are coinfection with BV and *C. trachomatis*, the inhibitory response to IFN-γ is not exactly the same, which may be related to the level of indole in the vaginal microenvironment (Lewis et al., 2014). In addition, the low oxygen environment formed under BV may result in insufficient energy supply for the IFN-γ signaling pathway, which further reduces its function (Roth et al., 2010). IFN-γ induces the production of nitric oxide and further inhibits the growth of *C. trachomatis* (Agrawal et al., 2011). The most recent *in vitro* experiments have confirmed that *C. trachomatis* induces the expression of ornithine decarboxylase (ODC), deprives the inducible nitric oxide synthase (iNOS) substrate arginine, and actively promotes polyamine synthesis while downregulating iNOS expression and inhibiting the activity of iNOS to reduce nitric oxide production in the host and further escape the host's innate immunity (Abu-Lubad et al., 2014; Olive and Sassetti, 2016). Studies have shown that the amino acids and sugars in the environment are critical to the ability of *C. trachomatis* to infect (Harper et al., 2000). However, the detailed metabolic and immune interaction mechanisms still need further study. After *C. trachomatis* escapes the host's immunity, the host's immune surveillance is reduced, and the environment is conducive to the growth of *C. trachomatis*, which is reactivated (Belland et al., 2003). After *C. trachomatis* reinfection or chronic infection, T helper (Th)1-, Th2- and Th17-type cells are triggered to mediate tissue destruction, fibrosis, and scarring, further leading to the progression of PID and its sequelae (Ziklo et al., 2016a; Molenaar et al., 2018).

AV

The incidence of AV in women of childbearing age is approximately 10% (Donders et al., 2017). Significantly different from BV patients and those with a normal flora, AV patients have increased aerobic bacteria or enterococci, such as *Escherichia coli*, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus anginosus*, and *Enterococcus faecalis*, in the vagina (Donders et al., 2017; Tao et al., 2019; Wang et al., 2020). Studies have also shown that BV-related bacteria often appear in the vaginal flora of AV patients, which may be related to the symbiotic relationship between BV- and AV-related bacteria in the state of flora disorder (Wang et al., 2020). Different from the clinical symptoms and signs of BV, AV mainly manifests as foul and yellow purulent discharge, but similar to BV, it easily causes adverse obstetrics and gynecology complications that may be related to bacterial ascending infection (Donders et al., 2017).

It is known that AV-related bacteria and their metabolites are involved in the host's inflammatory state and immune response, but their correlation with host disease phenotypes and diagnostic applications have not yet been studied. Previous studies have found that when *Streptococcus* sp. increase in the vaginal secretions of non-pregnant women, acetate also increases (Gajer et al., 2012) (**Table 2**). Acetate is a SCFAs that directly activates the host's immune-inflammatory pathway and promotes the expansion of T-regulatory (Treg) cells (Olive and Sassetti, 2016; Postler and Ghosh, 2017). However, the detailed mechanism by which acetic acid is produced by *Streptococcus* sp. and genital tract immunity still needs to be studied.

AV patients mainly present with a local immune imbalance in the reproductive tract caused by pathogens (Benner et al., 2018). In 2020, Budilovskaya et al. (2020) found that the expression of IL1β, IL-6, IL-8, IL10, tumor necrosis factor-α (TNFα) and CD68 messenger ribose nucleic acids (mRNAs) in AV patients was significantly increased, and this change was related to itching or burning as well as increases in leukocytes and parabasal epithelial cells under the microscope (Smith and Ravel, 2017). Purulent vaginal discharge and vaginal redness may be related to the toxic effect of the virulence gene *sag* of *Streptococcus anginosus* on epithelial cells, leading to epithelial cell lysis (Tao et al., 2019).

The molecular mechanism of inflammatory genital tract symptoms in AV patients may be related to the interaction of metabolism and immunity. Nitric oxide plays an important role in host resistance to pathogens. Nitric oxide is synthesized by iNOS in inflammatory cells (Richardson et al., 2006; Jones et al., 2010). After the cells secrete nitric oxide, they can kill pathogens directly beside the inflammatory cells. However, *Staphylococcus aureus* can evade the host nitric oxide response by changing metabolism (Olive and Sassetti, 2016). *Staphylococcus aureus* induces the expression of flavohaemoglobin (Hmp) through the SrrAB system, quickly and enzymatically hydrolyzes nitric oxide, and resists the host's inhibitory effect on pathogens (Richardson et al., 2006); at the same time, *Staphylococcus aureus* upregulates L-lactate dehydrogenase 1 (Ldh1), enabling it to survive lactic acid fermentation under aerobic and anaerobic conditions (Richardson et al., 2008). The virulence of *Staphylococcus aureus* requires hexose produced by glycolysis, and an increase in the glucose concentration will enhance the resistance of pathogens to nitric oxide and subsequently the host immune response (Vitko et al., 2015; Olive and Sassetti, 2016). Polyamines are toxic to *Staphylococcus aureus*. *Staphylococcus aureus* strains with arginine catabolic mobile element (ACME) encode the acetyltransferase SpeG, which makes the strains resistant to polyamines and facilitates colonization in host cells (Diep et al., 2008; Olive and Sassetti, 2016). Pathogens evade the killing effect of the host's immune system, facilitating the colonization of pathogens in the host's reproductive tract. The colonization of toxic shock syndrome toxin-1 (TSST-1) *Staphylooccus aureus strains* will increase the production of proinflammatory cytokines and chemokines in human vaginal epithelial cells, further destroying the mucosal barrier and increasing the penetrating effect of TSST-1, leading to severe symptoms and signs of vulvovaginitis (Pereira et al., 2013). This also explains why vaginal inflammation in AV is more serious than that in BV. However, previous studies have mainly focused on of BV-related bacteria, and there are few studies on AV-related bacteria, metabolites, and immunity. Future research may reveal the significance if it is used as a future research direction.

NORMAL PREGNANCY

Unlike non-pregnant women, healthy pregnant women are affected by estrogen-progesterone, and the vaginal microflora tends to be stable from the first trimester to the third trimester, that is, the low richness and low diversity dominated by *Lactobacillus* spp. inhibits the growth of CST IV pathogenic bacteria such as *Gardnerella vaginalis, Atopobium vaginae, Sneathia amnii*, *Prevotella Bivia*, and *Prevotella cluster 2* (MacIntyre et al., 2015; Brown et al., 2018; Serrano et al., 2019). The vaginal flora during pregnancy is less transformed, mostly between *Lactobacillus* species (Romero et al., 2014;

TABLE 3 | Current existing articles analyzing the correlation between the genital tract flora and metabolites in pregnant women.

Year	Author	Population	Sample	CST/ microorganisms	Metabolites
Normal pregnant women					
2016	(Prince et al., 2016)	Women who delivered at term (n = 27), women who delivered preterm (n = 44)	Placental membranes swabs	*Bradyrhizobium* spp., *streptococcus thermophilus*	Term cohorts: lower levels of the amino sugar and nucleotide sugar metabolism, butanoate metabolism, riboflavin metabolism, and amino-benzoate degradation
2015	(McMillan et al., 2015)	Pregnant women (n = 67) and non-pregnant women (n = 64)	Vaginal fluid	CST-I	Similar with non-pregnant women : Higher levels of succinate
				CST-IV	Similar with non-pregnant women : 1) Organic acid: higher levels of 2-hydroxyisovalerate, γ-hydroxybutyrate, 2-hydroxyglutarate and 2-hydroxyisocaproate; lower levels of lactate 2) Higher levels of amines: tyramine, putrescine, and cadaverine 3) Lower levels of amine precursors: tyrosine, lysine, ornithine
Preterm birth					
2016	(Prince et al., 2016)	Women who delivered at term (n = 27), women with spontaneous preterm birth (n = 44)	Placental membranes swabs	*Lactobacillus crispatus*, *Acinetobacter johnsonii*	Preterm cohorts: higher levels of pentose phosphate pathway, glycerophopholipid metabolism, and biosynthesis of the siderophore group non-ribosomal peptides
2014	(Aagaard et al., 2014)	Women who delivered with preterm birth (n = 16), women with remote antenatal infection (n = 16), and controls (n = 16)	Placenta tissue	*Burkholderia spp.*	1)Higher levels of methane metabolism, isoquinoline alkaloid biosynthesis, and glycine/serine/threonine metabolism 2) Lower levels of biotin metabolism and glycosylphosphatidylinositol anchor pathways

CST, Community state types.

Serrano et al., 2019). Pregnant women with CST I as the dominant bacteria have the most stable vaginal flora throughout pregnancy, followed by those with CST V, CST II, and CST IV (MacIntyre et al., 2015). The vaginal microbiota during the third trimester is similar to that of non-pregnant women. One week after delivery, estrogen decreases, and glycogen-supported *Lactobacillus* spp. also decreases sharply. The stability and compliance of the vaginal flora decreased significantly, and the diversity increases, especially that of CST IV, which leads to disorders of the postpartum vaginal flora and even postpartum endometritis and puerperal morbidity (DiGiulio et al., 2015; MacIntyre et al., 2015). There are also microflora in the placenta and amniotic fluid of women who experience normal-term delivery (Collado et al., 2016; Nuriel-Ohayon et al., 2016). Some studies suggest that placental bacteria may be derived from oral flora, mainly non-pathogenic symbiotic flora such as *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes*, and *Fusobacteria* phyla (Aagaard et al., 2014). The origin of the placental microbiota is also related to the migration of the intestinal flora to the fetus-placenta interface, which promotes colonization of the fetus after birth (Collado et al., 2016). However, some studies indicate that there are no microorganisms in the normal placenta and amniotic fluid, which may be caused by the contamination of laboratory reagents or equipment or by different methods used to obtaining specimens (Leiby et al., 2018; Lim et al., 2018; De Goffau et al., 2019).

Analysis of the metabolic characteristics of the vaginal microbiota revealed that the microbial metabolic activity in the first trimester is the highest to adapt to changes in pregnancy (Serrano et al., 2019) (**Table 3**). As pregnancy progresses, the vaginal microbiota tends to become stable, and its metabolic capacity tends to be simplified, mainly reflected in the low activity of carbohydrate metabolism, cell wall/membrane biochemical pathways, protein synthesis pathways, and nucleic acid metabolism pathways (Serrano et al., 2019). Another study also found that the carbohydrate metabolism and lipid metabolism of cervicovaginal secretions in full-term women were downregulated during the second and third trimesters (Ghartey et al., 2015). Carbohydrate metabolism was significantly downregulated, especially in the third trimester of pregnancy, and was related to the large amount of glycogen deposition and metabolization into lactic acid in a highly estrogen state (Ghartey et al., 2015). This change is conducive to the colonization of lactobacilli in the host reproductive tract and maintains the necessary acidic pH in the "healthy" reproductive tract. It also helps maintain the integrity of the cervix and is related to a reduction in adverse pregnancy outcomes. In women who give birth at term, the lipid metabolism of cervicovaginal secretions is significantly reduced in the third trimester, which may be related to the acidic environment inhibiting the growth of pathogenic bacteria, and the antimicrobial component of cervicovaginal secretions, methyl-4-hydroxybenzoate, increases by approximately 8.8 times from the second to the third trimester, helping maintain a stable vaginal microenvironment (Ghartey et al., 2015). The metabolic pathways of amniotic fluid and placental flora are mainly involved in membrane transport, carbohydrate metabolism, amino acid metabolism and energy metabolism, which are closely related to the life activities of the fetal placenta (Aagaard et al., 2014; Collado et al., 2016). Therefore, the metabolic function of the genital tract flora during pregnancy is of great significance for maintaining pregnancy stability.

In normal pregnancy, the mother has increased immune tolerance to fetal-expressed paternal antigens through "extended-self" antigens to maintain the growth of the fetus in the body (Bromfield et al., 2017; Deshmukh and Way, 2019). Maternal forkhead box P3 (FOXP3) Treg cells expand locally at the maternal-fetal interface and expand systemically during pregnancy to maintain allogeneic fetal tolerance (Agostinis et al., 2019; Deshmukh and Way, 2019; Ghaemi et al., 2019). Metabolites are closely related to host immunity during pregnancy. In humans, the metabolism of L-arginine is related to the temporary suppression of the maternal immune response during pregnancy (Kropf et al., 2007). The activity of arginase expressed in the full-term placenta of pregnant women increases significantly, and the high enzyme activity leads to a decrease in its substrate L-arginine, which in turn induces the downregulation of T-cell receptor (TCR) associated ζ-chain (CD3ζ) and the hyporesponsiveness of functional T cells (Ismail, 2018). IDO also uses a similar approach to silence T cells to induce and maintain immune tolerance (Kropf et al., 2007). The normal reproductive tract flora plays an important role in the establishment and consolidation of mother-placental-fetal immunity to resist the interference of external pathogenic bacteria (Mei et al., 2019). Studies have demonstrated a correlation between *Bacteroides* species and TCR$\gamma\delta$+ T cells (participating in mucosal immunity) (Ghaemi et al., 2019). The microbiota can induce the accumulation of Treg cells, which are essential for maintaining immune tolerance, timely endometrial receptivity, and correct placental implantation (Benner et al., 2018). However, dysbiosis in the reproductive genital tract can lead to immune disorders and participate in the occurrence of adverse pregnancy outcomes (Smith and Ravel, 2017). Therefore, a comprehensive interpretation of the reproductive tract microbes, metabolism, and immunity during normal pregnancy provides a reference for discovering the causes and mechanisms of adverse pregnancy outcomes (Wang et al., 2016).

PREGNANCY-RELATED ADVERSE OUTCOMES

Spontaneous Abortion and Infertility

Statistics from the Centers for Disease Control in the United States showed that among married women aged 15–44 years, 6% had infertility and 12% had impaired fecundity, and the incidence increased yearly (Prevention, 2019). RTI is a risk factor leading to reproductive dysfunction (such as infertility, miscarriage, and repeated fertility failures), which in turn leads to a clinical pregnancy rate of only 29.7–43.3% with embryo

transfer technology (Baker et al., 2010; Franasiak et al., 2016; Koedooder et al., 2019). For example, the prevalence of BV in infertile women is 19–28%, while the clinical pregnancy success rate is only 8% (Haahr et al., 2016; Bracewell-Milnes et al., 2018). This observation may be related to abnormal vaginal microbiota and pelvic pathogens (such as *C. trachomatis*) ascending to the upper genital tract through the cervix, leading to PID and reduced fertility (Witkin et al., 1995; Franasiak et al., 2016; Haahr et al., 2016).

The normal reproductive tract flora provides a favorable environment for embryo implantation, which can increase the success rate and live birth rate of *in vitro* fertilization-embryo transfer (IVFET) (Hyman et al., 2012; Sirota et al., 2014; Koedooder et al., 2019). The genital tract microbiota in females with fertility disorders is mainly manifested as a decrease in *Lactobacillus* spp., an increase in non-*Lactobacillus* spp., a high concentration of *Candida* spp., and an increase in the prevalence of asymptomatic BV (Franasiak et al., 2016; Babu et al., 2017; Campisciano et al., 2017; Wee et al., 2018; Koedooder et al., 2019). The live birth rate will be reduced if harmful bacteria, such as *Gardnerella vaginalis*, *Atopobium vaginae*, *Acidovorax* spp., *Enterococcus* spp., and *Streptococcus* spp., are found in the lower reproductive tract (Hyman et al., 2012; Haahr et al., 2016; Wee et al., 2018; Koedooder et al., 2019). Studies have shown that before spontaneous abortion, endometrial aspiration fluid has higher bacterial diversity and lower *Lactobacillus* abundance than before a healthy pregnancy (Moreno et al., 2020). The presence of CST IV microbiota in the endometrium is related to a significant reduction in the incidence of implantation, pregnancy, and continuous pregnancy (Moreno et al., 2016). In 2016, Verstraelen et al. (2016) performed endometrial biopsy on 19 women with fertility disorders (infertility, repeated implantation failures, and repeated miscarriages) and found that 90% of women with fertility disorders mainly had *Bacteroides* phylum as the dominant bacteria in their endometrium. In addition, when *Gardnerella* and *Streptococcus* genra are detected in the endometrium, they have a particularly adverse effect on reproductive outcomes (Moreno et al., 2016). The endometrium microbiota may be carried by sperm and affect the microbial composition of the female reproductive tract (Koedooder et al., 2019). For example, when the detection rate of *Mycoplasma hominis, Neisseria genus, Klebsiella genus* and *Pseudomonas genus* in semen increases, it is not only related to a low sperm concentration, abnormal sperm morphology, high semen viscosity, and oligospermia but also indirectly leads to a decline in female fertility (Ahmadi et al., 2017; Monteiro et al., 2018). Microorganisms also colonize in the follicular fluid, and the low success rate of embryo transfer is related to the colonization of *Propionibacterium* spp. and *Streptococcus* spp. in the follicular fluid (Pelzer et al., 2013). Therefore, the normal genital tract flora is of great significance to the maintenance of fertility.

Endometrial receptivity and follicle quality in people with reproductive disorders are closely related to metabolites in the reproductive tract. Lipid homeostasis is essential for maintaining health (Braga et al., 2019; Hernandez-Vargas et al., 2020). In 2019, Braga et al. (2019) analyzed the lipid metabolism of the endometrial secretions taken from patients with IVFET cycles before transplantation and found that phosphoethanolamine, phosphatidic acid, diacylglycerol, triacylglycerol, glycosyl diacylglycerol, phosphatidylcholine, neutral sphingolipid, and lysophosphatidylglycerol are possible biomarkers of endometrial receptivity and are associated with implantation failure (Altmäe et al., 2014). Follicular fluid is the microenvironment for the growth of oocytes, and the metabolism of follicular fluid indirectly affects the growth and development of oocytes (Bracewell-Milnes et al., 2017). In 2019, Song et al. (2019) conducted a targeted metabolomics analysis of the follicular fluid of patients with recurrent spontaneous abortion after IVFET treatment and found that eight metabolites, namely, dehydroepiandrosterone, lysophosphatidylcholine (lysoPC) (16:0), lysoPC(18:2), lysoPC (18:1), lysoPC(18:0), lysoPC(20:5), lysoPC(20:4), and lysoPC (20:3) were upregulated in the recurrent abortion group, and 10 metabolites, namely, phenylalanine, linoleate, oleic acid, docosahexaenoic acid, lithocholic acid, 25-hydroxyvitamin D3, hydroxycholesterol, 13-hydroxy-alpha-tocopherol, leucine, and tryptophan were downregulated. The above indicators can also predict the success rate of transplantation. Therefore, it is very meaningful to analyze metabolites in people with reproductive disorders. However the metabonomic analysis of cervicovaginal secretions in women with fertility disorders still needs more research to fully prove the role of metabolism in fertility disorders and the interaction between immunity and the flora.

Fertility dysfunction may be related to the destruction of immune tolerance caused by a decline in the number and function of Treg cells (Deshmukh and Way, 2019).Through endometrial biopsies of women with infertility in the mid-secretory phase of the menstrual cycle, it was found that the expression of Foxp3 mRNA was reduced, suggesting that the differentiation of uterine T cells into a Treg cell phenotype is impaired, which may lead to reduced endometrial receptivity (Jasper et al., 2006). Moreover, immunoglobulin-like transcript 4 + (ILT4+) DCs may be involved in the process of recurrent miscarriage and recurrent implantation failure induced by Foxp3 + Treg cells (Liu et al., 2018). A reduction in maternal Treg cell inhibitory ability caused by microbial infection can also cause placental inflammation, leading to the release and activation of fetal-specific maternal CD8+ T cells, which infiltrate the decidua and lead to abortion (Deshmukh and Way, 2019). The microbiota is important for basic CCL2 (monocyte chemotactic protein-1, MCP-1) secretion to control the homeostasis of plasmacytoid DCs, macrophage recruitment and polarization, and local T cell balance (Sierra-Filardi et al., 2014; Swiecki et al., 2017). DCs are a key regulator of immune tolerance during pregnancy. Patients with elevated dehydroepiandrosterone and dehydroepiandrosterone sulfate (DHEAS) in the follicular fluid have DC damage, which can cause infertility or spontaneous abortion by causing the abnormal immunity of oocytes or embryos (Song et al., 2019). When combined with a bacterial flora disorder, it may aggravate the dysfunction of DCs, and reproductive dysfunction is likely. It is known that plasma

tryptophan metabolism is closely related to abortion (Fei et al., 2016). While BV-related bacteria are involved in the metabolism of tryptophan and a variety of amino acids (Srinivasan et al., 2015), it is necessary to further study whether genital tract bacteria and their metabolites cause an imbalance of immune tolerance, affect plasma metabolite levels and participate in the occurrence of reproductive dysfunction. Future research should focus on the local immune effects of genital tract flora and metabolites on people with reproductive dysfunction as the main research direction to explore the impact of the three interactions on reproductive disorders.

Preterm Birth

Every year, 15 million babies are born premature worldwide, accounting for approximately 11% of the live birth population (Blencowe et al., 2012). Preterm birth caused by ascending genitourinary tract infection accounts for 40–50% of all preterm births (Goldenberg et al., 2008). CST-IV vaginal microflora is closely related to premature delivery (DiGiulio et al., 2015; Dunlop et al., 2015; Workowski and Bolan, 2015; Brown et al., 2018; Han et al., 2019). Additionally, studies have confirmed that pregnant women with BV have an increased risk of premature birth (Callahan et al., 2017; Anahtar et al., 2018; Chu et al., 2018; Fettweis et al., 2019; Serrano et al., 2019). Preterm birth is the second leading cause of neonatal death (Liu et al., 2016). Premature babies are prone to diabetes, chronic inflammation and cardiovascular disease in the long term (Snyers et al., 2020). Therefore, the prevention of premature birth is the top priority of medical work.

The Human Microbiome Project Multi-Omic Microbiome Study showed that *L. crispatus* decreased and that BVAB1, *Sneathia amnii*, TM7-H1 (BVAB-TM7), and partial *Prevotella* species increased in the first and second trimesters of women who deliver prematurely, thus, these factors can be used as markers for predicting preterm birth (Fettweis et al., 2019). Studies have also shown that the colonization of vaginal *Streptococcus agalactiae* and *Klebsiella pneumonia* in the second trimester is significantly associated with late miscarriage and very premature delivery (before 28 weeks) (Son et al., 2018; Koedooder et al., 2019). Changes in the cervicovaginal flora of women who deliver prematurely greatly alter the metabolome and are involved in premature cervical remodeling (**Table 3**). Ghartey et al. (2015; 2017) found that women with symptoms of preterm birth and eventually spontaneous preterm birth (sPTB) have significant changes in cervicovaginal metabolites. Lipid metabolism and carbohydrate metabolism in the cervicovaginal secretions of women who deliver prematurely are significantly upregulated, and peptide levels are significantly reduced (Ghartey et al., 2015; Ghartey et al., 2017). Upregulation of lipid and carbohydrate pathways is associated with positive energy utilization and may be related to early cervical remodeling and sPTB microbiota utilization (Ghartey et al., 2015; Ghartey et al., 2017). A decrease in dipeptides may reflect the decreased level of proteolysis in women who deliver prematurely and changes in the activities of proteases and are associated with asymptomatic sPTB (Ghartey et al., 2015). In addition, the level of N-acetylneuraminate in the cervix of women who deliver prematurely increased significantly (by 4.9 times), which may be related to the increased affinity of cells for infection and participate in host immunity (Ghartey et al., 2015).

Embryo development and growth depend to a large extent on placental function, and the placental microbiome may affect fetal and pregnancy outcomes. Chorioamnionitis and intrauterine infection are closely related to premature delivery (Chu et al., 2018). However, the specific source of infection may be the ascending infection of BV bacteria (Fettweis et al., 2019), the ascending carrying of sperm (Svenstrup et al., 2003), the colonization of endometrial bacteria (Cowling et al., 1992; Chu et al., 2018), the retrograde infection of salpingitis, and the blood-borne infection of oral bacteria (Chu et al., 2018). Studies have shown that the bacteria in the uterus of women who deliver prematurely are mainly derived from vaginal bacteria, such as *Burkholderia* taxa, which is significantly enriched in the placenta (Goldenberg et al., 2000; Aagaard et al., 2014). The metabolic enrichment of the lipopolysaccharide biosynthetic pathway of the microbiota in the placenta may be related to the expansion and reproduction of the microbiota (Aagaard et al., 2014). The premature birth rate of women with bacteria detected in amniotic fluid is higher, and the metabolomics of the amniotic fluid of women who deliver prematurely are significantly altered, contributing to the initiation of preterm birth (Menon et al., 2014; Collado et al., 2016). Studies have shown that there are flora on the fetal membranes and that the composition of the fetal membranes is closely related to the degree of inflammation of chorioamnionitis (Prince et al., 2016). Analysis of the metabolic function of the fetal membrane microbiome revealed that a reduction in the pentose phosphate pathway and glycerophospholipid metabolism is related to chorioamnionitis, and a reduction in glycerophosopholipid metabolism will lead to an increase in the production of arachidonic acid, which is related to inflammation and prostanoid synthesis and is involved in premature birth (Prince et al., 2016).

Inflammation and antimicrobial peptide reactions involved in certain vaginal microorganisms play a role in destroying and invading cervical mucus plugs or amniotic membranes and ultimately trigger proinflammatory reactions, leading to premature delivery (Goldenberg et al., 2000; Yarbrough et al., 2015; Smith and Ravel, 2017; Strauss et al., 2018). It is known that *Gardnerella vaginalis* ascends to infect the amniotic membrane and irritate the cervix, leading to premature delivery. *Gardnerella vaginalis* may activate the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasomes through monocyte NLRs; then, NLRP3 binds to and cleaves caspase-1, induces IL-1β, IL-18, and TNF-α secretion, and ultimately leads to premature delivery (Vick et al., 2014). Metabolites are involved in the occurrence and development of preterm labor. When *L. iners* and BV-related microorganisms are increased in the vagina, the ratio of D-type/L-type lactic acid decreases, and matrix metalloproteinase (MMP-8) increases (Witkin et al., 2013; MacIntyre et al., 2015). This process eventually leads to premature cervix maturation and ascending infection of the amniotic membrane and thus,

premature delivery (Yoon et al., 2001). Four proinflammatory cytokines, eotaxin, IL-1β, IL-6 and MIP-1β, are increased significantly in the vagina of women who deliver prematurely (Fettweis et al., 2019). BVAB1, *Sneathia amnii*, TM7-H1, *Prevotella timonensis*, and *Prevotella buccalis* are closely related to the levels of cytokines in vaginal secretions (Fettweis et al., 2019). Both BVAB1 and TM7-H1 can produce pyruvate, acetate, L-lactate and propionate. These SCFAs reduce antimicrobial activity and promote the production of host proinflammatory cytokines and are also involved in the occurrence of preterm labor (Fettweis et al., 2019). Therefore, there is a close correlation between the microbiota, metabolites and host immune status in reproductive tracts of women who deliver prematurely, and future researchers need to study the relevant pathogenesis. However, the relationship of microbial metabolites in the reproductive tract, preterm delivery immunity and the onset of preterm delivery still needs further exploration.

GYNECOLOGICAL ONCOLOGY

Dysbiosis is related to tumor carcinogenicity (Ilhan et al., 2019; Scott et al., 2019). An imbalance in specific microorganisms can lead to host epithelial barrier dysfunction, genome integration, genotoxicity, inflammatory activation, immune abnormalities and metabolic abnormalities, creating a microenvironment that allows tumor growth and further leading to the occurrence, development and/or transfer of gynecological malignancies (Scott et al., 2019; Laniewski et al., 2020). Among them, inflammation is the central feature of carcinogenesis and the main carcinogenic mechanism related to cancer (Scott et al., 2019). Microbial virulence factors can induce chronic inflammation in host tissues, stimulate cell proliferation, cause cell proliferation disorders, and combine with the failure of cell apoptosis to ultimately lead to a malignant phenotype (Scott et al., 2019; Laniewski et al., 2020). Metabolic changes in cancer are the core of tumorigenesis and phenotypic changes (Buckendahl et al., 2011; Zhang et al., 2012; Turkoglu et al., 2016; Hopkins and Meier, 2017; Icard et al., 2018). Human microorganisms are also involved in the formation of carcinogenic metabolites and even exert genotoxicity to cause host deoxyribonucleic acid (DNA) damage and participate in tumor carcinogenicity (Kassie et al., 2001; Scott et al., 2019). Flora disorders can also destroy the host-based anticancer immune monitoring to promote tumor development and progression (Di Pietro et al., 2018; Klein et al., 2019; Scott et al., 2019; Laniewski et al., 2020). Therefore, the balance of the microenvironment of the reproductive tract has a positive effect on maintaining the stability of the microbiota and antitumor effects.

Cervical Intraepithelial Lesions and Cervical Cancer

Cervical cancer (CC) is the most common human papillomavirus (HPV)-related malignant tumor and the fourth most common malignant tumor in women worldwide. In 2018, there were an estimated 570,000 new cases and 311,000 deaths from this disease (Bray et al., 2018). Approximately 85-90% of high-risk HPV infections can be cleared spontaneously, and only 10-15% that persist lead to cervical intraepithelial neoplasia (CIN) and invasive cervical cancer (ICC). HPV-16 and HPV-18 are the main pathogens of CC (Chase et al., 2015). The surface of the cervical mucosa is susceptible to environmental influences. When dysbiosis occurs, the local cervicovaginal microenvironment may promote the progression of malignant tumors together with HPV (Ma et al., 2014; Laniewski et al., 2018; Chorna et al., 2020).

A decrease in *Lactobacillus* spp. and an increase in vaginal pH are closely related to HPV infection, cervical lesions and CC (Laniewski et al., 2018; Ilhan et al., 2019; Laniewski et al., 2020). Cervical squamous intraepithelial lesions or CC also increase the

TABLE 4 | Current existing articles analyzing the correlation between the genital tract flora and metabolites in women with cervical intraepithelial lesions and cervical cancer.

Year	Author	Population	Sample	CST	Metabolites
2020	(Borgogna et al., 2020)	HPV-negative participants (n = 13) and HPV-positive participants (n = 26)	Vaginal swabs	CST-I	Higher concentrations of histamine, 3-n-acetyl-LL-cysteine-S-yl acetaminophen, and gammaaminobutyrate
				CST-III	High levels of 3-n-acetyl-L-cysteine-S-yl acetaminophen
				CST-IV	Low levels of heme, glycerophosphorylcholine, and oxidized glutathione
2019	(Ilhan et al., 2019)	78 premenopausal, non-pregnant women and grouped as follows: healthy HPV-negative (n = 18) and HPV-positive participants (n = 11), low-grade squamous intraepithelial lesions (n = 12), high-grade squamous intraepithelial lesions (n = 27) and invasive cervical carcinoma (n = 10)	Cervicovaginal lavages and vaginal swabs	CST-IV	1) Higher levels of cadaverine, putrescine, tyramine, tryptamine, agmatine, and glutathione synthesis intermediate, 2-hydroxybutyrate, branched chain amino acid metabolism product, alpha-hydroxy-isovalerate, and L-isoleucine metabolism product, 2-hydroxy-3-methyl-valerate 2) Lower levels of nucleotides adenosine and cytosine and xenobiotics such as 2-keto-3-deoxy-gluconate and 1,2,3-benzenetriol
2019	(Kwon et al., 2019)	Normal women (n = 18), cervical intraepithelial neoplasia two or three patients (n = 17), and cervical cancer patients (n = 12)	Cervical swabs	CST-IV	1) Cervical cancer patients: enriched in peptidoglycan biosynthesis (ko00550) pathway 2) Cervical intraeptithelia neoplasia 2/3 patients: enriched in ko00300 (lysine biosynthesis), ko00680 (methane metabolism), and ko05211 (renal cell carcinoma)

CST, Community state types; HPV, human papillomavirus.

diversity of the vaginal flora, limited not only to BV-related microorganisms but also to non-BV bacteria, such as *Streptococcus agalactiae*, *Clostridium* spp., *Pseudomonadales* order, and *Staphylococcus* spp. (Klein et al., 2019; Laniewski et al., 2020). An increase in the CIN stage is also related to an increase in the diversity of the vaginal microbiota, suggesting that microorganisms play a role in the regulation of persistent viral infection and disease progression (Van Ostade et al., 2018). Compared with patients with low-grade squamous intraepithelial lesions (LSILs), *Sneathia sanguinegens*, *Anaerococcus tetradius* and *Peptostreptococcus anaerobius* in patients with high-grade squamous intraepithelial lesions (HSILs) are more enriched in the vagina, and *Mycoplasmatales* order, *Pseudomonadales* order, and *Staphylococcus* spp. are more enriched in the cervix (Mitra et al., 2015; Klein et al., 2019; Laniewski et al., 2020). Another study found that *Sneathia* spp. and *Fusobacterium* spp. exist only in women with cervical lesions or cancer but not in women without lesions (Audirac-Chalifour et al., 2016). Therefore, the presence of *Sneathia* in the vaginal microbiome may be a characteristic microorganism of cervical lesions and CC (Laniewski et al., 2018; Laniewski et al., 2020). Analysis of the metabolic pathways of the bacterial flora in patients with CC showed that the peptidoglycan biosynthesis (ko00550) pathway is significantly enriched (Kwon et al., 2019). Research has also found that the bacterial cell wall peptidoglycan is not only essential for maintaining the overall antiosmotic pressure of the bacteria to ensure cell survival but also participates in the occurrence of inflammation, affecting the function of host neutrophils and the innate immune response. Therefore, cervical microbes may promote the development of CC and precancerous lesions by acting as a modulators of host inflammatory pathways.

The vaginal metabolism characteristics of HPV-infected and uninfected patients are different, and the vaginal CST status drives the metabolic characteristics of HPV-infected patients (Borgogna et al., 2020) (**Table 4**). In vaginal CST III, HPV-infected women have higher levels of biogenic amines than HPV uninfected women (Borgogna et al., 2020). In CST IV, HPV-infected women have lower concentrations of glutathione (GSH), oxidized glutathione (GSSG), glycogen, and phospholipid-related metabolites than uninfected women. There are also differences in the metabolic characteristics of HPV infection, cervical lesions, and CC. Several researchers conducted a study on the metabolome of cervicovaginal secretions in HPV-mediated cervical tumors and found that compared with HPV-negative group, HPV-positive group, and cervical lesion group, the number and diversity of cervical vaginal metabolites in CC patients were increased (Ilhan et al., 2019). Compared with the HPV-negative group, the HPV-positive, LSIL and HSIL groups had fewer amino acids, and their metabolites in cervical and vaginal secretions and the subpathways and depletion levels under the amino acid superpathway were different (Ilhan et al., 2019; Laniewski et al., 2020). In addition, in the vulvovaginal secretions of patients with CC, volatile organic compounds, such as alkanes, and methylated alkanes are different from those of healthy women, which may be related to the oxidation of cell membrane lipids and proteins during the carcinogenic process and the production of volatile organic compounds (Rodriguez-Esquivel et al., 2018; Ilhan et al., 2019). These metabolites can be used as potential biomarkers for CC. In addition, the unique metabolic characteristics in the cervicovaginal microenvironment can assist in the diagnosis and differentiation of health, HPV infection (Borgogna et al., 2020), HSILs, LSILs, and CC (Ilhan et al., 2019). For example, long chain fatty acids, ketone bodies, steroids, ceramides, and plasmalogens can distinguish individuals with ICC from those with HPV (−).

The interaction between the host and reproductive tract microorganisms forms a metabolic network that participates in the formation of the local tumor environment during the process of continuous HPV infection and cancer progression (Ilhan et al., 2019). In HSILs and CC, the vaginal microbial community disrupts amino acid and nucleotide metabolism in a manner similar to that in BV (Ilhan et al., 2019). Compared with healthy individuals, the abundance of lipid metabolites in the vaginas of ICC patients is higher (Ilhan et al., 2019; Szewczyk et al., 2019). This phenomenon may be related to the interaction between microbes and the host, which activates the carcinogenic pathways in the tumor microenvironment and increases cell proliferation and cell membrane synthesis, thus enhancing the carcinogenic activity of the microflora. Changes in the cervicovaginal microbial community cannot only change the cervicovaginal metabolome but also further affect immunity and participate in cancer progression (Ilhan et al., 2019). For example, glycochenodeoxycholate (GCDC) is a key metabolite of host-*Lactobacillus* cometabolism and can inhibit vaginal flora disorders (Ilhan et al., 2019). A decrease in GCDC and *Lactobacillus* species in CC patients leads to a decrease in the ability to induce inflammation and toxic reactions and further leads to a weakened antitumor effect.

The interaction between immunity and metabolites forms a special tumor microenvironment. In the CIN group, the concentrations of IL-8, IL-10, and nitric oxide in cervicovaginal secretions were higher than those in the control group, indicating that these mediators play a role in the tumor immune microenvironment (Tavares-Murta et al., 2008). Since IL-8 is a Th1-type cytokine and has a proinflammatory effect and IL-10 is a Th2-type cytokine and has an anti-inflammatory effect (Fernandes et al., 2015), the interaction mechanism between nitric oxide and the two needs to be further studied. ICC patients with high genital inflammation (high IL-1α, IL-1β, IL-8, MIP-1β, CCL20, regulation on activation normal T-cell expressed and secreted (RANTES), and TNFα expression) had the strongest correlation with lipids. An increase in plasmalogens and long chain polyunsaturated fatty acids in ICC not only indicates abnormal cell metabolism but also has a proinflammatory cytokine precursors effect, inducing abnormal gene expression and disordered cytokine production (Ilhan et al., 2019). Metabolites are also closely related to CC progression and tumor cell growth. CC is characterized by an immunosuppressive microenvironment and Th2-type cytokines (Bedoya et al., 2014). In females with CC, Th2-type cytokines (IL-10 and IL-13) induce the expression of arginase (ASE), which converts L-arginine into L-ornithine and polyamines, and a reduction in L-arginine is related to the downregulation of the immune response, further promoting tumor progression (Bedoya et al., 2014). Therefore, an increase in

polyamines in the vagina flora of CST IV HPV-positive patients is a metabolic feature that HPV uses to escape host immunity and promote tumor progression (Borgogna et al., 2020). More research is needed to support the impact of HPV infection, cervical lesions, and the direct mechanism of action between the bacterial flora, metabolites and immunity in the cervicovaginal secretions of patients with CC on tumor progression and tumor metastasis.

Endometrial Cancer

In 2018, there were an estimated 382,069 new cases and 89,929 deaths related to corpus uteri cancer (Bray et al., 2018). Endometrial cancer (EC) is a perimenopausal and postmenopausal tumor, divided into two categories: type I and type II (Troisi et al., 2018). Type I EC is the most common (Troisi et al., 2018). Environmental factors, including obesity, inflammation, postmenopausal estrogen metabolism imbalance and estrogen therapy, are the main risk factors for the development of type I EC (Laniewski et al., 2020). Type II EC is rare and is mainly related to endometrial atrophy (Troisi et al., 2018). Environmental factors are related to changes in the intestinal and vaginal microbiomes. The close relationship between the flora, estrogen metabolism and obesity indicates the potential role of the microbiome in the etiology of EC (Laniewski et al., 2020). EC is also closely related to PID, and an imbalance in the vaginal flora can cause PID through ascending infection, so an imbalance in the vaginal flora may be indirectly related to EC (Ness et al., 2005; Yang et al., 2015; Champer et al., 2018).

The reproductive tract microbiota is involved in the pathogenesis of EC (Laniewski et al., 2020). In 2016, Walther-Antonio et al. (2016) analyzed the genital tract flora of 17 patients with EC, four with endometrial hyperplasia and 10 with benign uterine diseases and found that in the EC cohort, *Porphyromonas* sp. was common in the vagina and cervix, *Bacteroides* and *Faecalibacterium* sp. were common in the endometrium, and *Bacteroides* sp.was common in the ovary. The endometrial microbiota of the EC and hyperplasia cohorts was similar but differed to some degree from that of the benign cohort. The endometrial hyperplasia and benign cohorts had different microbiota structures, indicating that the microbiota plays a role in the early stages of cell transformation (Walther-Antonio et al., 2016). EC patients usually have a high vaginal pH. The detection of vaginal *Atopobium vaginae* and *Porphyromonas* sp. combined with a high vaginal pH is statistically correlated with EC (Walther-Antonio et al., 2016). Studies have found that *Atopobium vaginae* can induce proinflammatory cytokines and antimicrobial peptides, cause chronic inflammation and local immune disorders, promote *Porphyromonas* sp. infection in cells, destroy normal cell regulatory functions, and ultimately lead to carcinogenic processes (Walther-Antonio et al., 2016; Laniewski et al., 2020). The link between *Atopobium vaginae* and *Porphyromonas* sp. supports the link between BV-related bacteria, immunity and EC (Laniewski et al., 2020).

EC has a unique endometrial metabolic signature. In 2017, Altadill et al. (2017) studied the metabolomics of endometrial tissue samples from 39 EC patients and 17 healthy women and found lipids, kynurenine, endocannabinoids and RNA editing pathway disorders in EC patients. Through further research on RNA editing pathways, we found that adenosine deaminases acting on RNA2 (ADAR2) are overexpressed in EC and are positively correlated with tumor aggressiveness. ADAR2 may contribute to the carcinogenicity of EC and can be used as a potential marker for EC treatment. However, whether the genital tract flora metabolites involved in EC carcinogenesis remains to be studied. It is known that the concentration of hydroxybutyric acid in the reproductive tract of BV patients is elevated. In the intestine, SCFAs (such as hydroxybutyrate) induce Treg cells through histone deacetylases (HDACs) and exert an immunosuppressive effect in innate immune cells (McMillan et al., 2015; Chen and Stappenbeck, 2019). Whether hydroxybutyrate in the reproductive tract induces immune suppression through HDACs and promotes the growth of endometrial tumors requires further *in vitro* experiments.

Ovarian Cancer

Ovarian cancer (OC) is one of the deadliest malignant tumors in women and the main cause of death from gynecological malignancies (Turkoglu et al., 2016; Laniewski et al., 2020). In 2018, an estimated 295,414 new cases of OC were diagnosed worldwide, and 184,799 women died from the disease, ranking fifth among cancer-related deaths (Bray et al., 2018). More than 80% of patients have advanced disease, and the five-year overall survival rate is between 15 and 45% (Turkoglu et al., 2016). Similar to EC, chronic infection of sexually transmitted pathogens and ascending infection of genital tract inflammation are related to the occurrence of OC (Shanmughapriya et al., 2012; Idahl et al., 2020).

In 2019, Nene et al. (2019) first published a study on the presence of abnormal uterine flora in women with OC or at risk of OC and found a strong correlation of OC or the *breast cancer susceptibility gene 1* (*BRCA1*) mutation status with participants aged <50 years and those with a non-*Lactobacillus* dominant microbiota. Women who have used oral contraceptive pills or combined hormones for more than 5 years are more likely to have *Lactobacillus* dominance and a lower risk of OC than women who are using oral contraceptive pills or women who have used combined hormones for less than 5 years. Compared with the healthy surrounding ovarian tissue of the same individual, OC tissue has unique microbial characteristics (Banerjee et al., 2017). Potentially pathogenic intracellular microorganisms, such as *Brucella* spp., *Chlamydia* spp. and *Mycoplasma* spp., are present in 60–76% of ovarian tumors (Banerjee et al., 2017). In addition, an increase in *Proteobacteria* and *Firmicutes* phyla in ovarian tumors, especially an increase in *Actinobacteria* phyla, may cause double-stranded breaks by releasing bacterial toxins (such as colibactin and cytolethal distending toxin) and directly damage cellular DNA (Banerjee et al., 2017; Nene et al., 2019). In addition, several pathogenic viruses, intracellular bacteria, fungi and parasites also exist in ovarian tissue (Banerjee et al., 2017). These microorganisms may induce cancer through direct or indirect mechanisms (Laniewski et al., 2020). It is known that the integration of the HPV genome into the human genome is an important reason for the development of CC. In 2017,

Banerjee et al. (2017) found HPV signals in the tumor tissues of OC patients, and there was also an integration phenomenon. For example, HPV16 has the largest number of viral integration sites in human chromosomes. It can be integrated into various intronic regions and genetic regions within 56 kb upstream of many cancer-related human genes. In addition, the coding sequence of the E1 gene of HPV18 is integrated in the intronic region of the non-coding RNA gene of the host chromosomes. All of the above factors may lead to the dysregulation of gene expression and participate in the occurrence and development of cancer (Banerjee et al., 2017). This research provides new ideas for exploring the molecular mechanism of OC.

It is known that the metabolic characteristics of OC cells, OC tissues, and ascites are significantly changed and are closely related to tumor tissue energy utilization, reproductive tract inflammation, the invasion and migration of OC cells, and the chemotherapy resistance of OC (Fong et al., 2011; Poisson et al., 2015). However, there are still few studies on the correlation between the characteristics of microbial metabolism in the reproductive tract and OC. The ovary is the end organ of the upper genital tract and is affected by the ascending bacteria of the genital tract, the bacteria of the ovary and the flora in the abdominal cavity. Whether the metabolic characteristics of the above bacteria are related to the occurrence and development of ovarian tumors and the carcinogenicity of tumors, leading to a unique tumor microenvironment, needs further research. Metabolites are closely related to tumor immunity and tumor development (Turkoglu et al., 2016; Clifford et al., 2018; Szewczyk et al., 2019). Whether the metabolites of the genital tract flora of OC patients participate in tumor immunity and host antitumor immunity, which affects the occurrence, development and metastasis of OC, still needs further research.

CONCLUSION

The host reproductive tract microenvironment includes microorganisms, metabolites and immunity, and the balance of the interactions among them is essential to maintain reproductive health. Existing research on the relationship between female reproductive tract microbes or immunity and reproductive tract inflammation, pregnancy, and tumors is becoming increasingly detailed. In contrast to research on intestinal metabolites, research on female reproductive tract metabolites is still in the preliminary stage. Moreover, the reproductive tract metabolic characteristics of AV, reproductive dysfunction, EC, and OC still need more research at present, as relevant data are lacking. Additionally, the relationship between microbial metabolites and host immunity in inflammation, pregnancy, and tumors of the female reproductive tract is relatively unclear, and it may become a new direction for future research. According to the current research, BV-related/CST IV bacteria and the microenvironment formed by the reproductive tract have the most comprehensive research on the adverse pregnancy outcomes and tumor pathogenesis. The reproductive tract microenvironment produced by BV/CST IV not only participates in the ascending infection causing PID, which leads to an increased risk of infertility, miscarriage, and premature delivery, but also participates in the increased risks of precancerous lesions and malignancy of the reproductive tract. The pathogenic mechanism of adverse pregnancy outcomes and tumor diseases in the AV microenvironment of the reproductive tract needs to be further explored. Molecular detection technology combined with immune and metabolomics can be used to better describe the function and metabolic status of the flora, infer the possible pathogenic pathways and immune response status, and analyze the complicated relationship of the local microenvironment with inflammation, pregnancy, and tumor diseases. This method is also the best way to study the pathogenic mechanism and disease characteristics of female reproductive tract inflammation, pregnancy, and tumor diseases in the reproductive tract microenvironment. The study of microorganisms, metabolites, and immunity in the microenvironment of the reproductive tract under different diseases (and then the development of targeted therapies for the above three) was the main purpose of this article. At present, there are many studies on the treatment of microorganisms with antibiotics and probiotics. Studies have proven that probiotic supplementation is very helpful in reducing the risk of inflammation, adverse pregnancy outcomes, and cancers (Nene et al., 2019; Laniewski et al., 2020) and improving the ability to respond to cancer treatments and quality of life (Postler and Ghosh, 2017; Champer et al., 2018; Laniewski et al., 2020). However, microbial therapy is still prone to relapse and associated with a high risk of recurrence. Whether targeted therapy for metabolites (Sévin et al., 2015) and immunity (Ventriglia et al., 2017; Deshmukh and Way, 2019) can be used to treat diseases or improve the effect of microbial therapy still needs further research. Therefore, studying the role and mechanism of reproductive tract flora, metabolites, and immunity in disease pathogenesis will aid in disease diagnosis and treatment and improve female reproductive health.

AUTHOR CONTRIBUTIONS

HL, CH, and FX conceived the study question, and all authors were involved in the study design. HL created the first draft of the manuscript. YZ, CW, HYL, and AF made substantial contributions to drafting the article and revising it critically. All authors contributed to the article and approved the submitted version.

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REFERENCES

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., and Versalovic, J. (2014). The placenta harbors a unique microbiome. *Sci. Transl. Med.* 6, 237ra265. doi: 10.1126/scitranslmed.3008599

Abu-Lubad, M., Meyer, T. F., and Al-Zeer, M. A. (2014). Chlamydia trachomatis inhibits inducible NO synthase in human mesenchymal stem cells by stimulating polyamine synthesis. *J. Immunol.* 193, 2941–2951. doi: 10.4049/jimmunol.1400377

Agostinis, C., Mangogna, A., Bossi, F., Ricci, G., Kishore, U., and Bulla, R. (2019). Uterine Immunity and Microbiota: A Shifting Paradigm. *Front. Immunol.* 10, 2387. doi: 10.3389/fimmu.2019.02387

Agrawal, T., Bhengraj, A. R., Vats, V., Salhan, S., and Mittal, A. (2011). Expression of TLR 2, TLR 4 and iNOS in cervical monocytes of Chlamydia trachomatis-infected women and their role in host immune response. *Am. J. Reprod. Immunol.* 66, 534–543. doi: 10.1111/j.1600-0897.2011.01064.x

Ahmadi, M. H., Mirsalehian, A., Sadighi Gilani, M. A., Bahador, A., and Talebi, M. (2017). Asymptomatic Infection With Mycoplasma hominis Negatively Affects Semen Parameters and Leads to Male Infertility as Confirmed by Improved Semen Parameters After Antibiotic Treatment. *Urology* 100, 97–102. doi: 10.1016/j.urology.2016.11.018

Aiyar, A., Quayle, A. J., Buckner, L. R., Sherchand, S. P., Chang, T. L., Zea, A. H., et al. (2014). Influence of the tryptophan-indole-IFNγ axis on human genital Chlamydia trachomatis infection: role of vaginal co-infections. *Front. Cell Infect. Microbiol.* 4, 72. doi: 10.3389/fcimb.2014.00072

Aldunate, M., Srbinovski, D., Hearps, A. C., Latham, C. F., Ramsland, P. A., Gugasyan, R., et al. (2015). Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis. *Front. Physiol.* 6, 164. doi: 10.3389/fphys.2015.00164

Al-Mushrif, S., Eley, A., and Jones, B. M. (2000). Inhibition of chemotaxis by organic acids from anaerobes may prevent a purulent response in bacterial vaginosis. *J. Med. Microbiol.* 49, 1023–1030. doi: 10.1099/0022-1317-49-11-1023

Altadill, T., Dowdy, T. M., Gill, K., Reques, A., Menon, S. S., Moiola, C. P., et al. (2017). Metabolomic and Lipidomic Profiling Identifies The Role of the RNA Editing Pathway in Endometrial Carcinogenesis. *Sci. Rep.* 7, 8803. doi: 10.1038/s41598-017-09169-2

Altmäe, S., Esteban, F. J., Stavreus-Evers, A., Simón, C., Giudice, L., Lessey, B. A., et al. (2014). Guidelines for the design, analysis and interpretation of 'omics' data: focus on human endometrium. *Hum. Reprod. Update* 20, 12–28. doi: 10.1093/humupd/dmt048

Anahtar, M. N., Gootenberg, D. B., Mitchell, C. M., and Kwon, D. S. (2018). Cervicovaginal Microbiota and Reproductive Health: The Virtue of Simplicity. *Cell Host Microbe* 23, 159–168. doi: 10.1016/j.chom.2018.01.013

Audirac-Chalifour, A., Torres-Poveda, K., Bahena-Román, M., Téllez-Sosa, J., Martínez-Barnetche, J., Cortina-Ceballos, B., et al. (2016). Cervical Microbiome and Cytokine Profile at Various Stages of Cervical Cancer: A Pilot Study. *PloS One* 11, e0153274. doi: 10.1371/journal.pone.0153274

Babu, G., Singaravelu, B. G., Srikumar, R., Reddy, S. V., and Kokan, A. (2017). Comparative Study on the Vaginal Flora and Incidence of Asymptomatic Vaginosis among Healthy Women and in Women with Infertility Problems of Reproductive Age. *J. Clin. Diagn. Res.* 11, Dc18–dc22. doi: 10.7860/JCDR/2017/28296.10417

Baker, V. L., Jones, C. E., Cometti, B., Hoehler, F., Salle, B., Urbancsek, J., et al. (2010). Factors affecting success rates in two concurrent clinical IVF trials: an examination of potential explanations for the difference in pregnancy rates between the United States and Europe. *Fertil. Steril.* 94, 1287–1291. doi: 10.1016/j.fertnstert.2009.07.1673

Banerjee, S., Tian, T., Wei, Z., Shih, N., Feldman, M. D., Alwine, J. C., et al. (2017). The ovarian cancer oncobiome. *Oncotarget* 8, 36225–36245. doi: 10.18632/oncotarget.16717

Baqui, A. H., Lee, A. C. C., Koffi, A. K., Khanam, R., Mitra, D. K., Dasgupta, S. K., et al. (2019). Prevalence of and risk factors for abnormal vaginal flora and its association with adverse pregnancy outcomes in a rural district in north-east Bangladesh. *Acta Obstet. Gynecol. Scand.* 98, 309–319. doi: 10.1111/aogs.13492

Beatty, W. L., Belanger, T. A., Desai, A. A., Morrison, R. P., and Byrne, G. I. (1994). Tryptophan depletion as a mechanism of gamma interferon-mediated chlamydial persistence. *Infect. Immun.* 62, 3705–3711. doi: 10.1128/IAI.62.9.3705-3711.1994

Bedoya, A. M., Tate, D. J., Baena, A., Cordoba, C. M., Borrero, M., Pareja, R., et al. (2014). Immunosuppression in cervical cancer with special reference to arginase activity. *Gynecol. Oncol.* 135, 74–80. doi: 10.1016/j.ygyno.2014.07.096

Belland, R. J., Nelson, D. E., Virok, D., Crane, D. D., Hogan, D., Sturdevant, D., et al. (2003). Transcriptome analysis of chlamydial growth during IFN-gamma-mediated persistence and reactivation. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15971–15976. doi: 10.1073/pnas.2535394100

Benner, M., Ferwerda, G., Joosten, I., and Van Der Molen, R. G. (2018). How uterine microbiota might be responsible for a receptive, fertile endometrium. *Hum. Reprod. Update* 24, 393–415. doi: 10.1093/humupd/dmy012

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A. B., Narwal, R., et al. (2012). National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet* 379, 2162–2172. doi: 10.1016/S0140-6736(12)60820-4

Borgogna, J. C., Shardell, M. D., Santori, E. K., Nelson, T. M., Rath, J. M., Glover, E. D., et al. (2020). The vaginal metabolome and microbiota of cervical HPV-positive and HPV-negative women: a cross-sectional analysis. *Bjog* 127, 182–192. doi: 10.1111/1471-0528.15981

Bracewell-Milnes, T., Saso, S., Abdalla, H., Nikolau, D., Norman-Taylor, J., Johnson, M., et al. (2017). Metabolomics as a tool to identify biomarkers to predict and improve outcomes in reproductive medicine: a systematic review. *Hum. Reprod. Update* 23, 723–736. doi: 10.1093/humupd/dmx023

Bracewell-Milnes, T., Saso, S., Nikolaou, D., Norman-Taylor, J., Johnson, M., and Thum, M. Y. (2018). Investigating the effect of an abnormal cervico-vaginal and endometrial microbiome on assisted reproductive technologies: A systematic review. *Am. J. Reprod. Immunol.* 80, e13037. doi: 10.1111/aji.13037

Braga, D., Borges, E.Jr., Godoy, A. T., Montani, D. A., Setti, A. S., Zanetti, B. F., et al. (2019). Lipidomic profile as a noninvasive tool to predict endometrial receptivity. *Mol. Reprod. Dev.* 86, 145–155. doi: 10.1002/mrd.23088

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68, 394–424. doi: 10.3322/caac.21492

Bromfield, J. J., Rizo, J. A., and Ibrahim, L. A. (2017). Paternal priming of maternal tissues to optimise pregnancy success. *Reprod. Fertil. Dev.* 30, 50–55. doi: 10.1071/RD17345

Brown, R. G., Marchesi, J. R., Lee, Y. S., Smith, A., Lehne, B., Kindinger, L. M., et al. (2018). Vaginal dysbiosis increases risk of preterm fetal membrane rupture, neonatal sepsis and is exacerbated by erythromycin. *BMC Med.* 16, 9. doi: 10.1186/s12916-017-0999-x

Brunham, R. C., and Rey-Ladino, J. (2005). Immunology of Chlamydia infection: implications for a Chlamydia trachomatis vaccine. *Nat. Rev. Immunol.* 5, 149–161. doi: 10.1038/nri1551

Buckendahl, A. C., Budczies, J., Fiehn, O., Darb-Esfahani, S., Kind, T., Noske, A., et al. (2011). Prognostic impact of AMP-activated protein kinase expression in ovarian carcinoma: correlation of protein expression and GC/TOF-MS-based metabolomics. *Oncol. Rep.* 25, 1005–1012. doi: 10.3892/or.2011.1162

Budilovskaya, O. V., Shipitsina, E. V., Spasibova, E. V., Pereverzeva, N. A., Vorob'eva, N. E., Tsypurdeeva, N. D., et al. (2020). Differential Expression of Local Immune Response Genes in the Vagina: Implication for the Diagnosis of Vaginal Infections. *Bull. Exp. Biol. Med.* 168, 646–650. doi: 10.1007/s10517-020-04771-3

Byrne, G. I., Carlin, J. M., Merkert, T. P., and Arter, D. L. (1989). Long-term effects of gamma interferon on chlamydia-infected host cells: microbicidal activity follows microbistasis. *Infect. Immun.* 57, 1318–1320. doi: 10.1128/IAI.57.4.1318-1320.1989

Callahan, B. J., Digiulio, D. B., Goltsman, D. S. A., Sun, C. L., Costello, E. K., Jeganathan, P., et al. (2017). Replication and refinement of a vaginal microbial signature of preterm birth in two racially distinct cohorts of US women. *Proc. Natl. Acad. Sci. U.S.A.* 114, 9966–9971. doi: 10.1073/pnas.1705899114

Campisciano, G., Florian, F., D'eustacchio, A., Stankovic, D., Ricci, G., De Seta, F., et al. (2017). Subclinical alteration of the cervical-vaginal microbiome in women with idiopathic infertility. *J. Cell Physiol.* 232, 1681–1688. doi: 10.1002/jcp.25806

Ceccarani, C., Foschi, C., Parolin, C., D'antuono, A., Gaspari, V., Consolandi, C., et al. (2019). Diversity of vaginal microbiome and metabolome during genital infections. *Sci. Rep.* 9, 14095. doi: 10.1038/s41598-019-50410-x

Champer, M., Wong, A. M., Champer, J., Brito, I. L., Messer, P. W., Hou, J. Y., et al. (2018). The role of the vaginal microbiome in gynaecological cancer. *Bjog* 125, 309–315. doi: 10.1111/1471-0528.14631

Chase, D., Goulder, A., Zenhausern, F., Monk, B., and Herbst-Kralovetz, M. (2015). The vaginal and gastrointestinal microbiomes in gynecologic cancers: a review of applications in etiology, symptoms and treatment. *Gynecol. Oncol.* 138, 190–200. doi: 10.1016/j.ygyno.2015.04.036

Chen, C., Song, X., Wei, W., Zhong, H., Dai, J., Lan, Z., et al. (2017). The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat. Commun.* 8, 875. doi: 10.1038/s41467-017-00901-0

Chen, F., and Stappenbeck, T. S. (2019). Microbiome control of innate reactivity. *Curr. Opin. Immunol.* 56, 107–113. doi: 10.1016/j.coi.2018.12.003

Chorna, N., Romaguera, J., and Godoy-Vitorino, F. (2020). Cervicovaginal Microbiome and Urine Metabolome Paired Analysis Reveals Niche Partitioning of the Microbiota in Patients with Human Papilloma Virus Infections. *Metabolites* 10, 36. doi: 10.3390/metabo10010036

Chu, D. M., Seferovic, M., Pace, R. M., and Aagaard, K. M. (2018). The microbiome in preterm birth. *Best Pract. Res. Clin. Obstet. Gynaecol.* 52, 103–113. doi: 10.1016/j.bpobgyn.2018.03.006

Clifford, C., Vitkin, N., Nersesian, S., Reid-Schachter, G., Francis, J. A., and Koti, M. (2018). Multi-omics in high-grade serous ovarian cancer: Biomarkers from genome to the immunome. *Am. J. Reprod. Immunol.* 80, e12975. doi: 10.1111/aji.12975

Collado, M. C., Rautava, S., Aakko, J., Isolauri, E., and Salminen, S. (2016). Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Sci. Rep.* 6, 23129. doi: 10.1038/srep23129

Consortium, H. M. P. (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

Cowling, P., Mccoy, D. R., Marshall, R. J., Padfield, C. J., and Reeves, D. S. (1992). Bacterial colonization of the non-pregnant uterus: a study of pre-menopausal abdominal hysterectomy specimens. *Eur. J. Clin. Microbiol. Infect. Dis.* 11, 204–205. doi: 10.1007/BF01967084

De Goffau, M. C., Lager, S., Sovio, U., Gaccioli, F., Cook, E., Peacock, S. J., et al. (2019). Human placenta has no microbiome but can contain potential pathogens. *Nature* 572, 329–334. doi: 10.1038/s41586-019-1451-5

Delgado-Diaz, D. J., Tyssen, D., Hayward, J. A., Gugasyan, R., Hearps, A. C., and Tachedjian, G. (2019). Distinct Immune Responses Elicited From Cervicovaginal Epithelial Cells by Lactic Acid and Short Chain Fatty Acids Associated With Optimal and Non-optimal Vaginal Microbiota. *Front. Cell Infect. Microbiol.* 9, 446. doi: 10.3389/fcimb.2019.00446

Deshmukh, H., and Way, S. S. (2019). Immunological Basis for Recurrent Fetal Loss and Pregnancy Complications. *Annu. Rev. Pathol.* 14, 185–210. doi: 10.1146/annurev-pathmechdis-012418-012743

Di Pietro, M., Filardo, S., Porpora, M. G., Recine, N., Latino, M. A., and Sessa, R. (2018). HPV/Chlamydia trachomatis co-infection: metagenomic analysis of cervical microbiota in asymptomatic women. *New Microbiol.* 41, 34–41.

Diep, B. A., Stone, G. G., Basuino, L., Graber, C. J., Miller, A., Des Etages, S. A., et al. (2008). The arginine catabolic mobile element and staphylococcal chromosomal cassette mec linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant Staphylococcus aureus. *J. Infect. Dis.* 197, 1523–1530. doi: 10.1086/587907

DiGiulio, D. B., Callahan, B. J., Mcmurdie, P. J., Costello, E. K., Lyell, D. J., Robaczewska, A., et al. (2015). Temporal and spatial variation of the human microbiota during pregnancy. *Proc. Natl. Acad. Sci. U.S.A.* 112, 11060–11065. doi: 10.1073/pnas.1502875112

Donders, G. G. G., Bellen, G., Grinceviciene, S., Ruban, K., and Vieira-Baptista, P. (2017). Aerobic vaginitis: no longer a stranger. *Res. Microbiol.* 168, 845–858. doi: 10.1016/j.resmic.2017.04.004

Dunlop, A. L., Mulle, J. G., Ferranti, E. P., Edwards, S., Dunn, A. B., and Corwin, E. J. (2015). Maternal Microbiome and Pregnancy Outcomes That Impact Infant Health: A Review. *Adv. Neonatal Care* 15, 377–385. doi: 10.1097/ANC.0000000000000218

Fehlner-Gardiner, C., Roshick, C., Carlson, J. H., Hughes, S., Belland, R. J., Caldwell, H. D., et al. (2002). Molecular basis defining human Chlamydia trachomatis tissue tropism. A possible role for tryptophan synthase. *J. Biol. Chem.* 277, 26893–26903. doi: 10.1074/jbc.M203937200

Fei, H., Hou, J., Wu, Z., Zhang, L., Zhao, H., Dong, X., et al. (2016). Plasma metabolomic profile and potential biomarkers for missed abortion. *BioMed. Chromatogr.* 30, 1942–1952. doi: 10.1002/bmc.3770

Fernandes, J. V., Ta, D. E. M. F., Jc, D. E. A., Cobucci, R. N., Mg, D. E. C., Andrade, V. S., et al. (2015). Link between chronic inflammation and human papillomavirus-induced carcinogenesis (Review). *Oncol. Lett.* 9, 1015–1026. doi: 10.3892/ol.2015.2884

Fettweis, J. M., Serrano, M. G., Brooks, J. P., Edwards, D. J., Girerd, P. H., Parikh, H. I., et al. (2019). The vaginal microbiome and preterm birth. *Nat. Med.* 25, 1012–1021. doi: 10.1038/s41591-019-0450-2

Fong, M. Y., Mcdunn, J., and Kakar, S. S. (2011). Identification of metabolites in the normal ovary and their transformation in primary and metastatic ovarian cancer. *PloS One* 6, e19963. doi: 10.1371/journal.pone.0019963

Franasiak, J. M., Werner, M. D., Juneau, C. R., Tao, X., Landis, J., Zhan, Y., et al. (2016). Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit. *J. Assist. Reprod. Genet.* 33, 129–136. doi: 10.1007/s10815-015-0614-z

France, M. T., Mendes-Soares, H., and Forney, L. J. (2016). Genomic Comparisons of Lactobacillus crispatus and Lactobacillus iners Reveal Potential Ecological Drivers of Community Composition in the Vagina. *Appl. Environ. Microbiol.* 82, 7063–7073. doi: 10.1128/AEM.02385-16

Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schutte, U. M., Zhong, X., et al. (2012). Temporal dynamics of the human vaginal microbiota. *Sci. Transl. Med.* 4, 132ra152. doi: 10.1126/scitranslmed.3003605

Ghaemi, M. S., Digiulio, D. B., Contrepois, K., Callahan, B., Ngo, T. T. M., Lee-Mcmullen, B., et al. (2019). Multiomics modeling of the immunome, transcriptome, microbiome, proteome and metabolome adaptations during human pregnancy. *Bioinformatics* 35, 95–103. doi: 10.1093/bioinformatics/bty537

Ghartey, J., Bastek, J. A., Brown, A. G., Anglim, L., and Elovitz, M. A. (2015). Women with preterm birth have a distinct cervicovaginal metabolome. *Am. J. Obstet. Gynecol.* 212, 776.e771–776.e712. doi: 10.1016/j.ajog.2015.03.052

Ghartey, J., Anglim, L., Romero, J., Brown, A., and Elovitz, M. A. (2017). Women with Symptomatic Preterm Birth Have a Distinct Cervicovaginal Metabolome. *Am. J. Perinatol.* 34, 1078–1083. doi: 10.1055/s-0037-1603817

Goldenberg, R. L., Hauth, J. C., and Andrews, W. W. (2000). Intrauterine infection and preterm delivery. *N. Engl. J. Med.* 342, 1500–1507. doi: 10.1056/NEJM200005183422007

Goldenberg, R. L., Culhane, J. F., Iams, J. D., and Romero, R. (2008). Epidemiology and causes of preterm birth. *Lancet* 371, 75–84. doi: 10.1016/S0140-6736(08)60074-4

Gong, Z., Luna, Y., Yu, P., and Fan, H. (2014). Lactobacilli inactivate Chlamydia trachomatis through lactic acid but not H2O2. *PloS One* 9, e107758. doi: 10.1371/journal.pone.0107758

Haahr, T., Jensen, J. S., Thomsen, L., Duus, L., Rygaard, K., and Humaidan, P. (2016). Abnormal vaginal microbiota may be associated with poor reproductive outcomes: a prospective study in IVF patients. *Hum. Reprod.* 31, 795–803. doi: 10.1093/humrep/dew026

Han, C., Li, H., Han, L., Wang, C., Yan, Y., Qi, W., et al. (2019). Aerobic vaginitis in late pregnancy and outcomes of pregnancy. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 233–239. doi: 10.1007/s10096-018-3416-2

Harper, A., Pogson, C. I., Jones, M. L., and Pearce, J. H. (2000). Chlamydial development is adversely affected by minor changes in amino acid supply, blood plasma amino acid levels, and glucose deprivation. *Infect. Immun.* 68, 1457–1464. doi: 10.1128/IAI.68.3.1457-1464.2000

Hernandez-Vargas, P., Munoz, M., and Dominguez, F. (2020). Identifying biomarkers for predicting successful embryo implantation: applying single to multi-OMICs to improve reproductive outcomes. *Hum. Reprod. Update* 26, 264–301. doi: 10.1093/humupd/dmz042

Hooper, L. V., Littman, D. R., and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science* 336, 1268–1273. doi: 10.1126/science.1223490

Hopkins, M. M., and Meier, K. E. (2017). Free fatty acid receptor (FFAR) agonists inhibit proliferation of human ovarian cancer cells. *Prostaglandins Leukot. Essent. Fatty Acids* 122, 24–29. doi: 10.1016/j.plefa.2017.06.013

Hyman, R. W., Herndon, C. N., Jiang, H., Palm, C., Fukushima, M., Bernstein, D., et al. (2012). The dynamics of the vaginal microbiome during infertility therapy with in vitro fertilization-embryo transfer. *J. Assist. Reprod. Genet.* 29, 105–115. doi: 10.1007/s10815-011-9694-6

Icard, P., Shulman, S., Farhat, D., Steyaert, J. M., Alifano, M., and Lincet, H. (2018). How the Warburg effect supports aggressiveness and drug resistance of cancer cells? *Drug Resist. Update* 38, 1–11. doi: 10.1016/j.drup.2018.03.001

Idahl, A., Le Cornet, C., González Maldonado, S., Waterboer, T., Bender, N., Tjønneland, A., et al. (2020). Serologic markers of Chlamydia trachomatis and other sexually transmitted infections and subsequent ovarian cancer risk: Results from the EPIC cohort. *Int. J. Cancer* 147, 2042–2052. doi: 10.1002/ijc.32999

Ilhan, Z. E., Laniewski, P., Thomas, N., Roe, D. J., Chase, D. M., and Herbst-Kralovetz, M. M. (2019). Deciphering the complex interplay between microbiota, HPV, inflammation and cancer through cervicovaginal metabolic profiling. *EBioMedicine* 44, 675–690. doi: 10.1016/j.ebiom.2019.04.028

Ismail, A. Q. T. (2018). Does placental MDSC-mediated modulation of arginine levels help protect the foetus from auxotrophic pathogens? *J. Matern. Fetal Neonatal Med.* 31, 1667–1669. doi: 10.1080/14767058.2017.1319935

Jasper, M. J., Tremellen, K. P., and Robertson, S. A. (2006). Primary unexplained infertility is associated with reduced expression of the T-regulatory cell transcription factor Foxp3 in endometrial tissue. *Mol. Hum. Reprod.* 12, 301–308. doi: 10.1093/molehr/gal032

Johnson, R. M. (2004). Murine oviduct epithelial cell cytokine responses to Chlamydia muridarum infection include interleukin-12-p70 secretion. *Infect. Immun.* 72, 3951–3960. doi: 10.1128/IAI.72.7.3951-3960.2004

Jones, M. L., Ganopolsky, J. G., Labbé, A., Wahl, C., and Prakash, S. (2010). Antimicrobial properties of nitric oxide and its application in antimicrobial formulations and medical devices. *Appl. Microbiol. Biotechnol.* 88, 401–407. doi: 10.1007/s00253-010-2733-x

Kassie, F., Rabot, S., Kundi, M., Chabicovsky, M., Qin, H. M., and Knasmüller, S. (2001). Intestinal microflora plays a crucial role in the genotoxicity of the cooked food mutagen 2-amino-3-methylimidazo [4,5-f]quinoline. *Carcinogenesis* 22, 1721–1725. doi: 10.1093/carcin/22.10.1721

Klein, C., Gonzalez, D., Samwel, K., Kahesa, C., Mwaiselage, J., Aluthge, N., et al. (2019). Relationship between the Cervical Microbiome, HIV Status, and Precancerous Lesions. *mBio* 10, e02785-18. doi: 10.1128/mBio.02785-18

Koedooder, R., Mackens, S., Budding, A., Fares, D., Blockeel, C., Laven, J., et al. (2019). Identification and evaluation of the microbiome in the female and male reproductive tracts. *Hum. Reprod. Update* 25, 298–325. doi: 10.1093/humupd/dmy048

Kropf, P., Baud, D., Marshall, S. E., Munder, M., Mosley, A., Fuentes, J. M., et al. (2007). Arginase activity mediates reversible T cell hyporesponsiveness in human pregnancy. *Eur. J. Immunol.* 37, 935–945. doi: 10.1002/eji.200636542

Kwon, M., Seo, S. S., Kim, M. K., Lee, D. O., and Lim, M. C. (2019). Compositional and Functional Differences between Microbiota and Cervical Carcinogenesis as Identified by Shotgun Metagenomic Sequencing. *Cancers (Basel)* 11, 309. doi: 10.3390/cancers11030309

Kyongo, J. K., Jespers, V., Goovaerts, O., Michiels, J., Menten, J., Fichorova, R. N., et al. (2012). Searching for lower female genital tract soluble and cellular biomarkers: defining levels and predictors in a cohort of healthy Caucasian women. *PloS One* 7, e43951. doi: 10.1371/journal.pone.0043951

Laniewski, P., Barnes, D., Goulder, A., Cui, H., Roe, D. J., Chase, D. M., et al. (2018). Linking cervicovaginal immune signatures, HPV and microbiota composition in cervical carcinogenesis in non-Hispanic and Hispanic women. *Sci. Rep.* 8, 7593. doi: 10.1038/s41598-018-25879-7

Laniewski, P., Ilhan, Z. E., and Herbst-Kralovetz, M. M. (2020). The microbiome and gynaecological cancer development, prevention and therapy. *Nat. Rev. Urol.* 17, 232–250. doi: 10.1038/s41585-020-0286-z

Leiby, J. S., Mccormick, K., Sherrill-Mix, S., Clarke, E. L., Kessler, L. R., Taylor, L. J., et al. (2018). Lack of detection of a human placenta microbiome in samples from preterm and term deliveries. *Microbiome* 6, 196. doi: 10.1186/s40168-018-0575-4

Lewis, M. E., Belland, R. J., Abdelrahman, Y. M., Beatty, W. L., Aiyar, A. A., Zea, A. H., et al. (2014). Morphologic and molecular evaluation of Chlamydia trachomatis growth in human endocervix reveals distinct growth patterns. *Front. Cell Infect. Microbiol.* 4, 71. doi: 10.3389/fcimb.2014.00071

Lim, E. S., Rodriguez, C., and Holtz, L. R. (2018). Amniotic fluid from healthy term pregnancies does not harbor a detectable microbial community. *Microbiome* 6, 87. doi: 10.1186/s40168-018-0475-7

Liu, L., Oza, S., Hogan, D., Chu, Y., Perin, J., Zhu, J., et al. (2016). Global, regional, and national causes of under-5 mortality in 2000-15: an updated systematic analysis with implications for the Sustainable Development Goals. *Lancet* 388, 3027–3035. doi: 10.1016/S0140-6736(16)31593-8

Liu, S., Wei, H., Li, Y., Huang, C., Lian, R., Xu, J., et al. (2018). Downregulation of ILT4(+) dendritic cells in recurrent miscarriage and recurrent implantation failure. *Am. J. Reprod. Immunol.* 80, e12998. doi: 10.1111/aji.12998

Ma, Y., Madupu, R., Karaoz, U., Nossa, C. W., Yang, L., Yooseph, S., et al. (2014). Human papillomavirus community in healthy persons, defined by metagenomics analysis of human microbiome project shotgun sequencing data sets. *J. Virol.* 88, 4786–4797. doi: 10.1128/JVI.00093-14

MacIntyre, D. A., Chandiramani, M., Lee, Y. S., Kindinger, L., Smith, A., Angelopoulos, N., et al. (2015). The vaginal microbiome during pregnancy and the postpartum period in a European population. *Sci. Rep.* 5, 8988. doi: 10.1038/srep08988

Macklaim, J. M., Fernandes, A. D., Di Bella, J. M., Hammond, J. A., Reid, G., and Gloor, G. B. (2013). Comparative meta-RNA-seq of the vaginal microbiota and differential expression by Lactobacillus iners in health and dysbiosis. *Microbiome* 1, 12. doi: 10.1186/2049-2618-1-12

McMillan, A., Rulisa, S., Sumarah, M., Macklaim, J. M., Renaud, J., Bisanz, J. E., et al. (2015). A multi-platform metabolomics approach identifies highly specific biomarkers of bacterial diversity in the vagina of pregnant and non-pregnant women. *Sci. Rep.* 5, 14174. doi: 10.1038/srep14174

Mei, C., Yang, W., Wei, X., Wu, K., and Huang, D. (2019). The Unique Microbiome and Innate Immunity During Pregnancy. *Front. Immunol.* 10, 2886. doi: 10.3389/fimmu.2019.02886

Menon, R., Jones, J., Gunst, P. R., Kacerovsky, M., Fortunato, S. J., Saade, G. R., et al. (2014). Amniotic fluid metabolomic analysis in spontaneous preterm birth. *Reprod. Sci.* 21, 791–803. doi: 10.1177/1933719113518987

Mirmonsef, P., Gilbert, D., Zariffard, M. R., Hamaker, B. R., Kaur, A., Landay, A. L., et al. (2011). The effects of commensal bacteria on innate immune responses in the female genital tract. *Am. J. Reprod. Immunol.* 65, 190–195. doi: 10.1111/j.1600-0897.2010.00943.x

Mitchell, C., Manhart, L. E., Thomas, K., Fiedler, T., Fredricks, D. N., and Marrazzo, J. (2012). Behavioral predictors of colonization with Lactobacillus crispatus or Lactobacillus jensenii after treatment for bacterial vaginosis: a cohort study. *Infect. Dis. Obstet. Gynecol.* 2012, 706540. doi: 10.1155/2012/706540

Mitra, A., Macintyre, D. A., Lee, Y. S., Smith, A., Marchesi, J. R., Lehne, B., et al. (2015). Cervical intraepithelial neoplasia disease progression is associated with increased vaginal microbiome diversity. *Sci. Rep.* 5, 16865. doi: 10.1038/srep16865

Molenaar, M. C., Singer, M., and Ouburg, S. (2018). The two-sided role of the vaginal microbiome in Chlamydia trachomatis and Mycoplasma genitalium pathogenesis. *J. Reprod. Immunol.* 130, 11–17. doi: 10.1016/j.jri.2018.08.006

Monteiro, C., Marques, P. I., Cavadas, B., Damião, I., Almeida, V., Barros, N., et al. (2018). Characterization of microbiota in male infertility cases uncovers differences in seminal hyperviscosity and oligoasthenoteratozoospermia possibly correlated with increased prevalence of infectious bacteria. *Am. J. Reprod. Immunol.* 79, e12838. doi: 10.1111/aji.12838

Moreno, I., Codoner, F. M., Vilella, F., Valbuena, D., Martinez-Blanch, J. F., Jimenez-Almazan, J., et al. (2016). Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am. J. Obstet. Gynecol.* 215, 684–703. doi: 10.1016/j.ajog.2016.09.075

Moreno, I., Garcia-Grau, I., Bau, D., Perez-Villaroya, D., Gonzalez-Monfort, M., Vilella, F., et al. (2020). The first glimpse of the endometrial microbiota in early pregnancy. *Am. J. Obstet. Gynecol.* 222, 296–305. doi: 10.1016/j.ajog.2020.01.031

Nene, N. R., Reisel, D., Leimbach, A., Franchi, D., Jones, A., Evans, I., et al. (2019). Association between the cervicovaginal microbiome, BRCA1 mutation status, and risk of ovarian cancer: a case-control study. *Lancet Oncol.* 20, 1171–1182. doi: 10.1016/S1470-2045(19)30340-7

Ness, R. B., Kip, K. E., Hillier, S. L., Soper, D. E., Stamm, C. A., Sweet, R. L., et al. (2005). A cluster analysis of bacterial vaginosis-associated microflora and

pelvic inflammatory disease. *Am. J. Epidemiol.* 162, 585–590. doi: 10.1093/aje/kwi243

Nuriel-Ohayon, M., Neuman, H., and Koren, O. (2016). Microbial Changes during Pregnancy, Birth, and Infancy. *Front. Microbiol.* 7, 1031. doi: 10.3389/fmicb.2016.01031

Olive, A. J., and Sassetti, C. M. (2016). Metabolic crosstalk between host and pathogen: sensing, adapting and competing. *Nat. Rev. Microbiol.* 14, 221–234. doi: 10.1038/nrmicro.2016.12

Organization, W. H. (2020). *World Health Organization Global adult estimates of chlamydia, gonorrhoea, trichomoniasis and syphilis including maternal and congenital syphilis, 2016*. Available at: https://www.who.int/reproductivehealth/topics/rtis/STIs-Estimates.pdf?ua=1 (Accessed 2020).

Parolin, C., Foschi, C., Laghi, L., Zhu, C., Banzola, N., Gaspari, V., et al. (2018). Insights Into Vaginal Bacterial Communities and Metabolic Profiles of Chlamydia trachomatis Infection: Positioning Between Eubiosis and Dysbiosis. *Front. Microbiol.* 9, 600. doi: 10.3389/fmicb.2018.00600

Peebles, K., Velloza, J., Balkus, J. E., Mcclelland, R. S., and Barnabas, R. V. (2019). High Global Burden and Costs of Bacterial Vaginosis: A Systematic Review and Meta-Analysis. *Sex Transm. Dis.* 46, 304–311. doi: 10.1097/OLQ.0000000000000972

Pelzer, E. S., Allan, J. A., Waterhouse, M. A., Ross, T., Beagley, K. W., and Knox, C. L. (2013). Microorganisms within human follicular fluid: effects on IVF. *PloS One* 8, e59062. doi: 10.1371/journal.pone.0059062

Pereira, N., Edlind, T. D., Schlievert, P. M., and Nyirjesy, P. (2013). Vaginal toxic shock reaction triggering desquamative inflammatory vaginitis. *J. Low. Genit. Tract Dis.* 17, 88–91. doi: 10.1097/LGT.0b013e3182656991

Poisson, L. M., Munkarah, A., Madi, H., Datta, I., Hensley-Alford, S., Tebbe, C., et al. (2015). A metabolomic approach to identifying platinum resistance in ovarian cancer. *J. Ovarian Res.* 8, 13. doi: 10.1186/s13048-015-0140-8

Postler, T. S., and Ghosh, S. (2017). Understanding the Holobiont: How Microbial Metabolites Affect Human Health and Shape the Immune System. *Cell Metab.* 26, 110–130. doi: 10.1016/j.cmet.2017.05.008

Prevention, C.F.D.C.A. (2019). *Infertility Frequently Asked Questions [Online]. Centers for Disease Control and Prevention*. Available at: https://www.cdc.gov/reproductivehealth/infertility/index.htm (Accessed January 16 2019).

Prince, A. L., Ma, J., Kannan, P. S., Alvarez, M., Gisslen, T., Harris, R. A., et al. (2016). The placental membrane microbiome is altered among subjects with spontaneous preterm birth with and without chorioamnionitis. *Am. J. Obstet. Gynecol.* 214, 627.e621–627.e616. doi: 10.1016/j.ajog.2016.01.193

Pruski, P., Lewis, H. V., Lee, Y. S., Marchesi, J. R., Bennett, P. R., Takats, Z., et al. (2018). Assessment of microbiota:host interactions at the vaginal mucosa interface. *Methods* 149, 74–84. doi: 10.1016/j.ymeth.2018.04.022

Rasmussen, S. J., Eckmann, L., Quayle, A. J., Shen, L., Zhang, Y. X., Anderson, D. J., et al. (1997). Secretion of proinflammatory cytokines by epithelial cells in response to Chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis. *J. Clin. Invest.* 99, 77–87. doi: 10.1172/JCI119136

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., Mcculle, S. L., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U.S.A.* 108 Suppl 1, 4680–4687. doi: 10.1073/pnas.1002611107

Richardson, A. R., Dunman, P. M., and Fang, F. C. (2006). The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. *Mol. Microbiol.* 61, 927–939. doi: 10.1111/j.1365-2958.2006.05290.x

Richardson, A. R., Libby, S. J., and Fang, F. C. (2008). A nitric oxide-inducible lactate dehydrogenase enables Staphylococcus aureus to resist innate immunity. *Science* 319, 1672–1676. doi: 10.1126/science.1155207

Rodriguez-Esquivel, M., Rosales, J., Castro, R., Apresa-Garcia, T., Garay, O., Romero-Morelos, P., et al. (2018). Volatolome of the Female Genitourinary Area: Toward the Metabolome of Cervical Cancer. *Arch. Med. Res.* 49, 27–35. doi: 10.1016/j.arcmed.2018.04.004

Romanik, M., Martirosian, G., Wojciechowska-Wieja, A., Cieślik, K., and Kaźmierczak, W. (2007). [Co-occurence of indol-producing bacterial strains in the vagina of women infected with Chlamydia trachomatis]. *Ginekol. Pol.* 78, 611–615.

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Nikita, L., et al. (2014). The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2, 4. doi: 10.1186/2049-2618-2-4

Roth, A., König, P., Van Zandbergen, G., Klinger, M., Hellwig-Bürgel, T., Däubener, W., et al. (2010). Hypoxia abrogates antichlamydial properties of IFN-γ in human fallopian tube cells in vitro and ex vivo. *Proc. Natl. Acad. Sci. U.S.A.* 107, 19502–19507. doi: 10.1073/pnas.1008178107

Rotstein, O. D., Pruett, T. L., Fiegel, V. D., Nelson, R. D., and Simmons, R. L. (1985). Succinic acid, a metabolic by-product of Bacteroides species, inhibits polymorphonuclear leukocyte function. *Infect. Immun.* 48, 402–408. doi: 10.1128/IAI.48.2.402-408.1985

Sasaki-Imamura, T., Yoshida, Y., Suwabe, K., Yoshimura, F., and Kato, H. (2011). Molecular basis of indole production catalyzed by tryptophanase in the genus Prevotella. *FEMS Microbiol. Lett.* 322, 51–59. doi: 10.1111/j.1574-6968.2011.02329.x

Scott, A. J., Alexander, J. L., Merrifield, C. A., Cunningham, D., Jobin, C., Brown, R., et al. (2019). International Cancer Microbiome Consortium consensus statement on the role of the human microbiome in carcinogenesis. *Gut* 68, 1624–1632. doi: 10.1136/gutjnl-2019-318556

Serrano, M. G., Parikh, H. I., Brooks, J. P., Edwards, D. J., Arodz, T. J., Edupuganti, L., et al. (2019). Racioethnic diversity in the dynamics of the vaginal microbiome during pregnancy. *Nat. Med.* 25, 1001–1011. doi: 10.1038/s41591-019-0465-8

Sévin, D. C., Kuehne, A., Zamboni, N., and Sauer, U. (2015). Biological insights through nontargeted metabolomics. *Curr. Opin. Biotechnol.* 34, 1–8. doi: 10.1016/j.copbio.2014.10.001

Shanmughapriya, S., Senthilkumar, G., Vinodhini, K., Das, B. C., Vasanthi, N., and Natarajaseenivasan, K. (2012). Viral and bacterial aetiologies of epithelial ovarian cancer. *Eur. J. Clin. Microbiol. Infect. Dis.* 31, 2311–2317. doi: 10.1007/s10096-012-1570-5

Shemer, Y., and Sarov, I. (1985). Inhibition of growth of Chlamydia trachomatis by human gamma interferon. *Infect. Immun.* 48, 592–596. doi: 10.1128/IAI.48.2.592-596.1985

Sherrard, J., Wilson, J., Donders, G., Mendling, W., and Jensen, J. S. (2018). 2018 European (IUSTI/WHO) International Union against sexually transmitted infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge. *Int. J. STD AIDS* 29, 1258–1272. doi: 10.1177/0956462418785451

Shipitsyna, E., Khusnutdinova, T., Budilovskaya, O., Krysanova, A., Shalepo, K., Savicheva, A., et al. (2020). Bacterial vaginosis-associated vaginal microbiota is an age-independent risk factor for Chlamydia trachomatis, Mycoplasma genitalium and Trichomonas vaginalis infections in low-risk women, St. Petersburg, Russia. *Eur. J. Clin. Microbiol. Infect. Dis.* 39, 1221–1230. doi: 10.1007/s10096-020-03831-w

Sierra-Filardi, E., Nieto, C., Domínguez-Soto, A., Barroso, R., Sánchez-Mateos, P., Puig-Kroger, A., et al. (2014). CCL2 shapes macrophage polarization by GM-CSF and M-CSF: identification of CCL2/CCR2-dependent gene expression profile. *J. Immunol.* 192, 3858–3867. doi: 10.4049/jimmunol.1302821

Sirota, I., Zarek, S. M., and Segars, J. H. (2014). Potential influence of the microbiome on infertility and assisted reproductive technology. *Semin. Reprod. Med.* 32, 35–42. doi: 10.1055/s-0033-1361821

Smith, S. B., and Ravel, J. (2017). The vaginal microbiota, host defence and reproductive physiology. *J. Physiol.* 595, 451–463. doi: 10.1113/JP271694

Snyers, D., Lefebvre, C., Viellevoye, R., and Rigo, V. (2020). [Late preterm : high risk newborns despite appearances]. *Rev. Med. Liege* 75, 105–110.

Son, K. A., Kim, M., Kim, Y. M., Kim, S. H., Choi, S. J., Oh, S. Y., et al. (2018). Prevalence of vaginal microorganisms among pregnant women according to trimester and association with preterm birth. *Obstet. Gynecol. Sci.* 61, 38–47. doi: 10.5468/ogs.2018.61.1.38

Song, J., Wang, X., Guo, Y., Yang, Y., Xu, K., Wang, T., et al. (2019). Novel high-coverage targeted metabolomics method (SWATHtoMRM) for exploring follicular fluid metabolome alterations in women with recurrent spontaneous abortion undergoing in vitro fertilization. *Sci. Rep.* 9, 10873. doi: 10.1038/s41598-019-47370-7

Song, S. D., Acharya, K. D., Zhu, J. E., Deveney, C. M., Walther-Antonio, M. R. S., Tetel, M. J., et al. (2020). Daily Vaginal Microbiota Fluctuations Associated with Natural Hormonal Cycle, Contraceptives, Diet, and Exercise. *mSphere* 5, e00593–20. doi: 10.1128/mSphere.00593-20

Spiegel, C. A., Amsel, R., Eschenbach, D., Schoenknecht, F., and Holmes, K. K. (1980). Anaerobic bacteria in nonspecific vaginitis. *N. Engl. J. Med.* 303, 601–607. doi: 10.1056/NEJM198009113031102

Srinivasan, S., Morgan, M. T., Fiedler, T. L., Djukovic, D., Hoffman, N. G., Raftery, D., et al. (2015). Metabolic signatures of bacterial vaginosis. *mBio* 6, e00204-15. doi: 10.1128/mBio.00204-15

Stanek, R., Gain, R. E., Glover, D. D., and Larsen, B. (1992). High performance ion exclusion chromatographic characterization of the vaginal organic acids in women with bacterial vaginosis. *BioMed. Chromatogr.* 6, 231–235. doi: 10.1002/bmc.1130060506

Strauss, J. F., Romero, R., Gomez-Lopez, N., Haymond-Thornburg, H., Modi, B. P., Teves, M. E., et al. (2018). Spontaneous preterm birth: advances toward the discovery of genetic predisposition. *Am. J. Obstet. Gynecol.* 218, 294–314.e292. doi: 10.1016/j.ajog.2017.12.009

Svenstrup, H. F., Fedder, J., Abraham-Peskir, J., Birkelund, S., and Christiansen, G. (2003). Mycoplasma genitalium attaches to human spermatozoa. *Hum. Reprod.* 18, 2103–2109. doi: 10.1093/humrep/deg392

Swiecki, M., Miller, H. L., Sesti-Costa, R., Cella, M., Gilfillan, S., and Colonna, M. (2017). Microbiota induces tonic CCL2 systemic levels that control pDC trafficking in steady state. *Mucosal Immunol.* 10, 936–945. doi: 10.1038/mi.2016.99

Szewczyk, G., Maciejewski, T. M., and Szukiewicz, D. (2019). Current progress in the inflammatory background of angiogenesis in gynecological cancers. *Inflammation Res.* 68, 247–260. doi: 10.1007/s00011-019-01215-1

Tao, Z., Zhang, L., Zhang, Q., Lv, T., Chen, R., Wang, L., et al. (2019). The Pathogenesis Of Streptococcus anginosus In Aerobic Vaginitis. *Infect. Drug Resist.* 12, 3745–3754. doi: 10.2147/IDR.S227883

Tavares-Murta, B. M., De Resende, A. D., Cunha, F. Q., and Murta, E. F. (2008). Local profile of cytokines and nitric oxide in patients with bacterial vaginosis and cervical intraepithelial neoplasia. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 138, 93–99. doi: 10.1016/j.ejogrb.2007.06.015

Troisi, J., Sarno, L., Landolfi, A., Scala, G., Martinelli, P., Venturella, R., et al. (2018). Metabolomic Signature of Endometrial Cancer. *J. Proteome Res.* 17, 804–812. doi: 10.1021/acs.jproteome.7b00503

Turkoglu, O., Zeb, A., Graham, S., Szyperski, T., Szender, J. B., Odunsi, K., et al. (2016). Metabolomics of biomarker discovery in ovarian cancer: a systematic review of the current literature. *Metabolomics* 12, 60. doi: 10.1007/s11306-016-0990-0

Van Der Veer, C., Hertzberger, R. Y., Bruisten, S. M., Tytgat, H. L. P., Swanenburg, J., De Kat Angelino-Bart, A., et al. (2019). Comparative genomics of human Lactobacillus crispatus isolates reveals genes for glycosylation and glycogen degradation: implications for in vivo dominance of the vaginal microbiota. *Microbiome* 7, 49. doi: 10.1186/s40168-019-0667-9

Van Houdt, R., Ma, B., Bruisten, S. M., Speksnijder, A., Ravel, J., and De Vries, H. J. C. (2018). Lactobacillus iners-dominated vaginal microbiota is associated with increased susceptibility to Chlamydia trachomatis infection in Dutch women: a case-control study. *Sex Transm. Infect.* 94, 117–123. doi: 10.1136/sextrans-2017-053133

Van Ostade, X., Dom, M., Tjalma, W., and Van Raemdonck, G. (2018). Candidate biomarkers in the cervical vaginal fluid for the (self-)diagnosis of cervical precancer. *Arch. Gynecol. Obstet.* 297, 295–311. doi: 10.1007/s00404-017-4587-2

Ventriglia, J., Paciolla, I., Pisano, C., Cecere, S. C., Di Napoli, M., Tambaro, R., et al. (2017). Immunotherapy in ovarian, endometrial and cervical cancer: State of the art and future perspectives. *Cancer Treat Rev.* 59, 109–116. doi: 10.1016/j.ctrv.2017.07.008

Verstraelen, H., Vilchez-Vargas, R., Desimpel, F., Jauregui, R., Vankeirsbilck, N., Weyers, S., et al. (2016). Characterisation of the human uterine microbiome in non-pregnant women through deep sequencing of the V1-2 region of the 16S rRNA gene. *PeerJ* 4, e1602. doi: 10.7717/peerj.1602

Vick, E. J., Park, H. S., Huff, K. A., Brooks, K. M., Farone, A. L., and Farone, M. B. (2014). Gardnerella vaginalis triggers NLRP3 inflammasome recruitment in THP-1 monocytes. *J. Reprod. Immunol.* 106, 67–75. doi: 10.1016/j.jri.2014.08.005

Vitali, B., Cruciani, F., Picone, G., Parolin, C., Donders, G., and Laghi, L. (2015). Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 2367–2376. doi: 10.1007/s10096-015-2490-y

Vitko, N. P., Spahich, N. A., and Richardson, A. R. (2015). Glycolytic dependency of high-level nitric oxide resistance and virulence in Staphylococcus aureus. *mBio* 6, e00045-15. doi: 10.1128/mBio.00045-15

Walther-Antonio, M. R., Chen, J., Multinu, F., Hokenstad, A., Distad, T. J., Cheek, E. H., et al. (2016). Potential contribution of the uterine microbiome in the development of endometrial cancer. *Genome Med.* 8, 122. doi: 10.1186/s13073-016-0368-y

Wang, Q., Wurtz, P., Auro, K., Makinen, V. P., Kangas, A. J., Soininen, P., et al. (2016). Metabolic profiling of pregnancy: cross-sectional and longitudinal evidence. *BMC Med.* 14, 205. doi: 10.1186/s12916-016-0733-0

Wang, C., Fan, A., Li, H., Yan, Y., Qi, W., Wang, Y., et al. (2020). Vaginal bacterial profiles of aerobic vaginitis: a case-control study. *Diagn. Microbiol. Infect. Dis.* 96, 114981. doi: 10.1016/j.diagmicrobio.2019.114981

Watson, E., and Reid, G. (2018). Metabolomics as a clinical testing method for the diagnosis of vaginal dysbiosis. *Am. J. Reprod. Immunol.* 80, e12979. doi: 10.1111/aji.12979

Wee, B. A., Thomas, M., Sweeney, E. L., Frentiu, F. D., Samios, M., Ravel, J., et al. (2018). A retrospective pilot study to determine whether the reproductive tract microbiota differs between women with a history of infertility and fertile women. *Aust. N. Z. J. Obstet. Gynaecol.* 58, 341–348. doi: 10.1111/ajo.12754

Witkin, S. S., Kligman, I., Grifo, J. A., and Rosenwaks, Z. (1995). Chlamydia trachomatis detected by polymerase chain reaction in cervices of culture-negative women correlates with adverse in vitro fertilization outcome. *J. Infect. Dis.* 171, 1657–1659. doi: 10.1093/infdis/171.6.1657

Witkin, S. S., Mendes-Soares, H., Linhares, I. M., Jayaram, A., Ledger, W. J., and Forney, L. J. (2013). Influence of vaginal bacteria and D- and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: implications for protection against upper genital tract infections. *mBio* 4, e00460-13. doi: 10.1128/mBio.00460-13

Wolrath, H., Boren, H., Hallen, A., and Forsum, U. (2002). Trimethylamine content in vaginal secretion and its relation to bacterial vaginosis. *Apmis* 110, 819–824. doi: 10.1034/j.1600-0463.2002.1101108.x

Wood, H., Fehlner-Gardner, C., Berry, J., Fischer, E., Graham, B., Hackstadt, T., et al. (2003). Regulation of tryptophan synthase gene expression in Chlamydia trachomatis. *Mol. Microbiol.* 49, 1347–1359. doi: 10.1046/j.1365-2958.2003.03638.x

Workowski, K. A., and Bolan, G. A. (2015). Sexually transmitted diseases treatment guidelines 2015. *MMWR Recomm. Rep.* 64, 1–137.

Yang, T. K., Chung, C. J., Chung, S. D., Muo, C. H., Chang, C. H., and Huang, C. Y. (2015). Risk of Endometrial Cancer in Women With Pelvic Inflammatory Disease: A Nationwide Population-Based Retrospective Cohort Study. *Medicine (Baltimore)* 94, e1278. doi: 10.1097/MD.0000000000001278

Yarbrough, V. L., Winkle, S., and Herbst-Kralovetz, M. M. (2015). Antimicrobial peptides in the female reproductive tract: a critical component of the mucosal immune barrier with physiological and clinical implications. *Hum. Reprod. Update* 21, 353–377. doi: 10.1093/humupd/dmu065

Yeoman, C. J., Thomas, S. M., Miller, M. E., Ulanov, A. V., Torralba, M., Lucas, S., et al. (2013). A multi-omic systems-based approach reveals metabolic markers of bacterial vaginosis and insight into the disease. *PloS One* 8, e56111. doi: 10.1371/journal.pone.0056111

Yoon, B. H., Oh, S. Y., Romero, R., Shim, S. S., Han, S. Y., Park, J. S., et al. (2001). An elevated amniotic fluid matrix metalloproteinase-8 level at the time of mid-trimester genetic amniocentesis is a risk factor for spontaneous preterm delivery. *Am. J. Obstet. Gynecol.* 185, 1162–1167. doi: 10.1067/mob.2001.117678

Zhang, T., Wu, X., Yin, M., Fan, L., Zhang, H., Zhao, F., et al. (2012). Discrimination between malignant and benign ovarian tumors by plasma metabolomic profiling using ultra performance liquid chromatography/mass spectrometry. *Clin. Chim. Acta* 413, 861–868. doi: 10.1016/j.cca.2012.01.026

Ziklo, N., Huston, W. M., Hocking, J. S., and Timms, P. (2016a). Chlamydia trachomatis Genital Tract Infections: When Host Immune Response and the Microbiome Collide. *Trends Microbiol.* 24, 750–765. doi: 10.1016/j.tim.2016.05.007

Ziklo, N., Huston, W. M., Taing, K., Katouli, M., and Timms, P. (2016b). In vitro rescue of genital strains of Chlamydia trachomatis from interferon-γ and tryptophan depletion with indole-positive, but not indole-negative Prevotella spp. *BMC Microbiol.* 16, 286. doi: 10.1186/s12866-016-0903-4

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Lactobacillus-Depleted Vaginal Microbiota in Pregnant Women Living With HIV-1 Infection Are Associated With Increased Local Inflammation and Preterm Birth

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Background: Pregnant women living with HIV-1 infection (PWLWH) have an elevated risk of preterm birth (PTB) of unknown aetiology, which remains after successful suppression of HIV. Women at high risk for HIV have a common bacterial profile which has been associated with poor birth outcomes. We set out to explore factors associated with gestational age at delivery of PWLWH in a UK population.

Methods: Prospective study of PWLWH (n = 53) in whom the vaginal microbiota and cervicovaginal cytokine *milieu* were assessed using metataxonomics and multiplexed immunoassays, respectively. Cross-sectional characterisation of vaginal microbiota in PWLWH were compared with 22 HIV uninfected pregnant women (HUPW) at a similar second trimester timepoint. Within PWLWH the relationships between bacterial composition, inflammatory response, and gestational age at delivery were explored.

Findings: There was a high rate of PTB among PWLWH (12%). In the second trimester the vaginal microbiota was more diverse in PWLWH than in HUPW (Inverse Simpson Index, p = 0.0004 and Species Observed, p = 0.009). PWLWH had a lower prevalence of *L. crispatus* dominant vaginal microbiota group (VMB I, 15 vs 54%) than HUPW and higher prevalence of *L. iners* dominant (VMB III, 36 vs 9% and VMB IIIB, 15 vs 5%) and mixed anaerobes (VMB IV, 21 vs 0%). Across the second and third trimesters in PWLWH, VMB III/IIIB and IV were associated with PTB and with increased local inflammation [cervicovaginal fluid (CVF) cytokine concentrations in upper quartile]. High bacterial diversity and anaerobic bacterial abundance were also associated with CVF pro-inflammatory cytokines, most notably IL-1β.

Interpretation: There is an association between local inflammation, vaginal dysbiosis and PTB in PWLWH. Understanding the potential of antiretroviral therapies to influence this cascade will be important to improve birth outcomes in this population.

Keywords: HIV, preterm, microbiome, *Lactobacillus* sp. *Gardnerella* spp., bacterial diversity, inflammation, cytokines

INTRODUCTION

Preterm birth (<37 weeks completed gestation; PTB) affects in excess of 15 million women per year (Blencowe et al., 2012) and accounts for 35% of neonatal death worldwide (Liu et al., 2016). PWLWH are at greater risk of PTB than the general population (Uthman et al., 2017) with rates up to 40% reported in some cohorts (Grosch-Woerner et al., 2008; Short and Taylor, 2014). A high prevalence of recognised PTB risk factors such as Black race, low BMI, anaemia, and a past history of PTB are likely to contribute to this phenomenon. Additional factors associated with HIV-infection such as immune suppression and increased susceptibility to co-infections by pathogens such as cytomegalovirus, malaria, and human papillomavirus (HPV; +/- cervical interventions) may also have a role (Short and Taylor, 2014). Despite the advent of effective antiretroviral therapy (ART, usually three drugs combined) which enables immune reconstitution and reduces the rate of mother-to-child transmission of HIV (MTCT) to less than 1%, the rates of PTB have not declined (Thorne et al., 2004).

An association between ART and PTB has been repeatedly reported (Short and Taylor, 2014). The class of drugs and the timing of treatment in relation to conception appear to affect the risk, however, results from observational studies have been inconsistent (Short and Taylor, 2014; Uthman et al., 2017). Ritonavir-boosted lopinavir, a member of the protease inhibitor class, has been associated with a two-fold increase in PTB (Powis et al., 2011; Fowler et al., 2016). In spite this protease inhibitors remain a preferred option in many antenatal guidelines, including the UK, due to a greater experience in pregnancy with these agents compared to other newer drugs and their strong barrier to resistance (Gilleece et al., 2019). Conversely, zidovudine monotherapy, no longer used routinely, has been repeatedly associated with low rates of PTB (Short and Taylor, 2014). UNAIDS estimated that in 2017 there were 1 million pregnant women living with HIV in east and southern Africa of which 93% received antiretroviral therapy (UNAIDS, 2018). Lifelong ART is now recommended for all for its long-term health benefits, thus increasing the length of exposure, during pregnancy to all ART, including nucleoside analogues with antimicrobial properties, and the potential for adverse effects.

There is now substantial evidence that vaginal microbiota composition is an independent risk factor for PTB. Bacterial vaginosis (BV), microbiologically characterised by reduced levels of *Lactobacillus* species and overgrowth of anaerobic bacteria, has long been recognised to increase PTB risk (Hay et al., 1994). Recent studies using molecular-based metataxonomics profiling during pregnancy also show that vaginal microbiota dominated by *Lactobacillus* species, particularly *L. crispatus*, are stable and associate with protection against PTB, whereas increased prevalence of high-diversity communities depleted in *Lactobacillus* spp., and enriched for potential pathobionts such as *Gardnerella vaginalis, Sneathia* spp., *Prevotella* spp., and members of the Mollicutes associate with increased risk (Kindinger et al., 2016; Brown et al., 2018; Brown et al., 2019; Elovitz et al., 2019; Fettweis et al., 2019). The latter "sub-optimal" community compositions are also prevalent in women with, or at risk of, HIV infection (Spear et al., 2008; Hummelen et al., 2010; Pepin et al., 2011; Borgdorff et al., 2014; Reimers et al., 2016) and represent a risk factor for sexual transmission of HIV and other infections (e.g., HPV and Herpes Simplex Virus) (Borgdorff et al., 2014; Reimers et al., 2016). They also associate with enhanced HIV shedding in cervicovaginal fluid (CVF) (Borgdorff et al., 2014) and may substantially reduce efficacy of topical pre-exposure prophylaxis preparations such as 1% tenofovir gel (Klatt et al., 2017).

The vaginal microbiota of PWLWH have been under investigated. In this study, we set out to characterise the vaginal microbiota of a UK cohort of PWLWH sampled longitudinally throughout gestation compared to HIV uninfected pregnant women (HUPW) using metataxonomic profiling. We also aimed to determine if differences in vaginal microbiota composition of PWLWH influence local inflammation and subsequent risk of PTB.

MATERIALS AND METHODS

Study Design and Setting

This was a prospective and observational study of 53 PWLWH and 22 HUPW. Following written informed consent, women were recruited at 8–14 weeks gestation in HIV specialist and general antenatal clinics of ten London hospitals, UK between January 2013 and August 2017 (Barnet, Chelsea and Westminster, Homerton, Lewisham, North Middlesex, Northwick Park, Queen Charlotte, Queen Elizabeth, St Mary's and St Thomas' Hospital). The study was approved by the NHS Health Research Authority National Research Ethics Service (NRES) Committee approval (REC 13/LO/0107 (PWLWH) & REC 14/LO/0328 (HUPW).

Participants and Sample Collection

Women were eligible if 18 years of age or older and had known HIV status with a singleton pregnancy (confirmed on ultrasound). Exclusion criteria were: CD4 cell count <350 cells/

mm^3 if a PWLWH; or co-morbidities requiring immune modulating treatment (to limit bias introduced by immunosuppression); current injecting drug use and fertilization *in vitro*. Clinical data on medical and obstetric risk factors for PTB, intrapartum management and birth outcome were recorded for all women. The practice of vaginal douching, recent sexual intercouse and antibiotic use were also recorded. CD4 cell count, plasma HIV RNA concentration and ART regimen were additionally documented for PWLWH. Screening for syphilis, gonorrhea and chlaymdial infection was routinely offered as per national guidelines (Gilleece et al., 2019).

Clinician or self-sampling of the high lateral vaginal wall was undertaken using a BBL ™ CultureSwab™ MaxV Liquid amies swab. For PWLWH sampling of vagina was undertaken at three time points: 16.0–21.9 (t1), 22.0–26.9 (t2), and 27–31.9 weeks (t3) and for HUPW vaginal sampling occurred at one second trimester time point. An additional sample of CVF was obtained from PWLWH at all time points using a soft cup (Instead™). All samples were snap frozen and stored at −80°C within 2 h of collection until further processing.

DNA Extraction and 16S rRNA Gene Sequencing (Metataxonomics)

Bacterial DNA was extracted using a combination of enzymatic digestion and mechanical disruption of cell membranes and the QIAamp DNA Mini kit (Qiagen, Manchester, UK), as previously described (MacIntyre et al., 2015). The V1-V2 hypervariable regions of the 16s rRNA gene were amplified with a fusion primer set that includes four different 28F primers chosen to improve detection of *Bifidobacteriales* and a 388R primer (Frank et al., 2008). The 28F-YM forward primer (5′-GAGTTT GATCNTGGCTCAG-3′) was mixed in a ratio of 4:1:1:1 with 28F *Borrellia* (5′-GAGTTTGATCCTGGCTTAG-3′), 28F *Chloroflex* (5′-GAATTTGATCTTGGTTCAG-3′), and 28F *Bifido* (5′-GGGTTCGATTCTGGCTCAG-3′) (RTL Genomics Amplicon Diversity Assay List). The forward primers included an Illumina i5 adapter (5′-AATGATACGGCGACCACC GAGATCTACAC-3′), an 8-base-pair (bp) bar code and primer pad (forward, 5′-TATGGTAATT-3′). The 388R reverse primer (5′-TGCTGCCTCCCGTAGGAGT-3′) was constructed with an Illumina i7 adapter (5′-CAAGCAGAAGACGGCATA CGAGAT-3′), an 8-bp bar code, a primer pad (reverse, 5′-AGTCAGTCAG-3′). The pair end multiplex sequencing was performed on an Illumina MiSeq platform (Illumina Inc.) at Research and Testing Laboratory (Lubbock, TX, USA).

Sequence Analysis:

The MiSeq SOP pipeline and software package Mothur were used to analyse RNA sequence data. Highly similar amplicons were clustered into operational taxonomic units (OTUs) using the kmer searching method and the Silva bacterial database (www.arb-silva.de/). All OTUs had a taxonomic cut-off of ≥97%. Classification was performed using the Ribosomal Database Project (RDP) reference sequence files and the Wang method (Wang et al., 2007). The RDP MultiClassifier script was used for determination of OTUs (phylum to genus) and species level taxonomies were determined using USEARCH (Edgar, 2010). To account for potential bias introduced by differences in sequence depth, samples were rarefied to the smallest OTU read count (n = 1,750). OTUs with <10 reads across the dataset were considered rare taxa and were grouped (taxonomy_species X). Statistical modelling was performed using the top twenty species observed which accounted for >97% of the total reads. Diversity indices (e.g., non-parametric Shannon index, Inverse Simpson index and species observed (S_{Obs}) were calculated using the Vegan package within R.

Quantification of CVF Cytokine Concentrations and Leukocyte Counts

CVF was extracted from the soft cup as previously published by this group (Short et al., 2018). Multiplex chemiluminescent assays (V-plex Human Pro-inflammatory cytokine panel, Meso Scale Discovery® (MSD)) were used to measure concentrations of ten cytokines: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, and TNF-α according to the manufacturer's instructions on duplicate samples at a four-fold dilution in extraction buffer containing Protease-inhibitor cocktail, previously described (Short et al., 2018).

Light microscopy was performed on 21 samples for which a paired dry high vaginal smear was available to grade polymorphonuclear leucocyte count by ordinal scale: 0, 1–5, 6–10, 11–20, 21–30, and 31+ per high-powered field over an average of 3 fields.

Statistical Analyses

Hierarchical clustering analysis (Ward linkage) was performed on rarefied species level count data using ClustViz (Metsalu and Vilo, 2015) to facilitate the classification of all samples into six vaginal microbiota types (VMB 1–6) on the basis of relative abundance profiles. For comparison by HIV status analyses were conducted on a cross section of second trimester samples from the 53 PWLWH (16.0–26.9 weeks) and 22 HUPW (22.0–22.1 weeks). The Statistical Analysis of Metagenomic Profiles (STAMP) software package was used to explore mean proportions of vaginal bacterial genera and species by HIV status on in the second trimester samples (Parks and Beiko, 2010). Statistical significance of differences was tested using the Welsh test and corrected using Benjamini-Hochberg False Discovery Rate.

PWLWH Cohort Analyses

Within PWLWH, statistical comparison of the proportions of term and preterm delivery in each VMB were made using Fisher's exact test in the cross section of second trimester samples. For the 49 women for whom 79 matched CVF samples were available, the proportion with elevated cytokine concentrations [defined as the upper quartile of results (Arnold et al., 2016)] were compared by VMB group using Chi-Squared test in the statistical software package, SPSS (version 24; IBM, Armonk NY, USA). These analyses were performed for each of the three timepoints. Cytokine concentrations were log transformed to normalise their distribution and correlated with bacterial diversity indices and species abundance using Pearson's correlation co-efficient in SPSS. Leucocyte count was correlated with log transformed cytokine concentration using Spearman's correlation co-efficient in SPSS.

Longitudinal Data

For PWLWH women who had two or more samples, VMB stability was examined pictorially by plotting VMB type as a function of sample collection time point with the corresponding Inverse Simpson Index. The proportion of repeated samples where VMB transition was observed were compared by initial second trimester VMB classification using Chi-Squared test in SPSS. Median gestational age at delivery by occurrence of VMB transition were compared by the Mann Whitney U test

The relationship between bacterial species and gestational age at delivery was also explored using hierarchical multiple linear regression analysis in SPSS. The model was adjusted for maternal age, BMI and ethnicity, log transformed bacterial abundance was inputted as the second model factor, with patient ID included as a random effect. Fold increase of fit of the model of gestational age at delivery and individual bacterial species abundance is presented alongside the Beta Estimate value.

RESULTS

Study Participant Characteristics

In this prospective study of pregnant women consenting to genital tract sampling, metataxonomics profiling was undertaken on vaginal swabs collected from 53 PWLWH and 22 HUPW. The median age of PWLWH (34 years, range 21–42) was similar to that of HUPW (32 years, range 26–39), p = 0·06 (**Table 1**). Compared to HUPW, PWLWH were more likely to be Black [81% (43/53) vs 23% (5/22)], and less likely to be Asian [4% (2/53) vs 41% (9/22)] or Caucasian [9% (5/53) vs 36% (8/22)] (all comparisons p <0·0001). PWLWH and HUPW were both non-smokers. PWLWH a mean BMI that was in the overweight category [28 (range 18–44)], no BMI data were available for HUPW.

The mean CD4 count for PWLWH was within the normal range [668 cells/mcL (range 356–1,505)] and most women had a fully supressed infection with plasma HIV loads of <40 copies/mL at the time of sampling [79% (42/53)] (**Table 2**). Forty-one PWLWH had conceived on combination antiretroviral therapy (cART) with the non-nucleoside reverse transcriptase inhibitor, Efavirenz (EFZ) with the nucleotide analogue Tenofovir disoproxil fumarate (TDF) and the nucleoside analogue Emtricitabine (FTC) the most prescribed regimen (**Supplementary Table 1**). Twelve women initiated cART during pregnancy, with five initiating a Protease Inhibitor (PI) based and four Integrase-based regimen.

There were no preterm births to HUPW where as six (12%) PWLWH delivered preterm. Of these three delivered following the spontaneous onset of preterm labour at 31, 32, and 35 weeks gestational age (GA). Two were delivered by Caesarean section because of evidence of fetal compromise in the context of a small for gestational age baby. One was delivered by Caesarean section

TABLE 1 | Clinical characteristics of participants by HIV status.

Characteristic	HIV-1 infected pregnant women, n = 53	Uninfected pregnant women, n = 22	p value
Maternal age,			0.06
Median (range)	34 (21–42)	32 (26–39)	
Ethnicity, n (%)			
Caucasian	5 (9)	9 (41)	<0.0001
Black	43 (81)	5(23)	<0.0001
Asian	2 (4)	8 (36)	<0.0001
Other	3 (6)	0	
BMI, kg/m^2 (IQR)	27 (22–31)	Na	–
Missing, n	7		
Smoker, n (%)			
Yes	1 (2)	0	0.71
No	52 (98)	22 (100)	
Parity, n (%)			–
Nulliparous	20 (38)	na	
Multiparous	33 (62)		
PTB risk factors, n (%)			–
Yes	14 (26)	0	
No	39 (74)	22 (100)	
Prior PTB, n	13 (25)	0	–
Cervical surgery	4 (8)	na	
Fibroids	2 (4)	na	
HTN	1 (2)	0	
Diabetes	2 (4)	0	
Median gestational age at delivery,	39 (38–40)	40 (39–41)	0.03
weeks (IQR)	4		
Missing, n			
Birth outcome, n (%)			
Term	43 (89)	22 (100)	–
Preterm	6 (12)	0	
Other	1 IUD	0	
Missing, n	4	0	

TABLE 2 | Immune parameters and ART in HIV-1 infected pregnant women.

HIV infection specific clinical details	
CD4+ cell count at entry /mcL, median (IQR)	632 (505–770)
Percentage of total T cells expressing CD4+ at entry, median (IQR)	38 (32–43)
HIV viral load at entry copies/ml, median (range)	<40 (<20–16,113)
ART at conception, n (%)	
Yes	41 (77)
No	12 (23)
ART class, n (%)	
PI based	20 (38)
Non-PI based	33 (62)
NRTI backbone, n (%)	
FTC/TDF	33 (62)
ABC/3TC	20 (38)

because of evidence of fetal compromise, although her baby was normally grown. There was also one stillbirth at 39 weeks.

The Vaginal Microbiota During the 2nd Trimester Differs Between PWLWH and HUPW

The mean number of sequences per sample for PWLWH was 28,692 (range 8,015–206,599) and for HUPW 14,974 (range 1,750–27,111). A total of 102 bacterial taxa were identified across all samples with *L. iners*, *L. crispatus* and *G. vaginalis* being the three most abundant species observed in the dataset. Ward clustering of bacterial species data enabled participant samples (n = 139 samples, HIV = 117, uninfected = 22) to be assigned to one of six vaginal microbiota groups (VMB); I (*L. crispatus* dominant), II (*L. gasseri* dominant), III (*L. iners* dominant), IIIb (*L. iners* with presence of *Gardnerella vaginalis)*, IV (Diverse, high proportions of *Atopobium, Gardnerella, Prevotella* spp. and others), and V (*L. jensensii* dominant) (**Supplementary Figure 1**).

Analysis of a cross-section (n = 75; PWLWH = 53, HUPW = 22) of samples collected in the second trimester of pregnancy (**Figure 1A**) showed that vaginal microbiota from HUPW at this stage of gestation were largely dominated by *Lactobacillus* spp. with 54% (12/22) having VMB I-type communities, 32% (7/22) VMB III, 9% (2/22) VMB V with only one VMB IIIb sample observed. In contrast, the predominant VMB in PWLWH was VMB III (36%, 19/53), followed by VMB IV (21%,11/53), VMB IIIb (15%, 8/53) and VMB I (15%, 8/53) (**Figure 1B**). A similar proportion of self-reported Black and Caucasian women were observed to have VMB I, however, VMB III and IV compositions were mainly observed in Black women. Consistent with these observations, PWLWH had significantly greater vaginal bacterial diversity (**Figure 1C**) and richness (**Figure 1D**) compared to HUPW. PWLWH had higher relative abundance of *Gardnerella* and *Prevotella* species compared to HUPW and a lower relative abundance of *Lactobacillus* spp., which remained significant after correction for multiple comparisons (**Figure 1E**). At species level, these differences were largely driven by higher relative abundance of *G. vaginalis* ($p = 0{\cdot}0002$) and lower levels of *L. crispatus* ($p = 0{\cdot}0004$) (**Figures 1F, G**).

PWLWH COHORT

High Bacterial Diversity and Mixed Anaerobes are Associated with Increased Local Inflammation in PWLWH

Cervicovaginal cytokine levels were determined in a total of 79 matched samples collected from 49 PWLWH (t1: n = 37, t2: n = 15, and t3: n = 27). Pro-inflammatory cytokine concentrations of IL-1β and TNF-α were significantly elevated in VMB IIIb and IV compared to other VMBs at the first second trimester timepoint (t1) (**Figure 2A**) with a similar trend seen at the third trimester timepoint (t3) but not at t2 (**Supplementary Figure 2**). Consistent with this, a positive correlation between both IL-1β and IL-8 levels and vaginal bacterial diversity and richness was observed in PWLWH (**Figures 2B, C**).

Mean proportions of major anaerobic species (*Aerococcus christensenii*, *Atopobium vaginalis*, *BVAB1*, *Dialister* spp., *Gardnerella* spp., *Prevotella* spp. and *Sneathia* spp.) were positively correlated with CVF levels of pro-inflammatory cytokines IFN-γ, IL-1β, IL-8, and TNF-α [**Table 3** (t1, n = 37), **Supplementary Table 2** (t1–3, n = 79)]. IL-1β was also positively correlated with mean proportions of *L. gasseri* and *L. jensenii* at t1, which was maintained for *L. jensenii* in the longitudinal samples. In a subset of matched samples where high power field microscopy was available (n = 21), total leucocyte count was found to significantly correlate with pro-inflammatory IL-1β ($\rho = 0.506$, $p = 0{\cdot}023$) and a trend towards association with IFN-γ ($\rho = 0.423$, $p = 0{\cdot}063$) was observed.

VMB I Was the Most Stable and VMB IIIb Was the Least Stable During Pregnancy

Forty-three PWLWH had two or more consecutive vaginal samples (**Figure 3**). Women whose VMB were classified as I or V on their first second trimester sample, remained in the same microbiota group throughout the sampling period. PWLWH whose VMB was group III in the second trimester remained stable (13/19) or transitioned (6/19 (32%)) to IIIb (4) or other lactobacillus predominant VMBs I (1) or V (1). VMB IIIb during the second trimester was the least stable (4/6 (67%)) with transitions to III (3) and IV (1). Similarly (2/3 (67%) of VMB II transitioned to IV (2). Whilst there were three cases (3/11 (27%)) where VMB IV reverted from high diversity to lower diversity VMB III (2) and IIIb (1), more cases (8/11) remained in this high diversity microbiota type, many of which demonstrated an increase in α diversity (Inverse simpson index) through pregnancy. The observation that the proportion of PWLWH whose VMBs remained stable across repeated sampling differed according to their initial second trimester VMB grouping, approached but did not reach statistical significance, $p = 0.09$. The median gestational age at delivery for PWLWH in whom VMB transition occurred was slightly lower [38.2 weeks (IQR 37.1–40.1)] than in women whose VMB remained stable [39.0 weeks (IQR 38.2–40.4], this did not reach statistical significance $p = 0.11$.

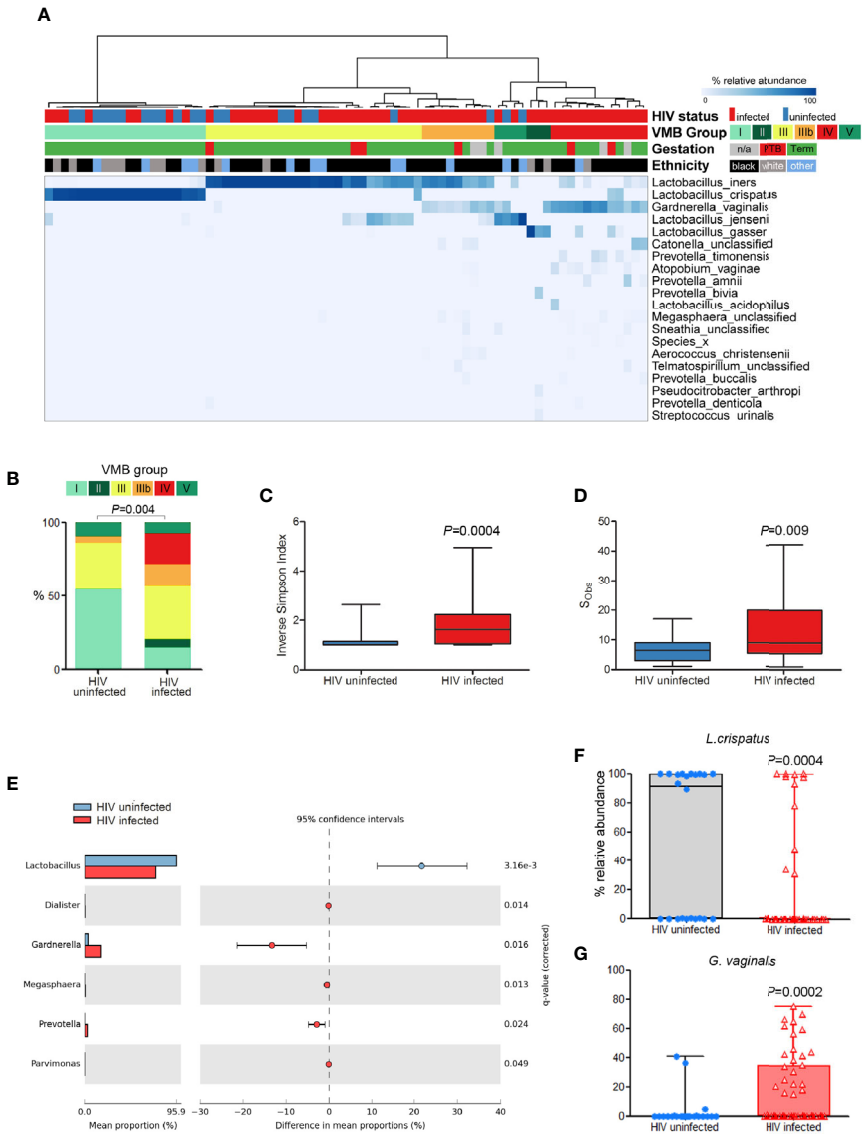

FIGURE 1 | Comparison of vaginal microbiota composition of PWLWH (n = 53) and HUPW (n = 22) sampled during the second trimester of pregnancy. **(A)** Hierarchical clustering (Ward linkage) of relative abundance data of vaginal bacterial species identified six major vaginal microbiota (VMB) groups, with HIV status, ethnicity, and gestation at delivery for each patient presented above the heat map. **(B)** Proportions of VMB types differed significantly between PWLWH and HUPW with the former characterised by increased bacterial **(C)** diversity and **(D)** richness. **(E)** Compared to HUPW, samples from PWLWH had lower relative abundance of *Lactobacillus* spp. and *Gardnerella* spp. which were largely driven by significantly lower proportions of *L crispatus* **(F)** and higher proportions of *G. vaginalis* **(G)**.

Associations Between Vaginal Microbiota and Gestational Age at Delivery in PWLWH

Examination of birth outcomes in PWLWH showed that all PTB in the cohort (n = 6) occurred in women with VMB III, IIIb, and IV vaginal microbiota compositions (**Figure 4**). All women with VMB I, II and V type profiles delivered at term. Hierarchical linear regression was used to model the relationship between relative abundance of vaginal bacterial species with gestational age at delivery, following adjustment for ethnicity, maternal age, BMI, with patient ID inputted as a random effect. *Prevotella* spp., *Sneathia* spp. and *Dialister* spp. were all found to inversely associate with gestational age at delivery, whereas *L. crispatus* had a positive association with gestational age at delivery (**Table 4**). A trend towards an inverse association between gestational age at delivery at *L.gasseri* and *L.jensenii* was also observed but did not reach significance level.

DISCUSSION

PWLWH are at increased risk of poor pregnancy outcomes such as PTB through a poorly defined pathogenesis. Substantial evidence now implicates vaginal microbiota composition during

A

VMB group	% with elevated CVF cytokines									
	IFNγ	IL10	IL12	IL13	IL1β	IL2	IL4	IL6	IL8	TNFα
I	14	0	29	14	14	14	29	14	29	14
III	16	21	5	16	5	16	16	10	5	11
IIIb	100	100	67	67	100	33	33	33	100	67
IV	50	67	33	33	100	50	33	67	33	50
P value	0.278	**0.033**	**0.009**	0.205	**0.003**	0.322	0.609	**0.028**	**0.003**	**0.036**

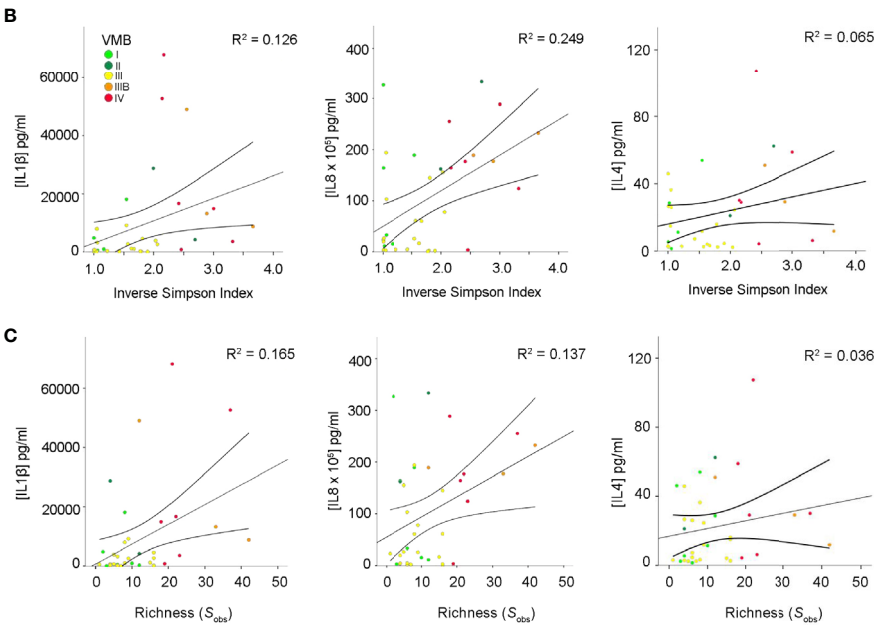

FIGURE 2 | Increased vaginal microbial diversity and richness associates with local inflammation in PWLWH. **(A)** Heat map displaying proportion of t1 samples with elevated CVF cytokines in each VMB group defined by the cytokine concentration being in the upper quartile. A positive correlation between both IL-1β and IL-8 was observed between **(B)** α-diversity (Inverse Simpson Index) and **(C)** richness (species observed; S_{obs}) in PWLWH. No such correlations were observed for IL-4 or the remaining cytokines assessed.

TABLE 3 | Association between vaginal microbiota and selected cervicovaginal pro-inflammatory cytokines during the second trimester of HIV-infected women.

	IL-1β		IL-8		IFN-γ		TNF-α	
	r	p	r	p	r	p	r	p
L. iners	−0.361	0.03	−0.406	0.013	−0.024	0.890	−0.210	0.213
L. crispatus	−0.165	0.328	−0.054	0.749	−0.102	0.547	−0.109	0.520
L. jensenii	0.583	< 0.0001	0.295	0.076	0.064	0.708	0.312	0.060
L. gasseri	0.350	0.034	0.329	0.047	0.434	0.007	0.307	0.065
BVAB1	0.080	0.639	0.108	0.526	−0.498	0.020	0.042	0.805
G. vaginalis	0.585	< 0.0001	0.296	0.075	0.066	0.697	0.313	0.059
Prevotella spp.	0.227	0.176	0.222	0.188	0.176	0.296	0.139	0.413
Atopobium vaginalis	0.406	0.013	0.261	0.919	−0.020	0.909	0.288	0.084
Megasphaera spp.	0.041	0.811	−0.032	0.853	−0.222	0.186	−0.040	0.813
L. acidophilus	0.184	0.276	0.145	0.392	−0.187	0.267	0.162	0.337
Snaethia spp.	0.211	0.209	0.202	0.229	0.085	0.619	0.070	0.681
Aerococcus christensenii	0.344	0.037	0.248	0.139	−0.120	0.478	0.265	0.115
Telmatospirillum unclass	−0.023	0.893	−0.064	0.704	−0.018	0.916	−0.190	0.260
Anaerococcus spp.	0.158	0.351	0.184	0.275	0.091	0.594	−0.028	0.872
Dialister spp.	0.370	0.04	0.370	0.024	0.188	0.264	0.340	0.039

Correlation analysis was performed using relative abundance data of vaginal bacterial species and matched log transformed CVF cytokine levels collected from HIV-infected women. r = Pearson's correlation co-efficient.

FIGURE 3 | Vaginal Microbiota Group (VMB) profiles throughout pregnancy in a UK PWLWH cohort. Each sample was assigned a VMB indicated by the left-sided coloured rectangles. Corresponding Inverse Simpson Indices are presented on the right-side (white- low diversity and dark blue- high diversity).

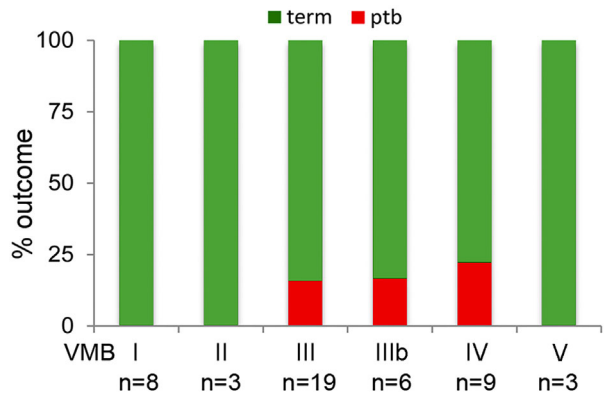

FIGURE 4 | Delivery outcomes by VMB group in PWLWH. In those PWLWH where delivery outcome were available (n = 46/53), 16% (3/19) of women in VMB III delivered preterm, 17% (1/6) of women in VMB IIIb and 22% (2/9) of women in VMB IV. No preterm births were recorded in those women with VMB I, II, and V.

pregnancy with subsequent risk of PTB (Beigi et al., 2007; Brown et al., 2018; Brown et al., 2019; Elovitz et al., 2019; Fettweis et al., 2019). In this study, we report that vaginal microbiota of PWLWH was characterised by increased prevalence of high diversity community compositions compared to uninfected women delivering at term. These communities were typically enriched for *G. vaginalis* and deplete in *Lactobacillus* species, particularly *L. crispatus*, which has been reported to provide protection against adverse pregnancy outcomes including PTB in a number of patient cohorts (Kindinger et al., 2017; Brown et al., 2018; Fettweis et al., 2019). In addition to our study of European-based PWLWH, increased vaginal bacterial diversity has been recently observed in PWLWH in Zimbabwe and Zambia (Price et al., 2019; Gudza-Mugabe et al., 2020), suggesting it is characteristic of HIV infection in both non-pregnant and pregnant women.

Longitudinal study of VMB composition in this cohort of PWLWH women revealed stability in the small proportion with VMB I but equally demonstrated high degrees of instability especially in VMB II, IIIB and IV between the second and third trimester. These data are consistent with earlier work in both pregnant and non-pregnant women that has shown the *L. crispatus* dominant communities are the most stable and *L. iners* and *L. gasseri* dominant compositions are more likely to transition to higher diversity communities, that associate with PTB (Verstraelen et al., 2009; MacIntyre et al., 2015; Kindinger et al., 2017).

High-diversity vaginal microbiota and/or colonisation by pathogens often associated with bacterial vaginosis have been widely reported to increase risk of PTB in uninfected pregnant women (Beigi et al., 2007; Brown et al., 2018; Brown et al., 2019; Elovitz et al., 2019; Fettweis et al., 2019). Such findings are consistent with the model of ascending vaginal pathogen colonisation and associated inflammation (infiltration of innate immune cells and expression of high concentrations of pro-inflammatory cytokines) in gestational tissues as a mechanistic driver of premature labour and birth (Goldenberg et al., 2008). Here we show in PWLWH that elevated cervicovaginal pro-inflammatory cytokines are associated with increased vaginal bacterial diversity and richness as well as the relative abundance of specific BV-associated pathobionts including *G. vaginalis, Atopobium, Dialister, Prevotella*, and *Sneathia* species and the number of polymorphonuclear leucocytes, most likely neutrophils. The association observed between IL-1β and *L.gasseri* and *L.jensenii* was unexpected especially as these species are typically considered part of a healthy vaginal microbiota. The significance of this finding is uncertain and could be the result of type 1 error as the number of PWLWH with these VMB was small.

Notably, all PTB in this cohort of PWLWH, regardless of being spontaneous or induced secondary to obstetric indications, occurred in women with high diversity or BV-type VMBs (III, IIIb, and IV). Exploring gestational age at delivery as a continuum, higher abundance of anaerobic bacteria *Prevotella*

TABLE 4 | Hierarchical linear regression modelling of vaginal bacterial species and gestational age at delivery.

Species	Fold Change	Estimate	Std.Error	F value	Q value
Lactobacillus iners	2.78	−0.27	0.16	−1.67	0.09
Lactobacillus crispatus	4.38	0.34	0.16	2.09	0.04
Lactobacillus jensenii	3.09	−0.29	0.16	−1.76	0.08
Lactobacillus gasseri	2.85	−0.43	0.26	−1.69	0.09
BVAB1	1.79	−0.85	0.63	−1.34	0.24
Gardnerella vaginalis	3.25	−0.29	0.16	−1.8	0.07
***Prevotella* spp.**	5.55	−0.52	0.22	−2.36	0.02
Atopobium vaginae	0.05	−0.1	0.46	−0.21	0.83
***Megasphaera* spp.**	0.02	−0.51	0.86	−0.59	0.56
Lactobacillus acidophilus	13.73	3.04	0.82	3.71	0.17
***Sneathia* spp.**	7.45	−1.24	0.45	−2.73	0.01
Aerococcus christensenii	1.85	−0.72	0.53	−1.36	0.18
Telmatospirillum unclass.	0.9	−1.77	1.86	−0.95	0.41
***Anearococcus* spp.**	1.48	−0.83	0.68	−1.22	0.26
***Dialister* spp.**	7.62	−1.27	0.46	−2.76	0.01

Beta estimate value is presented alongside fold increase of fit of the model of gestational age at delivery with individual bacterial species abundance inputted as a secondary factor. Following adjustment for ethnicity, maternal age, BMI, with patient ID inputted as a random effect, Prevotella spp., Sneathia spp., and Dialister spp. were all found to inversely associate with gestational age at delivery, whereas L. crispatus had a positive association with gestational age at delivery.

spp., *Sneathia* spp., and *Dialister* spp. were associated with earlier delivery whereas *L. crispatus* was associated with later delivery.

These findings differ to a recent study of PWLWH by Gudza-Mugabe and colleagues who concluded they did not observe a relationship between vaginal microbiota, vaginal cytokine levels, and PTB preterm birth (Gudza-Mugabe et al., 2020). This difference may be partly explained by differences in study design, particularly the gestational age of sampling and method of collection of genital tract fluid. While we focused on investigating samples collected in the second trimester [median 21 weeks (IQR 20–22)], the median sampling of patients in the Gudza-Mugabe and co-workers study was 29 weeks [IQR 25–33]. It is possible that any relationship between vaginal microbiota and local inflammatory status and gestational length is lost at these later gestational timepoints, however we were able to demonstrate similar trends in a smaller number of third trimester samples. In addition, our sampling method for cytokine measurement, the menstrual cup, collects a greater representative sample of cellular and cytokine expression in the lower female genital tract than a single vaginal swab. In spite of these differences, Gudza-Mugabe and colleagues were able to identify significant associations ($R > 0.3$) between six bacterial taxa and vaginal cytokine concentrations that withstood correction for multiple testing. Also in accordance with our findings, a recent study by Lopez and co-workers reported increased plasma concentrations of soluble CD14 and liposaccharide-binding-protein (markers of bacterial translocation) in the first trimester in PWLWH who went on to experience PTB compared to PWLWH delivering at term and uninfected pregnancies (Lopez et al., 2016).

The analysis of the vaginal microbiota at species level and integration of matched cytokine profiles in early gestation is a strength of our exploratory study. However, a relatively small number of PTB cases in PWLWH limited our ability to draw strong conclusions between microbiota composition, local inflammation and PTB risk in this cohort. We were not sufficiently powered to look at the effects of antiretroviral on PTB, an initial objective, but a strength of our study was the inclusion of a CD4 entry cut off of ≥350 cells/ml to limit any bias from underlying immune suppression. Differences in gestation age at delivery by individual drugs or drug classes could not be examined due to the diversity of therapies used. Most PWLWH conceived on cART limiting exploration of the potential role of initiation of therapy in PTB.

The PWLWH cohort was enriched for women self-reporting Black race compared to uninfected controls thus making it harder to attribute HIV status alone as the cause for differences in vaginal microbiota composition. Black race has previously been associated with increased colonisation of *L. iners* and high-diversity compositions during pregnancy, however recent data from the Human Microbiome Project suggests that women of African ancestry experience a rapid shift towards *Lactobacillus* spp. dominance early in pregnancy, which associates with simplification of the metabolic capacity of the microbiome (Serrano et al., 2019). Moreover, the linear regression model we used to identify a relationship between BV-associated pathobionts and earlier gestational age at delivery was corrected for ethnicity, maternal age and BMI.

Whilst spontaneous and iatrogenic labour are clearly distinguishable, the link observed here between dysbiosis and PTB regardless of its iatrogenic or spontaneous nature suggests that common pathways may underlie both. Fetal growth restriction is associated with some of the same biomarkers as spontaneous PTB (Kirkegaard et al., 2010). There is a well-known association between spontaneous PTB and growth restriction *in utero*; in general preterm babies tend to be small for gestational age (Lackman et al., 2001). Vaginal dysbiosis is also a risk factor for miscarriage (Al-Memar et al., 2020), which suggests a potential effect upon decidual function and placentation. It is possible that the adverse effect of having a specific bacterial profile may act very early in pregnancy. It is notable that of the four PTB in our cohort that did not follow spontaneous labour, three had clear evidence of abnormal fetal growth and fetal compromise and the fourth had fetal heart rate abnormalities despite normal fetal growth, each of which suggests significantly compromised placental function.

Generalisability and Conclusion

We conclude that high rates of unfavourable VMB are associated with cervico-vaginal inflammation in pregnancy which in turn contribute to the high rate of PTB experienced by PWLWH despite numerical restoration of CD4 T-cells.

Our women were recruited in the UK, however the high percentage of self-reported Black race, many from Sub-Saharan Africa, potentially widens the relevance of our results. Further elucidation of the infectious triggers to PTB in PWLWH, the interaction of antiretroviral therapy including the effects of new therapies on the microbiota, in addition to focusing of obstetric management of high risk women e.g. risk stratification tools, are imperative to reduce its global impact.

DATA AVAILABILITY STATEMENT

The RNASeq data are available in the SRA: PRJEB41429. The study name is ena-STUDY-CUMICRO-18-11-2020-15:38:11:823-609.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by NHS Health Research Authority National Research Ethics Service (NRES) Committee approval REC 13/LO/0107 (HIV infected pregnant women) and REC 14/LO/0328 (uninfected pregnant women). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

C-ES, PB, GT, and DM conceived and designed the study. Patient recruitment and sample collection were undertaken by

C-ES, RB, and RQ. Experiments and data collection were performed by C-ES, RB, RQ, and YL. Data processing, analyses, and interpretation were performed by C-ES, AS, PB, GT, and DM. All figures and tables were generated by C-ES and DM. C-ES wrote the first draft of the manuscript and all authors contributed critical revisions to the paper, interpretation of the results and approved the final version. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.596917/full#supplementary-material

REFERENCES

Al-Memar, M., Bobdiwala, S., Fourie, H., Mannino, R., Lee, Y. S., Smith, A., et al. (2020). The association between vaginal bacterial composition and miscarriage: a nested case-control study. *BJOG an Int. J. Obstetr. Gynaecol.* 127 (2), 264–274. doi: 10.1111/1471-0528.15972

Arnold, K. B., Burgener, A., Birse, K., Romas, L., Dunphy, L. J., Shahabi, K., et al. (2016). Increased levels of inflammatory cytokines in the female reproductive tract are associated with altered expression of proteases, mucosal barrier proteins, and an influx of HIV-susceptible target cells. *Mucosal Immunol.* 9 (1), 194–205. doi: 10.1038/mi.2015.51

Beigi, R. H., Yudin, M. H., Cosentino, L., Meyn, L. A., and Hillier, S. L. (2007). Cytokines, pregnancy, and bacterial vaginosis: comparison of levels of cervical cytokines in pregnant and nonpregnant women with bacterial vaginosis. *J. Infect. Diseases* 196 (9), 1355–1360. doi: 10.1086/521628

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A. B., Narwal, R., et al. (2012). National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet* 379 (9832), 2162–2172. doi: 10.1016/S0140-6736(12)60820-4

Borgdorff, H., Tsivtsivadze, E., Verhelst, R., Marzorati, M., Jurriaans, S., Ndayisaba, G. F., et al. (2014). Lactobacillus-dominated cervicovaginal microbiota associated with reduced HIV/STI prevalence and genital HIV viral load in African women. *ISME J.* 8 (9), 1781–1793. doi: 10.1038/ismej.2014.26

Brown, R. G., Marchesi, J. R., Lee, Y. S., Smith, A., Lehne, B., Kindinger, L. M., et al. (2018). Vaginal dysbiosis increases risk of preterm fetal membrane rupture, neonatal sepsis and is exacerbated by erythromycin. *BMC Med.* 16 (1), 9. doi: 10.1186/s12916-017-0999-x

Brown, R. G., Al-Memar, M., Marchesi, J. R., Lee, Y. S., Smith, A., Chan, D., et al. (2019). Establishment of vaginal microbiota composition in early pregnancy and its association with subsequent preterm prelabor rupture of the fetal membranes. *Transl. Res.* 207, 30–43. doi: 10.1016/j.trsl.2018.12.005

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26 (19), 2460–2461. doi: 10.1093/bioinformatics/btq461

Elovitz, M. A., Gajer, P., Riis, V., Brown, A. G., Humphrys, M. S., Holm, J. B., et al. (2019). Cervicovaginal microbiota and local immune response modulate the risk of spontaneous preterm delivery. *Nat. Commun.* 10 (1), 1305. doi: 10.1038/s41467-019-09285-9

Fettweis, J. M., Serrano, M. G., Brooks, J. P., Edwards, D. J., Girerd, P. H., Parikh, H. I., et al. (2019). The vaginal microbiome and preterm birth. *Nat. Med.* 25 (6), 1012–1021. doi: 10.1038/s41591-019-0450-2

Fowler, M. G., Qin, M., Fiscus, S. A., Currier, J. S., Flynn, P. M., Chipato, T., et al. (2016). Benefits and Risks of Antiretroviral Therapy for Perinatal HIV Prevention. *New Engl. J. Med.* 375 (18), 1726–1737. doi: 10.1056/NEJMoa1511691

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., and Olsen, G. J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl. Environ. Microbiol.* 74 (8), 2461–2470. doi: 10.1128/AEM.02272-07

Gilleece, D. Y., Tariq, D. S., Bamford, D. A., Bhagani, D. S., Byrne, D. L., Clarke, D. E., et al. (2019). British HIV Association guidelines for the management of HIV in pregnancy and postpartum 2018. *HIV Med.* 20 (Suppl 3), s2–s85. doi: 10.1111/hiv.12720

Goldenberg, R. L., Culhane, J. F., Iams, J. D., and Romero, R. (2008). Epidemiology and causes of preterm birth. *Lancet* 371 (9606), 75–84. doi: 10.1016/S0140-6736(08)60074-4

Grosch-Woerner, I., Puch, K., Maier, R. F., Niehues, T., Notheis, G., Patel, D., et al. (2008). Increased rate of prematurity associated with antenatal antiretroviral therapy in a German/Austrian cohort of HIV-1-infected women. *HIV Med.* 9 (1), 6–13. doi: 10.1111/j.1468-1293.2008.00520.x

Gudza-Mugabe, M., Havyarimana, E., Jaumdally, S., Garson, K. L., Lennard, K., Tarupiwa, A., et al. (2020). Human Immunodeficiency Virus Infection Is Associated With Preterm Delivery Independent of Vaginal Microbiota in Pregnant African Women. *J. Infect. Dis.* 221 (7), 1194–1203. doi: 10.1093/infdis/jiz584

Hay, P. E., Lamont, R. F., Taylor-Robinson, D., Morgan, D. J., Ison, C., and Pearson, J. (1994). Abnormal bacterial colonisation of the genital tract and subsequent preterm delivery and late miscarriage. *Bmj* 308 (6924), 295–298. doi: 10.1136/bmj.308.6924.295

Hummelen, R., Fernandes, A. D., Macklaim, J. M., Dickson, R. J., Changalucha, J., Gloor, G. B., et al. (2010). Deep sequencing of the vaginal microbiota of women with HIV. *PloS One* 5 (8), e12078. doi: 10.1371/journal.pone.0012078

Kindinger, L. M., MacIntyre, D. A., Lee, Y. S., Marchesi, J. R., Smith, A., McDonald, J. A., et al. (2016). Relationship between vaginal microbial dysbiosis, inflammation, and pregnancy outcomes in cervical cerclage. *Sci. Trans. Med.* 8 (350), 350ra102. doi: 10.1126/scitranslmed.aag1026

Kindinger, L. M., Bennett, P. R., Lee, Y. S., Marchesi, J. R., Smith, A., Cacciatore, S., et al. (2017). The interaction between vaginal microbiota, cervical length, and vaginal progesterone treatment for preterm birth risk. *Microbiome* 5 (1), 6. doi: 10.1186/s40168-016-0223-9

Kirkegaard, I., Uldbjerg, N., Petersen, O. B., Torring, N., and Henriksen, T. B. (2010). PAPP-A, free beta-hCG, and early fetal growth identify two pathways leading to preterm delivery. *Prenatal Diagnosis* 30 (10), 956–963. doi: 10.1002/pd.2593

Klatt, N. R., Cheu, R., Birse, K., Zevin, A. S., Perner, M., Noel-Romas, L., et al. (2017). Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* 356 (6341), 938–945. doi: 10.1126/science.aai9383

Lackman, F., Capewell, V., Richardson, B., daSilva, O., and Gagnon, R. (2001). The risks of spontaneous preterm delivery and perinatal mortality in relation to size at birth according to fetal versus neonatal growth standards. *Am. J. Obstetr. Gynecol.* 184 (5), 946–953. doi: 10.1067/mob.2001.111719

Liu, L., Hill, K., Oza, S., Hogan, D., Chu, Y., Cousens, S., et al. (2016). "Levels and Causes of Mortality under Age Five Years," in R. E. Black, R. Laxminarayan, M. Temmerman and N. Walker, editors. *Reproductive, Maternal, Newborn, and Child Health. Disease Control Priorities*, third edition, volume 2. Washington, DC: World Bank. doi: 10.1596/978-1-4648-0348-2

Lopez, M., Figueras, F., Coll, O., Gonce, A., Hernandez, S., Lonca, M., et al. (2016). Inflammatory Markers Related to Microbial Translocation Among HIV-Infected Pregnant Women: A Risk Factor of Preterm Delivery. *J. Infect. Dis.* 213 (3), 343–350. doi: 10.1093/infdis/jiv416

MacIntyre, D. A., Chandiramani, M., Lee, Y. S., Kindinger, L., Smith, A., Angelopoulos, N., et al. (2015). The vaginal microbiome during pregnancy and the postpartum period in a European population. *Sci. Rep.* 5, 8988. doi: 10.1038/srep08988

Metsalu, T., and Vilo, J. (2015). ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res.* 43 (W1), W566–W570. doi: 10.1093/nar/gkv468

Parks, D. H., and Beiko, R. G. (2010). Identifying biologically relevant differences between metagenomic communities. *Bioinformatics* 26 (6), 715–721. doi: 10.1093/bioinformatics/btq041

Pepin, J., Deslandes, S., Giroux, G., Sobela, F., Khonde, N., Diakite, S., et al. (2011). The complex vaginal flora of West African women with bacterial vaginosis. *PloS One* 6 (9), e25082. doi: 10.1371/journal.pone.0025082

Powis, K. M., Kitch, D., Ogwu, A., Hughes, M. D., Lockman, S., Leidner, J., et al. (2011). Increased risk of preterm delivery among HIV-infected women randomized to protease versus nucleoside reverse transcriptase inhibitor-based HAART during pregnancy. *J. Infect. Dis.* 204 (4), 506–514. doi: 10.1093/infdis/jir307

Price, J. T., Vwalika, B., Hobbs, M., Nelson, J. A. E., Stringer, E. M., Zou, F., et al. (2019). Highly diverse anaerobe-predominant vaginal microbiota among HIV-infected pregnant women in Zambia. *PloS One* 14 (10), e0223128. doi: 10.1371/journal.pone.0223128

Reimers, L. L., Mehta, S. D., Massad, L. S., Burk, R. D., Xie, X., Ravel, J., et al. (2016). The Cervicovaginal Microbiota and Its Associations With Human Papillomavirus Detection in HIV-Infected and HIV-Uninfected Women. *J. Infect. Dis.* 214 (9), 1361–1369. doi: 10.1093/infdis/jiw374

Serrano, M. G., Parikh, H. I., Brooks, J. P., Edwards, D. J., Arodz, T. J., Edupuganti, L., et al. (2019). Racioethnic diversity in the dynamics of the vaginal microbiome during pregnancy. *Nat. Med.* 25 (6), 1001–1011. doi: 10.1038/s41591-019-0465-8

Short, C. E., and Taylor, G. P. (2014). *Antiretroviral therapy and preterm birth in HIV-infected women* Vol. 12 (Expert review of anti-infective therapy). Taylor and Francis 293–306. doi: 10.1586/14787210.2014.885837

Short, C. S., Quinlan, R., Bennett, P., Shattock, R. J., and Taylor, G. P. (2018). Optimising the collection of female genital tract fluid for cytokine analysis in pregnant women. *J. Immunol. Methods* 458, 15–20. doi: 10.1016/j.jim.2018.03.014

Spear, G. T., Sikaroodi, M., Zariffard, M. R., Landay, A. L., French, A. L., and Gillevet, P. M. (2008). Comparison of the diversity of the vaginal microbiota in HIV-infected and HIV-uninfected women with or without bacterial vaginosis. *J. Infect. Diseases* 198 (8), 1131–1140. doi: 10.1086/591942

Thorne, C., Patel, D., and Newell, M. L. (2004). Increased risk of adverse pregnancy outcomes in HIV-infected women treated with highly active antiretroviral therapy in Europe. *Aids* 18 (17), 2337–2339. doi: 10.1097/00002030-200411190-00019

UNAIDS. (2018). *UNAIDS DATA 2018*. Available at: http://www.unaids.org/sites/default/files/media_asset/unaids-data-2018_en.pdf.

Uthman, O. A., Nachega, J. B., Anderson, J., Kanters, S., Mills, E. J., Renaud, F., et al. (2017). Timing of initiation of antiretroviral therapy and adverse pregnancy outcomes: a systematic review and meta-analysis. *Lancet HIV* 4 (1), e21–e30. doi: 10.1016/S2352-3018(16)30195-3

Verstraelen, H., Verhelst, R., Claeys, G., De Backer, E., Temmerman, M., and Vaneechoutte, M. (2009). Longitudinal analysis of the vaginal microflora in pregnancy suggests that L. crispatus promotes the stability of the normal vaginal microflora and that L. gasseri and/or L. iners are more conducive to the occurrence of abnormal vaginal microflora. *BMC Microbiol.* 9, 116. doi: 10.1186/1471-2180-9-116

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73 (16), 5261–5267. doi: 10.1128/AEM.00062-07

Conflict of Interest: PB reports personal fees and shares and stock ownership in ObsEva Pharmaceuticals, personal fees from GlaxoSmithKline that are both outside the submitted work. PB and DM have a patent for microRNA markers to predict cervical shortening and preterm birth issued again outside of the submitted work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Factors Associated With the Microbiome in Moderate–Late Preterm Babies: A Cohort Study From the DIAMOND Randomized Controlled Trial

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The gut microbiota of preterm infants is affected by perinatal factors and, in turn, may impact upon infant health. In this study, we collected fecal samples at Day-10 (D10) and 4-months corrected-age (4M) from 227 moderate–late preterm (MLPT) babies enrolled in a randomized controlled trial of nutritional management. A total of 320 samples underwent 16S amplicon sequencing, and shotgun metagenomic sequencing was performed on 94 samples from the 4M time point. The microbiome of babies whose families lived in lower socioeconomic status (SES) areas exhibited a significantly higher microbial alpha diversity at D10 (Wilcoxon test, $p = 0.021$), greater abundance of *Bifidobacterium* (linear model, q = 0.020) at D10 and *Megasphaera* (q = 0.031) at 4M. Hospital of birth explained 5.2% of the observed variance in 4M samples (PERMANOVA, $p = 0.038$), with *Staphylococcus aureus* more abundant in fecal samples from babies born in Middlemore hospital (linear model, q = 0.016). Maternal antibiotic (Wilcoxon test, $p = 0.013$) and probiotic ($p = 0.04$) usage within the four-week period before sample collection was associated with a reduction in the alpha diversity of D10 samples. Infant probiotic intake explained 2.1% (PERMANOVA, $p = 0.021$) of the variance in the D10 microbial profile with increased *Lactobacillus* (linear model, $q = 1.1 \times 10^{-10}$) levels. At 4M, the microbiome of infants who were breastmilk fed had reduced alpha diversity when compared to non-breastmilk fed infants (Wilcoxon test, $p < 0.05$). Although causality cannot be inferred within our study, we conclude that in MLPT babies, maternal socioeconomic factors, as well as the perinatal medical environment and nutrition impact on the development of the newborn microbiome.

Keywords: moderate–late preterm infant, socioeconomic status, ethnicity, gut microbiome, early life nutrition

INTRODUCTION

There are approximately 15 million babies born preterm each year (World Health Organization, 2017), with countries in Africa and South Asia accounting for more than 60% of all preterm births (World Health Organization, 2017). In New Zealand, between 2008 and 2017, 1.2% to 1.3% of all babies were born before 32 weeks of gestation, and an additional 5.9 to 6.3% of all babies were born moderate–late preterm (MLPT, between 32 and 36 weeks of gestation) (Ministry of Health, 2019), accounting for >80% of all preterm births (Ministry of Health, 2019). Preterm babies often require postnatal nutritional support to sustain growth following their early exposure to the extrauterine environment. However, there are no data from randomized trials to inform standardized nutritional practice for MLPT babies (Giannì et al., 2015; Harding et al., 2017; Alexander and Bloomfield, 2019), meaning that practice varies widely according to expert opinion.

Over the past decades, advances in molecular technology have led to the emergence of the microbiome and its importance in relationships between the environment and host characteristics (Blanton et al., 2016; Kamng'ona et al., 2019; Robertson et al., 2019). In preterm babies, alteration in the gut microbiome composition is hypothesized to be a determining factor leading to the development of necrotizing enterocolitis (NEC), a gut complication with high morbidity and mortality (Musemeche et al., 1986; Morowitz et al., 2010; Neu and Walker, 2011). A systematic review of 14 studies provided some support for this hypothesis, reporting that preterm infants diagnosed with NEC are characterized by an increased abundance of Proteobacteria and decreased abundance of Firmicutes and Bacteroidetes from 24 to 36 weeks corrected gestational age (Pammi et al., 2017).

Studies into the early life gut microbiota have been focused around extremely preterm, very preterm (Gregory et al., 2016; Arboleya et al., 2017), term, and healthy infants (Biasucci et al., 2010; Akagawa et al., 2019) or a mixture of term and preterm (Chernikova et al., 2018; Dahlgren et al., 2019; Fouhy et al., 2019). Feeding mode, chronological age, and birth weight have been reported to influence gut microbial composition in preterm infants <32 weeks of gestation over the first three months of life (Gregory et al., 2016; Cong et al., 2017). For example, very-low-birth-weight infants fed mother's own breastmilk have been reported to have higher microbial alpha diversity, improved feed tolerance and better growth 4–6 weeks after birth compared to infants fed donor human milk (Ford et al., 2019). It has previously been shown that the microbiomes of MLPT babies that were fed mothers' own breastmilk up to 15 days had similar alpha diversity, but significantly distinct beta diversity levels to formula fed MLPT babies (Wang et al., 2020). A meta-analysis of seven microbiome studies comparing the gut microbiome of exclusively and non-exclusively breastfed infants identified reduced alpha diversity in the former group (Ho et al., 2018).

There have been a number of studies that have focused on the acquisition and establishment of the infants' gut microbiome from birth (Dahlgren et al., 2019; Shao et al., 2019; Tauchi et al., 2019; Li et al., 2020). However, it remains uncertain how the microbiome of MLPT babies develops across the early life window. In this study, we focused on identifying associations between early life dietary nutrition, perinatal medical environments, and socioeconomic factors, and the gut microbiome of MLPT babies. Anthropometric measurements were collected at birth and 4-months corrected age (4M). Fecal samples were collected at Day-10 (D10), and 4M and were used to investigate associations with the anthropometric and social data. Our results provide insights that will contribute to the long-term optimization of health outcomes for MLPT babies.

MATERIALS AND METHODS

DIAMOND Trial Description and Ethics

The DIAMOND trial (Bloomfield et al., 2018), is a multi-center, factorial design, randomized, controlled clinical trial (Trials Registry: ACTRN12616001199404). Briefly, the DIAMOND trial enrols MLPT infants who have an intravenous line for clinical reasons and whose mothers intend to breastfeed to investigate the impact of current feeding strategies on feed tolerance, body composition, and developmental outcome (Bloomfield et al., 2018). Exclusion criteria are babies in whom a particular mode of nutrition is clinically indicated, or who have a congenital abnormality that is likely to affect growth, body composition, or neurodevelopmental outcome.

Ethical approval was obtained from the New Zealand Health and Disability Ethics Committee (number 16/NTA/90). Institutional approval for each site [Counties Manukau Health (Middlemore Hospital); Auckland District Health Board (Auckland City Hospital); and Waitemata District Health Board (North Shore and Waitakere Hospitals)] was obtained through local institutional review processes. Written, informed, consent was required from parents or legal guardians prior to enrolment. The DIAMOND trial is overseen by an independent data and safety monitoring committee.

Probiotics

Use of prophylactic probiotics was undertaken according to each hospital's policy. The probiotics that were given to babies during admission, if required, included: *Lactobacillus GG* (Dicoflor60 Dicofarm SpA), Infloran® (SIT, Laboratorio Farmaceutico, Mede, Italy) (*Bifidobacterium bifidum* and *Lactobacillus acidophilus*) or Labinic™ Drops (Biofloratech Ltd, UK) (*Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Bifidobacterium infantis*).

Sample Collection and Transportation

The fecal samples analyzed in this study were collected from babies enrolled in the DIAMOND trial between March 2017 and June 2019 from four hospitals in Auckland, New Zealand (*i.e.* Auckland City Hospital; Middlemore Hospital; North Shore Hospital, and Waitakere Hospital). Fecal samples were collected by the MLPT babies' parents or nursing staff at two time points, day-10 (D10) (chronological age) and at 4-months corrected age (4M). Protocols for stool collection were standardized at both time points (**Data Sheets 2** and **3** for D10

and 4M respectively) to minimize the introduction of uncontrolled variables (Vogtmann et al., 2017).

For D10 samples, all fecal samples were frozen (−20°C) immediately after collection. Frozen samples were transported, on ice, to The Liggins Institute within five days of collection.

4M fecal samples were collected by parents/legal guardians following a detailed protocol that included illustrations from collection through to storage (**Data Sheet 3**). Parents/legal guardians were requested to freeze the collected fecal sample before transporting it (on ice) to the follow-up appointment.

DNA Extraction

DNA was extracted from 200 mg of fecal matter per sample within 7 days of collection using the Allprep DNA/RNA Mini Kit (QIAGEN), using a modification of Giannoukos et al. (2012). The fat layer that floated on top of the supernatant, after cell lysis, was carefully removed to avoid clogging the extraction column. The quality and quantity of the extracted DNA were measured using a NanoPhotometer N60 (IMPLEN, Germany) prior to storage and Qubit (Invitrogen, US) prior to sequencing. Extracted DNA was stored at −80°C until use.

Metadata Processing

Socioeconomic status (SES) was assigned according to the census-based New Zealand geography deprivation index (NZDep2013) using the parents' self-reported postcode. NZDep index assigns a deprivation score ranging from 1 to 10 for each meshblock (a geographical unit that represent area where people live and containing a median of around 81 people) in New Zealand that applies to areas rather than individual people (Atkinson et al., 2014). SES was then categorized into three groups: higher SES (NZDep Index 1–3), moderate SES (NZDep Index 4–7), or lower SES (NZDep Index 8–10).

Maternal education level was classified as either university (*i.e.* bachelor, masters, or doctoral degree level) or no university (*i.e.* no education, primary, lower secondary (years 9–11), upper secondary (years 12–13), post-secondary non-tertiary course or short-cycle tertiary education).

Gestational age was classified into moderate (32^{+0} to 33^{+6} weeks gestation) or late gestation (34^{+0} to 35^{+6} weeks gestation).

Early life intravenous nutrition was classified as: intravenous nutrition (babies received both amino acid solution and dextrose), or only dextrose before stool collection at D10.

Types of milk feeding on day-10 was classified as: breastmilk feeding (breastmilk only, or a combination of breastmilk and bovine-origin fortifier); formula only (term and/or preterm formula); or mixed feeding (a mix of breastmilk, formula, and/or bovine-origin fortifier in breastmilk).

Types of milk feeding at 4-months corrected age was categorized into three groups: breastmilk feeding; formula only; or mixed feeding (including weaning foods) based on self-reported information provided by parents at the 4M follow-up appointment.

Anthropometric measurements were expressed as the delta z-score which corrected for gestational age and sex (z-score, based on Fenton and World Health Organisation (WHO) growth charts) using the following formula:

$$delta_{z-score} = 4\, month_{z-score} - birth_{z-score}$$

Amplicon Sequencing and Data Analysis

16S rRNA amplicon libraries were prepared using the Nextera XT kit (Illumina). The V3–V4 16S rRNA hypervariable region was amplified using the universal primers 341F (5′-CCTACG GGNGGCWGCAG-3′) and 805R (5′-GACTACHVG GGTATCTAATCC-3′). 16S rRNA amplicon sequencing was performed using an Illumina MiSeq sequencing platform (Auckland Genomics, School of Biological Sciences; The University of Auckland, New Zealand). Amplicon sequence information is available at the Sequence Read Archive (SRA) under BioProject Accession Number PRJNA645223.

Adapter trimming was performed using Cutadapt (Martin, 2011) and reads were fed into the DADA2 pipeline (version 1.13.3 (Callahan et al., 2016)) in R (version 3.5.0) for quality control, denoising, and sequence merging (including removal of PhiX reads and chimeric sequences). The resulting amplicon sequence variants (ASVs) were taxonomically annotated using the SILVA database (SSU release 132). Seven samples had fewer than 3,000 16S amplicon sequencing reads and were excluded from further analysis (**Supplementary Table S1**). The remainder of the samples had filtered sequence counts ranging from 6,098 to 74,597 reads (median reads = 33,026, **Supplementary Table S1**).

Metagenomics Sequencing and Initial Bioinformatics

Shotgun metagenomic libraries were generated using the NEBNext® Ultra™ DNA Library Prep Kit (Illumina). Shotgun metagenomic sequencing (150 bp paired-end reads) was performed on a NovaSeq 6000 platform (Annoroad Gene Technology Beijing Co Ltd). All metagenome sequence information was deposited in the SRA (https://www.ncbi.nlm.nih.gov/sra) under the BioProject Accession Number PRJNA648487. KneadData (http://huttenhower.sph.harvard.edu/kneaddata) was used for quality control and to remove contaminant reads (*e.g.* human genome; **Data Sheet 1**, **Supplementary Figure 1**) from the metagenomic data. Our samples contained <10% human-aligned reads (**Supplementary Table S2**), consistent with current estimates for stool samples (Marotz et al., 2018). MetaPhlAn2 was used for taxonomic profiling (Truong et al., 2015). Metabolic pathway reconstruction was performed using the MetaCyc database and the HUMAnN2 pipeline (The HMP Unified Metabolic Analysis Network 2; (Franzosa et al., 2018)).

Bioinformatic and Statistical Analyses

Statistical analyses were conducted using R (version 3.5.0 and 3.6.1). Microbial alpha diversity was measured using Shannon's diversity index. Permutational multivariate analysis of variance (PERMANOVA; adonis function in vegan R package, version 2.5-6 (Oksanen et al., 2019), 10,000 permutations) was used to quantify the contributions of covariates to the observed variance in microbial beta diversities. Associations between individual microbial taxa, that were present in ≥10% of the samples and other variables were tested using Multivariate Association with Linear Models (MaAsLin2) (Morgan et al., 2012).

Wilcoxon unpaired (R package rstatix version 0.3.0) (Kassambara, 2019) and Kruskal–Wallis tests (R Core Team, 2019) were used to compare two and ≥two independent groups, respectively. The Chi-square test of independence and Fisher's exact test (R Core Team, 2019) were used to determine the association between two categorical variables. All reported p-values in this study were corrected for multiple testing using the Benjamini–Hochberg procedure (p. adjust function) (R Core Team, 2019) (Benjamini & Hochberg, 1995; Chen et al., 2017). By convention, FDR corrected p-values from MaAsLin2 were reported as q-values.

RESULTS

Study Cohort

In total, 227 babies were included in this study. Five babies withdrew from the study and one died before sample collection (**Figure 1**). Of the 325 stool samples that were collected from 221 babies (n = 207 D10 and n = 118 4M), 320 underwent 16S amplicon sequencing, and 313 samples were analyzed in the downstream analysis. Specifically, 100 babies provided stool samples at both time points, 99 babies provided only D10 sample, 14 babies provided only 4M sample (**Table 1** and **Figure 1**). Three babies did not provide a fecal sample at either time point. Proportional analysis performed on the demographic data identified a significantly different sex distribution (test of proportions, $p = 0.026$) and Cesarean section rates ($p = 2.44 \times 10^{-8}$) between the time points but not gestation age. The difference in Cesarean section rates was also significant within sex strata (D10: $p = 7.86 \times 10^{-5}$ and 4M: $p = 0.0001$), but no significant difference was found in gestational age within the sex strata.

A subset of 94 4M fecal samples (36 female and 58 male), which contained sufficient DNA (≥155 ng), was sent for metagenomic shotgun sequencing (**Table 1** and **Figure 1**). As infants' growth differs by season (Gelander et al., 1994; Bozzola and Meazza, 2012) we ensured a representation of samples from individuals in each season. Specifically, among the 36 female babies, six babies were born in winter, 10 each in spring, summer, and autumn. Of the male babies: 23 were born in winter, 12 in spring, nine in summer, and 14 in autumn.

Longitudinal Changes in the Gut Microbiome

Longitudinal analysis of the fecal microbiome using 16S amplicon data from infants ($n = 100$) who were sampled at both D10 and 4M time points revealed that 5.7% of the variance in fecal microbial profile is explained by longitudinal changes (PERMANOVA, $p = 0.001$). The fecal microbial alpha diversity varied according to time point of sampling (Wilcoxon paired test, $p = 5.64 \times 10^{-10}$) with the 4M fecal samples showing significantly

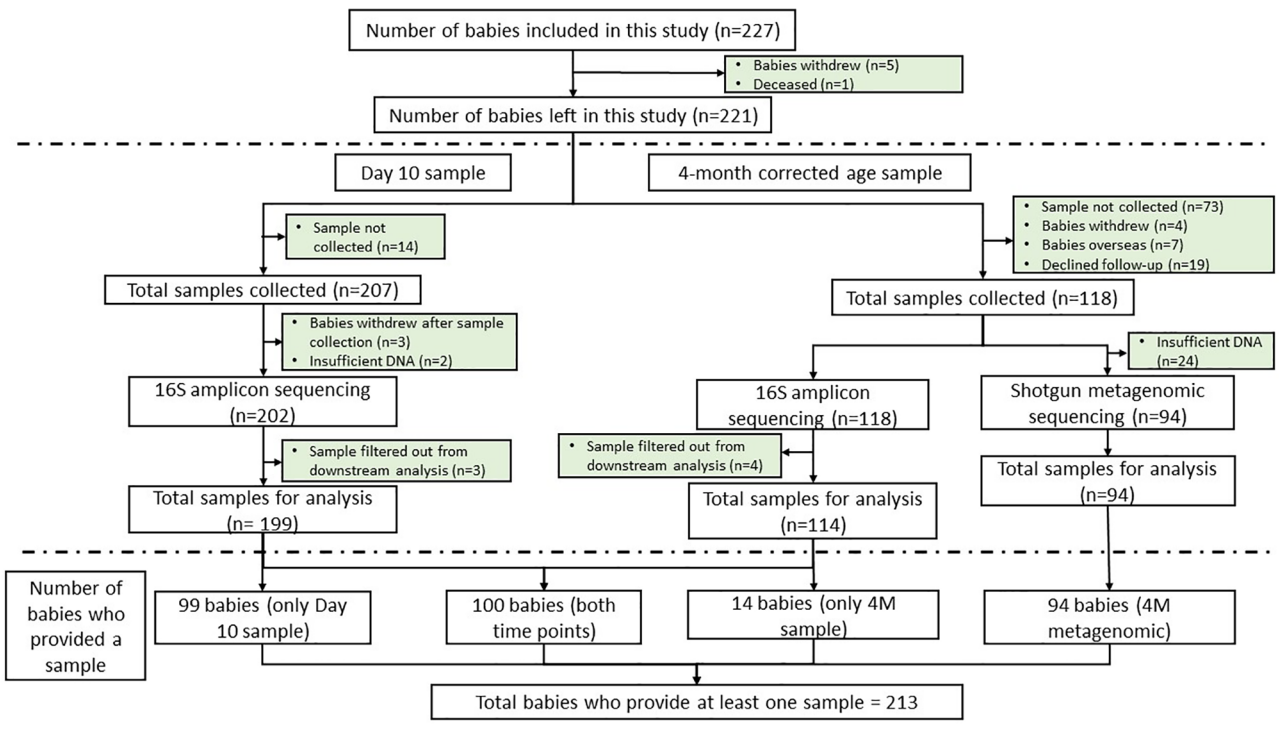

FIGURE 1 | Consort diagram showing the number of babies included in this study ($n = 227$), fecal samples collected ($n = 325$) and the number of babies excluded from the study at each time point. The consort diagram includes the numbers of samples that were available for downstream analysis and the equivalent number of babies that provided sample at each time point. Reasons for exclusion are stated.

TABLE 1 | Subject cohort demographic and clinical data.

Baby	
Birth weight (g)	2106 ± 426
Birth length (cm)	45 ± 3
Birth head circumference (cm)	31 ± 2
Weight at 4-months corrected age (g)	6,638 ± 926
Length at 4-months corrected age (cm)	64 ± 3
Head circumference at 4-months corrected age (cm)	42 ± 1
Neonatal intensive care unit (NICU) stay (days)	22 ± 11
Hospital stay (days)	24 ± 11
Mother	
Maternal age (years)	32 ± 6
Samples for 16S amplicon sequencing	
Sample collection (days)	
Day-10	10 ± 1
4-months corrected age	157 ± 14
Sex (Male/Female)	
Day-10	112/87
4-months corrected age	66/48
Mode of delivery (Vaginal/Caesarean section)	
Day-10	70/129
4-months corrected age	35/79
Gestational age (Moderate/Late)	
Day-10	112/87
4-months corrected age	57/57
Samples for shotgun metagenomics	
Sex (Male/Female)	58/36
Mode of delivery (Vaginal/Cesarean section)	28/66
Gestational age (Moderate/Late)	48/46

Gestational age: moderate preterm (32 + ^{0} to 33 + ^{6} weeks of gestational age) and late preterm (34 + ^{0} to 35 + ^{6} weeks of gestational age). Data are mean ± standard deviation. For birth weight, n = 213; birth head circumference, n = 213; birth length, n = 210; 4M-weight, n = 174; 4M-length, n = 172, 4M-head circumference, n = 174.

greater alpha diversity when compared to D10 (**Data Sheet 1**, **Supplementary Figure 2**). This observed increase in fecal microbial diversity is consistent with our current understanding of the development of the early life microbiome (Avershina et al., 2014; Fouhy et al., 2019). The abundance of all four major gut phyla—Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes—changed significantly between the two time points (linear model, q < 0.1). Within these phyla, eight taxa from the phylum Firmicutes (*i.e. Veillonellaceae, Ruminococcaceae, Erysipelotrichaceae, Peptostreptococcaceae*, and *Lactobacillaceae*), five taxa from Actinobacteria (*i.e. Bifidobacteriaceae, Eggerthellaceae, Coriobacteriaceae, Actinomycetaceae*, and *Atopobiaceae*), one taxon from Proteobacteria (*i.e. Enterobacteriaceae*) and one more taxon from Bacteroidetes (*i.e. Bacteroidaceae*) were more abundant in 4M fecal samples when compared to the D10 samples. By contrast, one taxon each from Firmicutes (*i.e. Staphylococcaceae*), Actinobacteria (*i.e. Corynebacteriaceae*) and Proteobacteria (*i.e. Pasteurellaceae*) were less abundant in 4M samples (**Supplementary Table S4**—MaAsLin2 longitudinal analysis, doi: 10.17608/k6.auckland.12793772).

Socioeconomic Factors Were Associated With Infants' Fecal Microbiome Diversity Up to 4-Months Corrected Age

In our MLPT cohort, we observed a correlation between early life microbial alpha diversity (D10, Shannon diversity index), SES, and self-reported maternal ethnicity. We observed a significantly higher microbial diversity in babies whose families lived in the lower SES areas (NZDep Index 8–10) when compared to the other two groups (**Figure 2A**, Wilcoxon test, $p = 0.021$). Notably, there was higher abundance for members of the *Bifidobacterium* (phylum Actinobacteria; linear model, q = 0.02) and *Megasphaera* (phylum Firmicutes; linear model, q = 0.031) genera in babies from lower SES group compared to the higher SES group at D10 and 4M, respectively (**Supplementary Table S4**—MaAsLin2—D10 (16S data) & 4M (16S data), doi: 10.17608/k6.auckland.12793772).

Mothers who self-reported as Māori were over-represented in lower SES areas (75%), compared to the other ethnicities in our MLPT cohort (**Supplementary Table S3**). Therefore, we tested for an association between self-reported maternal ethnicity and the infants' gut microbial diversity in D10 fecal samples. We observed that the alpha diversity of the D10 fecal microbiome of babies born to mothers self-reporting as Māori was the highest and significantly different to that from babies born to mothers self-reporting as European (Wilcoxon test, $p = 0.04$; **Figure 2B**). Linear modeling revealed that the genus *Rothia* (phylum Actinobacteria) (q = 0.061) was reduced at 4M in babies born to mothers self-reporting as Māori when compared to those born to mothers self-reporting as Asian. The genus *Staphylococcus* (phylum Firmicutes) was less abundant in fecal samples obtained at 4M from MLPT babies born to mothers self-reporting as Māori (q = 0.011) and Pacifica (q = 0.031) when compared to MLPT babies from mothers self-reporting as Asian.

We detected a negative correlation between infants' microbial alpha diversity at 4M with maternal education level (Wilcoxon test, $p = 0.038$). Specifically, babies whose mothers held a university degree had a reduced fecal microbial alpha diversity compared to babies whose mothers did not have a university degree (**Figure 2C**). Chi-square test of independence and Fisher's exact test identified a significant correlation between maternal education with both SES and maternal self-reported ethnicity at both time points (D10: SES, Chi-square test, $p = 0.0002$; ethnicity, Fisher's exact test, $p = 0.00002$; 4M: SES, Chi-square test, $p = 0.036$; ethnicity, Fisher's exact test, $p = 6.01 \times 10^{-7}$). Specifically, this indicates the inter-relatedness of SES, maternal self-reported ethnicity and maternal education at both D10 and 4M.

Breastmilk Feeding Was Associated With a Reduction in Microbial Diversity at 4 Months

Intravenous nutrition is an essential component of the medical care for preterm infants before they can tolerate full enteral feeds. No associations between the fecal microbiome and early life intravenous nutrition (a combination of amino acids and dextrose solution or only dextrose) were identified at D10 (PERMANOVA, $R^2 = 0.002$, $p = 0.91$) or 4M fecal samples (PERMANOVA, $R^2 = 0.013$, $p = 0.581$) by 16S amplicon data. Similarly, PERMANOVA analysis on the types of milk feeding in the early life from birth to D10 (*i.e.* breastmilk only, formula only, or mixed feeding) with fecal microbial composition in D10

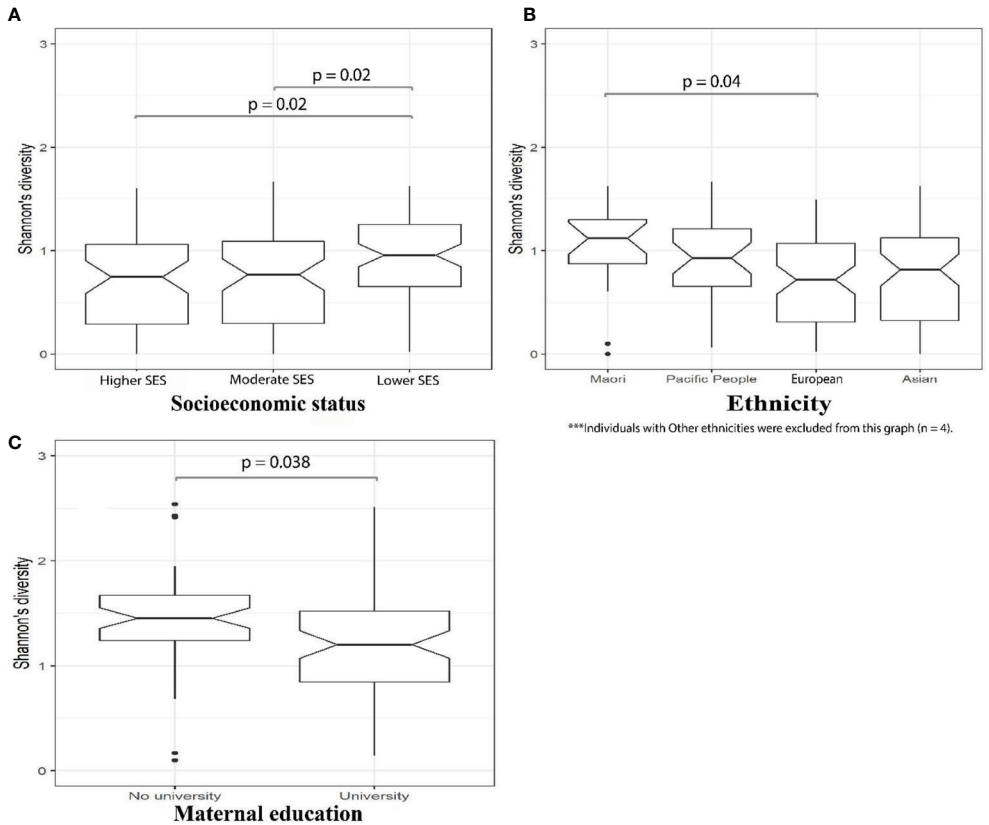

FIGURE 2 | 16S amplicon sequencing identified significantly (p = 0.02, Wilcoxon) greater microbial alpha diversity in D10 fecal samples from MLPT babies whose families lived in lower SES areas **(A)**; D10 fecal samples from MLPT babies born to mothers self-reporting as Māori exhibited significantly higher alpha diversity compared to babies from mothers self-reporting as European (p = 0.04, Wilcoxon) **(B)**; and 4M fecal samples from babies whose mothers hold a university degree had significantly (p = 0.038, Wilcoxon) lower microbial alpha diversity when compared to babies whose mothers do not have a university degree **(C)**.

(R^2 = 0.020, p = 0.172) or 4M (R^2 = 0.023, p = 0.581) samples did not identify any associations.

We then investigated the microbial alpha diversity at 4M in infants who were subject to different types of milk feeding (*i.e.* breastmilk, formula, or mixed). We observed that the types of milk feeding were associated with changes in diversity (Kruskal–Wallis test, p = 0.032). Specifically, significantly lower alpha diversity was observed in infants who received breastmilk when compared to mix fed babies (Wilcoxon test, p = 0.039), using 16S amplicon data. This observation was supported by metagenomics data at 4M where lower microbial alpha diversity was observed in 4M fecal samples collected from breastmilk fed infants when compared to infants who received only formula (Wilcoxon test, p = 0.029), or those who were mix fed (Wilcoxon test, p = 0.024; **Figure 3**). PERMANOVA analysis of 16S amplicon data also revealed that types of milk feeding explained 6.5% (p = 0.001) of the microbial taxonomic variation that was observed at 4M. Specifically, the genus *Megasphaera* from the Firmicutes phylum was more abundant in 4M fecal samples from breastmilk fed babies when compared to babies that were fed only formula (linear model, q = 0.051; **Supplementary Table S4**—MaAsLin2—4M (16S data), doi: 10.17608/k6.auckland.12793772).

Maternal and Infant Medical Factors Impact the Infants' Gut Microbial Diversity

Maternal use of antibiotics or probiotics within the four-week period before the D10 fecal sample collection was associated with a significant reduction (Wilcoxon test, p = 0.013 and p = 0.040, respectively) in MLPT infant fecal sample microbial alpha diversity (**Figures 4A, B**). By contrast, D10 fecal samples of infants who themselves received probiotics during their admission had significantly greater alpha diversity levels when compared to infants who did not (Wilcoxon test, p = 0.01, **Figure 4C**). PERMANOVA analysis confirmed that infant probiotic usage explained 2.1% (p = 0.021) of the variance in the D10 fecal microbial profile. *Lactobacillus*, a commonly used probiotic, was more abundant in the fecal sample collected from MLPT babies that received probiotics (n = 40) when compared to babies that did not receive probiotics (n = 159, linear model, q = 1.1 × 10^{-10}, **Supplementary Table S4**—MaAsLin2—D10 (16S data), doi: 10.17608/k6.auckland.12793772). No association was observed in the fecal microbial alpha diversity from babies that received probiotics (Wilcoxon test, p = 0.118, 10/113) within one month immediately preceding the 4-month follow-up appointment.

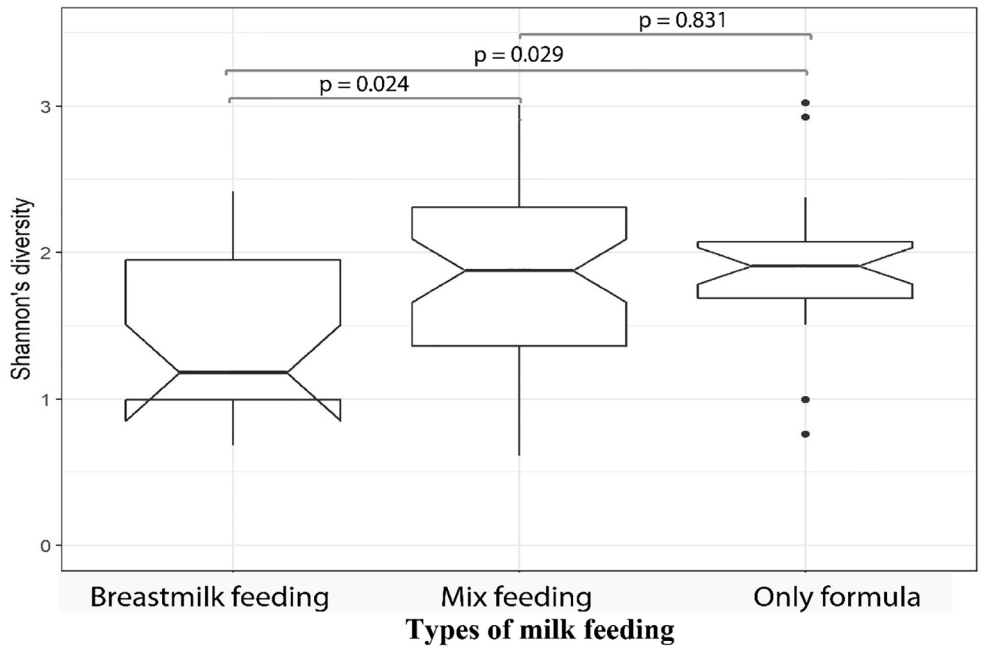

FIGURE 3 | Fecal samples from MLPT infants, at four-months corrected age, who were breastmilk fed exhibited lower microbial alpha diversities when compared to mixed fed (p = 0.024, Wilcoxon) and formula fed (p = 0.029, Wilcoxon) infants. The notch inversion indicates that the lower confidence level is less than the first quartile.

Infant antibiotic use during neonatal admission had no effect on fecal microbial alpha diversity at D10 (Wilcoxon test, p = 0.50, n = 102/199). Similarly, we observed no significant difference in alpha diversity levels in 4M fecal samples from the small number of babies (n = 5/113) that received antibiotics within the month immediately preceding the 4-month follow-up appointment (Wilcoxon test, p = 0.118). The levels of genus *Clostridium* were higher in 4M fecal samples from babies who received antibiotics within the month immediately preceding the 4-month follow-up appointment (linear model, q = 0.098, **Supplementary Table S4**—MaAsLin2—4M (16S data), doi: 10.17608/k6.auckland.12793772). However, the significance of these findings is limited by the numbers of individuals who received antibiotics (4.4%, n = 5/113, after excluding samples with missing data) and probiotics (8.8%, n = 10/113, after excluding samples with missing data) within the month immediately preceding the 4-month follow-up appointment.

We used HUMAnN2 to analyze the 4M shotgun metagenomics data to identify the gene complements of the samples. We identified a significant increase (linear model, q = 0.018) in the counts of genes involved in the allantoin degradation pathway (MetaCyc identifier PWY0-41) (**Supplementary Table S4**—HUMAnN2—4M (metagenomic data), doi: 10.17608/k6.auckland.12793772) in fecal samples from babies that received antibiotics (n = 4/93, after excluding samples with missing data) within the month immediately preceding the 4-month follow-up appointment when compared to babies that did not received antibiotics (n = 89/93, after excluding samples with missing data). The small number of babies who received antibiotics means this observation is underpowered.

The Gut Microbiome Composition Correlated With Growth Velocity

Evidence indicates there is an association between fecal microbial alpha diversity and the early growth rate of babies (Blanton et al., 2016; Gehrig et al., 2019; Vatanen et al., 2019). We examined the relationship between the fecal microbiome and change in MLPT weight, head circumference and length over the first four months of life. We computed the change (delta) z-score between birth and 4-months corrected age for the anthropometric growth measurements we collected. The change in weight and head circumference z-scores was associated with the beta diversity of the microbial metagenomic profiles from fecal samples collected at 4M in female (PERMANOVA, weight: R^2 = 0.050, p = 0.042; head circumference: R^2 = 0.051, p = 0.042), but not male, MLPT babies (PERMANOVA, weight: R^2 = 0.019, p = 0.566; head circumference: R^2 = 0.019, p = 0.566; **Supplementary Table S5**, doi: 10.17608/k6.auckland.12793811). No correlation was found between the delta z-score of length and the 4M fecal microbial profile in either male or female MLPT babies (male: R^2 = 0.009, p = 0.938; female: R^2 = 0.021, p = 0.735).

Gestational Age, Mode of Delivery, and Plurality Influence the Establishment of the Gut Microbiota

We performed a PERMANOVA analysis of Bray–Curtis dissimilarity to identify other factors that are associated with

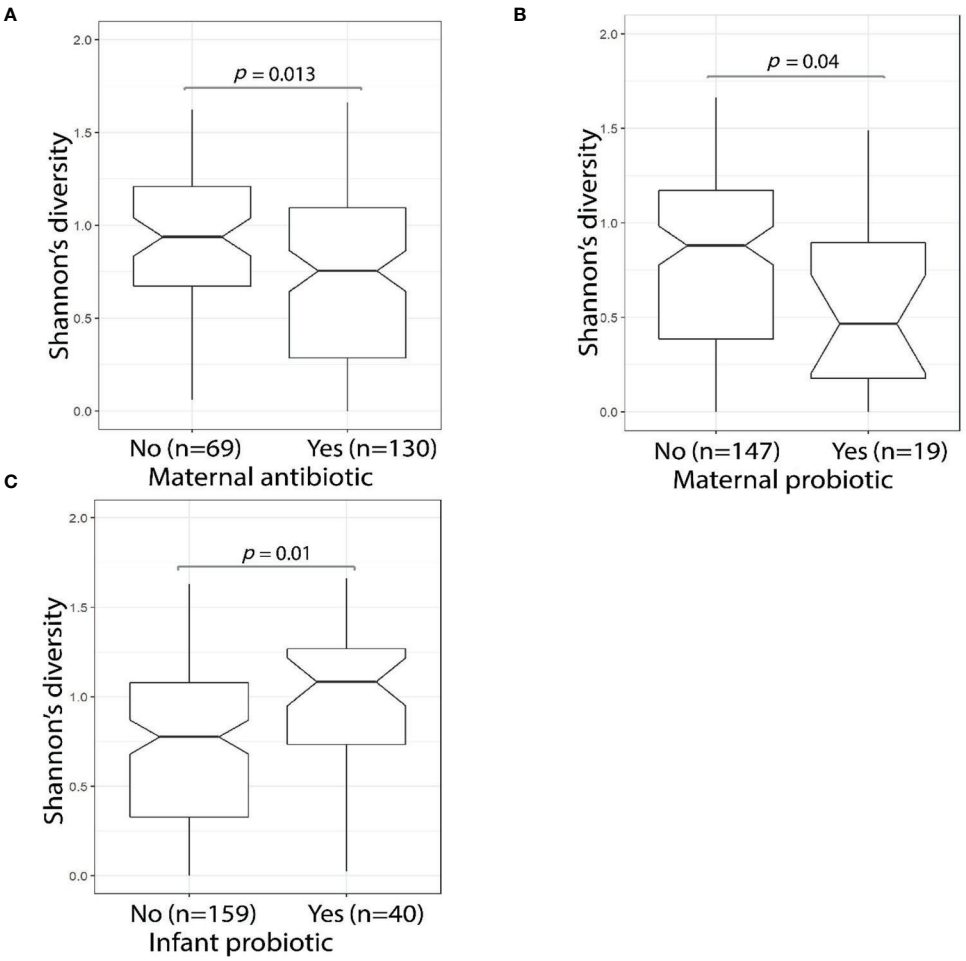

FIGURE 4 | MLPT fecal microbiome alpha diversity was affected by maternal antibiotic and probiotic usage. The Shannon's diversity index was calculated using 16S amplicon sequencing data obtained from MLPT infants' D10 fecal samples. Mother's antibiotic usage within the four weeks preceding the child's D10 sample collection was associated (p = 0.013, Wilcoxon) with a decrease in MLPT infants' fecal alpha diversity **(A)**; a significant reduction in microbial alpha diversity was also observed in infants whose mothers used probiotics (p = 0.04, Wilcoxon) in the four weeks preceding D10 sample collection **(B)**; and infants who received probiotics during admission had higher fecal microbial alpha diversity when compared to their counterparts who did not receive probiotics (p = 0.01, Wilcoxon) **(C)**.

changes in the MLPT fecal microbiome composition at D10 and 4M. Gestational age (*i.e.* moderate and late preterm) explained 1.2% of the variation in D10 fecal microbial beta diversity (p = 0.043). Mode of delivery contributed 2.1% of the variance (p = 0.002) within D10 fecal samples, with *Bacteroides* being more abundant in babies born vaginally (linear model, q = 0.002, **Supplementary Table S4**—MaAsLin2—D10 (16S data), doi: 10.17608/k6.auckland.12793772). However, neither gestational age (R^2 = 0.014, p = 0.173) nor mode of delivery (R^2 = 0.008, p = 0.452) were identified as contributing to the variation observed in the MLPT fecal microbial profile at 4M. Notably, plurality explained 2.6% of the variance (p = 0.034) in the microbial profile for samples collected at 4M, with a higher abundance of *Eggerthella lenta* observed in twins (n = 31/94) when compared to singletons (n = 63/94, linear model, q = 0.109, **Supplementary Table S4**—MaAsLin2—4M (metagenomic data), doi: 10.17608/k6.auckland.12793772).

The Impact of Hospital Environment and Maternal Associated Factors on the Establishment of the MLPT Fecal Gut Microbiota at 4M

The hospital environment was associated with changes to the microbial profile (Bray–Curtis) at 4M, where hospital of birth explained 5.2% of the observed variance (PERMANOVA, p = 0.038) in the shotgun metagenomic data. For example, *Staphylococcus aureus* was more abundant in the 4M microbiome of babies born at Middlemore hospital (linear model, q = 0.016) when compared to babies born at Auckland City hospital. By contrast, analyses of the 16S rRNA amplicon data did not identify an association between hospital of birth and infants D10 (PERMANOVA, R^2 = 0.024, p =0.084) and 4M fecal microbial profile (PERMANOVA, R^2 = 0.038, p = 0.150). Similarly, analyses of the 4M data using Fisher's exact test did not identify differences in either early life antibiotic (p = 0.725) or probiotic usage (p = 0.866)

between the birth hospitals. However, analyses of the 4M 16S rRNA amplicon data did identify an association between length of time that the MLPT baby stayed in hospital and microbial profile (Bray–Curtis), which explained 2.4% of the variance (PERMANOVA, $p = 0.034$) (**Supplementary Table S5**, doi: 10.17608/k6.auckland.12793811).

There was no association identified between the fecal microbial beta diversity at D10 (PERMANOVA, $R^2 = 0.003$, $p = 0.970$) or 4M (PERMANOVA, $R^2 = 0.014$, $p = 0.320$, **Supplementary Table S5**, doi: 10.17608/k6.auckland.12793811) and maternal age. Despite this, the level of the genus *Sutterella* (phylum Proteobacteria) in 4M fecal samples was inversely associated with maternal age (linear model, q = 0.051, **Supplementary Table S4**—MaAsLin2—4M (16S data), doi: 10.17608/k6.auckland.12793772). None of the other maternal factors we tested (*i.e.* stress and depression level) correlated with the alpha or beta diversity of the infants' gut microbiota at either time point (**Supplementary Table S5**, doi: 10.17608/k6.auckland.12793811).

DISCUSSION

Bifidobacterium is a common early life commensal in healthy-full term babies (Nagpal et al., 2017). Therefore, it was notable that *Bifidobacterium* species were at low abundance in fecal samples from our MLPT cohort at D10. The low abundance of *Bifidobacterium* observed in our D10 cohort could be explained by a number of factors. First, a majority (*i.e.* 63.8%) of the babies in our cohort were delivered by Cesarean section and *Bifidobacterium* has been reported to be in low abundance in term-born Cesarean section babies (Biasucci et al., 2010; Tannock et al., 2013). Secondly, *Bifidobacterium* is a genus that is well-known to be associated with breastfeeding (Tannock et al., 2013; Stewart et al., 2018). In MLPT babies, breastfeeding takes longer to be established. This delay may have contributed to the reduction observed. Thirdly, it might be related to the consequences of prematurity (*e.g.* composition of breastmilk and maturity of the infant gut) as in 45 preterm breastfed babies *Bifidobacterium* abundance has been reported to be associated with corrected postmenstrual age, increasing gradually after 30 weeks of postmenstrual age (Korpela et al., 2018). Therefore, we conclude that the combined effect of being born preterm and being born by Caesarean section delivery contributed to the observed low abundance of *Bifidobacterium* in MLPT children. With respect to a long-term effect, it is known that the numbers of *Bifidobacteria* peak after birth and progressively decrease to a stable number in adulthood (Arboleya et al., 2016). We speculate that the low abundance we observed in early life might not affect the level of *Bifidobacterium* that is attained in adulthood due to the compositional changes of *Bifidobacterium* species with respect to ageing (Gavini et al., 2001; Kato et al., 2017). However, this requires confirmation through long-term longitudinal studies of the MLPT population.

We observed higher alpha diversity in the D10 fecal microbiomes of babies whose mothers had lower socioeconomic status (SES) and babies born to mothers who self-reported as being of Māori ethnicity. Superficially, this would appear to be consistent with observations that associate socioeconomic disparity and diet with gut microbial composition and richness (Miller et al., 2016; Bowyer et al., 2019). However, the effect of SES on the gut microbiota remains controversial (Chong et al., 2015; Miller et al., 2016; Bowyer et al., 2019; Gschwendtner et al., 2019). In our study, this was further confounded by collinearities between ethnicity and SES rendering it difficult to untangle SES effects from a few other factors in our study. Further work needs to be undertaken to provide greater understanding of the effects of SES on the microbiome.

The differences observed at day 10 fecal microbiomes dissipated over time such that there was no association between the infants' gut microbiota composition and maternal ethnicity or SES in samples collected from our MLPT cohort at four months. This could be explained by a portion of maternal microbes that were vertically transferred to infants. However, we offer alternative explanations for this lack of differences at 4M. First, we observed a significant negative correlation between maternal education levels with infants' microbial alpha diversity at 4M, and maternal education levels are significantly colinear with both maternal self-reported ethnicity and SES in our MLPT cohort, similar to previous reports (Easton, 2013). Secondly, the common commensals present within the mothers' microbiomes might not be retained following early life vertical transmission due to a requirement for specific nutrients. Thirdly, the acquisition of these founder bacteria in early life might have been subject to a "dilution effect" as a result of nutrients present in the breastmilk or formula being bioavailable to bacteria that grow to outnumber these founder organisms.

No association was identified between infants' D10 fecal microbiome composition and hospital of birth. By contrast, an association between infants' 4M fecal microbiome composition with hospital of birth and length of hospital stay were observed. It is notable that D10 fecal sample was collected 10 days after birth yet, on average, babies in our cohort spent 22 days in the hospital. Therefore, it remains possible that the hospital of birth does impact the microbiome but that the establishment of this effect, by cross-transmission from other infants, or inoculation from hospital workers or environment is established over a longer period than the initial sampling time and thus not seen in the fecal sample collected at D10.

The influence of environmental factors on the gut microbiota composition has been shown to surpass that of the host genetics in two large cohorts of healthy adults [n= 1,046 (Rothschild et al., 2018) and n= 858 (Scepanovic et al., 2019)]. Support for the impact of environmental effects is further substantiated by Koo et al. (2019) who showed that twins separated for decades shared fewer bacteria strains compared to twins who cohabitated for a long time (Koo et al., 2019). Our findings demonstrated that plurality (*i.e.* singleton versus twins) was associated with 4M fecal microbial beta diversity. Notably, we did not observe a difference in D10 fecal sample microbial profile. Other than the vertical transfer of microbiome from mothers to infants, twins shared a common environment after hospital discharge, which

makes the environmental effect more prominent. We argue that the combined effect of interactions between genetic variation and environmental factors surfaced later in life when the infants are no longer sharing a common environment.

The increased abundance of Firmicutes in our longitudinal analysis of fecal microbiome development in MLPT infants from D10 to 4M was consistent with previous observations (Bäckhed et al., 2015) as a hallmark of the maturation of the gut microbiome (Stewart et al., 2018). Gestational age at birth and mode of delivery were observed to associated with D10 fecal microbial beta diversity. This observation is consistent with an earlier study that identified a significant effect of gestational age at birth on the microbiome composition (Fouhy et al., 2019). Our observation that this effect was not clearly identifiable at 4M indicates it is weak and transient and agrees with other studies that have indicated the microbiomes of preterm and term babies converge later in life (Jayasinghe et al., 2020). Similarly, delivery mode has a maximum impact on the infants' establishing gut microbiome during the first week of life (Reyman et al., 2019; Shao et al., 2019), consistent with our observation that the effect of delivery mode dissipated at 4M in the MLPT cohort. However, the demographic differences between the time points and potential sample collection biases between hospital (D10) and home sampling (4M) may confound these or any other findings of this study.

We saw an increase in *Staphylococcus* abundance in 4M fecal samples only in children born to mothers who self-reported as Asian. High levels of *Staphylococcus* were associated with vaginal birth and infants born early (Korpela et al., 2018). We contend that the increase we observed in the Asian subgroup was because a larger proportion of births were vaginal in this group (31/77, 40.3%). However, a recent study of 554 South African women has identified *Staphylococcus, Rothia*, and *Gemella* as among the most abundant genera present in human breast milk (Ojo-Okunola et al., 2019). Therefore, it remains possible that the increased abundance of *Staphylococcus, Gemella*, and *Rothia* that was associated with ethnicity at D10 and 4M was due to different types of milk feeding. For example, more Māori and Pacifica mothers practised breastmilk feeding compared to Asian mothers at D10. However, the opposite was observed at 4M. In other words, types of milk feeding, together with maternal lifestyle and cultural practice, contribute to the process of establishing the gut microbial composition during early life.

Feeding regime (*i.e.* breastfed and formula-fed) has been previously identified as significantly affecting the infants' gut microbiome composition (Azad et al., 2013; Praveen et al., 2015). In our MLPT cohort, we observed an increase in the abundance of *Megasphaera* spp. in breastmilk fed babies at 4M. This agrees with previous observations in a full-term Danish cohort where breastfeeding duration is positively associated with *Megasphaera* levels (Laursen et al., 2016). Lactate is more abundant in exclusively breastfed infants (Bridgman et al., 2017). The lactate utilization properties of *Megasphaera* are suggested to reduce lactate toxicity and to generate short-chain fatty acids (SCFAs) (Shetty et al., 2013). The low alpha diversity we observed in fecal samples from 4M breastmilk fed infants corroborates the findings of a previous meta-analysis on exclusively breastfed infants (Ho et al., 2018) of seven microbiome studies and a recent study on the exclusivity breastmilk feeding of infants at 3 months of age (Fehr et al., 2020). Therefore, we contend that HMOs and other compounds within breastmilk select for a specific, albeit low diversity microbiome.

The administration of prophylactic antibiotics to mothers undergoing Cesarean section is a common practice that is recommended by the World Health Organisation (Smaill and Grivell, 2014; World Health Organization, 2015; Liu et al., 2016). Maternal intrapartum antibiotic prophylaxis (IAP) was associated with changes in infants' fecal microbial beta diversity (Shao et al., 2019) and reduced infants' fecal microbial alpha diversity over the first three months of life (Nogacka et al., 2017). Our observation that maternal antibiotic and probiotic usage correlated with the infants' D10 gut microbial alpha diversities is consistent with a mechanism that affects vertical microbial transmission from mothers to infants after birth. Given the association of feeding regime and birth mode with diversity, it remains possible that this transmission occurs through breastmilk and mode of delivery. As such, treatment of maternal medical conditions during the perinatal period might impact on the infants' microbial profile during the most vulnerable postnatal period where breastmilk is the primary food source. However, the fact that the association with antibiotic usage was not seen at four-months corrected age indicates that it is not the dominant effect and is substitute by other environmental and feeding practices.

Healthy infants have predictable patterns of weight gain and growth (*i.e.* length and head circumference). Growth parameters are thus often used to reflect the overall health and nutritional status of an infant. However, infant growth does differ by seasons (greater in spring to summer compared to autumn to winter) (Gelander et al., 1994; Bozzola and Meazza, 2012) and is best calculated over at least a 6 month period. The ability of our study to address growth by seasons was limited by the fact that our growth records were: 1) only taken over a 4-month period; 2) only for MLPT infants; and 3) unevenly distributed over the seasons for the male babies. Despite these limitations, our results suggested a correlation of the gut microbiota composition with the growth velocity (delta z-score) in weight and head circumference of female babies. Kamng'ona et al. (2019) have identified an association between the gut microbiome and babies' weight gain but not in length and head circumference in a mixed genders cohort (Kamng'ona et al., 2019). The composition of breast milk differed when mothers gave birth to boy and girl (Galante et al., 2018). As both male and female babies in our cohort were not treated differently in terms of nutrition, it remains possible that these gender differences led to different nutritional needs that contributed to our observations in female but not male MLPT babies.

CONCLUSION

Our study demonstrates the complexity of factors (*i.e.* maternal socioeconomic factors, perinatal medical environment,

gestational age and delivery mode) that impact upon gut microbiota acquisition and establishment in MLPT infants over the first 10 days after birth. These factors are largely substituted by infants' immediate environment and types of milk feeding, which exert the dominant effects on the microbiome at 4-months corrected age. Finally, we highlighted the need to analyze male and female microbiomes separately, when looking at associations with growth.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the New Zealand Health and Disability Ethics Committee (number 16/NTA/90). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Conceptualization, FB, TA, and JO'S. Data curation, CC, TV, FB, and JO'S. Formal analysis, CC and TV. Funding acquisition, FB, JO'S, and TA. Investigation, CC, TV, FB, and JO'S. Methodology, CC, TV, FB, TA, and JO'S. Supervision, TV, FB, and JO'S. Visualization, CC, TV, FB, and JO'S. Writing—original draft, CC. Writing—review and editing, CC, TV, FB, and JO'S. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.595323/full#supplementary-material

REFERENCES

Akagawa, S., Tsuji, S., Onuma, C., Akagawa, Y., Yamaguchi, T., Yamagishi, M., et al. (2019). Effect of delivery mode and nutrition on gut microbiota in neonates. *Ann. Nutr. Metab.* 74 (2), 132–139. doi: 10.1159/000496427

Alexander, T., and Bloomfield, F. H. (2019). Nutritional management of moderate-late preterm infants: Survey of current practice. *J. Paediatr. Child Health* 55 (3), 338–342. doi: 10.1111/jpc.14201

Arboleya, S., Watkins, C., Stanton, C., and Ross, R. P. (2016). Gut bifidobacteria populations in human health and aging. *Front. Microbiol.* 7, 1204. doi: 10.3389/fmicb.2016.01204

Arboleya, S., Martinez-Camblor, P., Solís, G., Suárez, M., Fernández, N., de los Reyes-Gavilán, C. G., et al. (2017). Intestinal microbiota and weight-gain in preterm neonates. *Front. Microbiol.* 8, 183. doi: 10.3389/fmicb.2017.00183

Atkinson, J., Salmond, C., and Crampton, P. (2014). *NZDep2013 index of deprivation* (Dunedin). (Wellington: Department of Public Health, University of Otago). Retrieved from health.govt.nz/publication/nzdep2013-index-deprivation#:~:text=NZDep2013 is an updated version,each meshblock in New Zealand.

Avershina, E., Storrø, O., Øien, T., Johnsen, R., Pope, P., and Rudi, K. (2014). Major faecal microbiota shifts in composition and diversity with age in a geographically restricted cohort of mothers and their children. *FEMS Microbiol. Ecol.* 87 (1), 280–290. doi: 10.1111/1574-6941.12223

Azad, M. B., Konya, T., Maughan, H., Guttman, D. S., Field, C. J., Chari, R. S., et al. (2013). Gut microbiota of healthy Canadian infants: Profiles by mode of delivery and infant diet at 4 months. *Can. Med. Assoc. J.* 185 (5), 385–394. doi: 10.1503/cmaj.121189

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* 17 (6), 852. doi: 10.1016/j.chom.2015.05.012

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate - a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B (Methological)* 57 (1), 289–300. doi: 10.2307/2346101

Biasucci, G., Rubini, M., Riboni, S., Morelli, L., Bessi, E., and Retetangos, C. (2010). Mode of delivery affects the bacterial community in the newborn gut. *Early Hum. Dev.* 86 (1), 13–15. doi: 10.1016/j.earlhumdev.2010.01.004

Blanton, L. V., Charbonneau, M. R., Salih, T., Barratt, M. J., Venkatesh, S., Ilkaveya, O., et al. (2016). Gut bacteria that prevent growth impairments transmitted by microbiota from malnourished children. *Science* 351 (6275), aad3311–aad3311. doi: 10.1126/science.aad3311

Bloomfield, F. H., Harding, J. E., Meyer, M. P., Alsweiler, J. M., Jiang, Y., Wall, C. R., et al. (2018). The DIAMOND trial – DIfferent Approaches to MOderate & late preterm Nutrition: Determinants of feed tolerance, body composition and development: protocol of a randomised trial. *BMC Pediatr.* 18 (1), 220. doi: 10.1186/s12887-018-1195-7

Bowyer, R., Jackson, M., Le Roy, C., Ni Lochlainn, M., Spector, T., Dowd, J., et al. (2019). Socioeconomic status and the gut microbiome: A TwinsUK cohort study. *Microorganisms* 7 (1), 17. doi: 10.3390/microorganisms 7010017

Bozzola, M., and Meazza, C. (2012). "Growth velocity curves: What they are and how to use them," in *Handbook of Growth and Growth Monitoring in Health and Disease*. Ed. V. R. Preedy (New York, NY: Springer New York), 2999–3011. doi: 10.1007/978-1-4419-1795-9_180

Bridgman, S. L., Azad, M. B., Field, C. J., Haqq, A. M., Becker, A. B., Mandhane, P. J., et al. (2017). Fecal short-chain fatty acid variations by breastfeeding status in infants at 4 months: Differences in relative versus absolute concentrations. *Front. Nutr.* 4, 11. doi: 10.3389/fnut.2017.00011

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from

Illumina amplicon data. *Nat. Methods* 13 (7), 581–583. doi: 10.1038/nmeth.3869

Chen, S.-Y., Feng, Z., and Yi, X. (2017). A general introduction to adjustment for multiple comparisons. *J. Thorac. Dis.* 9 (6), 1725–1729. doi: 10.21037/jtd.2017.05.34

Chernikova, D. A., Madan, J. C., Housman, M. L., Zain-ul-abideen, M., Lundgren, S. N., Morrison, H. G., et al. (2018). The premature infant gut microbiome during the first 6 weeks of life differs based on gestational maturity at birth. *Pediatr. Res.* 84 (1), 71–79. doi: 10.1038/s41390-018-0022-z

Chong, C. W., Ahmad, A. F., Lim, Y. A. L., Teh, C. S. J., Yap, I. K. S., Lee, S. C., et al. (2015). Effect of ethnicity and socioeconomic variation to the gut microbiota composition among pre-adolescent in Malaysia. *Sci. Rep.* 5 (1), 13338. doi: 10.1038/srep13338

Cong, X., Judge, M., Xu, W., Diallo, A., Janton, S., Brownell, E. A., et al. (2017). Influence of feeding type on gut microbiome development in hospitalized preterm infants. *Nurs. Res.* 66 (2), 123–133. doi: 10.1097/NNR.0000000000000208

Dahlgren, A. F., Pan, A., Lam, V., Gouthro, K. C., Simpson, P. M., Salzman, N. H., et al. (2019). Longitudinal changes in the gut microbiome of infants on total parenteral nutrition. *Pediatr. Res.* 86 (1), 107–114. doi: 10.1038/s41390-019-0391-y

Easton, B. (2013). *Ethnicity, gender, socioeconomic status and educational achievement: An exploration*. (Wellington: Post-Primary Teachers' Association). Available at: http://www.eastonbh.ac.nz/2014/09/ethnicity-gender-socioeconomic-status-and-educational-achievement-an-exploration/.

Fehr, K., Moossavi, S., Sbihi, H., Boutin, R. C. T., Bode, L., Robertson, B., et al. (2020). Breastmilk feeding practices are associated with the co-occurrence of bacteria in mothers' milk and the infant gut: The CHILD cohort study. *Cell Host Microbe* 28 (2), 285–297.e4. doi: 10.1016/j.chom.2020.06.009

Ford, S. L., Lohmann, P., Preidis, G. A., Gordon, P. S., O'Donnell, A., Hagan, J., et al. (2019). Improved feeding tolerance and growth are linked to increased gut microbial community diversity in very-low-birth-weight infants fed mother's own milk compared with donor breast milk. *Am. J. Clin. Nutr.* 109 (4), 1088–1097. doi: 10.1093/ajcn/nqz006

Fouhy, F., Watkins, C., Hill, C. J., O'Shea, C.-A., Nagle, B., Dempsey, E. M., et al. (2019). Perinatal factors affect the gut microbiota up to four years after birth. *Nat. Commun.* 10 (1), 1517. doi: 10.1038/s41467-019-09252-4

Franzosa, E. A., McIver, L. J., Rahnavard, G., Thompson, L. R., Schirmer, M., Weingart, G., et al. (2018). Species-level functional profiling of metagenomes and metatranscriptomes. *Nat. Methods* 15 (11), 962–968. doi: 10.1038/s41592-018-0176-y

Galante, L., Milan, A., Reynolds, C., Cameron-Smith, D., Vickers, M., and Pundir, S. (2018). Sex-specific human milk composition: The role of infant sex in determining early life nutrition. *Nutrients* 10 (9), 1194. doi: 10.3390/nu10091194

Gavini, F., Cayuela, C., Antoine, J.-M., Lecoq, C., Lefebvre, B., Membré, J.-M., et al. (2001). Differences in the distribution of Bifidobacterial and Enterobacterial species in human faecal microflora of three different (children, adults, elderly) age groups. *Microb. Ecol. Health Dis.* 13 (1), 40–45. doi: 10.1080/089106001750071690

Gehrig, J. L., Venkatesh, S., Chang, H.-W., Hibberd, M. C., Kung, V. L., Cheng, J., et al. (2019). Effects of microbiota-directed foods in gnotobiotic animals and undernourished children. *Science* 365 (6449), eaau4732. doi: 10.1126/science.aau4732

Gelander, L., Karlberg, J., and Albertsson-Wikland, K. (1994). Seasonality in lower leg length velocity in prepubertal children. *Acta Paediatr.* 83 (12), 1249–1254. doi: 10.1111/j.1651-2227.1994.tb13006.x

Giannì, M. L., Roggero, P., Piemontese, P., Liotto, N., Orsi, A., Amato, O., et al. (2015). Is nutritional support needed in late preterm infants? *BMC Pediatr.* 15 (1), 194. doi: 10.1186/s12887-015-0511-8

Giannoukos, G., Ciulla, D. M., Huang, K., Haas, B. J., Izard, J., Levin, J. Z., et al. (2012). Efficient and robust RNA-seq process for cultured bacteria and complex community transcriptomes. *Genome Biol.* 13 (3), r23. doi: 10.1186/gb-2012-13-3-r23

Gregory, K. E., Samuel, B. S., Houghteling, P., Shan, G., Ausubel, F. M., Sadreyev, R. I., et al. (2016). Influence of maternal breast milk ingestion on acquisition of the intestinal microbiome in preterm infants. *Microbiome* 4 (1), 68. doi: 10.1186/s40168-016-0214-x

Gschwendtner, S., Kang, H., Thiering, E., Kublik, S., Fösel, B., Schulz, H., et al. (2019). Early life determinants induce sustainable changes in the gut microbiome of six-year-old children. *Sci. Rep.* 9 (1), 12675. doi: 10.1038/s41598-019-49160-7

Harding, J. E., Cormack, B. E., Alexander, T., Alsweiler, J. M., and Bloomfield, F. H. (2017). Advances in nutrition of the newborn infant. *Lancet* 389 (10079), 1660–1668. doi: 10.1016/S0140-6736(17)30552-4

Ho, N. T., Li, F., Lee-Sarwar, K. A., Tun, H. M., Brown, B. P., Pannaraj, P. S., et al. (2018). Meta-analysis of effects of exclusive breastfeeding on infant gut microbiota across populations. *Nat. Commun.* 9 (1), 4169. doi: 10.1038/s41467-018-06473-x

Jayasinghe, T. N., Vatanen, T., Chiavaroli, V., Jayan, S., McKenzie, E. J., Adriaenssens, E., et al. (2020). Differences in compositions of gut bacterial populations and bacteriophages in 5–11 Year-olds born preterm compared to full term. *Front. Cell. Infect. Microbiol.* 10, 276. doi: 10.3389/fcimb.2020.00276

Kamng'ona, A. W., Young, R., Arnold, C. D., Kortekangas, E., Patson, N., Jorgensen, J. M., et al. (2019). The association of gut microbiota characteristics in Malawian infants with growth and inflammation. *Sci. Rep.* 9 (1), 12893. doi: 10.1038/s41598-019-49274-y

Kassambara, A. (2019). *CRAN - Package rstatix*. Pipe-Friendly Framework for Basic Statistical Tests. Retrieved from cran.r-project.org/web/packages/rstatix/index.html.

Kato, K., Odamaki, T., Mitsuyama, E., Sugahara, H., Xiao, J., and Osawa, R. (2017). Age-related changes in the composition of gut Bifidobacterium species. *Curr. Microbiol.* 74 (8), 987–995. doi: 10.1007/s00284-017-1272-4

Koo, H., Hakim, J. A., Crossman, D. K., Lefkowitz, E. J., and Morrow, C. D. (2019). Sharing of gut microbial strains between selected individual sets of twins cohabitating for decades. *PloS One* 14 (12), e0226111. doi: 10.1371/journal.pone.0226111

Korpela, K., Blakstad, E. W., Moltu, S. J., Strømmen, K., Nakstad, B., Rønnestad, A. E., et al. (2018). Intestinal microbiota development and gestational age in preterm neonates. *Sci. Rep.* 8 (1), 2453. doi: 10.1038/s41598-018-20827-x

Laursen, M. F., Andersen, L. B. B., Michaelsen, K. F., Mølgaard, C., Trolle, E., Bahl, M. I., et al. (2016). Infant gut microbiota development is driven by transition to family foods independent of maternal obesity. *MSphere* 1 (1), e00069–e00015. doi: 10.1128/mSphere.00069-15

Li, W., Tapiainen, T., Brinkac, L., Lorenzi, H. A., Moncera, K., Tejesvi, M. V., et al. (2020). Vertical Transmission of Gut Microbiome and Antimicrobial Resistance Genes in Infants Exposed to Antibiotics at Birth. *J. Infect. Dis.* doi: 10.1093/infdis/jiaa155

Liu, R., Lin, L., and Wang, D. (2016). Antimicrobial prophylaxis in caesarean section delivery. *Exp. Ther. Med.* 12 (2), 961–964. doi: 10.3892/etm.2016.3350

Marotz, C. A., Sanders, J. G., Zuniga, C., Zaramela, L. S., Knight, R., and Zengler, K. (2018). Improving saliva shotgun metagenomics by chemical host DNA depletion. *Microbiome* 6 (1):42. doi: 10.1186/s40168-018-0426-3

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal* 17 (1), 10. doi: 10.14806/ej.17.1.200

Miller, G. E., Engen, P. A., Gillevet, P. M., Shaikh, M., Sikaroodi, M., Forsyth, C. B., et al. (2016). Lower neighborhood socioeconomic status associated with reduced diversity of the colonic microbiota in healthy adults. *PloS One* 11 (2), e0148952. doi: 10.1371/journal.pone.0148952

Ministry of Health (2019). *Report on maternity 2017* (Wellington: Wellington: Ministry of Health). Available at: https://webcache.googleusercontent.com/search?q=cache:xGIgafwpXZMJ:https://www.health.govt.nz/system/files/documents/publications/report-maternity-2017-may19.pdf+&cd=5&hl=en&ct=clnk&gl=nz.

Morgan, X. C., Tickle, T. L., Sokol, H., Gevers, D., Devaney, K. L., Ward, D. V., et al. (2012). Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol.* 13 (9), R79. doi: 10.1186/gb-2012-13-9-r79

Morowitz, M. J., Poroyko, V., Caplan, M., Alverdy, J., and Liu, D. C. (2010). Redefining the role of intestinal microbes in the pathogenesis of necrotizing enterocolitis. *Pediatrics* 125 (4), 777–785. doi: 10.1542/peds.2009-3149

Musemeche, C. A., Kosloske, A. M., Bartow, S. A., and Umland, E. T. (1986). Comparative efects of ischemia, bacteria, and substrate on the pathogenesis of intestinal necrosis. *J. Pediatr. Surg.* 21 (6), 536–538. doi: 10.1016/S0022-3468(86)80228-7

Nagpal, R., Kurakawa, T., Tsuji, H., Takahashi, T., Kawashima, K., Nagata, S., et al. (2017). Evolution of gut Bifidobacterium population in healthy Japanese

infants over the first three years of life: a quantitative assessment. *Sci. Rep.* 7 (1), 10097. doi: 10.1038/s41598-017-10711-5

Neu, J., and Walker, W. A. (2011). Necrotizing enterocolitis. *N Engl. J. Med.* 364 (3), 255–264. doi: 10.1056/NEJMra1005408

Nogacka, A., Salazar, N., Suárez, M., Milani, C., Arboleya, S., Solís, G., et al. (2017). Impact of intrapartum antimicrobial prophylaxis upon the intestinal microbiota and the prevalence of antibiotic resistance genes in vaginally delivered full-term neonates. *Microbiome* 5 (1), 93. doi: 10.1186/s40168-017-0313-3

Ojo-Okunola, C.-W., Mwaikono, G.-L., Stein, Z., and du Toit, (2019). Influence of socio-economic and psychosocial profiles on the human breast milk bacteriome of South African women. *Nutrients* 11 (6), 1390. doi: 10.3390/nu11061390

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2019). *vegan: Community Ecology Package version 2.5-6 from CRAN*. Available at: https://rdrr.io/cran/vegan/.

Pammi, M., Cope, J., Tarr, P. I., Warner, B. B., Morrow, A. L., Mai, V., et al. (2017). Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome* 5 (1), 31. doi: 10.1186/s40168-017-0248-8

Praveen, P., Jordan, F., Priami, C., and Morine, M. J. (2015). The role of breast-feeding in infant immune system: a systems perspective on the intestinal microbiome. *Microbiome* 3 (1), 41. doi: 10.1186/s40168-015-0104-7

R Core Team (2019). *R: A Language and Environment for Statistical Computing* (Vienna, Austria: R Foundation for Statistical Computing). Available at: https://www.r-project.org/.

Reyman, M., van Houten, M. A., van Baarle, D., Bosch, A. A. T. M., Man, W. H., Chu, M. L. J. N., et al. (2019). Impact of delivery mode-associated gut microbiota dynamics on health in the first year of life. *Nat. Commun.* 10 (1), 4997. doi: 10.1038/s41467-019-13014-7

Robertson, R. C., Manges, A. R., Finlay, B. B., and Prendergast, A. J. (2019). The human microbiome and child growth – First 1000 days and beyond. *Trends Microbiol.* 27 (2), 131–147. doi: 10.1016/j.tim.2018.09.008

Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., et al. (2018). Environment dominates over host genetics in shaping human gut microbiota. *Nature* 555 (7695), 210–215. doi: 10.1038/nature25973

Scepanovic, P., Hodel, F., Mondot, S., Partula, V., Byrd, A., Hammer, C., et al. (2019). A comprehensive assessment of demographic, environmental, and host genetic associations with gut microbiome diversity in healthy individuals. *Microbiome* 7 (1), 130. doi: 10.1186/s40168-019-0747-x

Shao, Y., Forster, S. C., Tsaliki, E., Vervier, K., Strang, A., Simpson, N., et al. (2019). Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature* 574 (7776), 117–121. doi: 10.1038/s41586-019-1560-1

Shetty, S. A., Marathe, N. P., Lanjekar, V., Ranade, D., and Shouche, Y. S. (2013). Comparative genome analysis of Megasphaera sp. reveals niche specialization and its potential role in the human gut. *PloS One* 8 (11), e79353. doi: 10.1371/journal.pone.0079353

Smaill, F. M., and Grivell, R. M. (2014). Antibiotic prophylaxis versus no prophylaxis for preventing infection after cesarean section. *Cochrane Database Syst. Rev.* 2014 (10), 1–219. doi: 10.1002/14651858.CD007482.pub3

Stewart, C. J., Ajami, N. J., O'Brien, J. L., Hutchinson, D. S., Smith, D. P., Wong, M. C., et al. (2018). Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature* 562 (7728), 583–588. doi: 10.1038/s41586-018-0617-x

Tannock, G. W., Lawley, B., Munro, K., Gowri Pathmanathan, S., Zhou, S. J., Makrides, M., et al. (2013). Comparison of the compositions of the stool microbiotas of infants fed goat milk formula, cow milk-based formula, or breast milk. *Appl. Environ. Microbiol.* 79 (9), 3040–3048. doi: 10.1128/AEM.03910-12

Tauchi, H., Yahagi, K., Yamauchi, T., Hara, T., Yamaoka, R., Tsukuda, N., et al. (2019). Gut microbiota development of preterm infants hospitalised in intensive care units. *Benef. Microbes* 10 (6), 641–651. doi: 10.3920/BM2019.0003

Truong, D. T., Franzosa, E. A., Tickle, T. L., Scholz, M., Weingart, G., Pasolli, E., et al. (2015). MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nat. Methods* 12 (10), 902–903. doi: 10.1038/nmeth.3589

Vatanen, T., Plichta, D. R., Somani, J., Münch, P. C., Arthur, T. D., Hall, A. B., et al. (2019). Genomic variation and strain-specific functional adaptation in the human gut microbiome during early life. *Nat. Microbiol.* 4 (3), 470–479. doi: 10.1038/s41564-018-0321-5

Vogtmann, E., Chen, J., Kibriya, M. G., Chen, Y., Islam, T., Eunes, M., et al. (2017). Comparison of fecal collection methods for microbiota studies in Bangladesh. *Appl. Environ. Microbiol.* 83 (10), e00361–e00317. doi: 10.1128/AEM.00361-17

Wang, Z., Neupane, A., Vo, R., White, J., Wang, X., and Marzano, S.-Y. L. (2020). Comparing gut microbiome in mothers' own breast milk- and formula-fed moderate-late preterm infants. *Front. Microbiol.* 11, 891. doi: 10.3389/fmicb.2020.00891

World Health Organization (2015). "WHO recommendation on routine antibiotic prophylaxis for women undergoing elective or emergency caesarean section," in *WHO recommendations for prevention and treatment of maternal peripartum infections* (Geneva: World Health Organization), 70. Available at: http://www.who.int/reproductivehealth/publications/maternal_perinatal_health/peripartum-infections-guidelines/en/.

World Health Organization (2017). *Preterm birth: fact sheet*. Available at: http://www.who.int/mediacentre/factsheets/fs363/en/.

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Comparison of Two Approaches for the Metataxonomic Analysis of the Human Milk Microbiome

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Recent work has demonstrated the existence of large inter-individual and inter-population variability in the microbiota of human milk from healthy women living across variable geographical and socio-cultural settings. However, no studies have evaluated the impact that variable sequencing approaches targeting different 16S rRNA variable regions may have on the human milk microbiota profiling results. This hampers our ability to make meaningful comparisons across studies. In this context, the main purpose of the present study was to re-process and re-sequence the microbiome in a large set of human milk samples (n = 412) collected from healthy women living at diverse international sites (Spain, Sweden, Peru, United States, Ethiopia, Gambia, Ghana and Kenya), by targeting a different 16S rRNA variable region and reaching a larger sequencing depth. Despite some differences between the results obtained from both sequencing approaches were notable (especially regarding alpha and beta diversities and Proteobacteria representation), results indicate that both sequencing approaches revealed a relatively consistent microbiota configurations in the studied cohorts. Our data expand upon the milk microbiota results we previously reported from the INSPIRE cohort and provide, for the first time across globally diverse populations, evidence of the impact that different DNA processing and sequencing approaches have on the microbiota profiles obtained for human milk samples. Overall, our results corroborate some similarities regarding the microbial communities previously reported for the INSPIRE cohort, but some differences were also detected. Understanding the impact of different sequencing approaches on

human milk microbiota profiles is essential to enable meaningful comparisons across studies.

Clinical Trial Registration: www.clinicaltrials.gov, identifier NCT02670278.

Keywords: human milk, microbiota, 16S rRNA, bacteria, sequencing reproducibility

INTRODUCTION

Despite the fact that human milk has long been considered sterile, research conducted over the last decade has provided convincing evidence that this biological fluid harbors a rich microbial community under all physiological circumstances (Martín et al., 2006; Martín et al., 2007; Solis et al., 2010; Fernández et al., 2013). Milk's microbial community contains an important arsenal of bacterial and fungal species of substantial interest as they likely play crucial roles in the maintenance of maternal and infant health (as reviewed in Boix-Amorós et al., 2019; Moossavi et al., 2020; Stinson et al., 2020). For instance, they likely seed the breastfed infant's gastrointestinal (GI) tract, initiating the assembly of a mature healthy human GI microbiota, and orchestrating the innate immunity maturation and programming that will condition infant health outcomes in the short and long term (as reviewed in Milani et al., 2017). The milk microbiota in conjunction with other bioactive factors present in human milk, such as oligosaccharides and immune factors, have been deemed responsible for many of the long-term, health promoting effects associated with exclusive breastfeeding in early life, which correlate with reduced incidence of chronic inflammatory and metabolic conditions in infancy and adulthood (Huërou-Luron et al., 2010; Roger et al., 2010; Musilova et al., 2017).

Indeed, a growing literature supporting the crucial roles exerted by the human milk microbiota in maternal and infant health, in conjunction with advances in high-throughput sequencing (HTS) technologies, have led to a rapidly growing interest in the study of this microbial community and its variation, mainly in relation to maternal and infant factors. In this regard evidence suggests the existence of a strong inter-individual variation in the composition of the human milk microbiota across different populations, in relation to variable delivery factors (Cabrera-Rubio et al., 2016; Hoashi et al., 2016; Asbury et al., 2020), lactation stage (Khodayar-Pardo et al., 2014), maternal conditions (either chronic pre-pregnancy situations or those developed during pregnancy) (LeMay-Nedjelski et al., 2020; Volery et al., 2020; Wan et al., 2020), lifestyle habits (Moossavi et al., 2019; Padilha et al., 2020), psycho-social and economic conditions (Ojo-Okunola et al., 2019), and infant health outcomes (Demmelmair et al., 2020) as summarized previously in Ruiz et al. (2019) and Lackey et al. (2019). More recently, a combination of HTS and culturomic approaches has further supported the existence of a diversity of viable bacterial cells in healthy human milk wider than previously anticipated, and has offered novel opportunities to conduct mechanistic studies on the metabolic potential of this microbial community (Schwab et al., 2019; Togo et al., 2019; Treven et al., 2019).

While most investigators have undertaken research on this field with the aim to identify imbalances or dysbiosis states under specific maternal/infant conditions or in relation to health outcomes, very little effort has been aimed at delineating the structure of a healthy human milk microbiota, even though defining the normal baseline of a given microbiota is essential for a comprehensive understanding into the variation associated with different health outcomes (Bäckhed et al., 2012). Moreover, early studies on the structure of the healthy human milk microbiota conducted on either US (Hunt et al., 2011), Finnish (Cabrera-Rubio et al., 2012), Mexican-American (Davé et al., 2016), Chinese (Li et al., 2017), and Canadian populations (Moossavi et al., 2019), suggested the existence of significant variation across populations. For instance while most studies concur regarding the identification of a few "core" and dominant bacterial genera including mainly *Staphylococcus* and *Streptococcus* species, other representative microbial organisms belonging to the lactic acid bacteria group (*Lactobacillus, Lactococcus, Leuconostoc, Weisella*), or typical skin inhabitants such as *Propionibacterium* (*Cutibacterium*) or *Corynebacterium* are not universally detected across all populations analyzed despite appearing as predominant in some. For instance, *Lactococcus, Leuconostoc*, and *Weisella* appear to be dominant taxa in Finnish women (Cabrera-Rubio et al., 2012), while in Chinese and Taiwanese women the dominant populations included *Pseudomonadaceae* and *Lactobacillaceae* (Li et al., 2017). In addition, some research such as that reported by Moosssavi and colleagues, have identified different milk biome "types" even within the same Canadian cohort, supporting the existence of intrapopulation variability (Moossavi et al., 2019). However, most of these studies have been conducted on a limited number of samples or population groups, and have employed non-standardized procedures which could have introduced important biases in the microbiota profiling (Panek et al., 2018). These facts have impeded our ability to make meaningful comparisons across studies and prevented our ability to understand genuine biological variation in the microbiota inherent in milk produced by healthy women across populations.

It is also worth remarking that, whereas some efforts to achieve standardization of sample processing and analysis in the context of the human GI microbiota have been reported as recently reviewed (Wu et al., 2019), such initiatives have not yet been tackled in the context of the human milk microbiota which, due to its intrinsic physiological and microbiological characteristics (Moossavi et al., 2019), might be strongly affected by variable collection and analytical processing.

We recently attempted to fill these knowledge gaps by reporting a large, cross-sectional study on the healthy human

milk microbiome across a cohort of over 400 healthy lactating women from selected geographically diverse populations living across three different continents, by using standardized sample collection and processing approaches (Lackey et al., 2019). As expected, we found substantial variation in the milk microbiome among cohorts. In the present work, we provide even more insight into the impact that variable sample processing and data analysis pipelines might have on the study and interpretation of the milk microbiota landscape, through extraction and sequencing the same set of milk samples using an amplicon approach targeting a different 16S rRNA variable region with greater sequencing depth, and performing a comparative analysis with the previously reported dataset.

MATERIALS AND METHODS

Design, Setting, and Sampling

The design of the cross-sectional, epidemiological, multi-cohort study has been described in detail (McGuire et al., 2017; Ruiz et al., 2017; Lackey et al., 2019; Lane et al., 2019). All study procedures were approved by the overarching Washington State University Institutional Review Board (#13264) and at each study location, and consent was obtained from each participating woman. Milk samples from 412 mothers were obtained from 11 different populations, including one cohort from Kenya (KE) (n=42), Ghana (GN) (n=40), Peru (PE) (n=43), Sweden (SW) (n=24), Spain (SP) (n=41), and two cohorts from Ethiopia (rural [ETR] [n=40]; and urban [ETU] [n=40], cohorts), two from Gambia (rural [GBR] [n=40] and urban [GBU] [n=40], cohorts), and two from the United States (San Diego, California [USC] [n=19] and Washington/Idaho [USW] [n=41] cohorts) (**Supplementary Figure 1**). Milk was collected as described previously (McGuire et al., 2017). Briefly, following skin cleaning twice with single use and using gloved hands, milk was manually expressed and collected into disposable sterile containers, with the exception of milk samples from USC, USW, SW and PE cohorts, which were pump-expressed by using an electric pump and sterile disposable containers. Milk from each woman was aliquoted into two samples for metataxonomic analysis; one was shipped on dry ice to the University of Idaho (USA), and the second was shipped on dry ice to the Complutense University of Madrid (Spain); in both locations the samples were immediately frozen at −20°C. Due to unreliable access to electricity supply and/or freezers, milk samples collected from the ETR cohort were immediately mixed at a 1:1 ratio with Milk Preservation Solution (Norgen Biotek, Ontario) and frozen within 6 days as it has previously been demonstrated capable to preserve bacterial DNA integrity for at least two weeks (Lackey et al., 2017). Whereas milk microbiome data garnered from methods utilized at the University of Idaho have been published (Lackey et al., 2019), here we report results from a companion analysis conducted in our laboratory in Spain. We also compare our results to those previously reported using different analytical and bioinformatic approaches.

DNA Extraction From Milk

For the DNA isolation from all sample cohorts, with the exception of ETR, approximately 1 mL of each sample was used for DNA extraction following the method described by Castro et al. (2019). Briefly, milk samples were thawed on ice and centrifuged (13,000 rpm, 10 min at 4°C), the lipid and supernatant layers were removed and the cell pellet was resuspended in 500 µl TE50 buffer. This solution was further processed for DNA isolation by performing an enzymatic lysis adding 100 µl of an enzymatic mix containing 5 mg/ml of lysozyme (Sigma-Aldrich), 1.5 KU/ml mutanolysin (Sigma-Aldrich) and 120 U/ml lysostaphyn and incubating the samples for 1 hour at 37°C. Subsequently samples were subjected to physical lysis by bead beating with FastPrep Fp120 (Thermo Scientific, Waltham, MA) and glass bead matrix tubes (3 cycles × 60 s, speed 6) in step 4. Finally, DNA was purified by using a modified version of the Qiamp DNA mini kit (Qiagen) columns whereby 100 µl of 3 M sodium acetate pH 5.5 were added to the lysate prior to its addition to the column. Extracted DNA was eluted in 22 µL of nuclease-free water and stored at -20 °C until further analysis. Purity and concentration of each extracted DNA was initially estimated using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, USA). Negative controls (blanks) were added during the extraction to account for possible contaminants introduced during sample manipulation and DNA isolation. In the particular case of ETR samples, only 250 µl of milk samples were used for DNA isolation since they were the only samples treated with a preservation solution and, under this circumstance, the manufacturer of the preservation solution and companion milk DNA isolation kit recommends not to use a volume larger than 250 µl (Milk DNA Preservation and Isolation Kit, NorgenBiotek, Throlod, Canada).

Sequencing of Microbial DNA and Bioinformatic Analysis

The V3-V4 hypervariable region of the 16S rRNA was amplified by PCR and sequenced as previously described (Klindworth et al., 2013). Briefly, universal primers S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-Bact-129 0785-a-A-21 (GACTACHVGGGTATCTAATCC) were used to amplify the V3-V4 hypervariable region of the 16S rRNA and then, barcodes were appended to 3' and 5' terminal ends of the PCR amplicons in a second PCR-reaction in order to allow separating forward and reverse sequences. The pooled, purified and barcoded DNA amplicons were sequenced using the Illumina MiSeq 2 x 300 bp paired-end protocol (Illumina Inc., San Diego, CA, USA) following the manufacturer's recommendations at the facilities of Parque Científico de Madrid (Tres Cantos, Spain) (Klindworth et al., 2013).

Raw sequence data were demultiplexed and quality filtered using Illumina MiSeq Reporter analysis software. Microbiome bioinformatics were performed with QIIME 2 2019.1 (Bolyen et al., 2019). Denoising was performed with DADA2 (Callahan et al., 2016). The forward reads were truncated at position 277 by trimming the last 15 nucleotides, while the reverse ones were

truncated at the 250 nucleotides by trimming the last 15 nucleotides, in order to discard positions for which nucleotide median quality were Q20 or below. Samples with less than 1000 sequences (n= 10) were excluded from further analysis.

Taxonomy was assigned to ASVs using the q2-feature-classifier (Bokulich et al., 2018) classify-sklearn naïve Bayes taxonomy classifier against the SILVA 138 reference database (Quast et al., 2013). Subsequent bioinformatic analysis was conducted using R version 3.5.1 (R Core Team, 2013; https://www.R-project.org). The decontam package version 1.2.1 (Davis et al., 2018) was used in order to identify, visualize and remove contaminating DNA with two negative control samples.

A set of "core" genera were characterized for each sample type both in the overall dataset and within each cohort. To be included in the core taxa, a genus must have been represented with a relative abundance higher than 0.1% in, at least, 90% of the samples from one or more cohorts. The 4 most abundant phyla from all the milk samples were selected as most abundant phyla, the rest were included in the "minor_phyla" group and the sequences whose phyla were unknown were grouped in the "unclassified_phyla" group. The 18 most abundant genera from all the milk samples were selected as most abundant genera, the rest were included in the "minor_genera" group and the sequences whose genera were unknown were grouped in the "unclassified_genera" group.

Comparison of the Results With Those Obtained With the Same Set of Samples but Using a Different Metataxonomic Approach

The results of a previous different metataxonomic approach with the same set of milk samples has been published (Lackey et al., 2019). Methods used for DNA extraction, amplification, assessment of DNA quality, sequencing and statistical analyses are detailed in that publication. Briefly, a dual-barcoded, two-step 30-cycle polymerase chain reaction (PCR) was conducted to amplify the V1-V3 hypervariable region of the 16S rRNA bacterial gene. For the first step, a 7-fold degenerate forward primer targeting position 27 and a reverse primer targeting position 534 (positions numbered according to the *Escherichia coli* rRNA gene) were used as described previously (Carrothers et al., 2015). For the second step, a unique barcoded primer pair with Illumina adaptors attached was added to each sample. Sequences were obtained using an Illumina MiSeq (San Diego, CA) v3 paired-end 300-bp protocol for 600 cycles at the University of Idaho IBEST Genomics Resources Core. However, due to the quality of the ends of the reverse reads, few reads were able to be merged and thus only the forward reads were used in the analyses.

A comparison of data obtained from both metataxonomic studies (V1-V3 reported by Lackey and colleagues (2019) versus V3-V4 studies conducted in this work) was made at genus and phylum levels using the SILVA 132 reference database since it was the one used in the first study. A schematic representation highlighting the main differences in the methodological and analytical strategies followed in the study reported by Lackey and colleagues, as compared to the sequencing approach conducted herein with the same dataset of samples is provided in **Figure 1**.

Statistical Analysis

Quantitative data were expressed as the median and interquartile range (IQR). Differences between groups were assessed using Kruskal-Wallis tests and pairwise Wilcoxon rank sum tests to calculate comparisons between groups. Bonferroni corrections were made to control for multiple comparisons. A table of amplicon sequence variants (ASVs) counts per sample was generated, and bacterial taxa abundances were normalized to the total number of sequences in each sample. Alpha diversity was studied with the Shannon and Simpson diversity indexes with the R vegan package (Version:2.5.6) (Oksanen et al., 2012). Principal coordinates analysis (PCoA) was used to evaluate beta diversity and to plot patterns of bacterial community diversity through a distance matrix containing a dissimilarity value for each pairwise sample comparison. Quantitative (relative abundance) and qualitative (presence/absence) analyses were performed with the Bray-Curtis index and binary Jaccard index, respectively. Analysis of variance of the distance matrices were performed with the "nonparametric MANOVA test" Adonis with 999 permutations as implemented in the R vegan package to reveal statistical significance. For multilevel pairwise Adonis comparisons, the method used for p-value correction was Holm–Bonferroni method with "pairwiseAdonis" R package (version 0.4) (Martínez, 2020). Heatmap hierarchical clustering was performed by using the Euclidean distance and complete hclust_method.

RESULTS

Metataxonomic Analysis Targeting the V3-V4 Hypervariable Region of the 16S rRNA Gene With the SILVA 138 Database

The 16S rRNA gene sequencing analysis of the milk samples conducted in this study (n = 392) (**Supplementary Figure 1**) yielded 17,622,545 high quality filtered sequences, ranging from 12,981 to 437,280 reads per sample [mean=44,956 reads per sample; median (IQR)=35,754 (26,438-50,539) sequences per sample].

The median values for the Shannon diversity index oscillated between 3.48 (ETR) and 2.44 (USC) while those for the Simpson diversity index ranged between 0.91 (ETR) and 0.93 (GBU) (**Supplementary Table 1**). Overall, there was a significant effect of cohort on both diversity indices ($p = 0.001$ and $p = 0.001$, respectively). A comparison at the continent level showed significant differences on both diversity indices when the African cohorts were compared ($p<0.002$ and $p<0.0002$, respectively) since samples collected in ETR and GBU exhibited a higher diversity than those obtained in KE. In contrast, no significant differences were found when the different European and US cohorts were compared ($p = 0.099$ and $p = 0.33$, respectively) (**Supplementary Table 1**).

The overall analysis of the beta diversity, calculated according to the relative abundance of ASVs (Bray-Curtis distance) and the presence/absence of ASVs sequences (binary Jaccard distance matrix), indicated that the profiles of bacterial genera of the different cohorts apparently clustered into different groups ($p < 0.001$ and $p < 0.001$, respectively; PERMANOVA) and the ETR cohort was observed to be more clearly separated from the other locations with both distance metrics (**Figures 2A, B**). Besides, when beta diversity of the samples was evaluated according to the continent where the different samples were collected from, the ordination based on relative abundance of ASVs (Bray-Curtis distance) revealed differences between the African and European samples ($p < 0.001$) and, also, between the African and American samples ($p < 0.001$). In contrast, differences between the European and American cohorts did not reach statistical significance ($p = 0.059$). In relation to the analysis of the beta diversity according to the presence/absence of ASVs (binary Jaccard distance matrix), all the comparisons (African *vs.* European samples, African *vs.* American samples, and European *vs.* American samples) revealed the existence of significant differences ($p < 0.001$). Among African countries all the cohorts showed significant differences for both the relative abundance and the presence/absence of ASVs ($p < 0.05$). In the European and American countries, only the pairwise comparisons between the SW and USC (for both the relative abundance and the presence/absence); and SW and USW (for relative abundance) showed no statistical differences. The *post-hoc* Holmes-Bonferroni correction of the multilevel pairwise Adonis comparisons increased the *p*-values of most of the previously significant pairwise comparisons from <0.05 to 0.05, with the exception of the comparison between GBU and GBR, which *p*-value changed from 0.04195804 to 0.17 and, therefore, it lost the statistical significance (**Supplementary Table 2**). It must be highlighted that the Holmes-Bonferroni correction involved a high number of comparisons (n=55), rendering it as a very exigent statistical test.

Comparison of the mean distances of samples to the centroids using PCoA plots based either on the Bray-Curtis dissimilarity index or on the Jaccard's coefficient of each cohort, also revealed the existence of significant differences ($p<0.001$) (**Figures 2C, D**). Notably, GN was the cohort displaying a higher beta-dispersion, in terms of relative abundance while ETU displayed the highest beta-dispersion in terms of presence/absence of ASVs, suggesting the existence of a greater intrapopulation heterogeneity of microbiota profiles among the samples from these two cohorts, as compared to other populations analyzed in this study; whereas ETR, SW and USC displayed the smallest distances to centroids in terms of relative abundance data, revealing these cohorts present the most homogeneous microbiota profiles.

A total of 46 phyla were identified in the milk samples, with Firmicutes, Proteobacteria, Actinobacteriota (formerly Actinobacteria) and Patescibacteria being the most abundant. There was a significant effect of cohort on Firmicutes, Proteobacteria, Actinobacteriota, Patescibacteria and the group of "unclassified_phyla" (**Table 1**). Again, the ETR cohort showed greater differences with respect to the rest of the cohorts since it exhibited the lowest relative abundance of Firmicutes and the highest concerning Proteobacteria, Actinobacteriota and Patescibacteria (**Table 1**). An initial assessment of potentially dominant patterns in the bacteriological profile of the milk samples is shown in the heatmap plot presented in **Figure 3**. Overall, there was no clear separation between the milk samples from women of the different cohorts; however, a clear separation could be observed on the basis of the relative abundance of sequences belonging to the genera *Staphylococcus* and *Streptococcus* (**Figure 3**), which seems to be independent of the continent or the cohort. In addition, the clustering analysis suggested that ETR samples separation could be driven by the genera *Rhizobium*, *Achromobacter*, *Corynebacterium* and *Stenotrophomonas* (**Figure 3**). A boxplot of the 9 most abundant genera (including the group of the unclassified ones) found in each location is shown in **Supplementary Figure 2**.

There was an effect of continent on the phyla Proteobacteria and Patescibacteria. More specifically, the abundance of

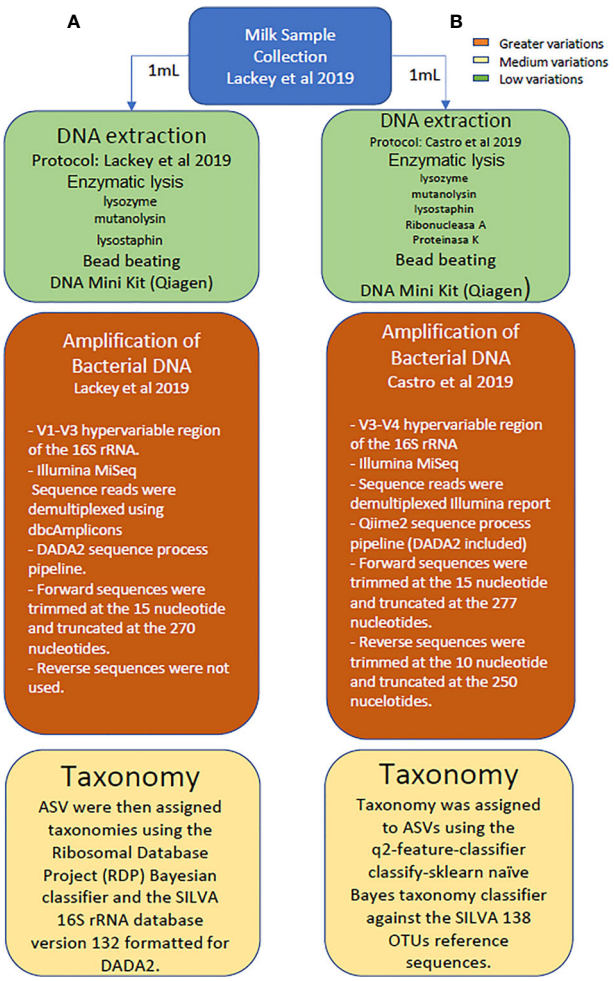

FIGURE 1 | Schematic representation highlighting the main differences in the methodological and analytical strategies followed in the study reported by Lackey and colleagues, as compared to the sequencing approach conducted herein with the same dataset of samples.

TABLE 1 | Relative frequencies, medians and interquartile range (IQR) of the most abundant bacterial phyla (bold) and genera detected in the milk samples analyzed in this work.

Phylum/Genus	ETR		ETU		GBR		GBU		GN		KE		PE		SP		SW		USC		USW		p-value†
	n (%)#	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	
Firmicutes	32 (100%)	34.13 (21.56-51.3)	40 (100%)	64.65 (36.57-82.26)	40 (100%)	63.34 (43.84-75.13)	39 (97.5%)	53.67 (44.13-62.98)	40 (100%)	37.29 (24.44-71.19)	42 (100%)	69.21 (48.17-74.24)	38 (100%)	73.74 (53.5-85.68)	40 (100%)	52.34 (41.38-80.31)	20 (100%)	63.1 (52.14-80.37)	19 (100%)	72.95 (18.73-83.59)	41 (100%)	60.58 (48.72-70.05)	<0.001
Staphylococcus	32 (100%)	9.67 (5.42-16.39)	40 (100%)	29.92 (8.78-48.42)	39 (97.5%)	14.81 (6.96-35.69)	40 (100%)	22.98 (16.06-34.3)	39 (97.5%)	9.59 (3.86-24.52)	42 (100%)	28.49 (13.47-52.45)	36 (94.74%)	18.12 (8.19-31.03)	40 (100%)	14.78 (7.51-33.91)	20 (100%)	26.32 (20.33-49.74)	19 (100%)	10.49 (4.69-51.83)	41 (100%)	25.26 (12.76-45.55)	<0.001
Streptococcus	30 (93.75%)	7.3 (3.28-13.29)	35 (87.5%)	5.91 (0.15-16.78)	39 (97.5%)	9.33 (3.59-32.28)	37 (92.5%)	10.25 (4.75-21.88)	36 (90%)	2.49 (0.08-8.88)	40 (95.24%)	15.43 (5.38-29.94)	38 (100%)	37.8 (22.74-57.26)	40 (100%)	12.55 (5.39-52.6)	20 (100%)	11.97 (7.84-28.44)	19 (100%)	11.38 (1.82-31.85)	41 (100%)	15.57 (6.59-28.15)	<0.001
Lactobacillus	26 (81.25%)	1.03 (0.25-2.93)	24 (60%)	0.13 (<0.01-0.51)	31 (77.5%)	1.57 (0.06-3.68)	28 (70%)	1.7 (<0.01-5.58)	30 (75%)	1.24 (0.01-5.01)	28 (66.67%)	0.79 (<0.01-2.77)	23 (60.53%)	0.08 (<0.01-1.01)	18 (45%)	<0.01 (<0.01-0.41)	17 (85%)	0.3 (0.05-1.49)	11 (57.89%)	0.02 (<0.01-0.25)	31 (75.61%)	0.44 (0.01-1.28)	<0.001
Veillonella	24 (75%)	0.75 (0.04-4.11)	18 (45%)	<0.01 (<0.01-0.28)	24 (60%)	0.3 (<0.01-1.52)	18 (45%)	<0.01 (<0.01-2.31)	12 (30%)	<0.01 (<0.01-0.13)	18 (42.86%)	<0.01 (<0.01-1.18)	27 (71.05%)	1.43 (<0.01-4.08)	18 (45%)	<0.01 (<0.01-0.59)	14 (70%)	0.15 (<0.01-0.72)	12 (63.16%)	0.06 (<0.01-1.54)	22 (53.66%)	0.01 (<0.01-0.6)	<0.001
Anaerococcus	23 (71.88%)	1.21 (<0.01-3.15)	21 (52.5%)	0.05 (<0.01-0.38)	24 (60%)	0.14 (<0.01-1.21)	20 (50%)	0.03 (<0.01-1.38)	24 (60%)	0.2 (<0.01-0.53)	18 (42.86%)	<0.01 (<0.01-0.92)	26 (68.42%)	0.3 (<0.01-0.95)	18 (45%)	<0.01 (<0.01-0.78)	13 (65%)	0.1 (<0.01-0.56)	12 (63.16%)	0.01 (<0.01-0.22)	30 (73.17%)	0.15 (<0.01-1.06)	0.062
Gemella	18 (56.25%)	0.07 (<0.01-0.31)	14 (35%)	<0.01 (<0.01-0.06)	15 (37.5%)	<0.01 (<0.01-0.22)	15 (37.5%)	<0.01 (<0.01-0.61)	13 (32.5%)	<0.01 (<0.01-0.07)	13 (30.95%)	<0.01 (<0.01-0.1)	24 (63.16%)	0.26 (<0.01-1.22)	18 (45%)	<0.01 (<0.01-0.42)	15 (75%)	0.69 (<0.01-1.55)	12 (63.16%)	0.13 (<0.01-0.9)	28 (68.29%)	0.15 (<0.01-0.68)	<0.001
Proteobacteria	32 (100%)	33.57 (27.59-51.89)	40 (100%)	8.49 (3.53-16.01)	40 (100%)	14.35 (10.5-26.58)	40 (100%)	17.66 (11.49-26.26)	40 (100%)	29.81 (8.48-47.97)	42 (100%)	15.25 (8.85-24.36)	38 (100%)	10.62 (3.99-22.95)	40 (100%)	11.5 (4.71-20.89)	20 (100%)	6.74 (2.67-21.41)	19 (100%)	6.9 (2.79-62.38)	41 (100%)	8.25 (3.31-14.05)	<0.001
Rhodanobacter	9 (28.12%)	<0.01 (<0.01-0.05)	35 (87.5%)	0.47 (0.06-2.2)	37 (92.5%)	3.38 (1.08-6.81)	31 (77.5%)	3.04 (0.41-5.52)	32 (80%)	0.77 (0.11-6.6)	31 (73.81%)	2.63 (0.28-6.21)	35 (92.11%)	2.55 (0.71-4.43)	27 (67.5%)	0.34 (<0.01-1.49)	11 (55%)	<0.01 (<0.01-0.2)	7 (36.84%)	<0.01 (<0.01-0.05)	30 (73.17%)	0.32 (<0.01-1.66)	<0.001
Rhizobium	31 (96.88%)	18.28 (11.16-29.48)	5 (12.5%)	<0.01 (<0.01-<0.01)	4 (10%)	<0.01 (<0.01-<0.01)	2 (5%)	<0.01 (<0.01-<0.01)	10 (25%)	<0.01 (<0.01-0.01)	3 (7.14%)	<0.01 (<0.01-<0.01)	6 (15.79%)	<0.01 (<0.01-<0.01)	4 (10%)	<0.01 (<0.01-<0.01)	2 (10%)	<0.01 (<0.01-<0.01)	7 (36.84%)	<0.01 (<0.01-0.05)	2 (4.88%)	<0.01 (<0.01-<0.01)	<0.001
Stenotrophomonas	22 (68.75%)	0.11 (<0.01-0.31)	25 (62.5%)	0.1 (<0.01-0.54)	28 (70%)	0.68 (<0.01-1.89)	24 (60%)	0.45 (<0.01-2.56)	29 (72.5%)	0.27 (<0.01-0.86)	29 (69.05%)	0.4 (<0.01-2.75)	25 (65.79%)	0.08 (<0.01-0.81)	25 (62.5%)	0.31 (<0.01-2.36)	15 (75%)	0.12 (<0.01-0.63)	12 (63.16%)	0.04 (<0.01-0.41)	29 (70.73%)	0.08 (<0.01-0.84)	0.4
Acinetobacter	29 (90.62%)	0.95 (0.55-2.91)	25 (62.5%)	0.17 (<0.01-1.12)	30 (75%)	1.31 (0.03-2.39)	27 (67.5%)	0.63 (<0.01-2.48)	29 (72.5%)	0.26 (<0.01-1.25)	21 (50%)	0.03 (<0.01-1.7)	28 (73.68%)	0.82 (0.02-3.94)	24 (60%)	0.14 (<0.01-0.99)	14 (70%)	0.17 (<0.01-0.7)	11 (57.89%)	0.08 (<0.01-1.08)	32 (78.05%)	0.26 (0.01-0.8)	0.004
Klebsiella	1 (3.12%)	<0.01 (<0.01-<0.01)	13 (32.5%)	<0.01 (<0.01-0.05)	5 (12.5%)	<0.01 (<0.01-<0.01)	6 (15%)	<0.01 (<0.01-<0.01)	25 (62.5%)	0.64 (<0.01-7.67)	15 (35.71%)	<0.01 (<0.01-0.16)	7 (18.42%)	<0.01 (<0.01-<0.01)	2 (5%)	<0.01 (<0.01-<0.01)	1 (5%)	<0.01 (<0.01-<0.01)	3 (15.79%)	<0.01 (<0.01-<0.01)	1 (2.44%)	<0.01 (<0.01-<0.01)	<0.001
Achromobacter	18 (56.25%)	0.62 (<0.01-14.63)	24 (60%)	0.04 (<0.01-0.24)	29 (72.5%)	0.3 (<0.01-1.06)	22 (55%)	0.08 (<0.01-1.22)	22 (55%)	0.02 (<0.01-0.31)	28 (66.67%)	0.13 (<0.01-0.74)	22 (57.89%)	0.07 (<0.01-0.98)	35 (87.5%)	0.45 (0.1-1.39)	13 (65%)	0.09 (<0.01-0.55)	14 (73.68%)	0.08 (<0.01-0.26)	25 (60.98%)	0.07 (<0.01-0.27)	0.005
Pseudomonas	15 (46.88%)	<0.01 (<0.01-0.17)	23 (57.5%)	0.09 (<0.01-0.41)	18 (45%)	<0.01 (<0.01-0.72)	23 (57.5%)	0.18 (<0.01-0.68)	26 (65%)	0.08 (<0.01-0.31)	14 (33.33%)	<0.01 (<0.01-0.09)	24 (63.16%)	0.07 (<0.01-0.43)	29 (72.5%)	0.21 (<0.01-0.43)	14 (70%)	0.32 (<0.01-1.04)	15 (78.95%)	0.27 (0.05-1.77)	31 (75.61%)	0.18 (<0.01-0.55)	0.005
Actinobacteriota	32 (100%)	16.77 (11.82-28.57)	39 (97.5%)	7.28 (0.58-17.16)	40 (100%)	10.79 (7.5-16.33)	40 (100%)	12.81 (6.21-19.35)	35 (87.5%)	9.37 (1.31-15)	42 (100%)	7.75 (4.95-13.1)	38 (100%)	8.07 (5.04-12.45)	40 (100%)	8.46 (5.72-13.89)	20 (100%)	8.72 (5.78-12.69)	19 (100%)	3.62 (1.45-7.7)	41 (100%)	12.5 (9.21-19.75)	<0.001
Corynebacterium	30 (93.75%)	5.04 (2.76-13.33)	28 (70%)	0.63 (<0.01-2.34)	32 (80%)	1.02 (0.31-3.44)	33 (82.5%)	1.12 (0.44-2.49)	30 (75%)	0.78 (0.02-1.49)	35 (83.33%)	2.05 (0.4-3.23)	34 (89.47%)	1.94 (0.55-3.12)	32 (80%)	0.74 (0.09-2.76)	20 (100%)	0.82 (0.44-2.05)	15 (78.95%)	0.3 (0.05-2.53)	37 (90.24%)	1.81 (0.5-4.07)	<0.001
Cutibacterium	28 (87.5%)	0.24 (0.11-0.51)	35 (87.5%)	0.36 (0.07-0.73)	37 (92.5%)	1.37 (0.42-2.11)	39 (97.5%)	1.24 (0.54-2.3)	29 (72.5%)	0.57 (<0.01-1.47)	32 (76.19%)	0.37 (0.03-1.24)	36 (94.74%)	0.67 (0.19-1.97)	36 (90%)	0.7 (0.16-2.03)	19 (95%)	1.87 (0.76-3.76)	18 (94.74%)	0.22 (0.08-1.26)	41 (100%)	2.74 (1.14-4.77)	<0.001
Rothia	17 (53.12%)	0.04 (<0.01-0.22)	25 (62.5%)	0.17 (<0.01-1.15)	19 (47.5%)	<0.01 (<0.01-0.6)	23 (57.5%)	0.22 (<0.01-1.24)	16 (40%)	<0.01 (<0.01-0.26)	23 (54.76%)	0.3 (<0.01-1.36)	28 (73.68%)	0.82 (<0.01-2.41)	24 (60%)	0.54 (<0.01-2.57)	13 (65%)	0.21 (<0.01-1.27)	10 (52.63%)	0.06 (<0.01-2.05)	27 (65.85%)	0.26 (<0.01-3.53)	0.01
Bifidobacterium	30 (93.75%)	1.62 (0.87-2.77)	29 (72.5%)	0.36 (<0.01-1.6)	28 (70%)	0.32 (<0.01-0.95)	25 (62.5%)	0.41 (<0.01-1.44)	29 (72.5%)	0.71 (<0.01-1.69)	30 (71.43%)	0.52 (<0.01-1.22)	25 (65.79%)	0.15 (<0.01-0.49)	29 (72.5%)	0.42 (<0.01-1.36)	15 (75%)	0.54 (0.03-1.36)	12 (63.16%)	0.1 (<0.01-0.42)	29 (70.73%)	0.13 (<0.01-0.37)	<0.001
Kocuria	26 (81.25%)	1.28 (0.42-3)	21 (52.5%)	0.03 (<0.01-0.82)	28 (70%)	0.57 (<0.01-1.86)	28 (70%)	0.68 (<0.01-2.24)	22 (55%)	0.09 (<0.01-0.68)	22 (52.38%)	0.25 (<0.01-0.95)	16 (42.11%)	<0.01 (<0.01-0.2)	14 (35%)	<0.01 (<0.01-0.5)	6 (30%)	<0.01 (<0.01-0.36)	2 (10.53%)	<0.01 (<0.01-<0.01)	12 (29.27%)	<0.01 (<0.01-0.14)	<0.001
Patescibacteria	16 (50%)	0.02 (<0.01-0.22)	28 (70%)	0.75 (<0.01-5.89)	28 (70%)	0.68 (<0.01-1.63)	40 (100%)	1.37 (<0.01-4.09)	29 (72.5%)	1.35 (<0.01-5.47)	28 (66.67%)	0.25 (<0.01-1.14)	29 (76.32%)	0.55 (0.06-1.57)	38 (95%)	3.21 (1.17-7.57)	19 (95%)	1.97 (0.24-4.31)	14 (73.68%)	0.14 (0.01-1.38)	36 (87.8%)	1.26 (0.37-2.71)	<0.001
Minor_phyla	31 (96.88%)	2.49 (1.55-4.07)	33 (82.5%)	1.53 (0.03-6.13)	39 (97.5%)	4.58 (1.89-6.81)	29 (72.5%)	4.84 (1.86-7.85)	32 (80%)	1.31 (0.03-4.11)	36 (85.71%)	1.85 (0.64-4.91)	38 (100%)	1.9 (0.59-4.78)	39 (97.5%)	4.99 (0.93-6.7)	19 (95%)	1.99 (0.28-4.84)	18 (94.74%)	1.41 (0.26-5.94)	41 (100%)	2.65 (1.2-5.84)	0.003
Minor_genera	32 (100%)	16.89 (11.62-23.67)	36 (90%)	9.93 (2.86-19.82)	40 (100%)	17.28 (9.17-31.39)	40 (100%)	21.36 (12.66-28.95)	38 (95%)	15.51 (3.54-23.46)	42 (100%)	12.57 (8.07-19.24)	38 (100%)	9.16 (3.71-17.33)	40 (100%)	14.46 (5.47-21.38)	20 (100%)	11.31 (3.68-21.06)	18 (94.74%)	8.28 (2.9-15.12)	41 (100%)	13.83 (7.79-22.19)	0.001
Unclassified_phyla	18 (56.25%)	0.01 (<0.01-0.12)	31 (77.5%)	0.39 (0.01-2.01)	40 (100%)	1.46 (0.76-3.29)	40 (100%)	2.09 (0.65-6.13)	32 (80%)	0.54 (0.03-3.65)	42 (100%)	3.04 (1.08-5.12)	38 (100%)	1 (0.4-3.34)	40 (100%)	2.2 (0.69-8.7)	20 (100%)	4.74 (2.56-9.72)	18 (94.74%)	1.21 (0.12-3.77)	41 (100%)	8.44 (4.3-14.33)	<0.001
Unclassified_genera	32 (100%)	3.11 (1.58-4.39)	38 (95%)	5.26 (1.11-16.07)	40 (100%)	8.33 (5.19-11.97)	40 (100%)	10.16 (7.82-14.23)	35 (87.5%)	13.27 (0.29-24.43)	42 (100%)	5.61 (2.98-10.7)	38 (100%)	3.88 (1.52-9.35)	40 (100%)	12.89 (3.42-23.39)	20 (100%)	11.72 (4.08-21.29)	19 (100%)	6.3 (2.52-11.63)	41 (100%)	12.18 (6.07-19.29)	<0.001

#*n (%): number of samples in which the phylum/genus was detected (relative frequency of detection).*

†*Kruskal-Wallis rank tests with Bonferroni correction.*

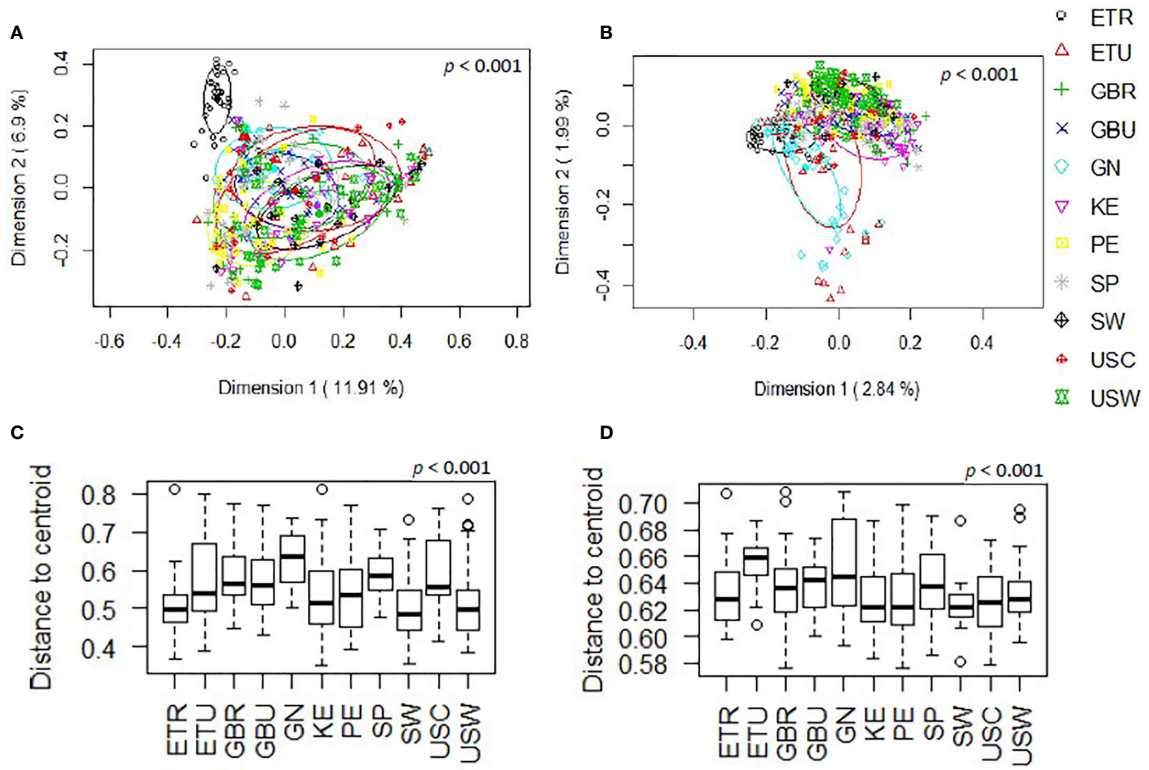

FIGURE 2 | Comparison, at the genus level, of the beta diversity of the different cohorts included in this work. **(A)** Principal coordinate analysis (PCoA) plots of bacterial profiles based on the Bray-Curtis similarity analysis (relative abundance). **(B)** Principal coordinate analysis (PCoA) plots of bacterial profiles at the genus level based on the Jaccard's coefficient for binary data (presence or absence). The values on each axis label in graphs **(A, B)** represent the percentage of the total variance explained by that axis. The differences between groups of milk samples were analyzed using the PERMANOVA test with 999 permutations. **(C)** Comparison of the mean distances of samples to the centroids in the PCoA plots based on the Bray-Curtis dissimilarity index of each group. **(D)** Comparison of the mean distances of samples to the centroids in the PCoA plots based on the Jaccard's coefficient of each group.

Proteobacteria was higher in African cohorts than the other cohorts ($p < 0.001$) while that of Patescibacteria was higher in the European samples than in those from the two other continents ($p < 0.001$). At the genus level, American and European cohorts seemed to be more similar to each other than to the African ones (**Table 2**). African cohorts were characterized by a lower *Streptococcus* abundance ($p < 0.001$) and a higher *Lactobacillus* abundance ($p < 0.001$) (**Tables 2** and **3**). The relative abundances of the genera *Rhizobium* and *Corynebacterium* in ETR samples was statistically higher than in any other cohort ($p < 0.001$) while the contrary was observed for the genus *Rhodanobacter* ($p < 0.001$) (**Tables 1** and **3**). When European and American cohorts were compared, the relative abundance of the phylum Actinobacteriota was found to be significantly higher in USW cohort than in PE, SP and USC cohorts ($p < 0.001$), while the relative abundance of the phylum Patescibacteria was higher in SP cohort than in the PE, USW and USC cohorts ($p < 0.001$) (**Table 4**). At the genus level, the relative abundance of the genus *Streptococcus* was significantly higher in the samples from the PE cohort than in those from the USC, SW and USW cohorts ($p < 0.001$) (**Table 4**).

Comparison of the Results Obtained With the Two Strategies (Sequencing of V1-V3 Versus V3-V4 Region) With the Same Database (SILVA 132)

Overall, the comparison of the results obtained by Lackey et al. (2019) targeting the V1-V3 region and those obtained in this work, targeting the V3-V4 region, showed notable differences among the most abundant phyla and genera. However, some of these differences were the result of different nomenclatures used by SILVA 132 (used in Lackey et al., 2019) and SILVA 138 (used in the present study) database versions, as is the case of Actinobacteriota and Actinobacteria; or the genera *Propionibacterium* and *Cutibacterium*, in which the pipeline considers them as different microorganisms.

To avoid this bias and facilitate comparison among both studies, we re-analyzed our sequences on the V3-V4 regions with the same database used by Lackey and colleagues (SILVA 132). For this comparison reads that could not be classified to the genus level were included. Finally, a total of 26,461,984 high quality reads were used to perform this comparison (**Supplementary Table 3**).

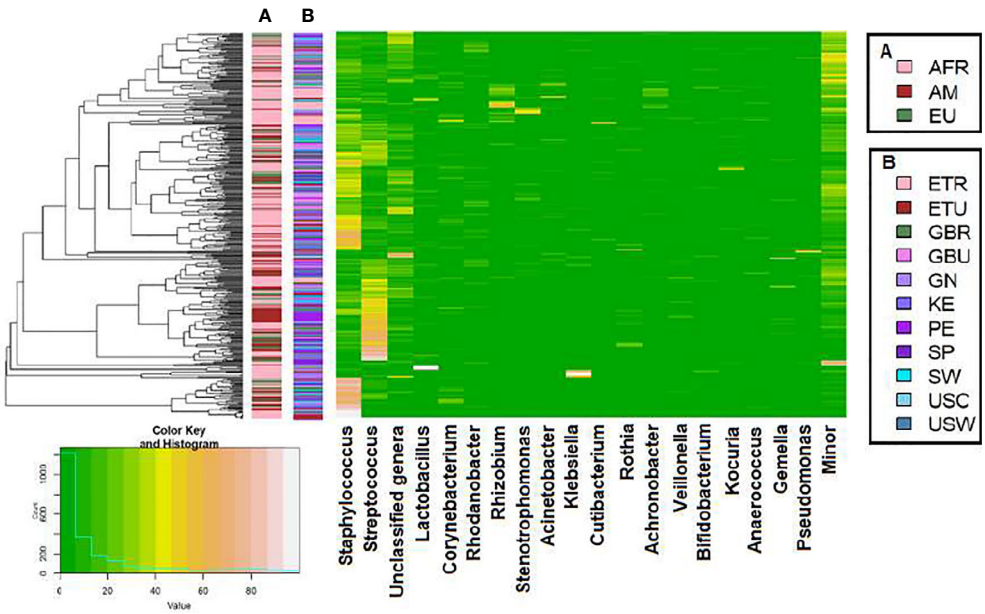

FIGURE 3 | Heatmap plot representing the hierarchical clustering, at the genus level, of the milk samples by continent cohorts **(A)** and by location cohorts **(B)** analyzed in this work with the SILVA 138 database.

TABLE 2 | Relative frequencies, medians and interquartile range (IQR) of the most abundant bacterial phyla (bold) and genera detected in milk samples from women in different continents.

Phylum/Genus	AFR		AM		EU		p- value[†]
	n (%)[#]	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	
Firmicutes	234 (100%)	52.26 (33.5-72.45)	98 (100%)	67.56 (49.7-81.27)	60 (100%)	59.42 (42.99-80.31)	<0.001
Staphylococcus	232 (99.15%)	17.89 (7.1-36.2)	96 (97.96%)	20.8 (9.97-35.87)	60 (100%)	22.68 (9.36-38.95)	0.450
Streptococcus	217 (92.74%)	8.35 (2.25-19.86)	98 (100%)	21.72 (7.64-43.59)	60 (100%)	12.55 (6.49-35.52)	<0.001
Lactobacillus	167 (71.37%)	0.79 (<0.01-3.25)	65 (66.33%)	0.18 (<0.01-0.79)	35 (58.33%)	0.07 (<0.01-0.6)	<0.001
Veillonella	114 (48.72%)	<0.01 (<0.01-1.19)	61 (62.24%)	0.16 (<0.01-1.87)	32 (53.33%)	0.02 (<0.01-0.64)	0.089
Anaerococcus	130 (55.56%)	0.13 (<0.01-1.17)	68 (69.39%)	0.16 (<0.01-0.89)	31 (51.67%)	0.02 (<0.01-0.69)	0.310
Gemella	88 (37.61%)	<0.01 (<0.01-0.2)	64 (65.31%)	0.17 (<0.01-0.93)	33 (55%)	0.03 (<0.01-0.89)	<0.001
Proteobacteria	234 (100%)	16.82 (8.65-32.95)	98 (100%)	8.36 (3.32-19.19)	60 (100%)	9.93 (3.74-21.04)	<0.001
Rhodanobacter	175 (74.79%)	1.27 (<0.01-5.11)	72 (73.47%)	0.5 (<0.01-2.42)	38 (63.33%)	0.19 (<0.01-1.32)	0.001
Rhizobium	55 (23.5%)	<0.01 (<0.01-<0.01)	15 (15.31%)	<0.01 (<0.01-<0.01)	6 (10%)	<0.01 (<0.01-<0.01)	0.012
Stenotrophomonas	157 (67.09%)	0.25 (<0.01-1.21)	66 (67.35%)	0.07 (<0.01-0.81)	40 (66.67%)	0.15 (<0.01-1.81)	0.310
Acinetobacter	161 (68.8%)	0.51 (<0.01-2.09)	71 (72.45%)	0.3 (<0.01-1.98)	38 (63.33%)	0.15 (<0.01-0.99)	0.170
Klebsiella	65 (27.78%)	<0.01 (<0.01-0.05)	11 (11.22%)	<0.01 (<0.01-<0.01)	3 (5%)	<0.01 (<0.01-<0.01)	<0.001
Achromobacter	143 (61.11%)	0.07 (<0.01-0.85)	61 (62.24%)	0.07 (<0.01-0.31)	48 (80%)	0.31 (0.03-1.3)	0.017
Pseudomonas	119 (50.85%)	0.02 (<0.01-0.4)	70 (71.43%)	0.13 (<0.01-0.56)	43 (71.67%)	0.23 (<0.01-0.78)	0.002
Actinobacteriota	228 (97%)	10.5 (5.21-17.74)	98 (100%)	9.21 (5.04-14.75)	60 (100%)	8.46 (5.78-13.89)	0.370
Corynebacterium	188 (80.34%)	1.29 (0.21-3.58)	86 (87.76%)	1.69 (0.32-3.46)	52 (86.67%)	0.82 (0.25-2.76)	0.270
Cutibacterium	200 (85.47%)	0.56 (0.12-1.61)	95 (96.94%)	1.23 (0.22-2.75)	55 (91.67%)	0.87 (0.25-2.7)	<0.001
Rothia	123 (52.56%)	0.08 (<0.01-0.79)	65 (66.33%)	0.41 (<0.01-2.56)	37 (61.67%)	0.37 (<0.01-2.34)	<0.001
Bifidobacterium	171 (73.08%)	0.59 (<0.01-1.61)	66 (67.35%)	0.13 (<0.01-0.44)	44 (73.33%)	0.47 (<0.01-1.36)	<0.001
Kocuria	147 (62.82%)	0.46 (<0.01-1.5)	30 (30.61%)	<0.01 (<0.01-0.14)	20 (33.33%)	<0.01 (<0.01-0.5)	<0.001
Patescibacteria	158 (68%)	0.51 (<0.01-2.38)	79 (81%)	0.73 (0.07-2.07)	57 (95%)	2.44 (0.69-5.87)	<0.001
Minor_phyla	211 (90%)	2.67 (0.8-6.08)	97 (99%)	2.21 (0.77-5.54)	58 (97%)	2.8 (0.59-6.36)	0.680
Minor_genera	228 (97.44%)	15.08 (8.34-25.41)	97 (98.98%)	10.65 (4.73-18.84)	60 (100%)	12.2 (5.18-21.38)	0.025
Unclassified_phyla	203 (87%)	1.03 (0.12-3.61)	97 (99%)	3.22 (0.59-8.42)	60 (100%)	3.66 (0.76-8.7)	<0.001
Unclassified_genera	227 (97.01%)	7.38 (2.88-13.82)	98 (100%)	8.26 (3.11-13.89)	60 (100%)	12.82 (3.96-22.24)	0.030

[#]*n (%): number of samples in which the phylum/genus was detected (relative frequency of detection).*
[†]*Kruskal-Wallis rank tests with Bonferroni correction.*

TABLE 3 | Relative frequencies, medians and interquartile range (IQR) of the most abundant bacterial phyla (bold) and genera detected in milk samples from African cohorts.

Phylum/Genus	ETR		ETU		GBR		GBU		GN		KE		p-value†
	n (%)#	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	
Firmicutes	32 (100%)	34.13 (21.56-51.3)	40 (100%)	64.65 (36.57-82.26)	40 (100%)	63.34 (43.84-75.13)	39 (97.5%)	53.67 (44.13-62.98)	40 (100%)	37.29 (24.44-71.19)	42 (100%)	69.21 (48.17-74.24)	<0.001
Staphylococcus	32 (100%)	9.67 (5.42-16.39)	40 (100%)	29.92 (8.78-48.42)	39 (97.5%)	14.81 (6.96-35.69)	40 (100%)	22.98 (16.06-34.3)	39 (97.5%)	9.59 (3.86-24.52)	42 (100%)	28.49 (13.47-52.45)	<0.001
Streptococcus	30 (93.75%)	7.3 (3.28-13.29)	35 (87.5%)	5.91 (0.15-16.78)	39 (97.5%)	9.33 (3.59-32.28)	37 (92.5%)	10.25 (4.75-21.88)	36 (90%)	2.49 (0.08-8.88)	40 (95.24%)	15.43 (5.38-29.94)	<0.001
Lactobacillus	26 (81.25%)	1.03 (0.25-2.93)	24 (60%)	0.13 (<0.01-0.51)	31 (77.5%)	1.57 (0.06-3.68)	28 (70%)	1.7 (<0.01-5.58)	30 (75%)	1.24 (0.01-5.01)	28 (66.67%)	0.79 (<0.01-2.77)	0.003
Veillonella	24 (75%)	0.75 (0.04-4.11)	18 (45%)	<0.01 (<0.01-0.28)	24 (60%)	0.3 (<0.01-1.52)	18 (45%)	<0.01 (<0.01-2.31)	12 (30%)	<0.01 (<0.01-0.13)	18 (42.86%)	<0.01 (<0.01-1.18)	<0.001
Anaerococcus	23 (71.88%)	1.21 (<0.01-3.15)	21 (52.5%)	0.05 (<0.01-0.38)	24 (60%)	0.14 (<0.01-1.21)	20 (50%)	0.03 (<0.01-1.38)	24 (60%)	0.2 (<0.01-0.53)	18 (42.86%)	<0.01 (<0.01-0.92)	0.025
Gemella	18 (56.25%)	0.07 (<0.01-0.31)	14 (35%)	<0.01 (<0.01-0.06)	15 (37.5%)	<0.01 (<0.01-0.22)	15 (37.5%)	<0.01 (<0.01-0.61)	13 (32.5%)	<0.01 (<0.01-0.07)	13 (30.95%)	<0.01 (<0.01-0.1)	0.010
Proteobacteria	32 (100%)	33.57 (27.59-51.89)	40 (100%)	8.49 (3.53-16.01)	40 (100%)	14.35 (10.5-26.58)	40 (100%)	17.66 (11.49-26.26)	40 (100%)	29.81 (8.48-47.97)	42 (100%)	15.25 (8.85-24.36)	<0.001
Rhodanobacter	9 (28.12%)	<0.01 (<0.01-0.05)	35 (87.5%)	0.47 (0.06-2.2)	37 (92.5%)	3.38 (1.08-6.81)	31 (77.5%)	3.04 (0.41-5.52)	32 (80%)	0.77 (0.11-6.6)	31 (73.81%)	2.63 (0.28-6.21)	<0.001
Rhizobium	31 (96.88%)	18.28 (11.16-29.48)	5 (12.5%)	<0.01 (<0.01-<0.01)	4 (10%)	<0.01 (<0.01-<0.01)	2 (5%)	<0.01 (<0.01-<0.01)	10 (25%)	<0.01 (<0.01-0.01)	3 (7.14%)	<0.01 (<0.01-<0.01)	<0.001
Stenotrophomonas	22 (68.75%)	0.11 (<0.01-0.31)	25 (62.5%)	0.1 (<0.01-0.54)	28 (70%)	0.68 (<0.01-1.89)	24 (60%)	0.45 (<0.01-2.56)	29 (72.5%)	0.27 (<0.01-0.86)	29 (69.05%)	0.4 (<0.01-2.75)	<0.001
Acinetobacter	29 (90.62%)	0.95 (0.55-2.91)	25 (62.5%)	0.17 (<0.01-1.12)	30 (75%)	1.31 (0.03-2.39)	27 (67.5%)	0.63 (<0.01-2.48)	29 (72.5%)	0.26 (<0.01-1.25)	21 (50%)	0.03 (<0.01-1.7)	<0.001
Klebsiella	1 (3.12%)	<0.01 (<0.01-<0.01)	13 (32.5%)	<0.01 (<0.01-0.05)	5 (12.5%)	<0.01 (<0.01-<0.01)	6 (15%)	<0.01 (<0.01-<0.01)	25 (62.5%)	0.64 (<0.01-7.67)	15 (35.71%)	<0.01 (<0.01-0.16)	<0.001
Achromobacter	18 (56.25%)	0.62 (<0.01-14.63)	24 (60%)	0.04 (<0.01-0.24)	29 (72.5%)	0.3 (<0.01-1.06)	22 (55%)	0.08 (<0.01-1.22)	22 (55%)	0.02 (<0.01-0.31)	28 (66.67%)	0.13 (<0.01-0.74)	<0.001
Delftia	23(71.88%)	0.37 (<0.01-2.37)	22(55.00%)	0.01 (<0.01-0.15)	22(55.00%)	0.28 (<0.01-1.18)	21(52.50%)	0.02 (<0.01-1.06)	23(57.50%)	0.01 (<0.01-0.39)	33(78.57%)	0.43 (0.07-1.31)	0.005
Actinobacteriota	32 (100%)	16.77 (11.82-28.57)	39 (97.5%)	7.28 (0.58-17.16)	40 (100%)	10.79 (7.5-16.33)	40 (100%)	12.81 (6.21-19.35)	35 (87.5%)	9.37 (1.31-15)	42 (100%)	7.75 (4.95-13.1)	<0.001
Corynebacterium	30 (93.75%)	5.04 (2.76-13.33)	28 (70%)	0.63 (<0.01-2.34)	32 (80%)	1.02 (0.31-3.44)	33 (82.5%)	1.12 (0.44-2.49)	30 (75%)	0.78 (0.02-1.49)	35 (83.33%)	2.05 (0.4-3.23)	<0.001
Cutibacterium	28 (87.5%)	0.24 (0.11-0.51)	35 (87.5%)	0.36 (0.07-0.73)	37 (92.5%)	1.37 (0.42-2.11)	39 (97.5%)	1.24 (0.54-2.3)	29 (72.5%)	0.57 (<0.01-1.47)	32 (76.19%)	0.37 (0.03-1.24)	<0.001
Rothia	17 (53.12%)	0.04 (<0.01-0.22)	25 (62.5%)	0.17 (<0.01-1.15)	19 (47.5%)	<0.01 (<0.01-0.6)	23 (57.5%)	0.22 (<0.01-1.24)	16 (40%)	<0.01 (<0.01-0.26)	23 (54.76%)	0.3 (<0.01-1.36)	0.18
Bifidobacterium	30 (93.75%)	1.62 (0.87-2.77)	29 (72.5%)	0.36 (<0.01-1.6)	28 (70%)	0.32 (<0.01-0.95)	25 (62.5%)	0.41 (<0.01-1.44)	29 (72.5%)	0.71 (<0.01-1.69)	30 (71.43%)	0.52 (<0.01-1.22)	<0.001
Kocuria	26 (81.25%)	1.28 (0.42-3)	21 (52.5%)	0.03 (<0.01-0.82)	28 (70%)	0.57 (<0.01-1.86)	28 (70%)	0.88 (<0.01-2.24)	22 (55%)	0.09 (<0.01-0.68)	22 (52.38%)	0.25 (<0.01-0.95)	<0.001
Patescibacteria	16 (50%)	0.02 (<0.01-0.22)	28 (70%)	0.75 (<0.01-5.89)	28 (70%)	0.68 (<0.01-1.63)	40 (100%)	1.37 (<0.01-4.09)	29 (72.5%)	1.36 (<0.01-5.47)	28 (66.67%)	0.25 (<0.01-1.14)	<0.001
Minor_Phyla	31 (96.88%)	2.49 (1.55-4.07)	33 (82.5%)	1.53 (0.03-6.13)	39 (97.5%)	4.58 (1.89-6.81)	29 (72.5%)	4.84 (1.86-7.85)	32 (80%)	1.31 (0.03-4.11)	36 (85.71%)	1.85 (0.64-4.91)	<0.001
Minor_genera	32 (100%)	16.89 (11.62-23.67)	36 (90%)	9.93 (2.86-19.82)	40 (100%)	17.28 (9.17-31.39)	40 (100%)	21.36 (12.66-28.95)	38 (95%)	15.51 (3.54-23.46)	42 (100%)	12.57 (8.07-19.24)	0.002
Unclassified_phyla	18 (56.25%)	0.01 (<0.01-0.12)	31 (77.5%)	0.39 (0.01-2.01)	40 (100%)	1.46 (0.76-3.29)	40 (100%)	2.09 (0.65-6.13)	32 (80%)	0.54 (0.03-3.65)	42 (100%)	3.04 (1.08-5.12)	<0.001
Unclassified_genera	32 (100%)	3.11 (1.58-4.39)	38 (95%)	5.26 (1.11-16.07)	40 (100%)	8.33 (5.19-11.97)	40 (100%)	10.16 (7.82-14.23)	35 (87.5%)	13.27 (0.29-24.43)	42 (100%)	5.61 (2.98-10.7)	<0.001

#n (%): number of samples in which the phylum/genus was detected (relative frequency of detection).
†Kruskal-Wallis rank tests with Bonferroni correction.

Using the same reference database (SILVA 132) and post-taxonomic bioinformatic analysis pipeline yielded some specific differences in the alpha diversity results. In general, the V3-V4 16S rRNA region study showed a higher alpha-diversity [Shannon and Simpson indices 2.03 (1.46-2.49) and 0.77 (0.59-0.85) respectively] than V1-V3 study [Shannon and Simpson indices 1.74 (1.21-2.28) and 0.71 (0.53-0.81) respectively] ($p < 0.001$) (**Figures 4A, B**). In addition, PCoA plots based on the Bray-Curtis dissimilarity index (**Figure 4C**) and on the Jaccard's coefficient (**Figure 4D**) revealed differences in the beta diversity results.

In contrast, both sequencing approaches led to highly concordant results in relation to some individual phyla and genera, allowing similar comparisons across cohorts. For instance, in terms of individual phyla, in both studies Firmicutes, Proteobacteria, and Actinobacteriota collectively represented >90% of those identified (**Table 5**). Besides, both 16S region studies showed that the relative abundance of Firmicutes was lower in ETR than in all cohorts ($p < 0.001$), that Proteobacteria was relatively more abundant in milk collected in ETR than in all other cohorts ($p < 0.001$), and that Actinobacteriota was more abundant in ETR, ETU, GBR, and GBU than in GN, USC, SP, and PE ($p \leq 0.001$). The higher relative abundance of Bacteroidetes in KE than GN was also detected in both studies ($p < 0.001$). There was also an effect of cohort on the "other" (Lackey et al., 2019) and "minor_phyla" (this work) category; which relative abundance in GBU was higher than in ETR, ETU, GN, PE, SP, SW, and USC ($p \leq 0.001$) according to both strategies.

At the individual genus analysis, both approaches found a higher relative abundance of *Rhizobium, Achromobacter*, and *Corynebacterium* in milk collected in ETR than all other cohorts. Besides, statistical differences were found in almost all pairwise comparisons ($p < 0.05$) with ETR V1-V3 region strategy, except for *Corynebacterium* with ETU. Both V1-V3 and V3-V4 approaches showed that African cohorts had the lowest abundance of *Streptococcus* ($p < 0.05$) while Peruvian milk bacterial communities had the highest relative abundance of this genus as compared to all the other cohorts ($p < 0.05$) in a pairwise comparison.

In our work, *Lactobacillus* had a higher relative abundance among some African cohorts (particularly in ETR, GBR, GBU and GN) than all other cohorts but these differences were not statistically significant. Interestingly, in agreement with these observations, Lackey et al. (2019) also analyzed the fecal microbiome of the breastfed babies and found a higher relative abundance of *Lactobacillus* in feces of ETR, GBR and GBU than in samples from the PE, SP, SW and US cohorts.

Finally, in relation to alpha and beta diversity analysis, both approaches found that African samples, and particularly those from ETR, displayed the highest alpha diversity as assessed by the Shannon and Simpson indices (**Figures 5A, B**). Comparison of the mean distances of samples to the centroids using PCoA plots based either on the Bray-Curtis dissimilarity index or on the Jaccard's coefficient of each cohort, revealed no differences between both approaches (p=0.87 and p=0.37, respectively).

TABLE 4 | Relative frequencies, medians and interquartile range (IQR) of the most abundant bacterial phyla (bold) and genera detected in milk samples from European and American cohorts.

Phylum/Genus	SP		SW		PE		USC		USW		P-value[†]
	n (%)	n (%)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	
Firmicutes	40 (100%)	52.34 (41.38-80.31)	20 (100%)	63.1 (52.14-80.37)	38 (100%)	73.74 (53.5-85.68)	19 (100%)	72.95 (18.73-83.59)	41 (100%)	60.58 (48.72-70.05)	0.074
Staphylococcus	40 (100%)	14.78 (7.51-33.91)	20 (100%)	26.32 (20.33-49.74)	36 (94.74%)	18.12 (8.19-31.03)	19 (100%)	10.49 (4.69-51.83)	41 (100%)	25.26 (12.76-45.55)	0.12
Streptococcus	40 (100%)	12.55 (5.39-52.6)	20 (100%)	11.97 (7.84-28.44)	38 (100%)	37.8 (22.74-57.26)	19 (100%)	11.38 (1.82-31.85)	41 (100%)	15.57 (6.59-28.15)	<0.001
Lactobacillus	18 (45%)	<0.01 (<0.01-0.41)	17 (85%)	0.3 (0.05-1.49)	23 (60.53%)	0.08 (<0.01-1.01)	11 (57.89%)	0.02 (<0.01-0.25)	31 (75.61%)	0.44 (0.01-1.28)	0.016
Veillonella	18 (45%)	<0.01 (<0.01-0.59)	14 (70%)	0.15 (<0.01-0.72)	27 (71.05%)	1.43 (<0.01-4.08)	12 (63.16%)	0.06 (<0.01-1.54)	22 (53.66%)	0.01 (<0.01-0.6)	0.006
Anaerococcus	18 (45%)	<0.01 (<0.01-0.78)	13 (65%)	0.1 (<0.01-0.56)	26 (68.42%)	0.3 (<0.01-0.95)	12 (63.16%)	0.01 (<0.01-0.22)	30 (73.17%)	0.15 (<0.01-1.06)	0.33
Gemella	18 (45%)	<0.01 (<0.01-0.42)	15 (75%)	0.69 (<0.01-1.55)	24 (63.16%)	0.26 (<0.01-1.22)	12 (63.16%)	0.13 (<0.01-0.9)	28 (68.29%)	0.15 (<0.01-0.68)	0.18
Proteobacteria	40 (100%)	11.5 (4.71-20.89)	20 (100%)	6.74 (2.67-21.41)	38 (100%)	10.62 (3.99-22.95)	19 (100%)	6.9 (2.79-62.38)	41 (100%)	8.25 (3.31-14.05)	0.81
Rhodanobacter	27 (67.5%)	0.34 (<0.01-1.49)	11 (55%)	<0.01 (<0.01-0.2)	35 (92.11%)	2.55 (0.71-4.43)	7 (36.84%)	<0.01 (<0.01-0.05)	30 (73.17%)	0.32 (<0.01-1.66)	<0.001
Rhizobium	4 (10%)	<0.01 (<0.01-<0.01)	2 (10%)	<0.01 (<0.01-<0.01)	6 (15.79%)	<0.01 (<0.01-<0.01)	7 (36.84%)	<0.01 (<0.01-0.05)	2 (4.88%)	<0.01 (<0.01-<0.01)	<0.001
Stenotrophomonas	25 (62.5%)	0.31 (<0.01-2.36)	15 (75%)	0.12 (<0.01-0.63)	25 (65.79%)	0.08 (<0.01-0.81)	12 (63.16%)	0.04 (<0.01-0.41)	29 (70.73%)	0.08 (<0.01-0.84)	0.87
Acinetobacter	24 (60%)	0.14 (<0.01-0.99)	14 (70%)	0.17 (<0.01-0.7)	28 (73.68%)	0.82 (0.02-3.94)	11 (57.89%)	0.08 (<0.01-1.08)	32 (78.05%)	0.26 (0.01-0.8)	0.28
Delftia	34 (85%)	<0.01 (<0.01-<0.01)	15 (75%)	0.1 (0.01-0.41)	17 (44.74%)	<0.01 (<0.01-0.89)	10 (52.63%)	0.01 (<0.01-0.14)	25 (60.98%)	0.09 (<0.01-0.45)	0.002
Achromobacter	35 (87.5%)	0.45 (0.1-1.39)	13 (65%)	0.09 (<0.01-0.55)	22 (57.89%)	0.07 (<0.01-0.98)	14 (73.68%)	0.08 (<0.01-0.26)	25 (60.98%)	0.07 (<0.01-0.27)	0.007
Pseudomonas	29 (72.5%)	0.21 (<0.01-0.43)	14 (70%)	0.32 (<0.01-1.04)	24 (63.16%)	0.07 (<0.01-0.43)	15 (78.95%)	0.27 (0.05-1.77)	31 (75.61%)	0.18 (<0.01-0.55)	0.36
Actinobacteriota	40 (100%)	8.46 (5.72-13.89)	20 (100%)	8.72 (5.78-12.69)	38 (100%)	8.07 (5.04-12.45)	19 (100%)	3.62 (1.45-7.7)	41 (100%)	12.5 (9.21-19.75)	<0.001
Corynebacterium	32 (80%)	0.74 (0.09-2.76)	20 (100%)	0.82 (0.44-2.05)	34 (89.47%)	1.94 (0.55-3.12)	15 (78.95%)	0.3 (0.05-2.53)	37 (90.24%)	1.81 (0.5-4.07)	0.1
Cutibacterium	36 (90%)	0.7 (0.16-2.03)	19 (95%)	1.87 (0.76-3.76)	36 (94.74%)	0.67 (0.19-1.97)	18 (94.74%)	0.22 (0.08-1.26)	41 (100%)	2.74 (1.14-4.77)	<0.001
Rothia	24 (60%)	0.54 (<0.01-2.57)	13 (65%)	0.21 (<0.01-1.27)	28 (73.68%)	0.82 (<0.01-2.41)	10 (52.63%)	0.06 (<0.01-2.05)	27 (65.85%)	0.26 (<0.01-3.53)	0.7
Bifidobacterium	29 (72.5%)	0.42 (<0.01-1.36)	15 (75%)	0.54 (0.03-1.36)	25 (65.79%)	0.15 (<0.01-0.49)	12 (63.16%)	0.1 (<0.01-0.42)	29 (70.73%)	0.13 (<0.01-0.37)	0.096
Kocuria	14 (35%)	<0.01 (<0.01-0.5)	6 (30%)	<0.01 (<0.01-0.36)	16 (42.11%)	<0.01 (<0.01-0.2)	2 (10.53%)	<0.01 (<0.01-<0.01)	12 (29.27%)	<0.01 (<0.01-0.14)	<0.001
Patescibacteria	38 (95%)	3.21 (1.17-7.57)	19 (95%)	1.97 (0.24-4.31)	29 (76.32%)	0.55 (0.06-1.57)	14 (73.68%)	0.14 (0.01-1.38)	36 (87.8%)	1.26 (0.37-2.71)	<0.001
Minor_Phyla	39 (97.5%)	4.99 (0.93-6.7)	19 (95%)	1.99 (0.28-4.84)	38 (100%)	1.9 (0.59-4.78)	18 (94.74%)	1.41 (0.26-5.94)	41 (100%)	2.65 (1.2-5.84)	<0.001
Flavobacterium	28 (70%)	14.46 (5.47-21.38)	6 (30%)	<0.01 (<0.01-0.02)	8 (21.05%)	<0.01 (<0.01-<0.01)	6 (31.58%)	<0.01 (<0.01-0.14)	11 (26.83%)	<0.01 (<0.01-0.01)	0.003
Minor_genera	40 (100%)	2.2 (0.69-8.7)	20 (100%)	11.31 (3.68-21.06)	38 (100%)	9.16 (3.71-17.33)	18 (94.74%)	8.28 (2.9-15.12)	41 (100%)	13.83 (7.79-22.19)	0.34
Unclassified_phyla	40 (100%)	12.89 (3.42-23.39)	20 (100%)	4.74 (2.56-9.72)	38 (100%)	1 (0.4-3.34)	18 (94.74%)	1.21 (0.12-3.77)	41 (100%)	8.44 (4.3-14.33)	<0.001
Unclassified_genera	40 (100%)	52.34 (41.38-80.31)	20 (100%)	11.72 (4.08-21.29)	38 (100%)	3.88 (1.52-9.35)	19 (100%)	6.3 (2.52-11.63)	41 (100%)	12.18 (6.07-19.29)	0.001

[#]*n (%): number of samples in which the phylum/genus was detected (relative frequency of detection).*

[†]*Kruskal-Wallis rank tests with Bonferroni correction.*

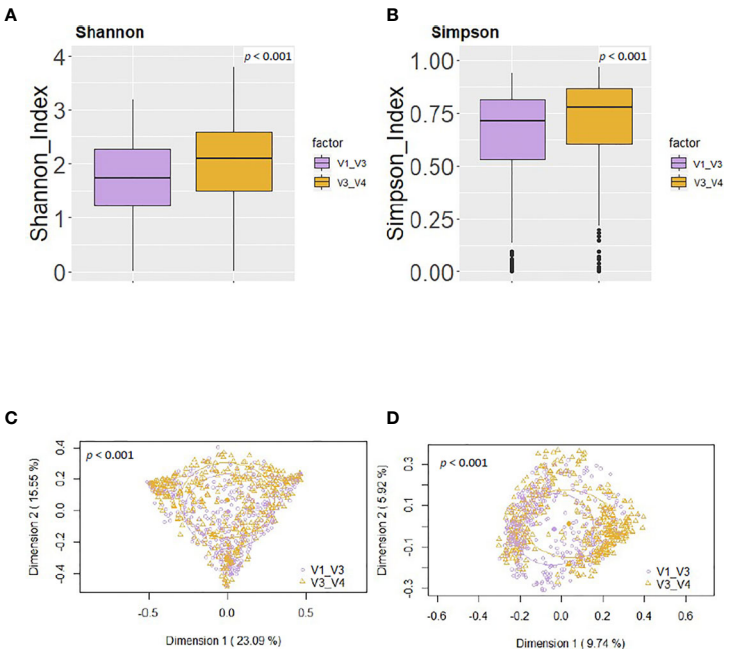

FIGURE 4 | Comparison between the values of alpha and beta diversity obtained after the analysis at the genus level of the same set of milk samples, either with the V3-V4 (this work) or the V1-V3 (Lackey et al., 2019) approach. **(A)** Shannon diversity index; **(B)** Simpson diversity index; **(C)** PCoA plots based on the Bray-Curtis dissimilarity index; **(D)** PCoA plots based on the Jaccard's coefficient with the SILVA 132 database.

TABLE 5 | Relative frequencies, medians and interquartile range (IQR) of the most abundant bacterial phyla (bold) and genera detected in the milk samples analyzed either with the V3-V4 (this work) or the V1-V3 (Lackey et al., 2019) approach with SILVA 132 database.

Phylum (bold)/Genus	**V1-V3**		**V3-V4**		**P-value†**
	n (%)#	**Median (IQR)**	**n (%)**	**Median (IQR)**	
Firmicutes	394 (100%)	63.03 (34.37-84.33)	392 (100%)	56.69 (41.15-76.09)	0.23
Staphylococcus	389 (98.73%)	12.49 (3.63-33.51)	389 (99.23%)	19.3 (8.03-36.84)	<0.001
Streptococcus	387 (98.22%)	15.61 (4.81-41.6)	377 (96.17%)	10.75 (3.62-28.16)	0.001
Lactobacillus	185 (46.95%)	<0.01 (<0.01-0.62)	280 (71.43%)	0.46 (<0.01-2.3)	<0.001
Veillonella	229 (58.12%)	0.25 (<0.01-1.62)	207 (52.81%)	0.04 (<0.01-1.28)	0.051
Gemella	189 (47.97%)	<0.01 (<0.01-0.74)	186 (47.45%)	<0.01 (<0.01-0.61)	0.380
Proteobacteria	394 (100%)	9.18 (3.47-25.32)	392 (100%)	13.84 (6.24-26.73)	0.002
Acinetobacter	204 (51.78%)	0.06 (<0.01-0.59)	270 (68.88%)	0.37 (<0.01-1.97)	<0.001
Rhizobium	115 (29.19%)	<0.01 (<0.01-0.05)	79 (20.15%)	<0.01 (<0.01-<0.01)	0.004
Klebsiella	51 (12.94%)	<0.01 (<0.01-<0.01)	82 (20.92%)	<0.01 (<0.01-<0.01)	0.004
Rhodanobacter	3 (0.76%)	<0.01 (<0.01-<0.01)	286 (72.96%)	0.66 (<0.01-3.68)	<0.001
Stenotrophomonas	137 (34.77%)	<0.01 (<0.01-0.2)	264 (67.35%)	0.14 (<0.01-1.1)	<0.001
Achromobacter	51 (12.94%)	<0.01 (<0.01-<0.01)	255 (65.05%)	0.1 (<0.01-0.76)	<0.001
Dyella	272 (69.04%)	0.83 (<0.01-2.57)	2 (0.51%)	<0.01 (<0.01-<0.01)	<0.001
Actinobacteria	381 (96.7%)	13.23 (3.95-25.09)	386 (98.47%)	9.93 (5.39-16.65)	0.003
Corynebacterium_1	295 (74.87%)	1.03 (<0.01-5.49)	324 (82.65%)	1.19 (0.27-3.33)	0.88
Rothia	256 (64.97%)	0.38 (<0.01-2.32)	224 (57.14%)	0.15 (<0.01-1.29)	<0.001
Propionibacterium	307 (77.92%)	0.49 (0.07-2.19)	0 (0%)	<0.01 (<0.01-<0.01)	<0.001
Kocuria	175 (44.42%)	<0.01 (<0.01-0.75)	196 (50%)	0.01 (<0.01-1.05)	0.120
Bifidobacterium	198 (50.25%)	0.02 (<0.01-0.94)	285 (72.7%)	0.42 (<0.01-1.32)	<0.001
Cutibacterium	0 (0%)	<0.01 (<0.01-<0.01)	355 (90.56%)	0.75 (0.18-2.1)	<0.001
Bacteroidetes	334 (84.77%)	0.65 (0.16-1.96)	321 (81.89%)	1.02 (0.12-2.74)	0.049
Minor_phyla	319 (80.96%)	0.4 (0.04-1.32)	350 (89.29%)	2.34 (0.52-5.91)	<0.001
Minor_genera	393 (99.75%)	9.98 (3.69-20.51)	387 (98.72%)	18.86 (8.74-30.12)	<0.001
Unclassified_phyla	283 (71.83%)	0.24 (<0.01-2.03)	360 (91.84%)	1.53 (0.28-5.31)	<0.001
Unclassified_genera	376 (95.43%)	3.33 (1.32-11.6)	383 (97.7%)	4.49 (1.76-10.51)	0.29

#*n (%): number of samples in which the phylum/genus was detected (relative frequency of detection).*
†*Wilconxon rank tests with Bonferroni correction.*

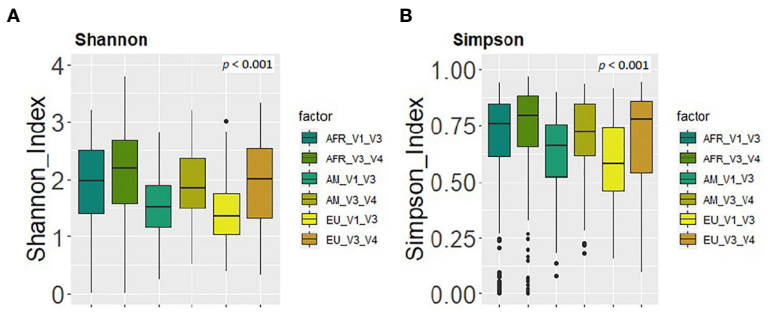

FIGURE 5 | Comparison between the values of alpha diversity obtained after the analysis at the genus level of the same set of milk samples, either with the V3-V4 (this work) or the V1-V3 (Lackey et al., 2019) approach and continent cohorts. **(A)** Shannon diversity index; **(B)** Simpson diversity index.

On the other hand, some differences were observed among the results obtained by Lackey et al. (2019) and those obtained in this work (**Table 5**). The relative abundance of the phylum Proteobacteria detected in our work was higher than in Lackey et al. (2019) [13.84 (6.24-26.73) *vs* 9.18 (3.47-25.32), p = 0.002]. On the contrary, the phylum Actinobacteriota was higher in Lackey et al. (2019) than in our work [13.23 (3.95-25.09) *vs* 9.93 (5.39-16.65), p = 0.003] (**Table 5**), whereas the medians of the Shannon or Simpson diversity indices were higher in this work ($p < 0.001$; Kruskal–Wallis tests with Bonferroni correction) (**Figures 4** and **5**). It is also worth noting that, despite finding comparable microbial community structures with both sequencing approaches, significant differences were found in the detection of the relative abundance of some bacterial genera which can be of great importance for the maternal-infant health, such as *Staphylococcus, Streptococcus, Bifidobacterium* and *Lactobacillus* (**Table 5**).

DISCUSSION

Findings from this study expand upon the milk microbiota profiling previously reported from the INSPIRE cohort (Lackey et al., 2019), which is the most comprehensive study to date on the topic. In addition, our findings provide, for the first time and across globally diverse populations, evidence of the impact of different DNA processing and sequencing approaches on the microbiota profiles obtained for human milk samples. While comparison of sequencing approaches and DNA isolation procedures has been long studied in the context of its impact on the GI microbiome (Hill et al., 2016; Rintala et al., 2017; Panek et al., 2018), little effort has been devoted to achieving standardization of optimal procedures to tackle the study of the human milk microbiome. However, such standardization is required to enable meaningful comparisons of the datasets generated across different studies of these important microbial communities, especially considering particular intrinsic characteristics which may impose additional challenges for microbiome studies. First, human milk has a relatively low density of bacterial cells (between 10^3 to 10^5 cfu mL^{-1}), can have a relatively high concentration of immune cells (and thus human DNA), and a high fat concentration that might entrap some bacterial cells. In addition, there exists an important risk of cross contamination of the samples with skin samples during collection or due to exposure to breast pumps; all of these factors can potentially introduce biases in microbiota studies (Boix-Amorós et al., 2016; Moossavi and Azad, 2019). In this context, our hypothesis is that DNA isolation, library generation, sequencing approach and data analysis can significantly impact the human milk microbiota profiles obtained through HTS surveys. For this purpose, the same set of human milk samples previously studied by means of sequencing the 16S rRNA V1-V3 regions reported by Lackey and colleagues (2019) was herein re-extracted and re-sequenced using different methods. For this study, the milk samples were extracted using the QIAamp DNA Stool Mini Kit with additional mechanical bead beating and using an amplicon sequencing approach that targeted a different 16S rRNA variable region (V3-V4) and that achieved higher sequencing depth. For the purposes of comparison, both datasets were downstream processed through identical post-taxonomy bioinformatics pipelines and reads generated in the present study (V3-V4 regions) were taxonomically re-assigned against the same reference database used in Lackey et al. (2019).

Overall, the dataset presented herein confirms the existence of large inter-individual and inter-populations variations in the healthy human milk microbiota across diverse geographical and ethnical populations as previously reported in several studies (Kumar et al., 2016; Vaidya et al., 2017). In agreement to the dataset previously reported by Lackey and colleagues on the same set of samples, this variation was evident between geographically distant but also, to some extent, between neighboring populations. For instance, both sequencing approaches found that the microbial communities present in African cohorts were dissimilar to those found in the European and American cohorts. The African cohorts also displayed higher diversity of microbial taxa, with ETR being the population harboring the most distinctive microbiota fingerprint. It must be highlighted that due to limitations in electricity access at ETR, this set of samples was preserved at room temperature in a preservation solution immediately following collection and thus this data must be interpreted with caution. While the utilization

of a different preservation and DNA isolation procedure might have introduced bias in the microbiota profiles detected for this particular cohort which could be partially responsible for the high dissimilarity exhibited by the ETR cohort as compared to the rest of the dataset, at this moment we cannot rule out to what extent these dissimilarities are due to genuinely biological differences. Nonetheless, it is worth remarking that other African cohorts also displayed significantly different beta-diversities as compared to American and European cohorts, particularly in terms of presence/absence of taxa, and that these observations were consistent among both 16S rRNA sequencing datasets. Besides, ETR together with GBU and GN were the cohorts displaying the highest alpha-diversity indices across all the studied cohorts. Overall, these observations agree with the overall reported loss of diversity in human microbiomes from populations with westernized and industrialized lifestyles (Segata, 2015), further suggesting that common genetic, environmental and/or lifestyle factors might have influenced a differential composition in the human milk microbiota in the cohorts under study. In line with "The Hygiene Hypothesis", these observations likely reflect a broad exposure to a wider array of microorganisms in some African cohorts as opposed to other European or American cohorts where westernized practices such as antibiotic utilization, water sanitation or reduced contact with animals, among others, may have reduced diversity in the human-associated microbial communities and the concomitant increase in non-communicable chronic diseases (Sonnenburg et al., 2016; Vandegrift et al., 2017). Further, even neighboring populations with different lifestyles, such as those represented by rural and urban communities of Ethiopia and rural and urban communities of Gambia, still exhibited significant differences in the diversity and/or structure of their respective milk microbiotas. These results support those reported by other authors when comparing the milk microbiota in urban and rural populations in India and China (Li et al., 2017; Vaidya et al., 2017). These observations reinforce the notion that microbial exposure, environmental factors and lifestyle habits strongly impact the assemblage of the human milk microbiome (Lackey et al., 2019) and, due to the influence of these microbial communities on seeding the infant GI microbiome; such factors might have decisive implications in infant health outcomes (Browne et al., 2019).

It is also worth noting that some African cohorts including ETR, GN and GBU presented taxa that were exclusively associated to their respective cohorts. For instance, GN samples were the only ones in which *Akkermansia* and *Butyricicoccus* were detected, both groups representing bacteria with attributed health promoting effects in models of inflammatory bowel disease (Eeckhaut et al., 2013; Ferrer-Picón et al., 2020), and likely representing candidates to develop prospective next-generation probiotics (O'Toole et al., 2017). The representation in the milk microbiota of other taxa traditionally including commensal microbes with attributed health promoting effects, such as *Lactobacillus* and *Bifidobacterium*, was also higher among African cohorts; and this result was independent on the sequencing approach employed. These observations strengthen recent research trends that defend the necessity to capture and preserve the microbial diversity from globally diverse human populations, as they might include taxa that could help mitigate non-communicable and chronic human diseases highly prevalent in industrialized and urbanized western populations (Dominguez-Bello et al., 2018; Sonnenburg and Sonnenburg, 2019).

The current dataset also supports the existence of a few universal core taxa consisting of dominant bacterial groups such as *Staphylococcus* and *Streptococcus*; as well as some other taxa that, despite being present in over 70% of the analyzed samples, exhibited minor relative abundances such as *Cutibacterium, Corynebacterium, Rhodanobacter* and *Bifidobacterium.* Some population-specific core taxa were also identified for some cohorts, in accordance with the results reported by Lackey et al., although population-specific core taxa appeared different depending on the sequencing approach. Moreover, the ETR core included *Rhizobium, Bifidobacterium* and *Acinetobacter*, the last two genera not present in the ETR core microbiota based on V1-V3 results. The prevalence of these bacterial groups likely drives the beta-diversity differentiation of this particular cohort from the other populations analyzed.

In conclusion, the V3-V4 approach enabled us to capture larger alpha-diversities, although the dissimilarity structures across cohorts, in terms of relative abundance of individual taxa, were relatively comparable in both datasets. Other reports have demonstrated that variable 16S rRNA regions can differently impact the taxa detected and thus the overall microbial community structures depicted, the largest differences being detected at lower taxonomic ranks (Bukin et al., 2019). Prior studies have described that V1-V3 regions may capture higher taxonomic diversities than V3-V4 when assessing oral and fecal samples (Zheng et al., 2015). This contrasts with the results observed in the present study with human milk samples, where V3-V4 revealed the highest alpha-diversity indices, which also yielded a higher representation of low abundance groups although at expenses of yielding a higher representation of unclassified minority phyla. However, at this point we cannot conclude whether this is due to a better resolution of this 16S rRNA region in this particular ecological niche, characterized by a relative lower complexity than the human gut, or to differences in the DNA extraction method, the sequencing depth and/or the criteria to exclude or trim the sequences after the quality analysis. For instance, differences in the levels of Proteobacteria or Actinobacteriota levels detected through both approaches, could be the result of differences in the selected primers' efficiency to amplify those groups, whereas an overall increased alpha diversity in the V3-V4 approach could either be the result of more efficient amplification of unrelated bacteria with selected universal primers and/or of the higher sequencing depth achieved. Although none of the short 16S rRNA hipervariable regions can provide the taxonomic resolution achieved by full length 16S rRNA sequencing, they can still provide meaningful information on the composition and structure of human associated microbial populations, specifically when reaching sufficient sequencing depths.

Remarkably, despite all the methodological differences between the two approaches, both were able to delineate a similar structure for the human milk microbiome of the different cohorts from which samples were collected, in terms of overall most abundant taxa and the differences these presented among cohorts. These results agree with previous results where patterns of predictions compared among different pipeline analysis were comparable provided that the sequencing depth and choice of NGS remain similar (Rajan et al., 2019), and suggest that the main inter-population and intra-population differences previously reported for the milk microbiome of the INSPIRE cohort are genuine as they could be corroborated through an independent different sequencing approach.

In addition, it must be highlighted that new versions of databases may introduce an important bias when trying to compare results within the same laboratory or among different laboratories. As an example, some phyla, such as Actinobacteriota or Patescibacteria, that appear as relevant for human milk microbiome using Silva 138 did not appear as such by using the previous version Silva 132. Discordances at the genus level may also arise, for example in relation to *Rhodanobacter* versus *Dyella* or to *Corynebacterium*_1 versus *Corynebacterium*. Thus, to conduct meaningful comparisons across datasets researchers should consider reanalyzing raw reads through common pipelines and reference databases.

DATA AVAILABILITY STATEMENT

The datasets generated in this study can be found in the SRA repository (https://www.ncbi.nlm.nih.gov) under Bioproject Accesion Number PRJNA693239.

ETHICS STATEMENT

All study procedures were approved by the overarching Washington State University Institutional Review Board (#13264) and at each study location, consent was obtained from each participating woman. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LR, CA, CG-C, EJ, KL, EK-M, EK, and SM conducted the research. MKM, CM, JF, DS, SEM, AP, DG, GO, RP, LB, MAM, JW, and JR designed the research. LR, CA, and JR wrote the manuscript. LR, CA, and JR had primary responsibility for the final content of the manuscript. LR and CA analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.622550/full#supplementary-material

REFERENCES

Asbury, M. R., Butcher, J., Copeland, J. K., Unger, S., Bando, N., Comelli, E. M., et al. (2020). Mothers of preterm infants have individualized breast milk that changes temporally based on maternal characteristics. *Cell Host Microbe* 31, S1931–3128(20)30424-8. doi: 10.1016/j.chom.2020.08.001

Bäckhed, F., Fraser, C. M., Ringel, Y., Sanders, M. E., Sartor, R. B., Sherman, P. M., et al. (2012). Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe* 12 (5), 611–622. doi: 10.1016/j.chom.2012.10.012

Boix-Amorós, A., Collado, M. C., and Mira, A. (2016). Relationship between milk microbiota, bacterial load, macronutrients, and human cells during lactation. *Front. Microbiol.* 7, 492. doi: 10.3389/fmicb.2016.00492

Boix-Amorós, A., Puente-Sánchez, F., du Toit, E., Linderborg, K. M., Zhang, Y., Yang, B., et al. (2019). Mycobiome profiles in breast milk from healthy women depend on mode of delivery, geographic location, and interaction with bacteria. *Appl. Environ. Microbiol.* 85 (9), e02994–e02918. doi: 10.1128/AEM.02994-18

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., et al. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* 6, 90. doi: 10.1186/s40168-018-0470-z

Bolyen, E., Rideout, J. R., Dillon, M. R., Boulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Browne, P. D., Aparicio, M., Alba, C., Hechler, C., Beijers, R., Rodríguez, J. M., et al. (2019). Human milk microbiome and maternal postnatal psychosocial distress. *Front. Microbiol.* 10, 2333. doi: 10.3389/fmicb.2019.02333

Bukin, Y. S., Galachyants, Y. P., Morozov, I. V., Bukin, S. V., Zakharenko, A. S., and Zemskaya, T. I. (2019). The effect of 16S rRNA region choice on bacterial community metabarcoding results. *Sci. Data* 6, 190007. doi: 10.1038/sdata.2019.7

Cabrera-Rubio, R., Collado, M. C., Laitinen, K., Salminen, S., Isolauri, E., and Mira, A. (2012). The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am. J. Clin. Nutr.* 96, 544–551. doi: 10.3945/ajcn.112.037382

Cabrera-Rubio, R., Mira-Pascual, L., Mira, A., and Collado, M. C. (2016). Impact of mode of delivery on the milk microbiota composition of healthy women. *J. Dev. Orig Health Dis.* 7 (1), 54–60. doi: 10.1017/S2040174415001397

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Carrothers, J. M., York, M. A., Brooker, S. L., Lackey, K. A., Williams, J. E., Shafii, B., et al. (2015). Fecal microbial community structure is stable over time and related to variation in macronutrient and micronutrient intakes in lactating women. *J. Nutr.* 145, 2379–2388. doi: 10.3945/jn.115.211110

Castro, I., Alba, C., Aparicio, M., Arroyo, R., Jiménez, L., Fernández, L., et al. (2019). Metataxonomic and immunological analysis of milk from ewes with or without a history of mastitis. *J. Dairy Sci.* 102 (10), 9298–9311. doi: 10.3168/jds.2019-16403

Davé, V., Street, K., Francis, S., Bradman, A., Riley, L., Eskenazi, B., et al. (2016). Bacterial microbiome of breast milk and child saliva from low-income Mexican-American women and children. *Pediatr. Res.* 79, 846–854. doi: 10.1038/pr.2016.9

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., and Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker gene and metagenomics data. *Microbiome* 6 (1), 226. doi: 10.1186/s40168-018-0605-2

Demmelmair, H., Jiménez, E., Collado, M. C., Salminen, S., and McGuire, M. K. (2020). Maternal and perinatal factors associated with the human milk microbiome. *Curr. Dev. Nutr.* 9;4 (4), nzaa027. doi: 10.1093/cdn/nzaa027

Dominguez-Bello, M. G., Knight, R., Gilbert, J. A., and Blaser, M. J. (2018). Preserving microbial diversity. *Science* 362 (6419), 33–34. doi: 10.1126/science.aau8816

Eeckhaut, V., Machiels, K., Perrier, C., Romero, C., Maes, S., Flahou, B., et al (2013). *Butyricicoccus pullicaecorum* in inflammatory bowel disease. *Gut* 62 (12), 1745–1752. doi: 10.1136/gutjnl-2012-303611

Fernández, L., Langa, S., Martín, V., Maldonado, A., Jiménez, E., Martín, R., et al. (2013). The human milk microbiota: origin and potential roles in health and disease. *Pharmacol. Res.* 69 (1), 1–10. doi: 10.1016/j.phrs.2012.09.001

Ferrer-Picón, E., Dotti, I., Corraliza, A. M., Mayorgas, A., Esteller, M., Perales, J. C., et al. (2020). Intestinal inflammation modulates the epithelial response to butyrate in patients with Inflammatory Bowel Disease. *Inflamm Bowel Dis.* 26 (1), 43–55. doi: 10.1093/ibd/izz119

Hill, C. J., Brown, J. R. M., Lynch, D. B., Jeffery, I. B., Ryan, C. A., Ross, R. P., et al. (2016). Effect of room temperature transport vials on DNA quality and phylogenetic composition of faecal microbiota of elderly adults and infants. *Microbiome* 4, Article number, 19. doi: 10.1186/s40168-016-0164-3

Hoashi, M., Meche, L., Mahal, L. K., Bakacs, E., Nardella, D., Naftolin, F., et al. (2016). Human milk bacterial and glycosylation patterns differ by delivery mode. *Reprod. Sci.* 23 (7), 902–907. doi: 10.1177/1933719115623645

Huërou-Luron, I. L., Blat, S., and Boudry, G. (2010). Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. *Nutr. Res. Rev.* 23 (1), 23–36. doi: 10.1017/S0954422410000065

Hunt, K. M., Foster, J. A., Forney, L. J., Schütte, U. M., Beck, D. L., Abdo, Z., et al. (2011). Characterization of the diversity and temporal stability of bacterial communities in human milk. *PloS One* 6, e21313. doi: 10.1371/journal.pone.0021313

Khodayar-Pardo, P., Mira-Pascual, L., Collado, M. C., and Martínez-Costa, C. (2014). Impact of lactation stage, gestational age and mode of delivery on breast milk microbiota. *J. Perinatol* 34 (8), 599–605. doi: 10.1038/jp.2014.47

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 7;41 (1), e1. doi: 10.1093/nar/gks808

Kumar, H., du Toit, E., Kulkarni, A., Aakko, J., Linderborg, K. M., Zhang, Y., et al. (2016). Distinct patterns in human milk microbiota and fatty acid profiles across specific geographic locations. *Front. Microbiol.* 7, 1619. doi: 10.3389/fmicb.2016.01619

Lackey, K. A., Williams, J. E., Price, W. J., Carrothers, J. M., Brooker, S. L., Shafii, B., et al. (2017). Comparison of commercially-available preservatives for maintaining the integrity of bacterial DNA in human milk. *J. Microbiol. Methods* 141, 73–81. doi: 10.1016/j.mimet.2017.08.002

Lackey, K. A., Williams, J. E., Meehan, C. L., Zachek, J. A., Benda, E. D., Price, W. J., et al. (2019). What's Normal? Microbiomes in human milk and infant feces are related to each other but vary geographically: The INSPIRE study. *Front. Nutr.* 6, 45. doi: 10.3389/fnut.2019.00045.

Lane, A. A., McGuire, M. K., McGuire, M. A., Williams, J. E., Lackey, K. A., Hagen, E. H., et al. (2019). Household composition and the infant fecal microbiome: The INSPIRE study. *Am. J. Phys. Anthropol* 169 (3), 526–539. doi: 10.1002/ajpa.23843

LeMay-Nedjelski, L., Butcher, J., Ley, S. H., Asbury, M. R., Hanley, A. J., Kiss, A., et al. (2020). Examining the relationship between maternal body size, gestational glucose tolerance status, mode of delivery and ethnicity on human milk microbiota at three months post-partum. *BMC Microbiol.* 20, 219. doi: 10.1186/s12866-020-01901-9

Li, S. W., Watanabe, K., Hsu, C. C., Chao, S. H., Yang, Z. H., Lin, Y. J., et al. (2017). Bacterial composition and diversity in breast milk samples from mothers living in Taiwan and mainland China. *Front. Microbiol.* 8, 965. doi: 10.3389/fmicb.2017.00965

Martín, R., Heilig, H. G., Zoetendal, E. G., Jiménez, E., Fernández, L., Smidt, H., et al. (2006). Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. *Res. Microbiol.* 158 (1), 31–37. doi: 10.1016/j.resmic.2006.11.004

Martín, R., Heilig, G. H., Zoetendal, E. G., Smidt, H., and Rodríguez, J. M. (2007) Diversity of the *Lactobacillus* group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. *J. Appl. Microbiol.* 103 (6), 2638–2644. doi: 10.1111/j.1365-2672.2007.03497.x.

Martinez, P. (2020). *pairwiseAdonis: Pairwise multilevel comparison using adonis. R package version 0.4.*

McGuire, M. K., Meehan, C. L., McGuire, M. A., Williams, J. E., Foster, J., Sellen, D. W., et al. (2017). What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. *Am. J. Clin. Nutr.* 105, 1086–1100. doi: 10.3945/ajcn.116.139980

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., et al. (2017). The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiol. Mol. Biol. Rev.* 81 (4), e00036–e00017. doi: 10.1128/MMBR.00036-17

Moossavi, S., and Azad, M. B. (2019). Origins of human milk microbiota: new evidence and arising uestions. *Gut Microbes*, 12 (1), 1667722. doi: 10.1080/19490976.2019.1667722

Moossavi, S., Sepehri, S., Robertson, B., Bode, L., Goruk, S., Field, C. J., et al. (2019). Composition and variation of the human milk microbiota are influenced by maternal and early-life factors. *Cell Host Microbe* 13;25 (2), 324–335.e4. doi: 10.1016/j.chom.2019.01.011

Moossavi, S., Fehr, K., Derakhshani, H., Sbihi, H., Robertson, B., Bode, L., et al. (2020). Human milk fungi: environmental determinants and inter-kingdom associations with milk bateria in the CHILD cohort study. *BMC Microbiol.* 20 (1), 146. doi: 10.1186/s12866-020-01829-0

Musilova, S., Modrackova, N., Hermanova, P., Hudcovic, T., Svejstil, R., Rada, V., et al. (2017). Assessment of the synbiotic properites of human milk oligosaccharides and *Bifidobacterium longum* subsp. *infantis* in vitro and in humanised mice. *Benef Microbes* 8 (2), 281–289. doi: 10.3920/BM2016.0138

Ojo-Okunola, A., Claassen-Weitz, S., Mwaikono, K. S., Gardner-Lubbe, S., Stein, D. J., Zar, H. J., et al. (2019). Influence of socio-economic and psychosocial profiles on the human breast milk bacteriome of South African women. *Nutrients* 11 (6), 1390. doi: 10.3390/nu11061390

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2012). *Vegan: Community Ecology Package. Software*. Available at: http://CRAN.R-project.org/package=vegan.

O'Toole, P. W., Marchesi, J. R., and Hill, C. (2017). Next-generation probiotics: The spectrum from probiotics to live biotherapeutics. *Nat. Microbiol.* 2, 17057. doi: 10.1038/nmicrobiol.2017.57

Padilha, M., Brejnrod, A., Danneskiold-Samsøe, N. B., Hoffmann, C., Iaucci, J. M., Cabral, V. P., et al. (2020). Response of the human milk microbiota to a maternal prebiotic intervention is individual and influenced by maternal age. *Nutrients* 13;12 (4), 1081. doi: 10.3390/nu12041081

Panek, M., Paljetak, H. C., Barešić, A., Perić, M., Matijašić, M., Lojkić, I., et al. (2018). Methodology challenges in studying human gut microbiota - effects of collection, storage, DNA extraction and next generation sequencing technologies. *Sci. Rep.* 8 (1), 5143. doi: 10.1038/s41598-018-23296-4

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41 (Database issue), D590–D596. doi: 10.1093/nar/gks1219

Rajan, S. K., Lindqvist, M., Brummer, R. J., Schoultz, I., and Repsilber, D. (2019). Phylogenetic microbiota profiling in fecal samples depends on combination of sequencing depth and choice of NGS analysis method. *PloS One* 14 (9), e0222171. doi: 10.1371/journal.pone.0222171

Rintala, A., Pietilä, S., Munukka, E., Eerola, E., Pursiheimo, J. P., Laiho, A., et al. (2017). Gut microbiota analysis results are highly dependent on the 16S rRNA gene target region, whereas the impact of DNA extraction is minor. *J. Biomol Tech* 28 (1), 19–30. doi: 10.7171/jbt.17-2801-003

Roger, L. C., Costabile, A., Holland, D. T., Hoyles, L., and McCartney, A. L. (2010). Examination of faecal *bifidobacterium* populations in breast- and formula-fed infants during the first 18 months of life. *Microbiology* 156 (Pt 11), 3329–3341. doi: 10.1099/mic.0.043224-0

Ruiz, L., Espinosa-Martos, I., García-Carral, C., Manzano, S., McGuire, M. K., Meehan, C. L., et al. (2017). What's Normal? Immune profiling of human milk from healthy women living in different geographical and socioeconomic settings. *Front. Immunol.* 8, 696. doi: 10.3389/fimmu.2017.00696

Ruiz, L., García-Carral, C., and Rodriguez, J. M. (2019). Unfolding the human milk microbiome landscape in the omics era. *Front. Microbiol.* 10, 1378. doi: 10.3389/fmicb.2019.01378

Segata, N. (2015). Gut microbiome: westernization and the disappearance of intestinal diversity. *Curr. Biol.* 25 (14), R611–R613. doi: 10.1016/j.cub.2015.05.040

Schwab, C., Voney, E., Ramirez Garcia, A., Vischer, M., and Lacroix, C. (2019). Characterization of the cultivable microbiota in fresh and stored mature human breast milk. *Front. Microbiol.* 10, 2666. doi: 10.3389/fmicb.2019.02666. eCollection 2019.

Solís, G., de Los Reyes-Gavilan, C. G., Fernández, N., Margolles, A., and Gueimonde, M. (2010). Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. *Anaerobe* 16 (3), 307–310. doi: 10.1016/j.anaerobe.2010.02.004

Sonnenburg, J. L., Smits, S. A., Tikhonov, M., Higginbottom, S. K., Wingreen, N. S., and Sonnenburg, J. L. (2016). Diet-induced extinctions in the gut microbiota compound over generations. *Nature* 529 (7585), 212–215. doi: 10.1038/nature16504.

Sonnenburg, J. L., and Sonnenburg, E. D. (2019). Vulnerability of the industrialized microbiota. *Science* 366, eaaw9255. doi: 10.1126/science.aaw9255

Stinson, L. F., Sindi, A. S. M., Cheema, A. S., Lai, C. T., Mühlhäusler, B. S., Wlodek, M. E., et al. (2020). The human milk microbiome: who, what, when, where, why, and how? *Nutr. Rev.*, nuaa029. doi: 10.1093/nutrit/nuaa029

Togo, A. H., Grine, G., Khelaifia, S., des Robert, C., Brevaut, V., Caputo, A., et al. (2019). Culture of methanogenic archaea from human colostrum and milk. *Sci. Rep.* 9 (1), 18653. doi: 10.1038/s41598-019-54759-x

Treven, P., Mahnič, A., Rupnik, M., Golob, M., Pirš, T., Matijašić, B. B., et al. (2019). Evaluation of human milk microbiota by 16S rRNA gene Next-Generation Sequencing (NGS) and cultivation/MALDI-TOF mass spectrometry identification. *Front. Microbiol.* 10, 2612. doi: 10.3389/fmicb.2019.02612

Vandegrift, R., Bateman, A. C., Siemens, K. N., Nguyen, M., Wilson, H. E., Green, J. L., et al. (2017). Cleanliness in context: reconciling hygiene with a modern microbial perspective. *Microbiome* 5 (1), 76. doi: 10.1186/s40168-017-0294-2.

Vaidya, Y. H., Patel, S. H., Patel, R. J., Pandit, R. J., Joshi, C. G., and Kunjadia, A. P. (2017). Human milk microbiome in urban and rural populations of India. *Meta Gene* 13, 13, 22. doi: 10.1016/j.mgene.2017.04.001

Volery, M., Scherz, V., Jakob, W., Bandeira, D., Deggim-Messmer, V., Lauber-Biason, A., et al. (2020). Study protocol for the ABERRANT study: antibiotic-induced disruption of the maternal and infant microbiome and adverse health outcomes - a prospective cohort study among children born at term. *BMJ Open* 23;10 (6), e036275. doi: 10.1136/bmjopen-2019-036275

Wan, Y., Jiang, J., Lu, M., Tong, W., Zhou, R., Li, J., et al. (2020). Human milk microbiota development during lactation and its relation to maternal geographic location and gestational hypertensive status. *Gut Microbes* 2;11 (5), 1438–1449. doi: 10.1080/19490976.2020.1760711

Wu, W. K., Chen, C. C., Panyod, S., Chen, R. A., Wu, M. S., Sheen, L. Y., et al (2019). Optimization of fecal sample processing for microbiome study - The journey from bathroom to bench. *J. Formos Med. Assoc.* 11 (2), 545–555. doi: 10.1016/j.jfma.2018.02.005

Zheng, W., Tsompana, M., Ruscitto, A., Sharma, A., Genco, R., Sun, Y., et al. (2015). An accurate and efficient experimental approach for characterization of the complex oral microbiota. *Microbiome* 3, 48. doi: 10.1186/s40168-015-0110-9

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Early Life Microbiota Colonization at Six Months of Age: A Transitional Time Point

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Background: Early life gut microbiota is involved in several biological processes, particularly metabolism, immunity, and cognitive neurodevelopment. Perturbation in the infant's gut microbiota increases the risk for diseases in early and later life, highlighting the importance of understanding the connections between perinatal factors with early life microbial composition. The present research paper is aimed at exploring the prenatal and postnatal factors influencing the infant gut microbiota composition at six months of age.

Methods: Gut microbiota of infants enrolled in the longitudinal, prospective, observational study "A.MA.MI" (*Alimentazione MAmma e bambino nei primi MIlle giorni*) was analyzed. We collected and analyzed 61 fecal samples at baseline (meconium, T0); at six months of age (T2), we collected and analyzed 53 fecal samples. Samples were grouped based on maternal and gestational weight factors, type of delivery, type of feeding, time of weaning, and presence/absence of older siblings. Alpha and beta diversities were evaluated to describe microbiota composition. Multivariate analyses were performed to understand the impact of the aforementioned factors on the infant's microbiota composition at six months of age.

Results: Different clustering hypotheses have been tested to evaluate the impact of known metadata factors on the infant microbiota. Neither maternal body mass index nor gestational weight gain was able to determine significant differences in infant microbiota composition six months of age. Concerning the type of feeding, we observed a low alpha diversity in exclusive breastfed infants; conversely, non-exclusively breastfed infants reported an overgrowth of *Ruminococcaceae* and *Flavonifractor*. Furthermore, we did not find any statistically significant difference resulting from an early introduction of solid foods (before 4 months of age). Lastly, our sample showed a higher abundance of clostridial patterns in firstborn babies when compared to infants with older siblings in the family.

Conclusion: Our findings showed that, at this stage of life, there is not a single factor able to affect in a distinct way the infants' gut microbiota development. Rather, there seems to be a complex multifactorial interaction between maternal and neonatal factors determining a unique microbial niche in the gastrointestinal tract.

Keywords: newborn, neonatal microbiota, maternal factors, breast milk, weaning, older siblings, delivery

INTRODUCTION

Research on the human gut microbiome has gained attention in the past years due to the vital contribution of microorganisms to host health across the life span. Microbial colonization indeed plays a pivotal role in stimulating immune system development, nutrient metabolism, and promoting differentiation of mucosal structure and function (Kumbhare et al., 2019). The interaction between host and microbiota is essential during the first years of life because substantial shifts in the abundance and structure occur in this critical stage of the infants' life. The intestinal microbiota undergoes dynamic development during the first years of life, determining adult microbiota composition and, consequently, impacting health. Many factors influence the shaping of gut microbiota in this critical window of plasticity, including gestational age, maternal pre-pregnancy BMI, weight gain during pregnancy, mode of birth, feeding types, weaning, birth environment, besides ethnic/geographical background (Nagpal et al., 2017; Raspini et al., 2020).

Among these factors overweight and obesity are of great concern since the number of pregnant women affected by overweight or obesity has increased both in high income and middle-income countries (Chen et al., 2018). In the US more than half of all pregnant women are affected by obesity (Flegal et al., 2012). These subjects are likely to face several complications such as gestational diabetes, hypertension, and delivery by cesarean section (Catalano and Shankar, 2017). Furthermore, it has been demonstrated that their offspring is likely to develop obesity in later life (Whitaker, 2004; Pirkola et al., 2010; Deierlein et al., 2011; Mehta et al., 2011; Gaillard et al., 2013) as well as non-communicable diseases (NCDs) (Glastras et al., 2018). These undesired effects are, at least partly, modulated by the related changes in gut microbial composition during pregnancy and lactation (Collado et al., 2012; Singh et al., 2017). Such changes impact on maternal and offspring health, altering host metabolic pathways and remodeling the expression of genes regulating them (Gaillard et al., 2013; Galley et al., 2014; Gallardo et al., 2015; Gohir et al., 2015; Kumbhare et al., 2019). Furthermore, evidence shows that breastfeeding is one of the key players in preventing alteration in gut microbiota composition, which likely contributes to the development of autoimmune and metabolic disorders later in life (Ho et al., 2018). Human milk contains remarkable bioactive compounds, including human milk oligosaccharides, HMOs, beneficial to infants as they not only promote a better growth but also strengthen the immune system of the newborn, reducing the risk of diarrhea and consequent dehydration, protecting against allergies and metabolic disorders (Ho et al., 2018). Longitudinal studies have indicated that the infant's microbial structure varies significantly with the suspension of breast/formula feeding and, consequently, with the introduction of solid foods (Thompson et al., 2015). The weaning process represents the final path of infant gut microbial shaping, characterized by significant shifts in taxonomic groups, and the increase in gut microbial diversity, into a stable adult composition. In this process, diet plays a key role in modulating the microbial community (Stewart et al., 2018; Kumbhare et al., 2019). As solid foods are included in the diet, the microbiota starts evolving from a simple environment, Bifidobacteria-rich (microorganisms that metabolize HMOs), to a different one, rich in species such as *Bacteroides*, able to metabolize starches present in a more complex dietary pattern (Moore and Townsend, 2019). Moreover, previous studies (Flores et al., 2014; Tamburini et al., 2016) have shown that the newborns' environment is also a natural source of germs that may colonize different body sites. For example, cohabitation boosts bacterial exchange probability from touching shared surfaces, using shared objects, and breathing indoor air (Flores et al., 2014; Tamburini et al., 2016). Other investigations, indeed, have examined the association between the presence of older siblings and increased diversity and richness of the gut microbial during early childhood, which could contribute to the substantiation of the hygiene hypothesis (Strachan, 1989; Azad et al., 2013; Laursen et al., 2015).

According to those findings, the present study explored prenatal factors, including maternal BMI and weight gain during pregnancy, as well as newborn postnatal exposure factors, including mode of feeding, time of weaning, and the presence of siblings in the family that might influence the infant gut microbiota composition at age 6 months to identify major active actors.

MATERIALS AND METHODS

Study Design

The present study is part of the longitudinal, prospective, observational study A.MA.MI (*Alimentazione MAmma e bambino nei primi MIlle giorni*), ClinicalTrials.gov identifier: NCT04122612. The study was approved by the Human Ethics Committee (EC) of *Fondazione IRCCS Policlinico S. Matteo of Pavia* (Protocol number: 20180022618; 6/12/2018), and it was conducted on a group of mother–infant pairs referred to the Neonatal Unit, Fondazione IRCCS Policlinico San Matteo, Pavia (Italy) from birth to 1 year of age, according to the Good Clinical Practice guidelines. Written informed consent of the parents/

legal guardian was provided. The Human EC of *Fondazione IRCCS Policlinico S. Matteo of Pavia* approved this procedure after ascertaining its compliance with the dictates of the Declaration of Helsinki (IV Adaptation).

The complete study design and the study protocol were previously described elsewhere (Raspini et al., 2020).

53 fecal samples were collected and analyzed at 6 months of infants' age, corresponding to the 3rd sampling of the A.MA.MI project (T2) (resuming data are presented in **Table S1**). Analyses were also performed at baseline (*meconium*, T0) on 61 samples (we did not receive eight T2 fecal samples). To evaluate the early microbiota colonization, we investigated maternal factors, such as pre-pregnancy body mass index (BMI) and gestational weight gain (WG) and perinatal factors as type of delivery, type of feeding, time of weaning, and environmental influences (due to the presence of older siblings in the household).

We collected anthropometric data as maternal height and pre-gestational weight to calculate body mass index (BMI; kg/m^2). Based on pre-gestational BMI, women were then stratified as Normal Weight (NW—BMI ≤ 24.9 kg/m^2) or Overweight/with Obesity (OW/OB—BMI ≥ 25 kg/m^2) while gestational weight gain (WG) was defined as body weight increase from pre-pregnancy to delivery and compared with recommended WG ranges by IOM guidelines for each pre-pregnancy BMI category (NW, 11.5–16 kg; OW, 7–11.5 kg; and OB, 5–9 kg) (Rasmussen and Yaktine, 2009).

The samples were grouped based on maternal pre-pregnancy BMI (NW: normal pre-pregnancy BMI or OW: excessive BMI), gestational weight gain (NWG normal gestational WG or EWG: excessive gestational WG), delivery mode (VD: vaginal delivery or CS: caesarean section) type of feeding ($_E$BF: exclusively breastfed or $_{Ne}$BF: not exclusively breastfed, which include exclusively formula-fed infants and mixed fed infants), weaning (evaluating the solid food introduction if before or after 4 months of age; ≤4 or >4, respectively), and presence (nFB)/absence (FB) of older siblings.

Samples Analysis

Stool samples were shipped on dry ice to Genomix4Life Srl (C/O Laboratory of Molecular and Genomic Medicine—Campus of Medicine and Surgery, Baronissi, Salerno, Italy, a spin-off of the University of Salerno, Fisciano, Italy) where 16S rRNA gene amplicon analysis was carried out. To ensure the personal privacy, samples had only the study ID number; no clinical or personal information was shipped.

16S rRNA Metagenomic Sequencing Library Preparation, Gene Amplicon Sequencing and Analysis

Next-generation sequencing (NGS) experiments, comprising DNA extraction and primary bioinformatics analysis, were performed by Genomix4life S.R.L. (Baronissi, Salerno, Italy). DNA extraction was performed with Invimag Stool kit (Stratec) using an extraction negative control. Final yield and quality of extracted DNA were determined by using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) and Qubit Fluorometer 1.0 (Invitrogen Co., Carlsbad, CA). 16S rRNA gene amplification was performed with primers: Forward: 5′-CCTACGGGNGGCWGCAG-3′ and Reverse: 5′-GACTACHVGGGTATCTAATCC-3′ (Klindworth et al., 2013), which target the hypervariable V3 and V4 regions of the 16S rRNA gene. Each PCR reaction was assembled according to Metagenomic Sequencing Library Preparation (Illumina, San Diego, CA). A negative control is included in the workflow; it consists of all reagents used during sample processing (16S amplification and library preparation) but does not contain a sample to assess potential contamination. Libraries were quantified using Qubit fluorometer (Invitrogen Co., Carlsbad, CA) and pooled to an equimolar amount of each index-tagged sample to a final concentration of 4 nM, including the Phix Control Library. Pooled samples were subject to cluster generation and sequenced on MiSeq platform (Illumina, San Diego, CA) in a 2 × 300 paired-end format. The raw sequence files generated (fast files) underwent quality control analysis with FastQC. The 16S metagenomics analysis performs the taxonomic classification of 16S rRNA targeted amplicon reads after OTU clustering based on the 97% of similarity (3% of divergence). The algorithm is a high-performance implementation of the Ribosomal Database Project (RDP) Classifier described in Wang et al., 2007 (http://dx.doi.org/10.1128%2FAEM.00062-07). Taxonomic databases used to perform taxonomic classification are RefSeq RDP 16S v3 May 2018 DADA2 32bp.

The obtained sequences were uploaded to a public database, and the following extremes refer to the submission to NCBI database (http://www.ncbi.nlm.nih.gov/bioproject/675753, extremes: Submission ID: SUB8491261; BioProject ID: PRJNA675753).

Statistical Analyses

Data were summarized using descriptive statistics, such as means and standard deviations, median, or interquartile range (IQR), as appropriate, for quantitative variables and relative frequencies for qualitative ones.

Multivariable association between 16S rRNA gene data abundances at different taxonomic levels occurring in infants' microbiota (relative to prenatal and postnatal factors) was performed using the MaAsLin2 R package (https://huttenhower.sph.harvard.edu/maaslin/). Meanwhile, unless specifically described, data and group differences were analyzed and compared by paired or unpaired, two-tailed Student's t-test.

To investigate the intestinal microbiota development of infants, principal component analysis (PCA) was used to evaluate the beta diversity occurring within our population to assess differences in the microbial composition [baseline (*meconium*), T0, *vs* six months of age, T2]. The dudi.pca function within the "ade4" R package (https://cran.r-project.org/web/packages/ade4/) was used to perform a PCA of data frames. The resulting PCA and dudi class objects were plotted with the "factoextra" R package (https://cran.r-project.org/web/packages/factoextra/index.html).

Looking for evidence of clustering among our samples, those genera with a median relative abundance lower than 0.1 were purged out and in first instance discriminant analysis of principal component (DAPC) without any *a priori* clustering condition was computed. The "DAPC" and the "find.clusters" functions

within the adegenet R package v2.1.1 (https://cran.r-project.org/web/packages/adegenet/index.html) were used to compute the DAPC and determine the optimal cluster assignment.

Then, the same multivariate analysis was run on genera abundances by superimposing as *a priori* condition; the belonging of each sample according to metadata information was on maternal factors (BMI and gestational WG), delivery mode (VD and CS), feeding (BF and FF), weaning (≤4 and >4 months), and presence of older siblings (FB and nFB). Thus, in order to ascertain if DAPC classification was consistent with the original clusters and based on the discriminant functions, the "assignplot" function in the R "adegenet" package was used to calculate the proportions of successful reassignments.

RESULTS

The amplicon 16S rRNA sequencing analysis, performed on the 53 fecal samples of infants at 6 months of age (T2), determined a number of reads singularly assigned taxonomy that passed the quality control (QC) filter corresponding to 97,753.21 ± 22,044.56 (mean ± standard deviation) *per* sample. Of these, the 91.70 ± 2.13% (mean ± s.d.) was assigned at least to the genus level. To investigate factors influencing early microbiota colonization of infants during the first 6 months of life, samples were grouped based on: maternal factors [pre-pregnancy body mass index (BMI) and gestational weight gain (WG)], type of delivery, diet-related factors (the type of feeding and weaning), and presence/absence of older siblings in the household.

Alpha diversity, evaluated using the Shannon index and the number of operational taxonomic units (OTUs), was determined according to the different aforementioned factors (**Figure 1**). We observed that only $_{E}$BF determined a lowering in Shannon index values (P = 0.018) when compared to not exclusively breastfed ones ($_{Ne}$BF). Any other of the evaluated factors determined significant differences concerning alpha diversity.

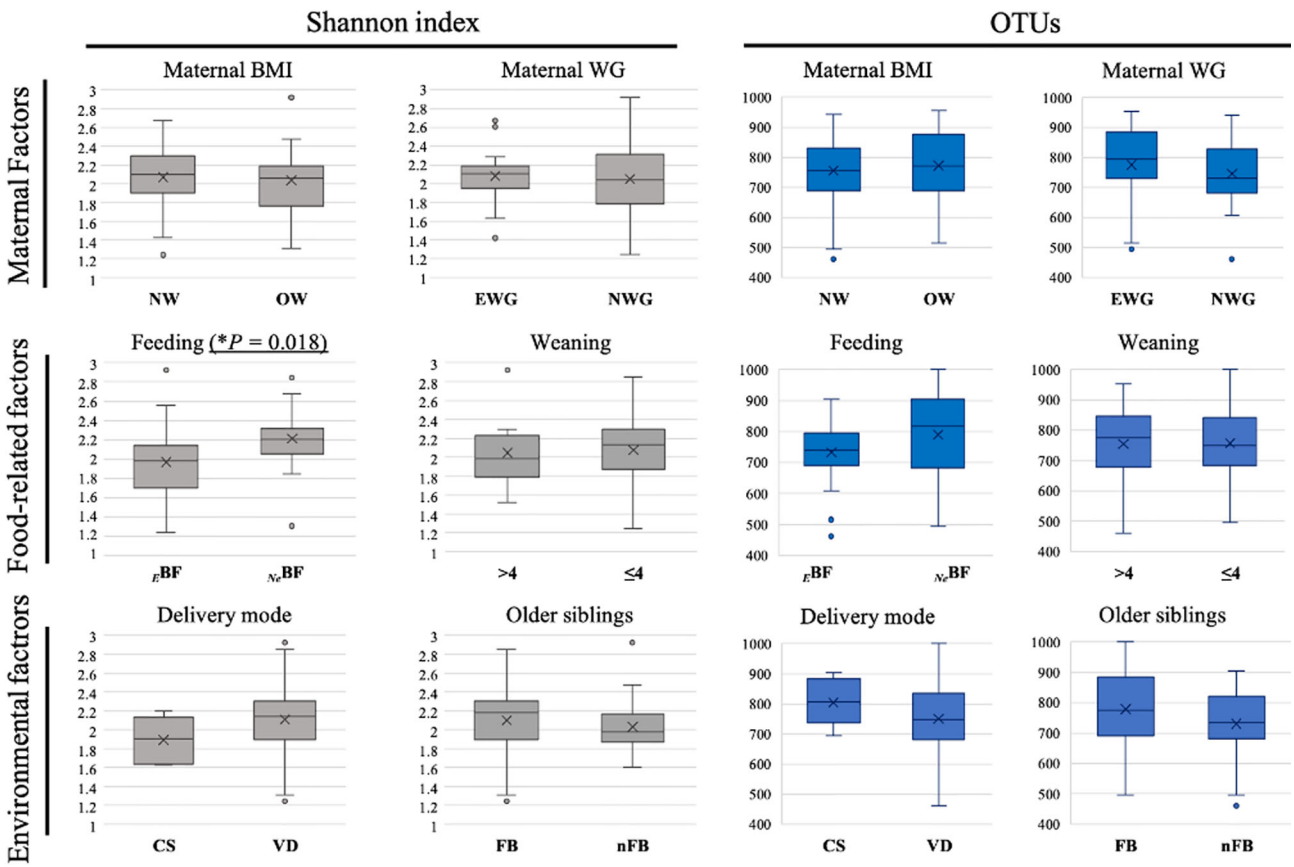

FIGURE 1 | Box plots of the alpha diversity (Shannon index and number of operational taxonomic units (OTUs) identified) among T2 fecal samples (infants at 6 months of age) grouped according to different factors influencing early microbiota composition. Maternal factors: maternal pre-pregnancy body mass index (BMI) and gestational weight gain (WG). NW *[n.37]*: normal pre-pregnancy BMI (BMI < 25 kg/m^2), OW *[n.12]*: excessive pre-pregnancy BMI (BMI ≥ 25 kg/m^2); EWG *[n.20]*: excessive gestational WG, NWG *[n.29]*: optimal gestational WG). Food-related factors: Feeding ($_{E}$BF *[n.31]*: exclusive breast-feeding, NeBF *[n.21]*: not exclusively breast fed, mixed fed or exclusively formula fed) and Weaning (≤4 *[n.41]*: before 4 months of age, >4 *[n.9]*: after 4 months of age). Environmental factors: Delivery mode (CS *[n.7]*: cesarean section, VD *[n.46]*: vaginal delivery) and presence of Older siblings (FB *[n.30]*: first-born, nFB *[n.22]*: not first-born). *P = p-value.

Factors Affecting Early Microbial Colonization of Infant Gut Microbiota

Starting from prenatal factors, maternal pre-pregnancy BMI within recommended values (BMI < 25 kg/m^2) or higher (BMI ≥ 25 kg/m^2) and gestational WG, optimal or excessive according to IOM reference values, were used to evaluate influence of maternal weight on gut microbiota composition in offspring till six months of age. At the phylum level, we did not find statistically significant differences concerning maternal features in both pre-pregnancy BMI and gestational WG (**Figures 2i, ii**). According to BMI and WG, any significant difference was found at the deeper taxonomic levels, specifically family and genus (data not shown).

Differently, evaluating infants' microbiota composition at six months of age considering delivery mode (VD *vs* CS), an increased amount of Bacteroidetes in VD was detected (P = 0.021; **Table 1**, **Figure 2iii**). Despite this result at the phylum level, no other statistically significant difference at deeper taxonomic levels was detected.

Evaluating type of feeding, specifically $_{E}$BF or not $_{Ne}$BF (mixed or exclusively formula-fed), no differences were found at the phylum level **Figure 2iv**. Interestingly, the family of *Ruminococcaceae* was strongly associated with the $_{Ne}$BF group (P < 0.0001, qvalue = 0.011; **Table 1**). According to the family abundances, *Ruminococcaceae* subclusters were more abundant in FF, specifically *Flavonifractor* and the *Clostridium cluster IV* of Firmicutes. Also, other genera of Firmicutes mainly characterized the microbiota of FF infants, specifically *Faecalicoccus* (and other taxa of *Erysipelotrichaceae Incertae Sedis*), *Romboutsia*, and *Oribacterium* (P < 0.005). Contrarily, *Staphylococcus* was higher in $_{E}$BF than in $_{Ne}$BF (P = 0.003; **Table 1**).

The introduction of solid foods at least at 2 months of age [infants weaned before 4 months of age (≤4)] was not sufficient to determine significant shifts in the microbiota composition when compared to infants weaned after the 4th month of age **Figure 2v**.

Among environmental factors, the presence of older siblings in the household did not determine differences at the phylum level **Figure 2vi**. The main feature of firstborn babies (FBs) was a higher abundance of *Clostridiaceae*, *Clostridiales Incertae Sedis XIII*, and *Peptostreptococcaceae* (P<0.003) than infants with older siblings (nFBs).

Thus, to evaluate the significant multivariable association among all infants, considering metadata (maternal BMI, gestational WG, type of delivery, feeding, weaning, and the presence of older siblings) as fixed effects in a regression model, we run MaAsLin2 software using family and genus relative abundances. Among all samples and relative metadata, *Ruminococcaceae* better discriminated $_{Ne}$BF samples (P < 10^{-3}; **Table S1**). However, this result was not confirmed for adjusted p-values (qval > 0.05). In the same line, *Flavonifractor* confirmed the previous discrimination in $_{Ne}$BF samples (**Table S2**).

As previously reported, prenatal factors (both maternal pre-pregnancy BMI and gestational WG) did not show any

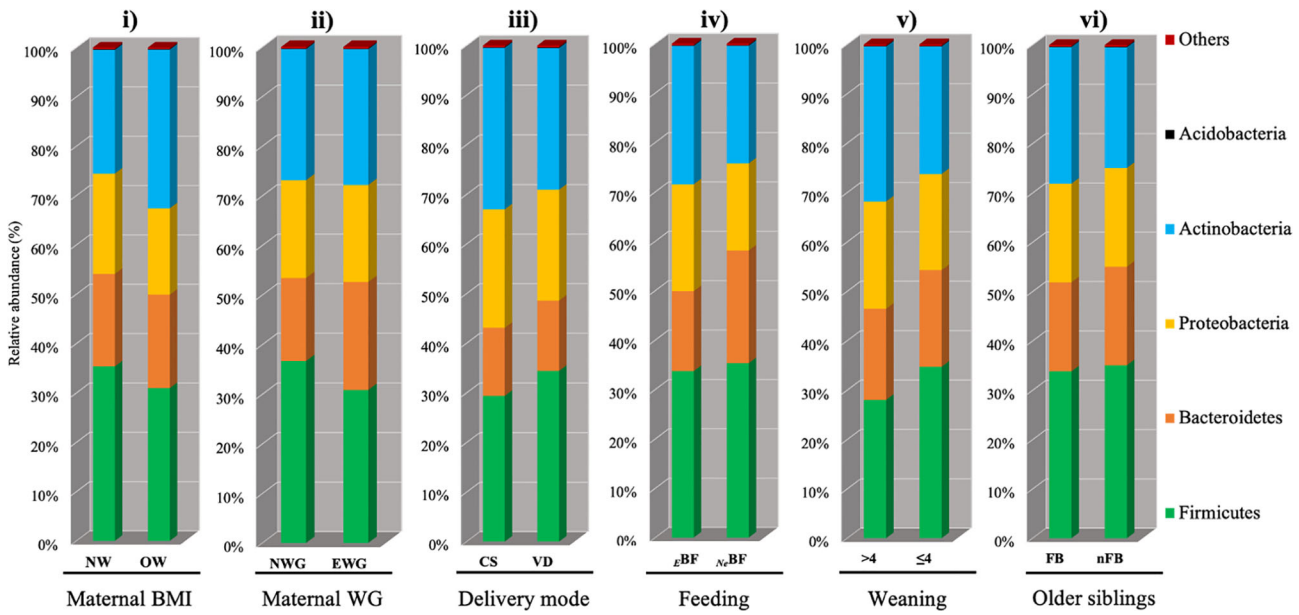

FIGURE 2 | Influence of different factors on early microbiota composition at the phylum level (16S rRNA gene amplicon sequencing) in T2 fecal samples (infants at 6 months of age). Starting from the left: i) and ii) Maternal factors: maternal pre-pregnancy body mass index (BMI) and gestational weight gain (WG), respectively. NW *[n.37]*: normal pre-pregnancy BMI (BMI < 25 kg/m^2), OW *[n.12]*: excessive pre-pregnancy BMI (BMI ≥ 25 kg/m^2); EWG *[n.20]*: excessive gestational WG, NWG *[n.29]*: optimal gestational WG). iii) Delivery mode (CS *[n.7]*: caesarean section, VD *[n.46]*: vaginal delivery). iv) Feeding ($_{E}$BF *[n.31]*: exclusive breast-feeding, NeBF *[n.21]*: not exclusively breast fed, mixed fed or exclusively formula fed). v) Weaning (≤4 *[n.41]*: before 4 months of age, >4 *[n.9*: after 4 months of age). vi) Presence of older siblings (FB *[n.30]*: first-born, nFB *[n.22]*: not first-born).

TABLE 1 | Statistically different phyla, families, and genera (16S rRNA gene amplicon sequencing) found in fecal samples of infants at 6 months of age (T2).

Taxon	metadata	feature	value	coef	Stderr	N	pval	qval	[factor] IQR (median)	[factor] IQR (median)
									[CS]	**[VD]**
Phylum	**Delivery**	Bacteroidetes	VD	0.85	0.36	53	0.021	0.209	0.38–0.87 (0.45)	0.44–37.1 (14.86)
									(FB)	**(nFB)**
Family	**Firstborn**	*Clostridiales Incertae Sedis XIII*	nFB	–0.12	0.03	53	0.001	0.108	0.01–0.01 (0.01)	0.00–0.01 (0.01)
	Firstborn	*Clostridiaceae*	nFB	–0.50	0.16	53	0.003	0.130	0.31–4.06 (0.81)	0.19–0.55 (0.23)
	Firstborn	*Peptostreptococcaceae*	nFB	–0.62	0.19	53	0.002	0.130	0.04–0.88 (0.32)	0.02–0.08 (0.04)
	Firstborn	*Planctomycetaceae*	nFB	0.10	0.03	53	0.003	0.130	*ND**	0.00–0.01 (0.00)
									[$_{E}$BF]	**[$_{Ne}$BF]**
	Feeding	*Ruminococcaceae*	**$_{Ne}$BF**	0.63	0.15	53	<0.0001	0.011	0.12–0.22 (0.15)	0.19–2.19 (1.41)
Genus	**Delivery**	*Propionibacterium*	VD	–0.22	0.05	53	<0.001	0.031	**[CS]**	**[VD]**
									0.00–0.01 (0.00)	*ND**
	Delivery	*Thiomicrospira*	VD	–0.15	0.04	53	<0.001	0.031	0.00–0.01 (0.01)	*ND**
	Delivery	*Streptacidiphilus*	VD	–0.23	0.07	53	0.002	0.210	0.00–0.02 (0.01)	0.00–0.01 (0.00)
									[$_{E}$BF]	**[$_{Ne}$BF]**
	Feeding	*Flavonifractor*	**$_{Ne}$BF**	0.99	0.25	53	<0.001	0.112	0.00–0.01 (0.00)	0.01–1.25 (0.06)
	Feeding	*Erysipelotrichaceae Incertae Sedis*	**$_{Ne}$BF**	0.79	0.25	53	0.003	0.218	0.01–0.01 (0.01)	0.01-1.24 (0.09)
	Feeding	*Romboutsia*	**$_{Ne}$BF**	0.59	0.18	53	0.002	0.218	0.00–0.01 (0.00)	0.00–0.22 (0.02)
	Feeding	*Staphylococcus*	**$_{Ne}$BF**	–0.51	0.17	53	0.003	0.218	0.01–0.15 (0.02)	0.00–0.01 (0.01)
	Feeding	*Faecalicoccus*	**$_{Ne}$BF**	0.21	0.06	53	0.002	0.218	*ND**	0.00–0.01 (0.00)
	Feeding	*Sulfurimonas*	**$_{Ne}$BF**	0.15	0.05	53	0.003	0.218	0.00–0.01 (0.00)	0.01–0.01 (0.01)
	Feeding	*Clostridium_IV*	**$_{Ne}$BF**	0.47	0.16	53	0.005	0.232	0.01–0.04 (0.01)	0.02–0.11 (0.06)
	Feeding	*Oribacterium*	**$_{Ne}$BF**	0.22	0.08	53	0.005	0.232	0.01–0.02 (0.01)	0.02–0.03 (0.02)

*VD, vaginal delivery; CS, caesarean section; $_{E}$BF, exclusive breastfed; $_{Ne}$BF, combined or exclusive formula-feeding; FB, first-born; nFB, not first-born; IQR, interquartile range (25th–75th percentile). *ND: not detected within all samples belonging to the considered group.*

significant difference. For this reason, we developed a statistical analysis using as fixed metadata only postnatal factors (mode of delivery, type of feeding, weaning, and the presence of older siblings). Among families, it was confirmed that *Ruminococcaeae* positively correlated with $_{Ne}$BF infants (**Figure 3**). Additionally, five families were negatively associated with vaginally delivered infants: *Cellulomonadaceae*, *Corynebacteriaceae*, *Actinomycetaceae*, *Streptomycetaceae*, and *Micromonosporaceae* (**Figure 3**). The presence of *Colwelliaceae* was negatively associated with infants weaned before 4 months of age. Meanwhile, *Staphylococcaceae* and *Clostridiales Incertae Sedis XIII* were negatively associated with nFB gut microbiota (**Figure 3**).

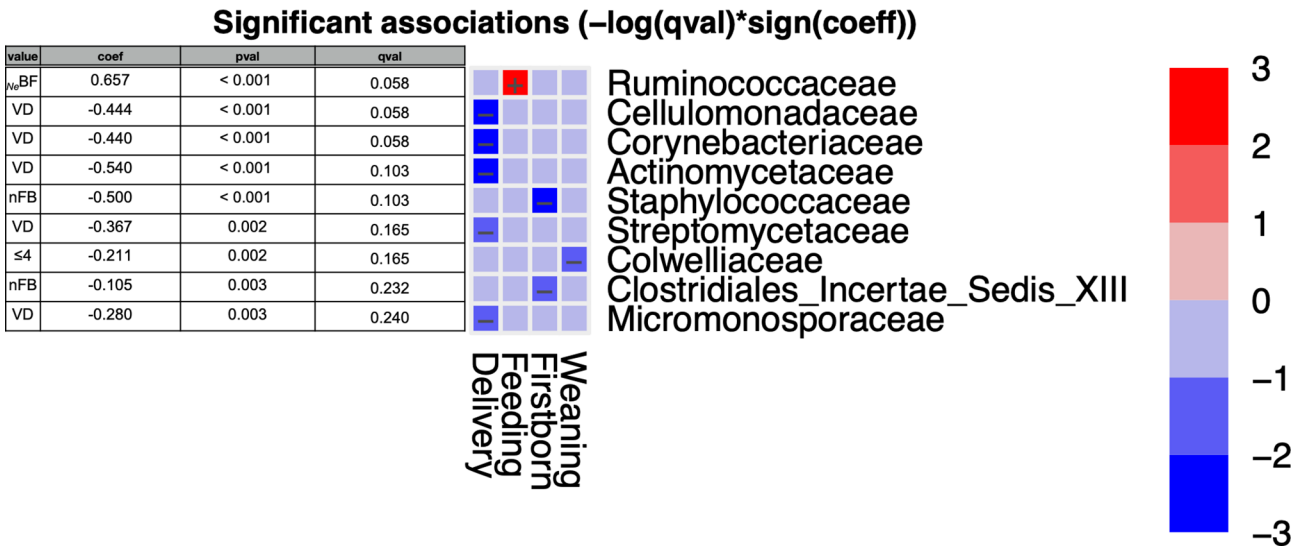

FIGURE 3 | MaAsLin2 significant results and associations between postnatal factors (delivery, feeding, weaning, and presence of older siblings) and gut microbiota composition at the family level of infants at six months of age (T2). Based on normalized obtained significant results, the color scale-bar showed a positive relationship (red) and a negative one (blue) between taxa and factors, ranging from the highest positive normalization (+3) to the lowest one (–3).

However, all the aforementioned results did not confirm the significance for adjusted p-values ($P < 0.05$; qval > 0.05).

Among $_{Ne}$BF (**Figure 4**), *Flavonifractor* exhibited a trend similar to *Ruminococcaceae* in $_{Ne}$BF. Moreover, three genera were negatively associated with vaginally delivered infants (*Corynebacterium*, *Propionibacterium*, and *Streptacidiphilus*), while *Staphylococcus* was negatively associated with nFB microbiota. Otherwise, as reported in family results, also at the genus level the significance was not confirmed for adjusted p-values ($P < 0.05$; qval > 0.05).

Multivariate Analyses

To compare infant microbiota composition from birth to six months of age, we performed a multivariate analysis (PCA) between T2 and baseline T0 (*meconium*) samples. We analyzed maternal pre-pregnancy BMI (**Figure 5A**), maternal gestational WG (**Figure 5B**), and delivery type (**Figure 5C**), using as variables the genera with a median relative abundance greater than 0.1% at least in one of the sampled times (T0 or T2).

As shown by the PCA group ellipses, in all of the three considered conditions, time of sampling impacts deeper on sample stratification than on related metadata (BMI, WG, and delivery). Among the variables, *Fusobacterium*, *Leptotrichia*, *Mycobacterium*, *Serratia*, and *Rothia* had a deeper impact on T0-sample spatial distribution, whereas the genera *Bifidobacterium*, *Siccibacter*, *Photorabdus*, *Veillonella*, and *Vibrio* mainly characterized infants' gut microbiota at T2.

Considering both T0 groups (NW and OW), the presence of four samples (three NW and one OW), that seem to be outliers, determined a not complete overlapping of both T0 clouds. On the other side, *Bifidobacterium*, *Siccibacter*, and *Photorabdus* relative abundances contributed to shifting a subset of NW samples at T2, therefore, determining elongation of the NW cloud (**Figure 5A**).

We also observed that at both times T0 and T2 the ellipses of NWG and EWG overlapped, and therefore, no PCA differences were associated with weight gain (**Figure 5B**).

Considering the type of delivery, CS and VD samples exhibited a more heterogeneous microbiota at T0, whereas at T2 the two delivery conditions allowed the construction of reduced and almost completely overlapped clouds (**Figure 5C**).

Therefore, a multivariate analysis was also run only on T2 fecal sample-set (infants at 6 months of age) performing a PCA and using bacterial genera (16S rRNA gene amplicon sequences) with a median value of relative abundance greater than 0.1% and therefore contributing to describe at least 50% of the whole population, resulting in 17 bacterial genera (**Figure S1b**). The two PCA principal components (Dim1 and Dim2; **Figure S1a**) described 52% of the total variance. The cos2 graduated scale values computed with R factoextra package described the quality of the sample. The best PCA sample quality is determined by high cos2 relative values. Observing the homogeneity of the sample in the PCA score plot where only a few linear distances marked some samples as outliers, it has been decided to perform another and more sensitive multivariate analysis. To understand how the aforementioned factors (maternal and diet-related ones, as well as environmental ones) affected infants' early microbial colonization, the samples were clustered. By using the same filtered (median > 0.1%) set of genera, (whose vectors have been plotted in PCA biplot pf samples and variables, **Figure S1b**), discriminant analysis of the principal components (DAPC) was considered. Hence, a DAPC without superimposing any *a priori* condition was performed. As a result of the best fit cluster number identification, the "find.clusters" R function provided that four was the best-supported cluster number for the sample set. This information was used to run the DAPC. In the DAPC scatter plot (**Figure S2**) only cluster "4" was poorly populated (three samples), while the other three clusters (1, 2, and 3) included at least nine samples and were all placed into different quarters of the axes.

In a second approach, each sample was assigned the membership group, as *a priori* condition, and the same was repeated for all metadata. The tested conditions (both maternal factors, type of delivery, dietary features, and presence of older

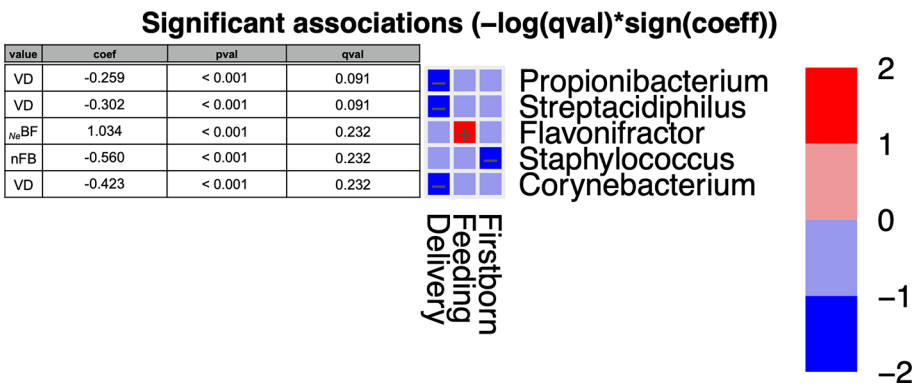

FIGURE 4 | MaAsLin2 significant results and associations between postnatal factors (delivery, feeding, weaning, and presence of older siblings) and gut microbiota composition at the genus level of infants at six months of age (T2). Based on normalized obtained significant results, the color scale-bar showed a positive relationship (red) and a negative one (blue) between taxa and factors, ranging from the highest positive normalization (+2) to the lowest one (–2).

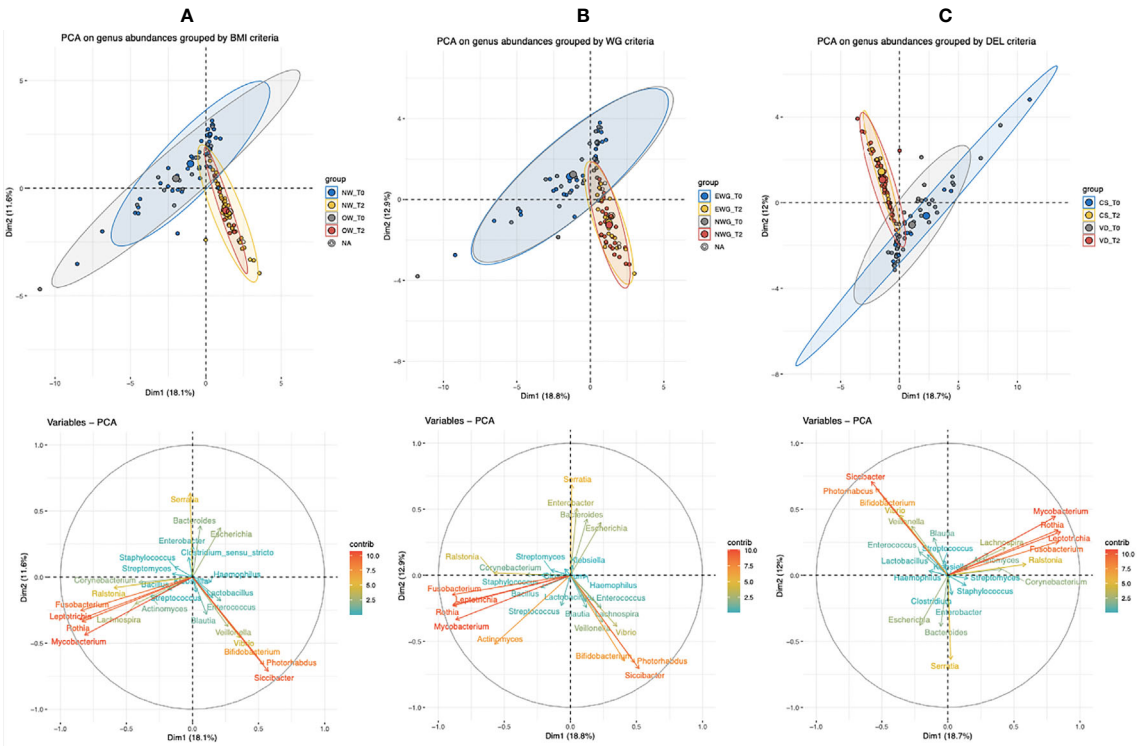

FIGURE 5 | Principal component analysis (PCA) of bacterial genera with a median abundance >0.1% (16S rRNA gene amplicon sequencing) of infants' T0 (meconium) and T2 (six months of age). Infants were grouped based on the relative metadata: **(A)** maternal pre-pregnancy BMI (NW, normal BMI, or OW, mothers with overweight/obesity), **(B)** maternal gestational weight gain (NWG, normal WG, or EWG, excessive WG), and **(C)** type of delivery (VD, vaginally delivered, or CS, by caesarean section).

sibling) resulted in the configuration of overlapping clusters (**Figures 6A–F**). Looking at the DAPC score plot concerning maternal BMI (**Figure 6A**), it was possible to observe that the samples belonging to NW were more divergent among themselves, ensuing an enlargement of the ellipse and, therefore, determining a partial overlapping on the OW-ellipse. Otherwise, none of the NW samples was assigned to the OW group; specifically 66% of OW samples should be included in the NW group (**Figure 6A1**).

Similarly, when evaluating the WG variable, we found that the EWG ellipse was included in the NWG one (**Figure 6B**). For 40% of the EWG samples, the *a priori* assignment was not verified, thus implying a better fit to the NWG group. On the opposite, 17% of EWG was not verified as part of the *a priori* assigned group (**Figure 6B1**).

The analysis based on the delivery mode reported a divergence of the ellipses of VD and CS (**Figure 6C**), displaying the relative centers in a different quarter of the DAPC system. Evaluating the "assign plot" scores in five out of seven CS samples, we observed a better fit to the VD group, while DAPC K-means analyses membership was not confirmed only in 6.5% of VD samples (**Figure 6C1**).

Concerning the feeding, the percentages of overlapping were only 19% in $_{E}$BF, whereas 50% of $_{Ne}$BF looked like they were fitted to the $_{E}$BF group (**Figure 6D1**); meanwhile, seven out of nine infants weaned after 4 months of life might be included in the ≤4 group and no one of this second group (≤4) showed a controversial membership (**Figure 6E1**).

We also analyzed the presence/absence of older siblings considering the "hygiene hypothesis" (Strachan, 1989). For these superimposed groups, the ellipsoid centers of the two clusters (FB and nFB) were placed into different plot quarters (**Figure 6F**). Also, by inspecting the relative "assign plot" sample membership probabilities, we observed the *a priori* clustering condition was not verified in 23 and 32% of FB and nFB, respectively (**Figure 6F1**).

DISCUSSION

A great relevance is widely assigned to the gastrointestinal (GI) microbiome composition's impact on health (Young, 2017), and it is well known that early microbial colonization could significantly contribute to long-term healthy and unhealthy consequences during lifespan (Tamburini et al., 2016). Infant GI microbiota colonization is crucial for healthy growth and is primarily involved in gut maturation and immune system development; indeed, altered colonization has been associated

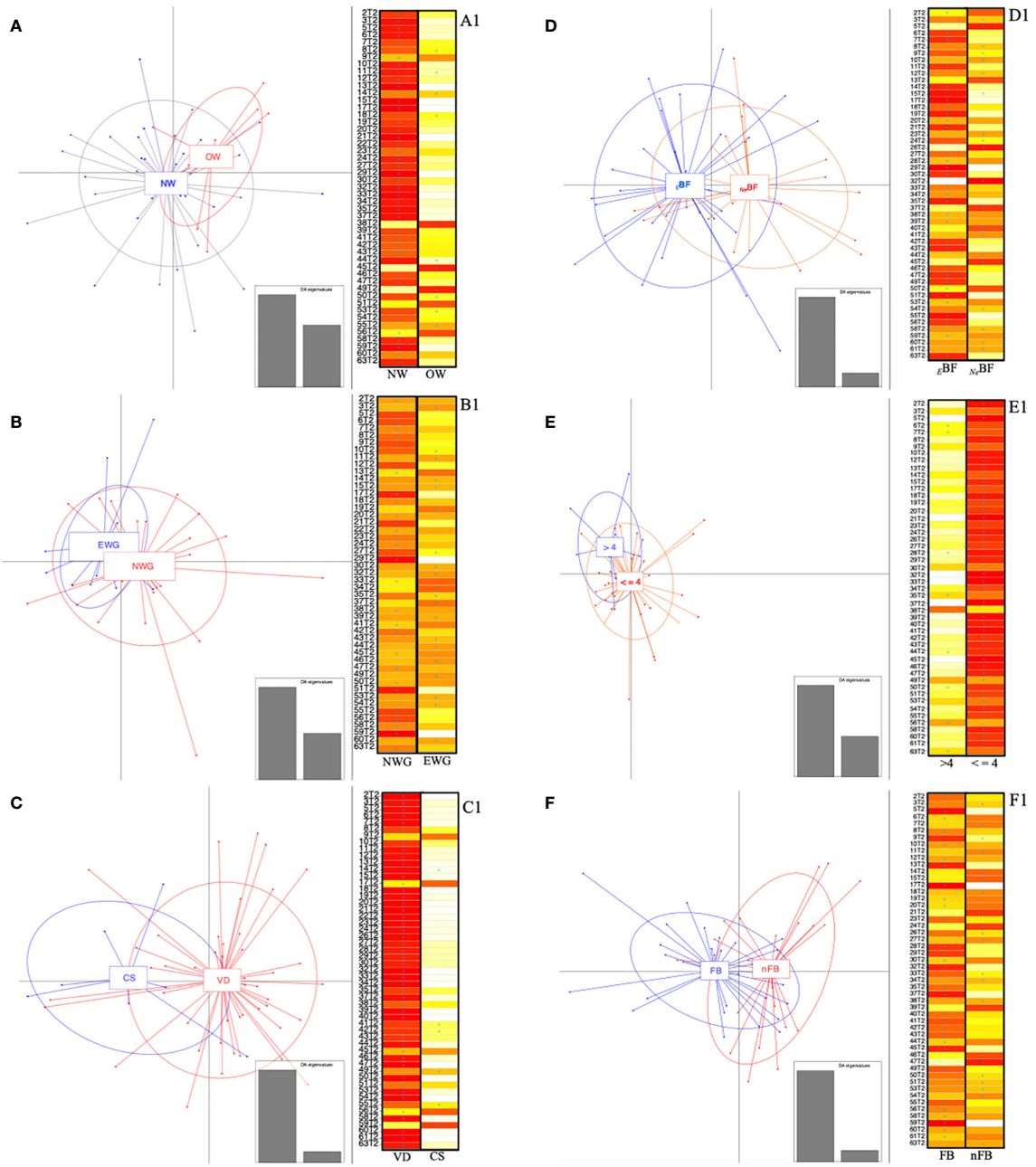

FIGURE 6 | Discriminant analysis of principal components (DAPC) and relative score-plots. **(A–F)** report the DAPC score plots based on the relative abundances (16S rRNA gene amplicon) of genera with median values >0.1% found in infants at 6 months. Panel: **(A)** NW, normal BMI (<25 kg/m^2); OW, women overweight/obesity (BMI ≥ 25 kg/m^2); **(B)** NWG, optimal weight gain; EWG, excessive weight gain; **(C)** VD, vaginally delivered; CS, delivered by cesarean section; **(D)** BF, exclusive breast-feeding; FF, combined or exclusive formula-feeding; **(E)** ≤4, infants weaned before 4 months of age; >4, infants weaned after 4 months of age. Panel **(F)** FB, first-born; nFB, not first-born. **(A1–F1)** respectively indicate whether the infants (rows) could be assigned based on discriminant functions by K-means analysis. The cell colors represent the membership probabilities (K-means analysis) to belong to each cluster (red = 1, orange = 0.75, yellow = 0.25, white = 0) and blue crosses indicate the originally belonging cluster.

with a high risk of disease outbreaks later in life (Stiemsma and Michels, 2018). In this line, the present longitudinal and observational study was aimed at evaluating the influence of maternal factors (pre-pregnancy BMI and gestational WG), perinatal factors such as type of delivery, infant's diet (the type of feeding and weaning timing), and the presence/absence of older siblings in the household, on GI microbiota composition of babies after six months of life (T2).

Concerning the prenatal factors, such as maternal pre-pregnancy BMI and gestational WG, evidence showed that

children born from mothers with overweight/obesity have an increased risk to develop obesity during their life (Ajslev et al., 2011) even if the factors behind this relationship are not fully understood. In our study, sampling infants at 6 months of age, we did not find differences in alpha diversity according to maternal factors (pre-pregnancy BMI and gestational WG). Additionally, we detected only partial and not significant differences in Firmicutes and Bacteroidetes abundances in offspring in line with previous studies, which reported no difference in F/B ratio in obese *versus* lean humans and rodents (Duncan et al., 2008; Jumpertz et al., 2011; Zhang et al., 2012). Other evidence observed Firmicutes overabundant in obese mice (Ley et al., 2005) and that microbiota of obese mice and lean littermates encoded different metabolic pathways (Turnbaugh et al., 2006). On the contrary, Collado and coworkers observed high abundances of Bacteroidetes subtaxa in overweight mothers and relative offspring (Collado et al., 2008; Collado et al., 2010); therefore, we emphasize that the reason stands behind the deeper taxonomic levels (*i.e.*, genus and species), where taxa and relative genes directly contribute to obesity onset.

In line with Collado et al., a significant overabundance of *Parabacteroides* and *Bacteroides* in fecal samples of children born from mothers with an excessive BMI has been reported also in another study (Cerdò et al., 2018). Both these Bacteroidetes genera are microbes that may frequently be vertically transmitted from mothers to offspring (Nayfach et al., 2016; Li et al., 2020), and for this reason, the presence of these taxa in the maternal GI microbiota may affect their presence also in newborns (Collado et al., 2010). Considering vertical transmission of microbes, we found that Bacteroidetes mainly characterized the gut microbiota of vaginally delivered infants. As Jakobsson et al. reported (Jakobsson et al., 2014), vaginally delivered infants were more characterized by high abundance of Bacteroidetes and relative subtaxa (*e.g.*, *Bacteroides*) than CS delivered ones. Interactions between Bacteroidetes and host seem to be crucial at the beginning of life due to the fact that *Bacteroides* may be able to correct the presence of the underdeveloped immune system in germ-free mice (Ivanov et al., 2008).

Overall, the main key factor able to improve and boost up the immune system certainly is exclusive breastfeeding. The World Health Organization (WHO) recommends that infants should be exclusively breastfed during the first 6 months of life (Butte et al., 2002) for the beneficial effects of breastfeeding on the immune system programming (Laouar, 2020). In the present study, $_{Ne}$BF samples reported higher values of Shannon index compared to $_{E}$BF ones. In adults' microbiota, high values of alpha diversity are positively related to ecosystem resistance, resilience, and health (Shade et al., 2012); however, in the early phases of life, it has been previously observed that formula-feeding determines high values of alpha diversity indices (Bezirtzoglou et al., 2011; Fan et al., 2013). Breastfed infants, indeed, are principally characterized by *Bifidobacterium* species (Gueimonde et al., 2007); therefore, their presence markedly affects, reducing, alpha diversity (Bezirtzoglou et al., 2011). This class of probiotics is markedly able to metabolize HMOs or HMO constituents (Duranti et al., 2019), prevailing on other taxa, and this is the power of human milk feeding. In the present study, we did not observe a high abundance of *Bifidobacteriaceae* (or relative subtaxa) in $_{E}$BF infants, probably due to the fact that our $_{Ne}$BF group included also infants fed with combine feeding (mixed feeding, *i.e.*, formula and breast milk). This condition could have reduced the clustering of our samples based on the type of feeding. However, despite this possible overlapping, we observed that our $_{Ne}$BF group was mainly characterized by high abundances of *Ruminococcaceae*, in particular *Flavonifractor*, and taxa assigned to the *Clostridium cluster IV*. This is in line with previous studies (Fan et al., 2013; Tannock et al., 2013; Sagheddu et al., 2016; Durrani et al., 2020), in which *Ruminococcacae* or *Lachnospiraceae* has been reported to be higher in the absence of competition with *Bifidobacteriaceae*, replacing them as butyrogenic bacteria in the gut environment of $_{Ne}$BF babies (Bui et al., 2020; Vacca et al., 2020). At the genus level, the high abundances of *Ruminococcaceae* determined a positive association between $_{Ne}$BF and *Flavonifractor*. The presence of *Flavonifractor* in $_{Ne}$BF infants has been previously found in different manuscripts (Zheng et al., 2016; Borewicz et al., 2019). This genus has been associated with a high level of circulating cytokines (Huang et al., 2019) and fecal microbiota of infants with food allergies (Ling et al., 2014). Recently, Bui et al. described an enrichment of the microbial pathways able to degrade *Nε*-fructosyllysine in stools of formula-fed infants, whereas fecal microbiota of exclusively breastfed was not able to grow on *Nε*-fructosyllysine medium (Bui et al., 2020). The authors also observed that in the gut microbiota of $_{E}$BF infants there was a lack of *Intestinimonas–Flavonifractor–Pseudoflavonifractor* group, a clade that mainly characterized stools of formula-fed infants. The transfer of the *Nε*-fructosyllysine/lysine pathway genes seems to be vertical, from mothers to offspring (Yassour et al., 2016), but the selective outgrowth of *Nε*-fructosyllysine/lysine-fermenting microorganisms predominated in formula-fed infants, suggesting that the type of milk might influences their outgrowth. We also found a positive association between $_{Ne}$BF and *Erysipelotrichaceae Incertae Sedis* (**Table 1**). In previous studies, also this taxon has been reported mainly in formula-fed gut microbiota (Wang et al., 2015; Borewicz et al., 2019) probably acting as *Ruminococcaceae* and *Lachnospiraceae* in the butyrate metabolism (Vital et al., 2017). Similar to *Flavonifractor*, Erysipelotrichaceae *Incertae Sedis* is likely linked to adverse outcomes concerning asthma and allergy later development (Dzidic et al., 2017).

Concerning weaning, previous research studies have shown that timing of solid food introduction likely plays a relevant role in the development of childhood overweight and obesity, with an increased risk when weaning process starts before 4 months of age (Wang et al., 2016; Differding et al., 2020). However, the aforementioned differences have been observed in babies aged at least 2 years. In our study on younger ones, we did not find any statistically significant difference resulting from an early introduction of solid foods. That result could undoubtedly emerge from the different feeding types (exclusive breast-, mixed-, or exclusive formula feeding) in the first six months of life, determining overlap in the microbiota composition. However, in our opinion, early weaning does not define any

advantage in terms of GI microbiota maturation at six months of age. Therefore, considering the literature cited above, breastfeeding remains the gold standard for optimal nutrition in the first six months of age and microbiota shaping interests.

As regards early microbiota colonization, other research studies have shown some differences according to the presence/absence of older siblings in the household. Starting from the "hygiene hypothesis" (Strachan, 1989), different hypothesis focused on the impact of exposure to infections during the first years on aberrant immune responses later in life. Strachan observed that the presence of older siblings in the household decreased the risk to develop allergies in infants (Strachan, 2000). According to the results so far collected, in our study, babies with an older sibling at six months of age displayed lower amounts ($P<0.05$) of *Clostridiaceae*, *Peptostreptococcaceae*, *Clostridiales Incertae Sedis XIII*, clusters that typically colonize the gut lumen in the first days after birth (Penders et al., 2014; Milani et al., 2017). Other evidence reported an early maturation of the microbial colonization mediated by the "adult"-associated genus *Faecalibacterium* (Laursen et al., 2017). Therefore, we suggest that the presence of older siblings in the household contributes to expose infants to "other and new" environmental bacterial patterns and indirectly reduces the abundances of the neonatal microbial colonizer. Analyzing all postnatal factors and their effects on the gut microbial composition, both *Staphylococcaceae* and *Staphylococcus* were reported to have a negative and significant relationship with nFB (**Figures 3** and **4**). *Staphylococcus* is an early gut colonizer in neonates and in particular in BF infants (Balmer and Wharton, 1989); thus, the presence of older siblings seems to influence an early shift towards an "adult" microbiota profile in nFB compared to FB.

Taking into account the low power of the considered variables in clustering our samples when T0 and T2 were compared (**Figure 5**) to discern if some factors determined a clear microbial clustering, we also run a multivariate analysis only on T2 samples. According to maternal factors, we observed that both BMI and WG reported the greatest overlapping of the relative ellipses (**Figures 6A, B**). Based on DAPC results, infants lost or reduced the significant relevance of prenatal features probably because of the postnatal events that occurred in the last 6 months of life. Also, the type of delivery did not show great strength in clustering our samples despite interesting results linked to over-presence of Bacteroidetes in VD were found. For those reasons the programmed T3 sampling of the A.MA.MI project (12 months of life of infants) will be essential to increase evidence of long-term effects linked to this finding.

As expected, the main finding obtained from the sampling of the gut microbiota of six-month-old babies is related to dietary features. One of the limits of our study is that the $_{Ne}$BF group includes both exclusive and combined formula-fed infants; therefore we were not able to observe how exclusive breastfeeding could be able to improve the gut environment harboring or could suppress specific bacterial patterns; we could only assume this effect by looking at alpha diversity findings. Indeed, it is clear how feeding has great relevance in this transitional time point, highlighting these primary results. Although *Ruminococcaceae* are often associated with beneficial outcomes (Liu et al., 2020) based on their roles in butyrate metabolism in adults (Biddle et al., 2013), their presence in our cohort was not necessarily beneficial. Thus, the different and widely reported involvement of *Bifidobacteriaceae* in gut health remains a key factor to be considered in formula feeding.

Further, considering that we have studied infants only until six months of age, we cannot give evidence about a positive or negative contribution of early weaning in the infant's gut microbiota.

Lastly, also DAPC performance based on the presence of older siblings showed interesting findings. Although no apparent clustering was found, DAPC determined a cluster separation of two groups (FB and nFB) into different system planes and associated admissible overlapping percentages with parents' care. Thus, it is easily presumed that, although parents of firstborns could be more apprehensive, the attention and care provided are strictly correlated to the family's cultural level and life experience. Moreover, the indirect contribution deriving from older siblings is not negligible under the point of view of the household "contamination" with other microbes.

Our study has some important strengths. First of all, our research was not based only on single-factor analysis able to shape early microbial composition. We adopted a multifactorial approach, taking into account the main prenatal and postnatal factors, providing a more extensive framework on this critical window of plasticity. Data analysis and result interpretation required multidisciplinary expertise in microbiology, clinical nutrition, pediatrics and biostatistics science, with a well-integrated approach. Nonetheless, some limitations need to be acknowledged. Only infant GI microbiota, and not maternal one, was analyzed. This determined a partial loss in fully interpreting the results, particularly those linked to maternal pre-pregnancy BMI, gestational WG, and the mode of delivery. Additionally, we did not investigate maternal antibiotic therapy during lactation, which might have influenced infant GI microbiota colonization. Lastly, the sample size was small and did not allow us to create homogenous groups for all the considered variables.

CONCLUSION

The first year of life is crucial for healthy growth; several factors affect gut microbiota development in newborns in this critical time window. Prenatal factors and postnatal ones directly contribute to infants' gut microbiota maturation determining possible disease outbreaks later in life. The type of milk feeding undoubtedly has a pivotal role in our population, preserving different microbiota composition after at least two months from solid food introduction. Although we found some differences in grouping samples considering maternal factors, diet-related ones, and presence/absence of older siblings, these did not help to identify specific actors to determine an absolute clustering of the belonging samples. Undoubtedly, exclusive breastfeeding preserved the gut microbiota composition mainly characterized by bifidobacteria, a condition that a formula feeding, both exclusive and combined, did not harbor. The risks associated

with overgrowth of specific and not beneficial taxa at six months of life could indirectly contribute to future disease onset, therefore determining adverse long-term effects.

Our findings contribute to add evidence of the complex multifactorial interaction of different maternal and neonatal factors on GI microbiota composition at this age. Based on these considerations, the programmed "at one year"-sampling becomes essential to bring evidence on how the different prenatal and postnatal factors drive the intestinal microbiota.

DATA AVAILABILITY STATEMENT

The data are available in the NCBI database: http://www.ncbi.nlm.nih.gov/bioproject/675753; https://www.ncbi.nlm.nih.gov/sra/PRJNA675753, BioProject ID: PRJNA675753.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Ethics Committee (EC) of Fondazione IRCCS Policlinico S. Matteo of Pavia. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

BR and MV equally contributed to the conception and design of the study, results' interpretation, and drafted the manuscript. DP and RD contributed to the conception, design of the study, results' interpretation, and revised the manuscript. MC and ML contributed to results' interpretation and revised the manuscript. RC, EC, and FG, contributed to the conception and design of the study and revised the manuscript. MD, MV, and FC conducted the statistical analysis, interpreted results, and revised the manuscript. HC drafted, revised, and approved the final version of the manuscript.

All authors have read and approved this version of the manuscript and declare that the content has not been published elsewhere. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.590202/full#supplementary-material

REFERENCES

Ajslev, T. A., Andersen, C. S., Gamborg, M., Sørensen, T. I. A., and Jess, T. (2011). Childhood overweight after establishment of the gut microbiota: the role of delivery mode, pre-pregnancy weight and early administration of antibiotics. *Int. J. Obes.* 35 (4), 522–529. doi: 10.1038/ijo.2011.27

Azad, M. B., Konya, T., Maughan, H., Guttman, D. S., Field, C. J., Sears, M. R., et al. (2013). Infant gut microbiota and the hygiene hypothesis of allergic disease: impact of household pets and siblings on microbiota composition and diversity. *Allergy Asthma Clin. Immunol.* 9 (1), 15. doi: 10.1186/1710-1492-9-15

Balmer, S. E., and Wharton, B. A. (1989). Diet and faecal flora in the newborn: breast milk and infant formula. *Arch. Dis. Child.* 64 (12), 1672–1677. doi: 10.1136/adc.64.12.1672

Bezirtzoglou, E., Tsiotsias, A., and Welling, G. W. (2011). Microbiota profile in feces of breast-and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe* 17 (6), 478–482. doi: 10.1016/j.anaerobe.2011.03.009

Biddle, A., Stewart, L., Blanchard, J., and Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. *Diversity* 5 (3), 627–640. doi: 10.3390/d5030627

Borewicz, K., Suarez-Diez, M., Hechler, C., Beijers, R., de Weerth, C., Arts, I., et al. (2019). The effect of prebiotic fortified infant formulas on microbiota composition and dynamics in early life. *Sci. Rep.* 9 (1), 1–13. doi: 10.1038/s41598-018-38268-x

Bui, T. P. N., Troise, A. D., Nijsse, B., Roviello, G. N., Fogliano, V., and de Vos, W. M. (2020). Intestinimonas-like bacteria are important butyrate producers that utilize Nε-fructosyllysine and lysine in formula-fed infants and adults. *J. Funct. Foods* 70, 103974. doi: 10.1016/j.jff.2020.103974

Butte, N. F., Lopez-Alarcon, M. G., Garza, C.& on the Optimal Duration of ExclusiveBreastfeeding (2001:Geneva, S.) (2002). Nutrient adequacy of exclusive breastfeeding forthe term infant during the first six months of life / Nancy F. Butte, Mardia G. Lopez-Alarcon, Cutberto Garza. *World Health Organ*. https://apps.who.int/iris/bitstream/handle/10665/42519/9241562110.pdf

Catalano, P. M., and Shankar, K. (2017). Obesity and pregnancy: mechanisms of short termand longterm adverse consequences for mother and child. *BMJ* 360:j1.

Cerdó, T., Ruiz, A., Jáuregui, R., Azaryah, H., Torres-Espínola, F. J., García-Valdés, L., et al. (2018). Maternal obesity is associated with gut microbial metabolic potential in offspring during infancy. *J. Physiol. Biochem.* 74 (1), 159–169. doi: 10.1007/s13105-017-0577-x

Chen, C., Xu, X., and Yan, Y. (2018). Estimated global overweight and obesity burden in pregnant women based on panel data model. *PloS One* 13 (8), e0202183. doi: 10.1371/journal.pone.0202183

Collado, M. C., Isolauri, E., Laitinen, K., and Salminen, S. (2008). Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. *Am. J. Clin. Nutr.* 88 (4), 894–899. doi: 10.1093/ajcn/88.4.894

Collado, M. C., Isolauri, E., Laitinen, K., and Salminen, S. (2010). Effect of mother's weight on infant's microbiota acquisition, composition, and activity during early infancy: a prospective follow-up study initiated in early pregnancy. *Am. J. Clin. Nutr.* 92 (5), 1023–1030. doi: 10.3945/ajcn.2010.29877

Collado, M. C., Cernada, M., Baüerl, C., Vento, M., and Pérez-Martínez, M. (2012). Microbial ecology and host-microbiota interactions during early lifestages. *Gut Microbes* 3 (4), 352–365. doi: 10.4161/gmic.21215

Deierlein, A. L., Siega-Riz, A. M., Chantala, K., and Herring, A. H. (2011). The association between maternal glucose concentration and child BMI at age 3 years. *Diabetes Care* 34 (2), 480–484. doi: 10.2337/dc10-1766

Differding, M. K., Doyon, M., Bouchard, L., Perron, P., Guérin, R., Asselin, C., et al. (2020). Potential interaction between timing of infantcomplementary feeding and breastfeeding duration in determination of early childhood gut microbiota composition and BMI. *Pediatr. Obes.* 15 (8), e12642. doi: 10.1111/ijpo.12642

Duncan, S. H., Lobley, G. E., Holtrop, G., Ince, J., Johnstone, A. M., Louis, P., et al. (2008). Human colonic microbiota associated with diet, obesity and weight loss. *Int. J. Obes.* 32 (11), 1720–1724. doi: 10.1038/ijo.2008.155

Duranti, S., Lugli, G. A., Milani, C., James, K., Mancabelli, L., Turroni, F., et al. (2019). Bifidobacterium bifidum and the infant gut microbiota: an intriguing case of microbe-host co-evolution. *Environ. Microbiol.* 21 (10), 3683–3695. doi: 10.1111/1462-2920.14705

Durrani, M., Nazli, R., Fatima, S., and Bakr, M. A. (2020). Impact of feeding practice on diversity pattern of the gut microbiome in infants. *J. Ayub Med. Coll. Abbottabad* 32 (4).

Dzidic, M., Abrahamsson, T. R., Artacho, A., Björkstén, B., Collado, M. C., Mira, A., et al. (2017). Aberrant IgA responses to the gut microbiota during infancy precede asthma and allergy development. *J. Allergy Clin. Immunol.* 139 (3), 1017–1025. doi: 10.1016/j.jaci.2016.06.047

Fan, W., Huo, G., Li, X., Yang, L., Duan, C., Wang, T., et al. (2013). Diversity of the intestinal microbiota in different patterns of feeding infants by Illumina high-throughput sequencing. *World J. Microbiol. Biotechnol.* 29 (12), 2365–2372. doi: 10.1007/s11274-013-1404-3

Flegal, K. M., Carroll, M. D., Kit, B. K., and Ogden, C. L. (2012). Prevalence of obesity and trends in the distribution of body mass index among US adults 1999-2010. *JAMA* 307 (5), 491–497. doi: 10.1001/jama.2012.39

Flores, G. E., Caporaso, J. G., Henley, J. B., Rideout, J. R., Domogala, D., Chase, J., et al. (2014). Temporal variability is a personalized feature of the human microbiome. *Genome Biol.* 15 (12), 531. doi: 10.1186/s13059-014-0531-y

Gaillard, R., Durmuş, B., Hofman, A., Mackenbach, J. P., Steegers, E. A., and Jaddoe, V. W. (2013). Risk factors and outcomes of maternal obesity and excessive weight gain during pregnancy. *Obesity* 21 (5), 1046–1055. doi: 10.1002/oby.20088

Gallardo, J. M., Gómez-López, J., Medina-Bravo, P., Juárez-Sánchez, F., Contreras-Ramos, A., Galicia-Esquivel, M., et al. (2015). Maternal obesity increases oxidative stress in the newborn. *Obesity* 23 (8), 1650–1654. doi: 10.1002/oby.21159

Galley, J. D., Bailey, M., Dush, C. K., Schoppe-Sullivan, S., and Christian, L. M. (2014). Maternal obesity is associated with alterations in the gut microbiome in toddlers. *PloS One* 9 (11), e113026. doi: 10.1371/journal.pone.0113026

Glastras, S. J., Chen, H., Pollock, C. A., and Saad, S. (2018). Maternal obesity increases the risk of metabolicdisease and impacts renal health in offspring. *Biosci. Rep.* 38 (2), BSR20180050. doi: 10.1042/BSR20180050

Gohir, W., Whelan, F. J., Surette, M. G., Moore, C., Schertzer, J. D., and Sloboda, D. M. (2015). Pregnancy-related changes in the maternal gut microbiota are dependent upon the mother's periconceptional diet. *Gut Microbes* 6 (5), 310–320. doi: 10.1080/19490976.2015.1086056

Gueimonde, M., Laitinen, K., Salminen, S., and Isolauri, E. (2007). Breast milk: a source of bifidobacteria for infant gut development and maturation? *Neonatology* 92 (1), 64–66. doi: 10.1159/000100088

Ho, N. T., Li, F., Lee-Sarwar, K. A., Tun, H. M., Brown, B. P., Pannaraj, P. S., et al. (2018). Meta-analysis of effects of exclusive breastfeeding on infant gut microbiota across populations. *Nat. Commun.* 9 (1), 1–13. doi: 10.1101/292755

Huang, S., Mao, J., Zhou, L., Xiong, X., and Deng, Y. (2019). The imbalance of gut microbiota and its correlation with plasma inflammatory cytokines in pemphigus vulgaris patients. *Scand. J. Immunol.* 90 (3), e12799. doi: 10.1111/sji.12799

Ivanov, I. I., de Llanos Frutos, R., Manel, N., Yoshinaga, K., Rifkin, D. B., Sartor, R. B., et al. (2008). Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 4 (4), 337–349. doi: 10.1016/j.chom.2008.09.009

Jakobsson, H. E., Abrahamsson, T. R., Jenmalm, M. C., Harris, K., Quince, C., Jernberg, C., et al. (2014). Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. *Gut* 63 (4), 559–566. doi: 10.1136/gutjnl-2012-303249

Jumpertz, R., Le, D. S., Turnbaugh, P. J., Trinidad, C., Bogardus, C., Gordon, J. I., et al. (2011). Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. *Am. J. Clin. Nutr.* 94 (1), 58–65. doi: 10.3945/ajcn.110.010132

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41 (1), e1. doi: 10.1093/nar/gks808

Kumbhare, S. V., Patangia, D. V., Patil, R. H., Shouche, Y. S., and Patil, N. P. (2019). Factors influencing the gut microbiome in children: from infancy to childhood. *J. Biosci.* 44 (2), 49. doi: 10.1007/s12038-019-9860-z

Laouar, A. (2020). Maternal leukocytes and infant immune programming during breastfeeding. *Trends Immunol.* 41 (3), 225–239. doi: 10.1016/j.it.2020.01.005

Laursen, M. F., Zachariassen, G., Bahl, M. I., Bergström, A., Høst, A., Michaelsen, K. F., et al. (2015). Having older siblings is associated with gut microbiota development during early childhood. *BMC Microbiol.* 15 (1), 154. doi: 10.1186/s12866-015-0477-6

Laursen, M. F., Laursen, R. P., Larnkjær, A., Mølgaard, C., Michaelsen, K. F., Frøkiær, H., et al. (2017). Faecalibacteriumgut colonization is accelerated by presence of older siblings. *mSphere* 2, e00448–e00417. doi: 10.1128/mSphere.00448-17

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., and Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11070–11075. doi: 10.1073/pnas.0504978102

Li, W., Tapiainen, T., Brinkac, L., Lorenzi, H. A., Moncera, K., Tejesvi, M., et al. (2020). Vertical transmission of gut microbiome andantimicrobial resistance genes in infants exposed to antibiotics at birth. *J. Infect. Dis.*, 2:jiaa155. doi: 10.1093/infdis/jiaa155

Ling, Z., Li, Z., Liu, X., Cheng, Y., Luo, Y., Tong, X., et al. (2014). Altered fecal microbiota composition associated with food allergy in infants. *Appl. Environ. Microbiol.* 80 (8), 2546–2554. doi: 10.1128/AEM.00003-14

Liu, H., Zhang, M., Ma, Q., Tian, B., Nie, C., and Chen, Z. (2020). Health beneficial effects of resistant starch on diabetes and obesity via regulation of gut microbiota: a review. *Food Funct.* 11 (7), 5749–5767.

Mehta, S. H., Kruger, M., and Sokol, R. J. (2011). Being too large for gestational age precedes childhood obesity in African Americans. *Am. J. Obstet. Gynecol.* 204 (3), 265–2e1. doi: 10.1016/j.ajog.2010.12.009

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., et al. (2017). The first microbial colonizers of the humangut: composition, activities, and health implications of the infant gut microbiota. *Microbiol. Mol. Biol. Rev.* 81 (4), e00036–17. doi: 10.1128/MMBR.00036-17

Moore, R. E., and Townsend, S. D. (2019). Temporal development of the infant gut microbiome. *Open Biol.* 9 (9), 190128. doi: 10.1098/rsob.190128

Nagpal, R., Tsuji, H., Takahashi, T., Nomoto, K., Kawashima, K., Nagata, S., et al. (2017). Ontogenesis of the gut microbiota composition in healthy, full-term, vaginally born and breast-fed infants over the first 3 years of life: A quantitative bird's-eye view. *Front. Microbiol.* 8, 1388. doi: 10.3389/fmicb.2017.01388

Nayfach, S., Rodriguez-Mueller, B., Garud, N., and Pollard, K. S. (2016). An integrated metagenomics pipeline for strain profiling reveals novel patterns of bacterial transmission and biogeography. *Genome Res.* 26 (11), 1612–1625. doi: 10.1101/gr.201863.115

Penders, J., Gerhold, K., Thijs, C., Zimmermann, K., Wahn, U., Lau, S., et al. (2014). New insights into the hygiene hypothesis in allergic diseases: mediation of sibling and birth mode effects by the gut microbiota. *Gut Microbes* 5 (2), 239–244. doi: 10.4161/gmic.27905

Pirkola, J., Pouta, A., Bloigu, A., Hartikainen, A. L., Laitinen, J., Järvelin, M. R., et al. (2010). Risks of overweight and abdominal obesity at age 16 years associated with prenatal exposures to maternal prepregnancy overweight and gestational diabetes mellitus. *Diabetes Care* 33 (5), 1115–1121. doi: 10.2337/dc09-1871

Rasmussen, K. M., and Yaktine, A. L. (2009) Institute of Medicine (US) and National Research Council (US) Committee to Reexamine IOM Pregnancy Weight Guidelines. Weight Gain During Pregnancy: Reexamining the

Guidelines. Rasmussen KM, Yaktine AL, editors. Washington, DC: National Academies Press (US).

Raspini, B., Porri, D., De Giuseppe, R., Chieppa, M., Liso, M., Cerbo, R. M., et al. (2020). Prenatal and postnatal determinants in shaping offspring's microbiome in the first 1000 days: study protocol and preliminary results at one month of life. *Ital. J. Pediatr.* 46, 1–14. doi: 10.1186/s13052-020-0794-8

Sagheddu, V., Patrone, V., Miragoli, F., Puglisi, E., and Morelli, L. (2016). Infant early gut colonization by Lachnospiraceae: High frequency of Ruminococcus gnavus. *Front. Pediatr.* 4, 57. doi: 10.3389/fped.2016.00057

Shade, A., Peter, H., Allison, S. D., Baho, D., Berga, M., Bürgmann, H., et al. (2012). Fundamentals of microbial community resistance and resilience. *Front. Microbiol.* 3, 417. doi: 10.3389/fmicb.2012.00417

Singh, S., Karagas, M. R., and Mueller, N. T. (2017). Charting the maternal and infant microbiome: what is the role of diabetes and obesity in pregnancy? *Curr. Diabetes Rep.* 17 (2), 11. doi: 10.1007/s11892-017-0836-9

Stewart, C. J., Ajami, N. J., O'Brien, J. L., Hutchinson, D. S., Smith, D. P., Wong, M. C., et al. (2018). Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature* 562 (7728), 583–588. doi: 10.1038/s41586-018-0617-x

Stiemsma, L. T., and Michels, K. B. (2018). The role of the microbiome in the developmentalorigins of health and disease. *Pediatrics* 141 (4), e20172437. doi: 10.1542/peds.2017-2437

Strachan, D. P. (1989). Hay fever, hygiene, and household size. *BMJ* 299 (6710), 1259. doi: 10.1136/bmj.299.6710.1259

Strachan, D. P. (2000). Family size, infection and atopy: the first decade of the 'hygiene hypothesis'. *Thorax* 55 (Suppl 1), S2. doi: 10.1136/thorax.55.suppl_1.S2

Tamburini, S., Shen, N., Wu, H. C., and Clemente, J. C. (2016). The microbiome in early life: implications for health outcomes. *Nat. Med.* 22 (7), 713–722. doi: 10.1038/nm.4142

Tannock, G. W., Lawley, B., Munro, K., Pathmanathan, S. G., Zhou, S. J., Makrides, M., et al. (2013). Comparison of the compositions of the stool microbiotas of infants fed goat milk formula, cow milk-based formula, or breast milk. *Appl. Environ. Microbiol.* 79 (9), 3040–3048. doi: 10.1128/AEM.03910-12

Thompson, A. L., Monteagudo-Mera, A., Cadenas, M. B., Lampl, M. L., and Azcarate-Peril, M. A. (2015). Milk-and solid-feeding practices and daycare attendance are associated with differences in bacterial diversity, predominant communities, and metabolic and immune function of the infant gut microbiome. *Front. Cell. Infect. Microbiol.* 5, 3. doi: 10.3389/fcimb.2015.00003

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444 (7122), 1027. doi: 10.1038/nature05414

Vacca, M., Celano, G., Calabrese, F. M., Portincasa, P., Gobbetti, M., and De Angelis, M. (2020). The Controversial Role of Human Gut Lachnospiraceae. *Microorganisms* 8 (4), 573. doi: 10.3390/microorganisms8040573

Vital, M., Karch, A., and Pieper, D. H. (2017). Colonic butyrate-producing communities inhumans: an overview using omics data. *Msystems* 2(6), e00130–17. doi: 10.1128/mSystems.00130-17

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73 (16), 5261–5267. doi: 10.1128/AEM.00062-07

Wang, M., Li, M., Wu, S., Lebrilla, C. B., Chapkin, R. S., Ivanov, I., et al. (2015). Fecal microbiota composition of breast-fed infants is correlated with human milk oligosaccharides consumed. *J. Pediatr. Gastroenterol. Nutr.* 60 (6), 825. doi: 10.1097/MPG.0000000000000752

Wang, J., Wu, Y., Xiong, G., Chao, T., Jin, Q., Liu, R., et al. (2016). Introduction of complementary feeding before 4 months of age increases the risk of childhood overweight or obesity: a meta-analysis of prospective cohort studies. *Nutr. Res.* 36 (8), 759–770. doi: 10.1016/j.nutres.2016.03.003

Whitaker, R. C. (2004). Predicting preschooler obesity at birth: the role of maternal obesity in early pregnancy. *Pediatrics* 114 (1), e29–e36. doi: 10.1542/peds.114.1.e29

Yassour, M., Vatanen, T., Siljander, H., Hämäläinen, A. M., Härkönen, T., Ryhänen, S. J., et al. (2016). Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. *Sci. Trans. Med.* 8 (343), 343ra81–343ra81. doi: 10.1126/scitranslmed.aad0917

Young, V. B. (2017). The role of the microbiome in human health and disease: an introduction for clinicians. *Bmj* 356, j831. doi: 10.1136/bmj.j831

Zhang, C., Zhang, M., Pang, X., Zhao, Y., Wang, L., and Zhao, L. (2012). Structural resilience of the gut microbiota in adult mice under high-fat dietary perturbations. *ISME J.* 6 (10), 1848–1857. doi: 10.1038/ismej.2012.27

Zheng, H., Liang, H., Wang, Y., Miao, M., Shi, T., Yang, F., et al. (2016). Altered gut microbiota composition associated with eczema in infants. *PloS One* 11 (11), e0166026. doi: 10.1371/journal.pone.0166026

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Vaginal Microbiome Composition in Early Pregnancy and Risk of Spontaneous Preterm and Early Term Birth Among African American Women

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Objective: To evaluate the association between the early pregnancy vaginal microbiome and spontaneous preterm birth (sPTB) and early term birth (sETB) among African American women.

Methods: Vaginal samples collected in early pregnancy (8-14 weeks' gestation) from 436 women enrolled in the Emory University African American Vaginal, Oral, and Gut Microbiome in Pregnancy Study underwent 16S rRNA gene sequencing of the V3-V4 region, taxonomic classification, and community state type (CST) assignment. We compared vaginal CST and abundance of taxa for women whose pregnancy ended in sPTB (N = 44) or sETB (N= 84) to those who delivered full term (N = 231).

Results: Nearly half of the women had a vaginal microbiome classified as CST IV (Diverse CST), while one-third had CST III (*L. iners* dominated) and just 16% had CST I, II, or V (non-iners *Lactobacillus* dominated). Compared to vaginal CST I, II, or V (non-iners *Lactobacillus* dominated), both CST III (*L. iners* dominated) and CST IV (Diverse) were associated with sPTB with an adjusted odds ratio (95% confidence interval) of 4.1 (1.1, infinity) and 7.7 (2.2, infinity), respectively, in multivariate logistic regression. In contrast, no vaginal CST was associated with sETB. The linear decomposition model (LDM) based on amplicon sequence variant (ASV) relative abundance found a significant overall effect of the vaginal microbiome on sPTB (p=0.034) but not sETB (p=0.320), whereas the LDM based on presence/absence of ASV found no overall effect on sPTB (p=0.328) but a significant effect on sETB (p=0.030). In testing for ASV-specific effects, the LDM found that no ASV was significantly associated with sPTB considering either relative abundance or

presence/absence data after controlling for multiple comparisons (FDR 10%), although in marginal analysis the relative abundance of *Gardnerella vaginalis* (p=0.011), non-iners *Lactobacillus* (p=0.016), and *Mobiluncus curtisii* (p=0.035) and the presence of *Atopobium vaginae* (p=0.049), BVAB2 (p=0.024), *Dialister microaerophilis* (p=0.011), and *Prevotella amnii* (p=0.044) were associated with sPTB. The LDM identified the higher abundance of 7 ASVs and the presence of 13 ASVs, all commonly residents of the gut, as associated with sETB at FDR < 10%.

Conclusions: In this cohort of African American women, an early pregnancy vaginal CST III or IV was associated with an increased risk of sPTB but not sETB. The relative abundance and presence of distinct taxa within the early pregnancy vaginal microbiome was associated with either sPTB or sETB.

Keywords: microbiome, microbiota, pregnancy, preterm birth, early term birth, gestational age at birth

INTRODUCTION

Nearly 1 in 10 US infants are born preterm (< 37 weeks gestation) (Martin et al., 2019), making preterm birth (PTB) a leading cause of infant morbidity and mortality (Callaghan et al., 2006; Mathews and Driscoll, 2017). African American (AA) women experience a PTB rate 1.5 times that of white women (Martin et al., 2019). While low socioeconomic status is a risk factor, less than half of the US black-white disparity in PTB is explained by socioeconomic status and other known risk factors (McGrady et al., 1992; Goldenberg et al., 2008; Kramer and Hogue, 2009). The Institute of Medicine has called for research aimed at understanding factors that contribute to the high US rates of PTB, particularly among AA women, as crucial for reducing US infant morbidity and mortality (Behrman and Butler, 2007).

A growing body of research has focused upon the prenatal vaginal microbiome in shaping risk for PTB. Most 16S rRNA gene sequencing surveys characterize the vaginal microbiome using a set of community state types (CST) that were defined *via* hierarchical clustering and consideration of predominant taxa, with communities clustered into five CST: four dominated by *Lactobacillus* spp., including *L. crispatus* (CST I), *L. gasseri* (CST II), *L. iners* (CST III), or *L. jensenii* (CST V), and a fifth with a lower proportion of lactic acid producing bacteria and higher proportion of anaerobes (CST IV) (Zhou et al., 2007; Ravel et al., 2011). A consistent finding is that the proportion of women classified into a specific CST varies by race, with AA women significantly more likely to have a diverse vaginal CST not dominated by *Lactobacillus* (Zhou et al., 2007; Ravel et al., 2011; MacIntyre et al., 2015). Among women whose vaginal microbiome is dominated by *Lactobacillus*, the predominant species also varies by race, with *L. crispatus* more commonly predominating among white women and *L. iners*, more commonly predominating among AA women (Hyman et al., 2014). Because *L. iners* produces less acid than other *Lactobacillus* spp., it is less effective at maintaining the low pH that typically characterizes vaginal health (Amabebe and Anumba, 2018). AA women are also significantly more likely than women of other races/ethnicities to have a vaginal microbiome that harbors *Gardnerella*, BV-associated bacterium-1 (BVAB1, candidate name *Candidatus Lachnocurva vaginae*), and other pathogenic bacteria associated with invasion of the amniotic cavity (Fettweis et al., 2014). Social, environmental, and behavioral exposures driving racial/ethnic differences in the vaginal microbiome remain to be explored, although factors associated with life course exposures to stress and adversity as well as socioeconomic status are likely to contribute (Cammack et al., 2011; Dunlop et al., 2019).

To date, studies of the vaginal microbiome and PTB have yielded somewhat conflicting findings both within and across racial/ethnic groups. To illustrate, lower *Lactobacillus* and higher *Gardnerella* abundance have been associated with PTB (DiGiulio et al., 2015) and preterm premature rupture of membranes (Brown et al., 2016) in two mostly white cohorts, whereas no such associations were found in mostly AA cohorts (Romero et al., 2014a; Nelson et al., 2016; Stout et al., 2017). A cohort of mostly white and Asian women with prior spontaneous PTB (sPTB) also found no association between diverse vaginal communities and PTB, but did identify that higher abundance of *L. iners* increased and *L. crispatus* decreased subsequent PTB risk (Kindinger et al., 2017). Application of a refined bioinformatics approach that improved *Lactobacillus* species classification found no association between *L. iners* and birth outcome in either the white or AA cohorts, a protective effect of *L. crispatus* in both cohorts, and an association between lower abundance of *L. gasseri* and *L. jensenii* and PTB only in the AA cohort (Callahan et al., 2017b). In a multi-race cohort, the presence of *Mycoplasma* was found to increase PTB risk and the presence of *Mageebacillus indoclicus* (previously referred to as BVAB3) to drastically decrease PTB risk for minority women, while no associations were observed for white women (Wen et al., 2013).

The discordance in findings across studies may reflect differences in study sample. Across studies, there is considerable variation in participant racial/ethnic and socioeconomic diversity, characteristics themselves that are linked with PTB (Dunlop et al., 2019), and many studies are small in size or had a limited number of AA women. In addition, across studies there is substantial

heterogeneity in the definition and classification of PTB, with many not distinguishing sPTB from any PTB, including those medically-indicated due to maternal or fetal complications. The clinical factors preceding PTB types vary; the spontaneous type is more often linked with intra-uterine infection or inflammation, whereas the indicated type is often related to medical complications (Behrman and Butler, 2007). There is also heterogeneity in classification of the term birth comparison group. Across microbiome-PTB studies, many classify women whose births fall into the early term category ($37^{0/7}$ through $38^{6/7}$ weeks) as term controls, whereas others use 39 weeks and greater, while some do not clearly define the gestational age limits of their comparison group. To date, no vaginal microbiome studies have specifically differentiated early term birth from full term birth despite recommendations to address risk factors for early term birth, as infants born early term experience excess morbidity relative to infants born full term (between $39^{0/7}$ and $40^{6/7}$ weeks) (American College of Obstetricians and Gynecologists, 2013). Finally, there is also considerable variability in the gestational age of vaginal sample collection across studies, and it is established that the composition of the vaginal microbiome changes with advancing gestational age (Romero et al., 2014a; Romero et al., 2014b) becoming more stable and less likely to shift from a diverse CST to one dominated by *Lactobacillus* spp.

The main objective of this study was to characterize the composition of the vaginal microbiome in early pregnancy (8-14 weeks' gestation) among a cohort of AA women and evaluate its association with the occurrence of sPTB and sETB in comparison to full term birth. We restricted the study to AA women based on a health disparity research framework that recommends as a first step to understanding health disparities to look within the high burden group to identify intra-group risk and protective factors. (Rowley et al., 1993) This is especially pertinent given that existing studies of the vaginal microbiome in pregnancy often do not consider socioeconomic status and hence are not able to parse the possibly competing or additive effects of race and socioeconomic status.

METHODS

Participants

Participants for this study were from the Emory University African American Vaginal, Oral, and Gut Microbiome in Pregnancy Study (Corwin et al., 2017). African American women were recruited from prenatal clinics of two hospitals in Atlanta, GA: a private hospital that provides services for a socioeconomically diverse group of women and a public facility that primarily provides services for low-income women. Inclusion criteria for enrollment into the cohort included: 1) being African American, defined as being of self-reported Black/African American race and born in the United States; 2) presenting with a singleton pregnancy between 8-14 weeks gestation (verified by prenatal record); 3) ability to comprehend written and spoken English; 4) age between 18-40 years; and 5) absence of the diagnosis of any chronic medical condition or the chronic use of prescription medication to manage a health condition (verified by prenatal record) in order to reduce the number of early deliveries attributed to medical indications and focus on spontaneous early births. Women who developed health conditions or pregnancy complications, including those who required prescription medications were retained in the study and these exposures and their gestational age of occurrence were recorded. Participants in the present study included the first consecutively enrolled 474 women whose pregnancy ended in a birth for whom at least one vaginal swab sample from the enrollment study visit was available for DNA extraction and 16S rRNA gene sequencing. The research protocol was reviewed and approved by the Emory University Institutional Review Board (protocol number 68441); all participants provided written informed consent.

Data Collection

The collection of biological samples, questionnaire and clinical at two points during pregnancy (at prenatal care visits occurring between 8-14 and 24-30 weeks' gestation), and clinical data (from the medical record) post-delivery has been described in detail previously (Corwin et al., 2017). Items relevant to this study are summarized below.

Questionnaire Data

Sociodemographic survey based on maternal self-report and prenatal administrative record review was used to ascertain maternal age upon entry into the study, years of education (less than high school, high school or GED, some college, college graduate), and prenatal health insurance type (categorized as low-income Medicaid, Right-from-the-Start Medicaid, private insurance). In Georgia, to be eligible for low-income Medicaid during pregnancy a woman must have a household income at or below 133 percent of the federal poverty level, whereas to be eligible for Right-from-the-Start Medicaid coverage during pregnancy, women must have a household income at or below of 200 percent of the federal poverty level (Georgia Medicaid, 2020).

A health survey was used to ascertain substance use (alcohol, tobacco, marijuana, other drugs) within the month prior to the visit the use of substances. For each item for which there is occurrence, the timeline follow back approach (Sobell and Sobell, 1996; Carey et al., 2001; Robinson et al., 2014); was used to ascertain the timing and frequency of occurrence. These data were used to classify women as users of alcohol, tobacco, and/or marijuana in the month prior to the enrollment visit.

Medical Record Data

Medical chart abstraction was completed by the research team using a standardized chart abstraction tool to ascertain the following characteristics, conditions and birth outcomes: (1) Parity, categorized according to whether the woman had any prior birth or not and if prior births were term or preterm; (2) First prenatal body mass index (BMI), calculated from measured height and weight at the first prenatal visit between 8-14 weeks gestation and categorized according to accepted definitions (obesity ≥ 30 kg/m^2, overweight 25-29.99 kg/m^2, healthy weight 18.5-24.99 kg/m^2, and underweight <18.5 kg/m^2);

(3) Genitourinary tract infections and antibiotic use were noted based on physician diagnosis and/or laboratory results in the record for bacterial vaginosis, chlamydia, gonorrhea, trichomoniasis, or urinary tract infection and any prescription for antibiotics; the gestational age of diagnosis of infections or prescription of antibiotics were ascertained by comparing the date of these occurrences to the estimated date of confinement based on the last menstrual period (LMP) and/or ultrasound before 14 weeks' gestation according to standard clinical criteria (American College of Obstetricians and Gynecologists, 2014); participants were coded as having been exposed to infection or antibiotics in the month prior to the enrollment visit if they had an infection or were prescribed an antibiotic in the four weeks prior; (4) Gestational age at birth was determined from the delivery record using the best obstetrical estimate (American College of Obstetricians and Gynecologists, 2014) based upon the date of delivery in relation to the estimated date of confinement established by the 8-14 week prenatal visit. All participants received early pregnancy dating by last menstrual period (LMP) and/or early ultrasound, given enrollment criteria. (5) Type of Labor (spontaneous, induced, none) and mode of delivery (vaginal, C-section) along with indication for induction and/or C-section were obtained and used to phenotype birth outcomes.

Pregnancy outcomes were classified into the following outcome categories: Preterm birth (between $22^{0/7}$ and $36^{6/7}$ weeks gestation) further classified as spontaneous (following spontaneous labor or premature ruptures of membranes) or medically-indicated (following C-section or induction for an indication) (Ananth and Vintzileos, 2008); early term birth (between $37^{0/7}$ and $38^{6/7}$ weeks gestation) further classified as spontaneous or medically-indicated; and full term birth ($39^{0/7}$ weeks or greater).

Vaginal Samples

Participants were provided verbal and pictorial instructions explaining how to obtain self-collected vaginal swabs. Vaginal microbiome sampling involved sampling the midportion of the vaginal vault (3-4 inches from introitus). Consistent with the protocols of the Human Microbiome Project (McInnes and Cutting, 2010), the sampling used a Sterile Catch-All™ Sample Collection Swab (Epicentre Biotechnologies, Madison WI) that was immediately handed to the study coordinator for placement in MoBio bead tubes (MoBio Laboratories, Inc.), that were frozen upright on ice until transported to the lab, where they were stored at −80°C until DNA extraction. Studies support that vaginal self-collection swabs sample the same microbial diversity as physician-collected swabs of the mid-vagina and have high overall morphotype-specific validity compared with provider-collected swabs (Forney et al., 2010). Additional self-collected vaginal swabs were obtained for pH (measured *via* pH-strips [Merck, Darmstadt, Germany] with a scale from 4.0-7.7), Nugent scoring *via* Gram staining, and measurement of vaginal cytokines. Gram staining was performed at the Emory Clinical Microbiology Laboratory, with smeared slides dried and heat-fixed prior to Gram staining and the slide scored *via* Nugent's criteria (scores 0-3 are categorized as normal, 4-6 as intermediate, and 7-10 as bacterial vaginosis) (Nugent et al., 1991). Vaginal swabs were analyzed for cytokines, including interferon-γ, interleukin (IL)-6, IL-8, IL-10, and TNF-α, using the MesoScale assay platform (MesoScale Diagnostics Rockville, Maryland), which uses electrochemiluminescence for high sensitivity and broad dynamic range, according to manufacturer protocols. Vaginal swabs were also analyzed for C-reactive protein using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, cat# SCRP00) following the manufacture protocol. Vaginal fluid was used undiluted after centrifugation. The concentration of C-reactive protein was calculated based on the four parameter calibration curves generated for each set of samples assayed using the BioTek Gen5 software.

DNA Extraction, Library preparation, Sequencing, and Bioinformatic Processing

DNA was extracted from participant swab samples using the DNeasy PowerSoil Kit (cat# 12888-100, Qiagen). DNA quantification based on a threshold of 5 ug/nL was used to identify samples that were borderline in terms of DNA yield; in cases that were borderline, DNA quality was assessed on a 2% agarose gel and quantitated with the Broad Range Quant-It kit from ThermoFisher Scientific (Q33130). Participant samples with DNA visible on the gel were sequenced as were 30 no-template controls (that contained all assay components except for DNA to verify lack of contamination across reagents and samples) and positive controls (that were a mixture of 20 vaginal specimens of known composition). Microbial composition was characterized by DNA sequencing of the 16S rRNA gene.

Amplification of the V3–V4 regions of the 16S rRNA gene was performed using a two step-PCR described previously (Elovitz et al., 2019). Briefly, the first PCR used the short 16S rRNA gene specific primers 319F (ACACTGACGACATGGTTCTACA[0–7]ACTCCTRCGGGAGGCAGCAG) and 806R (TACGGTAGCAGAGACTTGGTCT[0–7]GGACTACHVGGGTWTCTAAT) where the underlined sequence is the Illumina sequencing primer sequence and [0–7] indicate the presence of an heterogeneous pad sequence to improve sequencing quality (Fadrosh et al., 2014), for a total of 20 cycles. The second step extends the amplicon with the Illumina required adaptor sequences and the sample specific dual barcode system *via* 10 cycles with primers H1 (AATGATACGGCGACCACCGAGATCTACACNNNNNNNNACACTGACGACATGGTTCTACA) and H2 (CAAGCAGAAGACGGCATACGAGATNNNNNNNNTACGGTAGCAGAGACTTGGTCT) where NNNNNN indicates a sample specific barcode sequence and the underlined sequence corresponds to the Illumina sequencing primer for priming to the first step amplicon (Fadrosh et al., 2014). Amplicons were visualized on a 2% agarose gel, quantified, pooled in equimolar concentration, and purified prior to sequencing on an Illumina HiSeq 2500 (San Diego, CA, USA) modified to generate 300 bp paired-end reads (Holm et al., 2019). Extraction and PCR negative controls as well as a positive control composed of a mixture of 20 vaginal biological specimens of known composition were processed in parallel.

The sequences were de-multiplexed using the dual-barcode strategy, a mapping file linking barcode to samples and split_libraries.py, a QIIME-dependent script (Kuczynski et al., 2012). The resulting forward and reverse fastq files were split by sample using the QIIME-dependent script split_sequence_file_on_sample_ids.py, and primer sequences were removed using TagCleaner (version 0.16) (Schmieder et al., 2010). Further processing followed the DADA2 Workflow for Big Data and dada2 (v. 1.5.2) (https://benjjneb.github.io/dada2/bigdata.html) (Callahan et al., 2016). Forward and reverse reads were each trimmed using lengths of 255 and 225 bp, respectively, and were filtered to contain no ambiguous bases, have minimum quality score of 2, and were required to contain less than two expected errors based on their quality score. The relationship between quality scores and error rates were estimated for both sequencing runs to reduce batch effects arising from run-to-run variability. Reads were assembled and chimeras removed as per dada2 protocol.

Taxonomy was assigned to each amplicon sequence variant (ASV) generated by dada2 using PECAN (version 1.0), a rapid per sequence classifier (http://ravel-lab.org/speciateit), which classifies 16S rRNA gene sequences using Markov Chain models built from a curated set of reference sequences. Tables including total sequence counts for or relative abundances of 666 taxa for each sample with more than 2000 sequences were generated and used to calculate Shannon (richness) and Chao1 measures of alpha-diversity and rarefaction curves. A community state type (CST) was assigned to each sample using hierarchical clustering with the Jensen-Shannon divergence and Ward linkage (Ravel et al., 2011; Gajer et al., 2012). CST I is predominated by *L. crispatus*, CST II by *L. gasseri*, CST III by *L. iners*, CST IV was defined as lacking *Lactobacillus* predominance and comprising a diverse set of strict and facultative anaerobes, further split into CST IV-A (predominated by BVAB and *Gardnerella*), IV-B (predominated by *Atopobium* and *Gardnerella*), and IV-D (only anaerobes), while CST V is predominated by *L. jensenii*.

For association analyses, all taxa that were not assigned at the genus level were removed. Read counts for ASVs assigned to the same taxonomy (i.e., to a taxon defined by species and genus when species was available, or to a taxon defined only by genus when species was not available). All non-iners *Lactobacilli* were pooled; we assumed that any lactobacilli that could not be assigned at the genus level would not be *L. iners*, since it is well characterized. We further removed taxa found in fewer than 5 samples of the combined data from the 44 women who experienced sPTB and the 231 women who delivered full term. This trimming was conducted in two steps; first, taxa defined by genus *and* species were combined with the corresponding genus-only taxon if they occurred in fewer than 5 samples; then, genus-only taxa were removed if they occurred in fewer than 5 samples.

Statistical Analyses

We calculated the proportion of participating women whose early pregnancy vaginal sample (collected during the enrollment visit at 8-14 weeks gestation) was categorized into each CST. Due to the low proportion of samples assigned to CST I, II, and V, these CST were combined into a single category creating three CST categories: non-iners *Lactobacillus* dominated (CST I, II, V), *Lactobacillus iners* dominated (CST III), non-*Lactobacillus* dominated or Diverse (CST IV). We summarized characteristics of samples as well as sociodemographic and clinical characteristics of women according to CST categories and compared differences in characteristics using Chi-square or t-test, as appropriate. We also summarized sociodemographic and clinical characteristics of women according to birth outcome categories and compared differences in characteristics using Chi-square or t-test, as appropriate.

We tabulated the proportion of women whose early pregnancy vaginal sample was categorized into each CST and CST category and compared the proportion of women with sPTB and sETB (in separate models) vs. the proportion of women with a full term birth across the CST categories using the Chi-square test. We also displayed the relative abundance of the 25 taxa of highest relative abundance in the vaginal samples by CST and gestational age categories *via* a heat map. To evaluate the association between vaginal CST (I through IV) and CST category (non-iners *Lactobacillus*, *Lactobacillus iners*, or Diverse) in early pregnancy and gestational age at birth outcomes, we then performed Firth-corrected logistic regression, contrasting sPTB and sETB with full term birth in separate models, according to vaginal CST category (with non-iners *Lactobacillus* as the referent category). We used the Firth correction for the relatively rare occurrence of the gestational age outcomes of interest (especially sPTB) among those with non-iners *Lactobacillus* CST, which reduces small sample bias in maximum likelihood estimation (King and Zeng, 2001). In addition to CST, all logistic regression models included the following co-variates, selected on the basis of their association with gestational age at birth and vaginal microbiome composition based on the existing literature (Behrman and Butler, 2007; Dunlop et al., 2019) as well as bivariate associations within this cohort (**Tables 3**, **4**): maternal age, level of education, insurance type, marital-cohabitation status, parity, first prenatal BMI, tobacco use, marijuana use, and gestational age at sample acquisition. In some cases, the upper confidence limits for the odds of sPTB were very large and appeared to support a directional hypothesis; in these cases, we also give one-sided intervals at the same confidence level (Ruxton and Neuhäuser, 2010).

To test for both the global effect of the vaginal microbiome on the outcomes of interest as well as ASV-specific effects we used the linear decomposition model (LDM). The LDM allows for complex fixed-effects models, such as those that include multiple variables of interest whether continuous or categorical, their interactions, as well as other co-variates. Furthermore, it is permutation-based and, as such, can accommodate clustered data and maintain validity for small sample sizes and when data are subject to over-dispersion (Hu and Satten, 2020). The LDM analyses likewise adjusted for maternal age, level of education, insurance type, marital-cohabitation status, parity, first prenatal BMI, tobacco use, marijuana use, and gestational age at sample acquisition. The LDM provides an overall (global) test of

association between the microbiome and traits of interest such as PTB, as well as a list of taxa that are individually associated with the trait that controls false discovery rate (FDR) at a pre-specified level; in these analyses, we set the FDR at 10%. We performed LDM analyses using relative abundance data as well as using a binary variable indicating presence or absence of each taxon. Analyses based on presence-absence were rarefied to the minimum library size, and averaged over rarefactions (Hu and Satten, 2021). To test for the effect of particular taxa that have previously been reported as associated with preterm birth on the occurrence of sPTB in our cohort, we also report *p*-values from the LDM based on relative abundances and presence/absence of the following 19 taxa: *Aerococcus christensenii, Atopobium vaginae, BVAB1* (candidate name *Candidatus Lachnocurva vaginae*), BVAB2, *Dialister microaerophilus, Finegoldia magna, Gardnerella vaginalis, Lactobacillus iners*, non-*iners Lactobacillus, Megasphaera, Mobiluncus curtisii, Mycoplasma hominis, Prevotella amnii, Prevotella bivia, Prevotella buccalis, Prevotella timonensis, Ureaplasma urealyticum, Sneathia amnii, and Sneathia sanguinegens*.

RESULTS

16S rRNA Gene Sequencing Data

Among the 474 cohort participants with vaginal samples from early pregnancy (8-14 weeks gestation) that were included in this study, 38 samples were removed due to low library size (fewer than 2,000 reads). A rarefaction curve displaying rarefaction depth by richness is given in **Supplement Figure 1**. From the remaining 436 samples, a total of 20,097,432 reads were grouped into 666 ASVs. After removing taxa that were only identified to the family level or higher, we retained 19,571,748 reads in 601 ASVs. After the glomming and trimming operation, the data remaining comprised 19,544,087 reads in 324 taxa (with a range of 2,978 to 181,072 reads per sample). For samples run in duplicate (N=7), the sample with the higher read count was retained and the other dropped before creation of the biome table.

Sample and Participant Characteristics According to CST

Table 1 shows the distribution of vaginal CST at the early pregnancy enrollment visit (8-14 weeks gestation) among the 436 study participants. Nearly half of the women had a vaginal microbiome classified as CST IV (Diverse CST), with CST IV-A (26.8%) and CST IV-B (21.1%) being substantially more common than CST IV-C (1.1%). Approximately one-third had CST III (*L. iners* dominated), while just 16% classified as CST I, II, or V, with most of these being dominated by *L. crispatus* (11.5%) compared to *L. gasseri* (1.8%) and *L. jenseni* (3%). Because of the small proportion of women with CST I, II, and V, we combined these into a single CST category for statistical analyses (referred to as non-iners *Lactobacillus* CST).

In examining vaginal sample characteristics by CST category, there were significant differences in sample read count, pH, Nugent score, Shannon diversity and the $\log_{10}$-transformed vaginal concentrations of C-reactive protein and cytokines index, but no significant difference in vaginal white blood cell quantification or Chao1 diversity (**Table 2A**). Box plots of the alpha-diversity measures across the CST categories demonstrate that the Diverse CST, which had the smaller library size, had significantly higher Shannon index compared to the non-iners *Lactobacillus* CST and the *Lactobacillus iners* CST (**Figure 1A**, p<0.0001 for both comparisons). The combination of similar Chao1 diversity across CSTs (**Figure 1B**) but higher Shannon diversity (evenness) moving from the non-iners *Lactobacillus* CST to *Lactobacillus iners* CST to the Diverse CST suggests the main feature differentiating the CST categories is the increasing population of the rare taxa along this axis, rather than in increase in the number of taxa.

TABLE 1 | Distribution of Vaginal Microbiome Community State Type among Study Participants.

CST	Characteristic Taxa	No. (%) of Cohort N = 436
I	*Lactobacillus crispatus*	50 (11.5%)
II	*Lactobacillus gasseri*	8 (1.8%)
III	*Lactobacillus iners*	151 (34.6%)
IV	Diverse	214 (49.1%)
IV-A	*BVAB, Gardnerella*	*117 (26.8%)*
IV-B	*Atopobium, Gardnerella*	*92 (21.1%)*
IV-C	*Anaerobes*[1]	*5 (1.1%)*
V	*Lactobacillus jenseni*	13 (3.0%)

[1]*Common anaerobes include Aerococcus, Dialister, Eggerthella, Finegoldia, Megasphaera, Mobiluncus, and Prevotella.*

Box plots of the $\log_{10}$-transformed vaginal concentrations of C-reactive protein and cytokines across the CST categories are shown in **Figure 2** and the pairwise differences in $\log_{10}$-transformed vaginal concentrations of C-reactive protein and cytokines by CST category are given in **Figure 2** and **Table 2B**. When comparing the non-iners *Lactobacillus* to the *Lactobacillus iners* CST category, there was a pairwise significant difference in the concentration of C-reactive protein (p=0.004) but not the vaginal cytokines. When comparing the non-iners *Lactobacillus* CST to the Diverse CST, there were significant differences in vaginal concentrations of IL-6 (p<0.0001), TNF-α (p=p<0.0001) and C-reactive protein (p<0.0001). When comparing the *Lactobacillus iners* CST to the Diverse CST, there were significant differences in vaginal concentrations of IL-6 (p=0.006), IL-10 (p=0.005), TNF-α (p<0.0001) and C-reactive protein p<0.0001).

Sociodemographic and clinical characteristics of the 436 study participants according to vaginal CST category at the early pregnancy enrollment visit (8-14 weeks gestation) shown in **Table 3**. A number of sociodemographic characteristics varied significantly according to CST category, including maternal age (p=0.009), level of education (p=0.0001), marital or cohabitation status (p=0.001). When considering clinical characteristics, parity (prior birth, p=0.044)) status and the use of marijuana (p=0.001) in the month prior to the early pregnancy enrollment visit (8-14 weeks) were significantly different according to CST category, whereas no other clinical characteristics were, including whether the woman had a diagnosis of reproductive

TABLE 2A | Early Pregnancy Vaginal Sample Characteristics According to CST Category.

CHARACTERISTIC	Non-iners *Lactobacillus* (CST I, II, V) N = 71	*Lactobacillus iners* (CST III) N = 151	Diverse (CST IV) N = 214	p-value[1]
Gestational weeks, mean ± sd	11.5 ± 1.9	11.47 ± 2.7	11.0 ± 2.4	0.158
Read count, mean ± sd	50,053 ± 27,826	52,327 ± 26,624	40,385 ± 19,724	***0.0001***
pH, mean ± sd	4.61 ± 0.37	4.59 ± 0.33	4.75 ± 0.43	***0.001***
Nugent score, mean ± sd	1.75 ± 2.29	2.85 ± 2.67	7.19 ± 2.49	***0.0001***
`Nugent score				
Normal	57 (84%)	93 (65%)	20 (10%)	***0.0001***
Intermediate	5 (7%)	31 (22%)	33 (16%)	
Bacterial vaginosis	6 (9%)	20 (14%)	155 (75%)	
White blood cells				
None	40 (59%)	66 (46%)	102 (49%)	0.130
Rare	12 (18%)	41 (29%)	54 (26%)	
Few	10 (15%)	34 (24%)	44 (21%)	
Moderate	5 (7%)	3 (2%)	5 (2%)	
Many	1 (2%)	0 (0%)	3 (1%)	
Chao1 diversity	69.37 ± 31.98	68.57 ± 28.25	63.58 ± 27.59	0.159
Shannon diversity	1.02 ± 0.78	1.04 ± 0.80	1.79 ± 0.65	***0.0001***
Cytokines (log_{10}, mg/dL)				
Interferon-γ	-0.89 ± 0.23	- 0.88 ± 0.20	- 0.30 ± 0.17	***0.043***
Interleukin-6	-0.85 ± 0.27	- 0.22 ± 0.18	0.51 ± 0.15	***0.0001***
Interleukin-10	-3.48 ± 0.25	-3.69 ± 0.20	-2.96 ± 0.14	***0.005***
TNF-α	-2.54 ± 0.26	- 2.19 ± 0.18	-0.89 ± 0.13	***0.0001***
C-reactive protein (log_{10}, mg/dL)	-10.6 ± 0.19	-9.8 ± 0.16	-8.7 ± 0.12	***0.0001***

[1]*p-value for t-test (continuous variables) or Fisher's exact or Chi-square test (categorical variables); bold indicates statistical significance for* $\alpha = 0.05$.

or urinary tract infection or whether she was prescribed antibiotics (oral or parenteral) in the month prior to sampling.

Birth Outcome According to CST

Of the 436 participants, 401 had a pregnancy that ended in birth: 59 (15%) PTB, with 44 (11%) spontaneous and 15 (4%) medically-indicated; 111 (28%) ETB, with 84 (21%) spontaneous and 27 (7%) medically-indicated; 231 (58%) full term birth. The remaining 35 participants had a pregnancy that ended in spontaneous abortion and were excluded from analysis. Of note, the distribution of pregnancy outcomes among the 38 women whose vaginal samples were excluded due to low library size were not different from those whose samples were included (19/38 [50%] had a full term birth, 11/38 [29%] had ETB, 6/38 [16%] had PTB, and 2/38 had a spontaneous abortion. Similarly, in comparing the library size according to gestational age at birth outcome, there was no significant difference in library size for those with or sPTB *vs.* full term birth (p=0.90; **Supplement Figure 2A**) or sETB *vs.* full term birth (p=0.32; **Supplement Figure 2B**).

The 359 women included in the comparative analyses that follow are those whose pregnancy ended in either sPTB or sETB, with full term birth serving as the comparison group. Among the 44 women in the sPTB group, the range of gestational weeks were 23-1/7 weeks through 36-6/7 weeks (mean gestational weeks of 33-2/7 ± 3-3/7 days) with 11/44 (25%) being very preterm (< 32 weeks), 2/44 (5%) being moderate preterm (32 through 33-6/7 weeks) and 31/44 (70%) being late preterm (34 through 36-6/7 weeks). Among the 84 women in the sETB group, the range of gestational age were 37-0/7 weeks through 38-6/7 weeks,

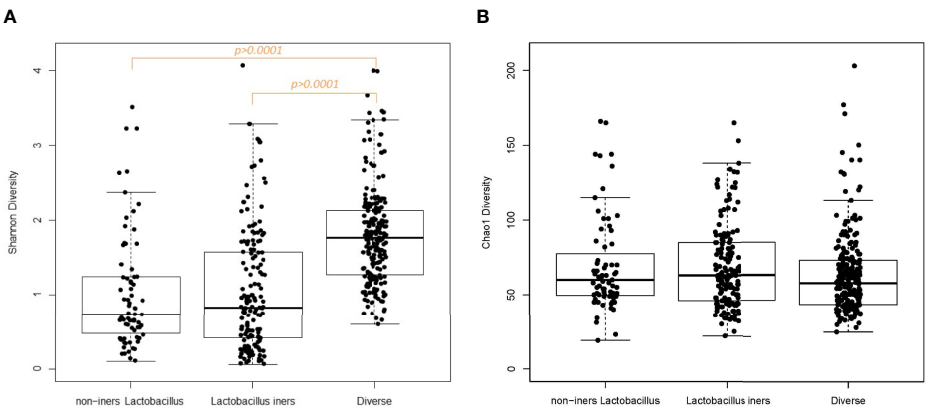

FIGURE 1 | **(A)** Box plot Shannon diversity according to CST category. **(B)** Box plot of Chao1 diversity according to CST category.

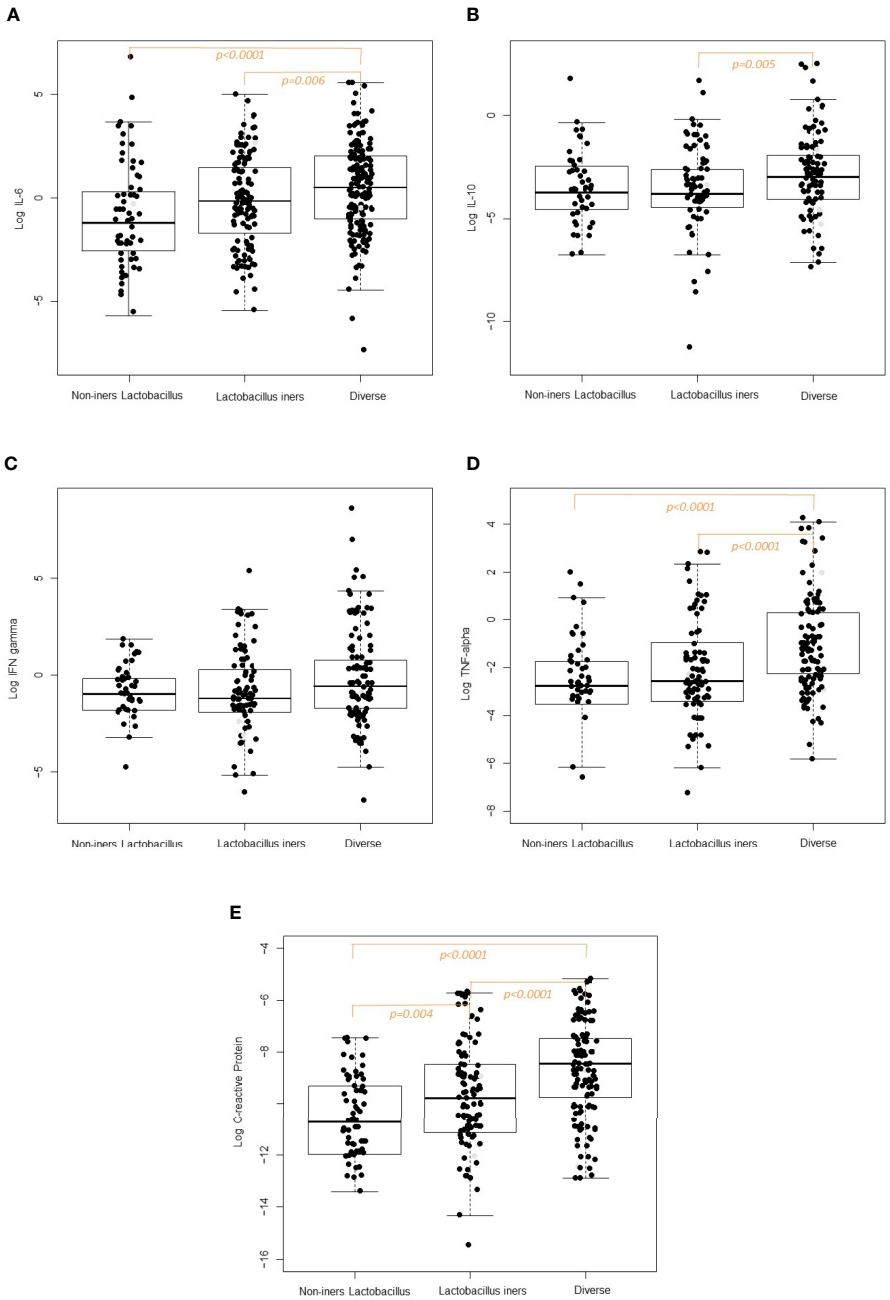

FIGURE 2 | **(A)** Vaginal Interleukin-6 concentration according to CST category. **(B)** Vaginal Interleukin-10 concentration according to CST category. **(C)** Vaginal Interferon-gamma concentration according to CST category. **(D)** Vaginal TFN-alpha concentration according to CST category. **(E)** Vaginal C-reactive concentration according to CST category.

with mean gestational weeks of 37-6/7 ± 3/7 weeks). **Table 4** shows the sociodemographic and clinical characteristics of these participants according to birth outcome. There were significant differences in maternal pregnancy insurance type, parity, body mass index at first prenatal visit, and marijuana use when comparing those with sPTB and sETB to those with full term birth; these variables were controlled for in multivariate analyses examining the relationship between the vaginal microbiome and birth outcome.

Table 5 shows the proportion of women in the five CST (top portion) and three CST categories (bottom portion) according to the birth outcomes of interest. Because of the low proportion of women with vaginal CST II and V, we collapsed these with CST I to create the non-iners *Lactobacillus* CST category. Likewise,

TABLE 2B | Vaginal C-reactive protein and Cytokine Concentrations According to CST Category.

Vaginal Measure	Non-iners *Lactobacillus* (CST I, II, V) N = 71	*Lactobacillusiners* (CST III) N = 151	Diverse (CST IV) N = 214
	p-value[1]		
Interferon-γ			
Non-iners Lactobacillus	–	1.0	0.256
Lactobacillus iners	1.0	–	0.073
Diverse	0.256	0.073	–
Interleukin-6			
Non-iners Lactobacillus	–	0.142	***0.0001***
Lactobacillus iners	0.142	–	***0.006***
Diverse	***0.0001***	***0.006***	–
Interleukin-10			
Non-iners Lactobacillus	–	1.0	0.239
Lactobacillus iners	1.0	–	***0.005***
Diverse	0.239	***0.005***	–
TNF- α			
Non-iners Lactobacillus	–	0.797	***0.0001***
Lactobacillus iners	0.797	–	***0.0001***
Diverse	***0.0001***	***0.0001***	–
C-reactive protein			
Non-iners Lactobacillus	–	***0.004***	***0.0001***
Lactobacillus iners	***0.004***	–	***0.0001***
Diverse	***0.0001***	***0.0001***	–

[1]*p-value for t-test with Bonferroni correction; bold indicates statistical significance for α = 0.05.*

TABLE 3 | Sociodemographic and Clinical Characteristics of Women According to CST Category.

CHARACTERISTIC	Non-iners *Lactobacillus* (CST I, II, V) N = 71	*Lactobacillusiners* (CST III) N = 151	Diverse (CST IV) N = 214	p-value[1]
Age in years, mean ± sd	26.4 ± 5.4	25.1 ± 4.7	24.4 ± 4.8	***0.009***
Pregnancy insurance				
Low-income Medicaid	14 (20%)	66 (44%)	78 (36%)	***0.0001***
Pregnancy Medicaid	27 (38%)	51 (34%)	105 (49%)	
Private	30 (42%)	34 (23%)	31 (14%)	
Education				
Less than high school	8 (9%)	18 (12%)	43 (20%)	***0.0001***
High school or GED	18 (25%)	59 (39%)	93 (44%)	
Some college	15 (21%)	56 (37%)	56 (26%)	
College graduate	32 (45%)	18 (12%)	22 (10%)	
Not Married or Cohabiting	29 (41%)	65 (43%)	129 (60%)	***0.001***
Obstetrical history				
Prior birth	36 (51%)	93 (62%)	104 (49%)	***0.044***
Prior preterm birth (among those with birth)	6/36 (17%)	20/93 (22%)	23 (22%)	0.779
First prenatal Body Mass Index				
Underweight	1 (1%)	9 (6%)	8 (4%)	0.266
Healthy weight	33 (47%)	60 (40%)	82 (38%)	
Overweight	19 (27%)	27 (18%)	43 (20%)	
Obese	18 (25%)	55 (36%)	81 (38%)	
Substance use in month prior				
Tobacco	5 (7%)	24 (16%)	40 (19%)	0.067
Marijuana	12 (17%)	45 (30%)	86 (40%)	***0.001***
Alcohol	6 (9%)	17 (11%)	11 (5%)	0.106
Antibiotic use in month prior				
Any parenteral or oral	4 (6%)	21 (14%)	32 (15%)	0.119
Diagnoses in month prior				
Bacterial vaginosis	3 (4%)	8 (5%)	15 (7%)	0.736
Chlamydia	2 (3%)	7 (5%)	9 (4%)	0.900
Gonorrhea	0	0	2 (0.9%)	0.659
Trichomonas	0	4 (3%)	1 (0.5%)	0.153
Urinary tract infection	1 (1%)	13 (9%)	17 (8%)	0.123

[1]*p-value for t-test (for continuous variables) or Fisher's exact test or Chi-square test (for categorical variables); bold indicates statistical significance for α = 0.05.*

TABLE 4 | Sociodemographic and Clinical Characteristics of Women According to Birth Outcome.

CHARACTERISTIC	Spontaneous Preterm N = 44	Spontaneous Early Term N = 84	Full Term (ref)[1] N = 231
Age, mean ± sd	*p=0.721*	*p=0.475*	
	24.9 ± 4.9	25.2 ± 4.9	24.7 ± 4.7
Pregnancy insurance	***p=0.022****	*p=0.793*	
Low-income Medicaid	21 (48%)	29 (34%)	82 (36%)
Pregnancy Medicaid	20 (46%)	36 (43%)	90 (39%)
Private	3 (7%)	19 (23%)	59 (26%)
Education	*p=0.167*	*p=0.286*	
Less than high school	8 (18%)	10 (12%)	35 (15%)
High school or GED	21 (48%)	38 (45%)	77 (33%)
Some college	11 (25%)	23 (27%)	76 (33%)
College graduate	4 (9%)	13 (16%)	43 (19%)
Not Married or Cohabiting	*p=0.088*	*p=0.760*	
	32 (64%)	42 (50%)	120 (52%)
Prior birth	*p=0.130*	***p=0.026****	
	27 (61%)	53 (63%)	113 (49%)
Body Mass Index	***p=0.019****	***p=0.047****	
Underweight	1 (2%)	0	11 (5%)
Healthy weight	20 (46%)	40 (48%)	86 (37%)
Overweight	15 (34%)	19 (23%)	44 (19%)
Obese	8 (18%)	25 (30%)	90 (39%)
Substance use in month prior			
	p=0.522	*p=0.583*	
Tobacco	6 (14%)	11 (13%)	36 (16%)
	p=0.601	***p=0.043***	
Marijuana	14 (32%)	20 (24%)	83 (36%)
	p=0.318	*p=0.585*	
Alcohol	2 (5%)	6 (7%)	21 (9%)
Antibiotics in month prior			
Any parenteral or oral	*p=0.473*	*p=0.712*	32 (14%)
	4 (9%)	10 (12%)	
Diagnosis in month prior			
	p=0.051	*p=0.296*	
Bacterial vaginosis	0	4 (5%)	19 (8%)
	p=0.999	P=0.999	
Chlamydia	1 (2%)	4 (5%)	10 (4%)
	p=0.160		
Gonorrhea	1 (2%)	0	0
	p=0.999	p=0.577	
Trichomonas	0	0	4 (1.7%)
	p=0.999	P=0.414	
Urinary tract infection	3 (7%)	4 (5%)	17 (7%)

[1]*p-value indicates the result of the Chi-square test of significance with full term births as referent category; bold indicates statistical significance for* $\alpha = 0.05$.

TABLE 5 | Proportion of Women with Vaginal Community State Type According to Birth Outcome.

Vaginal Community State Type	Spontaneous Preterm N = 44	Spontaneous Early Term N = 84	Full Term *(ref)*[1] N = 231
CST	*p=0.068*	*p=0.716*	
CST I *(L. crispatus)*	1 (2%)	14 (17%)	25 (11%)
CST II *(L. gasseri)*	0	1 (1%)	6 (3%)
CST V *(L. jenseni)*	0	4 (5%)	9 (4%)
CST III *(L. iners)*	12 (27%)	26 (31%)	86 (37%)
CST IV-A (Diverse-A)	17 (39%)	21 (25%)	58 (25%)
CST IV-B (Diverse-B)	14 (32%)	18 (21%)	44 (19%)
CST IV-C (Diverse-C)	0	0	3 (1%)
CST Category	***p=0.004****	*p=0.445*	
CST I, II, V (Non-iners *Lactobacillus*)	1 (2%)	10 (23%)	40 (17%)
CST III *(Lactobacillus iners)*	12 (27%)	26 (31%)	86 (37%)
CST IV-A, IV-B, IV-C (Diverse)	31 (71%)	39 (46%)	105 (45%)

[1]*p-value indicates the result of the Chi-square test of significance with full term births as referent category; bold indicates statistical significance for* $\alpha = 0.05$.

because of the low proportion of women with CST VI-C, and the non-significant differences in the birth outcomes of interest for women with CST IV-A vs. CST IV-B (p=0.840), we combined CST IV-A, IV-B, and IV-C into a single category (Diverse CST).

A heat map representing the composition of the vaginal microbiome, as classified by CST, according to gestational age at birth outcome is given in **Figure 3**.

Table 6 shows the results of multivariate logistic regression modeling, controlling for key co-variates that associate with gestational age at birth outcomes among our cohort or in the established literature. The upper portion of **Table 6** shows the Diverse CST (Diverse-A, Diverse-B, and Diverse-C combined) whereas the lower portion shows the Diverse CST subcategories. Compared to women in the non-iners *Lactobacillus* CST, those in the Diverse CST had substantially and significantly elevated adjusted odds of sPTB (aOR = 7.7 [1.8, 72.0]) but not sETB (aOR = 0.9 [0.4, 1.8]). Within the Diverse CST, both Diverse-A and Diverse-B were strongly significantly associated with sPTB with aOR = 7.6 (1.7, 73.1) and 8.2 (1.8, 79.3), respectively, whereas Diverse-C was not (aOR = 3.5 [0.01, 108.5]), acknowledging a very small sample size for the Diverse-C CST. The two-sided confidence intervals for the adjusted odds of sPTB or sETB for those in the *Lactobacillus iners* CST (CST III) compared to the non-iners *Lactobacillus* CST included the null value of 1. However, as the upper confidence limits for the odds of sPTB were very large and appeared to support a directional hypothesis, we also present one-sided 95% confidence intervals that recognize the upper limits of the two-sided intervals are essentially infinite (Ruxton and Neuhäuser, 2010). The one-sided confidence interval for the *Lactobacillus iners* CST excludes the null value of 1 (aOR=4.0 [1.1, inf]), thus showing significantly increased adjusted odds of sPTB relative to the non-iners *Lactobacillus* CST.

Birth Outcome According to Relative Abundance and Presence/Absence of Taxa

When applying the LDM to those with sPTB and full term birth (275 samples), there were 324 ASVs that remained after filtering out ASVs present in less than 5 samples. In the LDM that considered relative abundance, the global p-value for the effect of the vaginal microbiome on the outcome of sPTB was statistically significant (global p-value = 0.034), yet no ASVs were significant after controlling for multiple comparisons at a FDR of 10%. The top 10 smallest p-values (q-values ≥ 0.348) yielded by the LDM that considered relative abundance corresponded to a higher relative abundance of *Paraprevotella clara* (p=0.001), *Collinsella aerofaciens* (p=0.006), *Ruminococcus callidus* (p=0.008), *Gardnerella vaginalis* (p=0.011), *Paraeggerthella hongkongensis* (p=0.014), an ASV that classified to genus *Prevotella* (p=0.016), *Intestinibacter barlettii* (p=0.017), and *Alistipes putredinis* (p=0.019) and *Catenibacterium mituokai* (p=0.018) and a lower relative abundance of non-iners *Lactobacillus* (p=0.016). In this LDM that considered relative abundance, none of the 19 pre-specified taxa achieved significance when accounting for multiple comparisons (q-values ≥ 0.498) but the relative abundance of three taxa, namely, the higher relative abundance of *Gardnerella vaginalis* and *Mobiluncus curtisii* and the lower relative abundance of non-iners *Lactobacillus*, had significant raw p-values (**Table 7**) using the nominal 5% cutoff. Of note, in

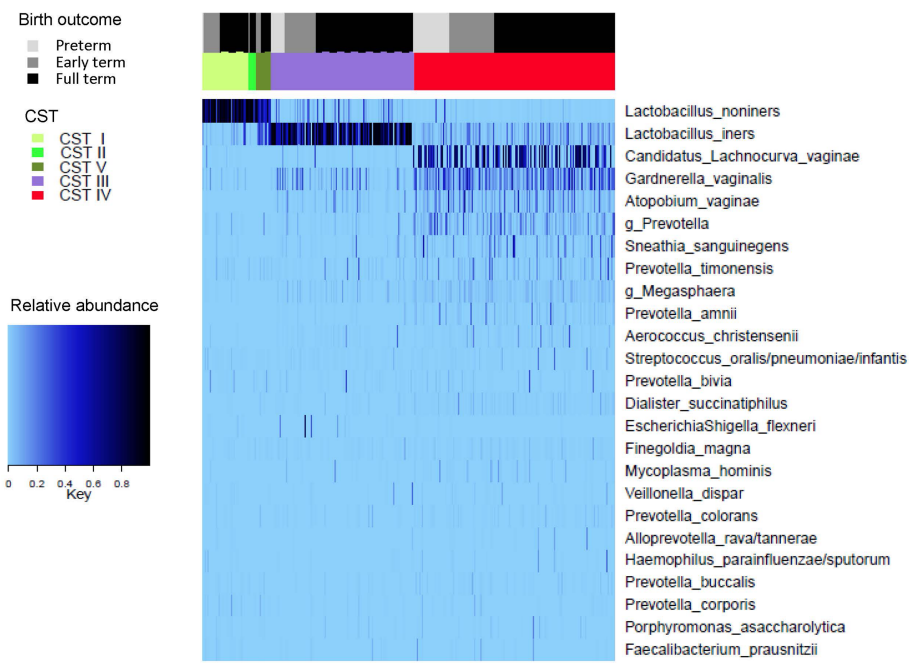

FIGURE 3 | Top 25 taxa, grouped according to Community State Type (CST) and Gestational age at birth outcome.

TABLE 6 | Odds of Birth Outcome According to CST (5-Category) *(Full Term = Referent)*.

Vaginal CST Category	OR (95% Confidence Interval)			
	Spontaneous Preterm N = 44		Spontaneous Early Term N = 84	
	unadjusted	**adjusted**[1]	**unadjusted**	**adjusted**[1]
Non-iners Lactobacillus	*Ref*	*Ref*	*Ref*	*Ref*
Lactobacillus iners	3.9 (0.89, 36.7) **(1.1, inf)**[#]	4.0 (0.8, 38) **(1.1, inf)**[#]	0.6 (0.3, 1.3)	0.6 (0.3, 1.2)
Diverse	**8.1 (2.0, 73.4) (2.4, inf)** [#]	**7.7 (1.8, 72.0) (2.2, inf)** [#]	0.8 (0.4, 1.5)	0.9 (0.4, 1.8)
Diverse-A	**8.1 (1.9, 75.1) (2.3, inf)** [#]	**7.6 (1.7, 73.1) (2.1, inf)** [#]	0.8 (0.4, 1.6)	0.8 (0.4, 1.9)
Diverse-B	**8.8 (2.0, 82.7) (2.5, inf)** [#]	**8.2 (1.8, 79.3) (2.2, inf)** [#]	0.9 (0.4, 1.9)	1.0 (0.4, 2.3)
Diverse-C	3.9 (0.02, 89.1)	3.5 (0.02, 108.5)	0.3 (0.002, 3.3)	0.3 (0.002, 4.0)

[1]*Co-variates included in the multivariate model include age, education level, insurance type, marital-cohabitation status, parity, first prenatal BMI category, tobacco use, marijuana use, gestational age of sample.*

[#]*One-sided confidence interval; bold indicates statistical significance for* $\alpha = 0.05$.

sensitivity analyses, we compared the relative abundance of dropped taxa for those whose pregnancy ended in full term birth vs. sPTB, and found no significant difference (p=0.15; **Supplement Figure 3**), supporting that the removal of taxa from analysis did not influence our findings. In addition, we re-ran the LDM both with the inclusion of read count as a co-variate and found no change in the global p-values as well as with the dropped taxa included and none were identified as significantly different according to gestational age at birth outcomes.

In the LDM that considered the presence/absence of ASV on the outcome of sPTB, the global p-value was 0.328. In this presence/absence LDM model, no ASVs were significantly associated with sPTB after controlling for multiple comparisons. The top 10 smallest p-values (q-values ≥ 0.661) yielded by the LDM model corresponded to the presence of these ASVs: *Dialister succinatiphilus* (p=0.002), *Paraprevotella clara* (p=0.006), *Dialister microaerophilis* (p=0.011), *Bifidobacterium breve* (p=0.011), *Paraeggerthella hongkongensis* (p=0.014), *Intestinibacter barlettii* (p=0.015), *Roseburia inolinivorans* (p=0.020), BVAB2 p= (0.024), *Atopobium deltae* (0.031), and *Ruminococcus callidus* (p=0.0345). In the LDM model that considered presence/absence, none of the 19 pre-specified taxa achieved significance when accounting for multiple comparisons (q-values ≥ 0.818) although the presence of four taxa, namely, *Atopobium vaginae*, BVAB2, *Dialister microaerophilis*, and *Prevotella amnii*, had significant raw p-values (**Table 7**) using the nominal 5% cutoff.

When applying the LDM to the sample that included sETB and full term birth (315 samples), we used the same 324 ASVs as the sPTB analysis. In the LDM that considered relative abundance, the global p-value for the effect of the vaginal microbiome on sETB was not significant (global p-value =

TABLE 7 | Raw p-values for Pre-specific Taxa in the LDM Model[1] for Spontaneous Preterm Birth and Spontaneous Early Term Birth.

Taxon	Spontaneous Preterm N = 44		Spontaneous Early Term N = 84	
	Relative Abundance Model	**Presence/Absence Model**	**Relative Abundance Model**	**Presence/Absence Model**
Aerococcus christensenii	0.170	0.291	0.719	0.195
Atopobium vaginae	0.211	***0.049***	0.810	0.137
BVAB1	0.209	0.091	0.697	0.062
BVAB2	0.077	***0.024***	0.903	0.622
Dialister microaerophilis	0.093	***0.011***	0.167	0.271
Finegoldia magna	0.924	0.629	0.970	0.981
Gardnerella vaginalis	***0.011***	0.382	0.132	0.530
non-iners Lactobacillus	***0.016***	0.307	0.654	0.449
Lactobacillus iners	0.308	0.834	0.123	0.318
g_Megasphaera	0.345	0.436	0.052	0.303
Mobiluncus curtisii	***0.035***	0.244	0.346	0.280
Mycoplasma hominis	0.737	0.423	0.274	0.141
Prevotella amnii	0.296	***0.044***	0.934	0.188
Prevotella bivia	0.848	0.517	0.906	0.915
Prevotella buccalis	0.391	0.498	0.053	0.443
Prevotella timonensis	0.722	0.853	0.849	0.689
Ureaplasma urealyticum	0.624	0.673	0.440	0.080
Sneathia amni	0.782	0.952	0.127	0.203
Sneathia sanguinegens	0.511	0.390	0.138	0.961

[1]*Co-variates included in the multivariate model include age, education level, insurance type, marital-cohabitation status, parity, first prenatal BMI category, tobacco use, marijuana use, gestational age of sample; bold indicates statistical significance for* $\alpha = 0.05$.

0.320), however, the higher relative abundance of seven ASVs were associated with sETB with FDR < 10% (q-value 0.0299-0.0471), including *Dialister invisus* (p=0.00016), *Blautia luti* (p=0.0002), *Collinsella aerofaciens* (p=0.0003), *Campylobacter hominis* (p=0.00042), *Bifobacterium kashiwanohense* (p=0.0005), *Bacteroides vulgatus* (p=0.001), *Bacteroides xylanisolvens* (p=0.0009). In the LDM that considered relative abundance, none of the 19 pre-specified taxa achieved significance when accounting for multiple comparisons (q-values ≥ 0.414) although two taxa had borderline significant raw p-values (**Table 7**) using the nominal 5% cutoff: *Prevotella buccalis* (p=0.053) and an ASV belonging to genus *Megasphaera* (p=0.052).

In the LDM that considered the presence/absence of ASV on the outcome of sETB, the global p-value was significant (p=0.030) and the presence of 13 ASVs were associated with sETB with FDR < 10% (q-value 0.017 – 0.084), including *Bifidobacterium breve* (p<0.0001), *Blautia luti* (p=0.0002), *Bifidobacterium longum* (p=0.0004), *Akkermansi muciniphila* (p=0.0005), *Citrobacter freundi/gillenii/youngae* (p=0.0006), *Bifidobacterium kashiwanohense* (p=0.0007), *Dialister invisus* (p=0.0014), *Klebsiella pneumonia/variicola/oxytoca* (p=0.0023), *Veillonella rogosae* (p=0.0037), *Parabacteroides distasonis* (p=0.0030), *Bacteroides xylanisolvens* (p=0.0028), *Collinsella aerofaciens* (p=0.003), *Alistipes putredinis* (p=0.0033). In the LDM that considered presence/absence, none of the 19 pre-specified taxa achieved significance when accounting for multiple comparisons (q-values ≥ 0.475) and none had a significant raw p-value (**Table 7**).

In order to discern whether particular ASV within the Diverse CST (CST IV) were associated with the occurrence of sPTB, we applied the LDM model to only those study participants with Diverse CST considering 11 taxa comprised of the 9 most abundant ASV within CST IV (BVAB1, *Garderella vaginalis, Atopbium vaginae*, an ASV in genus *Prevotella, Sneathia sanguinegens, Prevotella timonensis, Prevotella amnii, Aerococcus christensenii*, an ASV in genus *Megasphaera*) in addition to *L. iners* and non-iners *Lactobacillus*. For the relative abundance LDM for sPTB vs. full term birth (N = 136 samples) within the Diverse CST, the global p-value for the vaginal microbiome was not significant (p=0.888) and the relative abundance of none of these 11 ASV were associated with sPTB when accounting for multiple comparisons, although the raw p-value for one ASV was nominally significant (*Aerococcus christensenii*, p=0.0367) at the 5% level. For the presence/absence modeling for sPTB vs. full term birth within the Diverse CST, the global p-value also was not significant (p=0.448) and the presence/absence of none of the 11 ASVs were associated with sPTB in accounting for multiple comparisons although the raw p-value for one ASV was nominally significant (*Atopobium vaginae*, p=0.0392) at the 5% level. To further investigate whether the differential relative abundance of particular taxa within each CST category associated with sPTB we calculated the Bray-Curtis dissimilarity index for those with sPTB vs. full term birth; for each CST category, we show (**Figure 4**) that the within CST category composition did not significantly differ based on sPTB vs. full term birth status (non-iners *Lactobacillus* CST, p=0.84; *Lactobacillus iners* CST, p=0.81; Diverse CST, p=0.95).

DISCUSSION

This study adds to the growing literature characterizing the vaginal microbiome and sPTB among AA women, who have been underrepresented in research despite having higher rates of PTB relative to US women of other races/ethnicities. In this cohort of AA women, those with an early pregnancy vaginal CST IV (Diverse CST) had a substantially and significantly elevated risk for sPTB compared to AA women with a vaginal microbiome classified as CST I, II, or V (non-iners *Lactobacillus* dominated), and those with vaginal CST III (*L. iners* dominated) were of intermediate risk between CST IV and CST I, II, or V (non-iners *Lactobacillus* dominated). Notably, nearly half (49%) of women in the Atlanta African American cohort had an early pregnancy vaginal microbiome classified as CST IV (Diverse CST) while nearly one-third (35%) had CST III (*L. iners* dominated). Despite finding a strong association between vaginal CST IV and risk of sPTB in this AA cohort, neither the relative abundance nor presence/absence of any particular ASV was associated with sPTB when considering women across all CST categories or within CST IV. In particular, within the three CST groups, we found no evidence of differences in microbial composition between women who experienced sPTB and women who delivered full term, further suggesting that these CST groupings are sufficient to explain the patterns we observed.

In this cohort of AA women, there was no significant association between vaginal CST in early pregnancy and risk of sETB, however, the LDM identified 7 ASV (*Bacteroides vulgatus, Bacteroides xylanisolvens, Bifidobacterium kashiwanohense, Blautia luti, Campylobacter hominis, Collinsella aerofaciens Dialister invisus*) whose higher relative abundance and 13 ASV (*Akkermansi muciniphila, Alistipes putredinis, Bacteroides xylanisolvens, Bifidobacterium breve, Bifidobacterium kashiwanohense, Bifidobacterium longum, Blautia luti, Citrobacter freundi/gillenii/youngae, Collinsella aerofaciens, Dialister invisus, Klebsiella pneumonia/variicola/oxytoca, Parabacteroides distasonis, Veillonella rogosae*) whose presence was associated with sETB. Notably, these ASV identified in both the relative abundance and presence/absence model are those typically considered as gut-associated, although they are not the most common taxa found in fecal samples (Truong et al., 2017). Previous research has documented the presence of gut and oral bacterial populations in the vaginal microbiome, particularly in cases of bacterial vaginosis, and has suggested that their presence may be linked to fecal or oral transplantation (Africa et al., 2014; Fenollar and Raoult, 2016). Previous research has described *Bifidobacterium breve* and *B. longum* occurring as dominant members of the vaginal microbiome (Freitas and Hill, 2017) and that the vagina and gut are, in fact, colonized by a shared community of *Bifidobacterium* (Freitas and Hill, 2018). To rule out the possibility that these "gut-like" taxa that we observed as

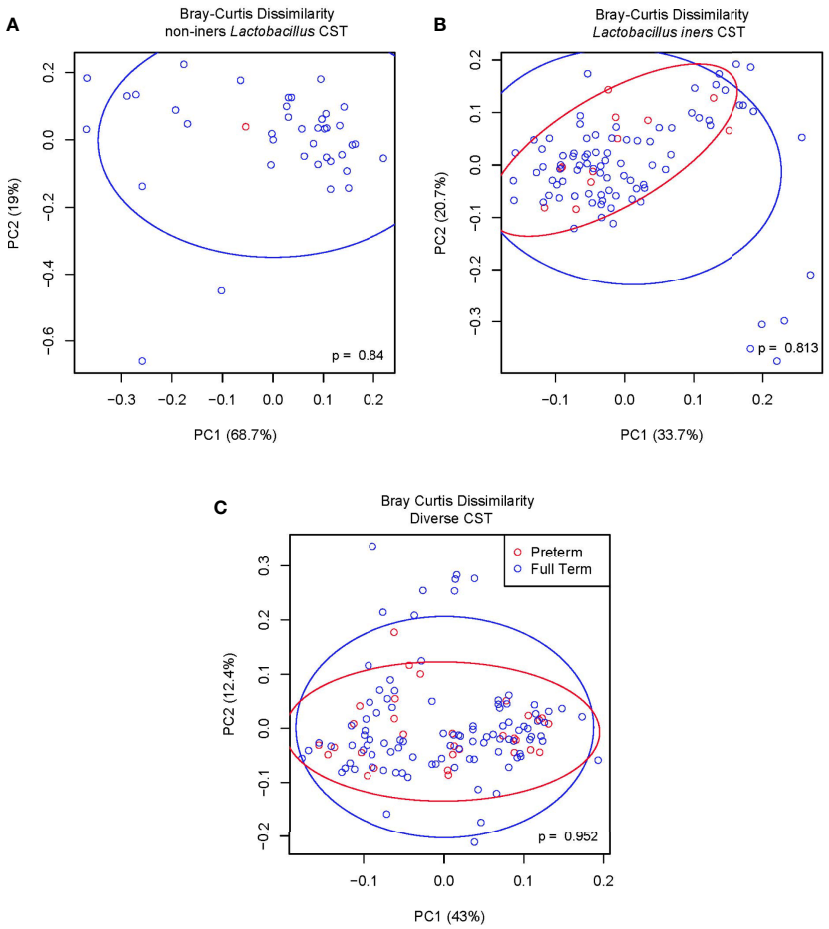

FIGURE 4 | **(A)** Bray-Curtis dissimilarity non-iners *Lactobacillus* CST. **(B)** Bray-Curtis dissimilarity *Lactobacillus iners* CST. **(C)** Bray Curtis dissimilarity diverse CST.

associated with sETB were artifactual, we conducted sensitivity analyses. First, we repeated our LDM analyses after removing data from three women with sETB that had the highest abundance of these "gut-like" taxa. Second, based on counts for these taxa observed in our no-template controls, we applied a threshold to require all ASVs to have a raw count of 20 or more to be included in the LDM analyses, which were repeated using both relative abundance and presence-absence data. In all of these instances, the conclusions were essentially unchanged and the taxa with the smallest raw p-values continued to be these "gut-like" taxa. Finally, we note that case samples (sPTB and sETB) were carefully balanced across extraction batches, PCR plates and sequencing lanes in these experiments.

In summary, the findings from this study support that the early pregnancy microbiome of AA women is important in the risk of both sPTB and sETB. In this cohort of AA women, an early pregnancy vaginal CST III or IV was associated with an increased risk of sPTB but not an increased risk of sETB. The LDM based on relative abundance found a significant overall effect of the vaginal microbiome on sPTB (p=0.034) but not sETB (p=0.320), whereas the LDM based on presence/absence of ASV found no overall effect on sPTB (p=0.328) but a significant effect on sETB (p=0.030). Using the LDM to test for ASV-specific effects, no ASV was significantly associated with sPTB considering either relative abundance or presence/absence data after controlling for multiple comparisons (FDR 10%), although in marginal analyses of taxa previously shown to be associated with sPTB, the relative abundance of *Gardnerella vaginalis* (p=0.011), non-iners *Lactobacillus* (p=0.016), and *Mobiluncus curtisii* (p=0.035) and the presence of *Atopobium vaginae* (p=0.049), BVAB2 (p=0.024), *Dialister microaerophilis* (p=0.011), and *Prevotella amnii* (p=0.044) were associated with sPTB. The LDM identified the higher abundance of 7 ASV and the presence of 13 ASV, all commonly residents of the gut, as associated with sETB at FDR of 10%. Although the clinical literature conveys that the occurrence and recurrence of PTB and ETB share common risk factors that suggests that these outcomes share a common etiology and reflect a continuum of risk related to shortened gestation (Delnord and Zeitlin, 2019), our findings support that the microbial risks may be unique. It is difficult to speculate as to why the presence of gut-associated bacteria would be associated with sETB but not sPTB, but this may relate to differences in inflammatory milieu or microbial products of the gut-like taxa. We plan to explore this area of

research further through a metagenomics approach that allows for functional profiling of these bacterial taxa and their virulence properties. Taken together, these findings suggest that it is the overall composition of the vaginal microbiome with respect to common taxa, particularly the low relative abundance of non-iners *Lactobacillus* spp. that is the relevant feature in shaping sPTB risk, whereas it is the relative abundance or presence/absence of rare, potentially pathogenic taxa that is relevant in shaping sETB risk. An alternative explanation for these findings, however, is that the quantitative level (qPCR concentrations) of minority and pathogenic bacterial taxa may be a driver of sPTB, but exploration of this issue is beyond the scope of this project.

To our knowledge, this is the first study to report on the association between the vaginal microbiome and risk of sETB. Our findings around the association between the vaginal microbiome and risk of sETB are important for contextualizing and interpreting earlier vaginal microbiome and birth outcome studies, many of which did not distinguish ETB as a category but rather included births in this category in the "term birth" category. While more research in a broader study population is warranted, our findings support that the inclusion of ETB in the comparison category of term births could result in bias.

Direct comparison of the findings of our study with those of other studies of the vaginal microbiome and PTB is difficult as there is substantial heterogeneity in methods across published studies, and differing methods of DNA extraction, PCR amplification (including the selection of particular primers), bioinformatic processing and taxonomic classification are known to impact the sensitivity for identifying particular taxa (Stackebrandt and Goebel, 1994; Gill et al., 2016; Gohl et al., 2016). However, it is notable that our findings around the importance of the relative abundance of non-iners *Lactobacillus* and *L. iners* are similar to those reported previously for an AA cohort, which found that a lower abundance of *L. crispatus*, *L. gasseri*, and *L. jensenii* were significantly associated with PTB risk. This agreement is more remarkable as that cohort was restricted to women with a prior history of sPTB who received progesterone therapy in the subsequent pregnancies that comprise their cohort (Callahan et al., 2017b). Also, in a study restricted to women with a prior sPTB, vaginal CST IV was found to be a significant risk factor for a recurrent sPTB (Gerson et al., 2020). Conversely, studies that have considered substantial numbers of cases of PTB among AA women did find particular taxa that increased the risk of PTB, whereas our study did not. Specifically, in a cohort focused on AA women (45 with sPTB, 90 with term birth matched for age and income) those with sPTB had significantly lower abundance of *L. crispatus*, higher abundance of BVAB1 (candidate name *Candidatus Lachnocurva vaginae*), *Sneathia amnii*, TM7-HI, and a group of Prevotella whereas women that delivered term were more likely to have *L. crispatus* and decreased prevalence of *A. vaginae* and *G. vaginalis* (Fettweis et al., 2019). In another cohort of predominantly AA women (107 with sPTB, 432 with term birth), seven specific taxa were significantly associated with increased risk of sPTB (*Sneathia sanguinegens, Mobiluncus curtisii/mulleris, Mageeibacillus indolicus, Megasphaera, Porphyromonas asaccaraolycia, Prevotella buccalis, Atopbium*) with a stronger effect among AA women (Elovitz et al., 2019). Also, in contrast to our study's findings, that same study found an association between CST IV and sPTB among non-AA women but no significant association among AA women (Elovitz et al., 2019). Another predominantly AA cohort study also found no association between vaginal CST and sPTB vs. term birth (Romero et al., 2014a). It is possible that differences in the timing of sampling collection (8-14 weeks gestation for our study in contrast to 24 weeks or fewer for the other studies) contribute to differences in findings among AA women, given that the vaginal microbiome composition does change across pregnancy (Romero et al., 2014a; Romero et al., 2014b). The significance of vaginal CST IV in early pregnancy upon sPTB risk suggests that the vaginal composition early in pregnancy is more influential upon PTB risk than that in mid-pregnancy.

Findings from this study also supports that the CST IV (Diverse CST) is associated with a vaginal cytokine profile that is more pro-inflammatory than that of CST I, II, or V (non-iners *Lactobacillus*) or CST III (*L. iners*), which is consistent with other research. Vaginal lactobacilli, including *L. crispatus*, *L. gasseri*, and *L. jensenii*, are known to play a critical role in regulating the inflammatory response in the female genitourinary tract (Anahtar et al., 2015), with *L. iners* having a comparatively upregulated inflammatory response (Doerflinger et al., 2014). A study that contrasted the inflammatory profile of a vaginal microbiome dominated by *L. crispatus* vs. a vaginal microbiome dominated by *A. vaginae* found unique species-specific innate immune signatures with *L. crispatus* colonization resulting in low epithelial cell activation and minimal disruption of the immune barrier properties and *A. vaginae* inducing a robust inflammatory profile that disrupts physiochemical barrier properties of the vaginal mucosa (Doerflinger et al., 2014). Experimental research also supports that cytokine production by vaginal epithelial cells in response to stimuli varies according to vaginal microbiome composition. Vaginal epithelial cells harvested from women with a vaginal microbiome not dominated by *Lactobacillus* have been found to produce less lactic acid and induce greater inflammatory cytokine production of IL-1α, IL-1β, IL-6, IL-8, macrophage inflammatory protein (MIP)-1α, and MIP-1β in response to the presence of *Gardnerella* relative to epithelial cells from women whose vaginal microbiome is *Lactobacillus* dominated (Manhanzva et al., 2020). The prevailing theory is that, when *Lactobacillus* dominance is lost and vaginal microbial diversity increases, the production of pro-inflammatory cytokines along with the recruitment of immune cells and reduced viscosity of the cervicovaginal fluid result in changes in immune and epithelial homeostasis, which ultimately affect the barrier properties of the genital epithelium and cervix, increasing the risk for ascending infection (Amabebe and Anumba, 2018; Torcia, 2019). Ascending intrauterine infection early in gestation accounts for an estimated 50% of spontaneous PTB (Goldenberg et al., 2008).

This study has substantial strengths for investigating the role of vaginal microbiome composition and PTB among AA

women. First, this study exclusively focused on AA women and, as such, had a relatively large number of cases of PTB across CST categories to consider the within-race risk of PTB according to CST. Second, our protocol allowed for the careful phenotypic differentiation of PTB into sPTB and medically-indicated categories based on in-depth review of the prenatal and labor and delivery record, minimizing the opportunity for misclassification which might affect studies of self-report of type of PTB. Although a related issue is that this study restricted enrollment to women without chronic medical conditions, in order to reduce the number of early deliveries that were attributable to medical indications, which may affect the generalizability of our findings to the broader population of African American women who give birth, a substantial proportion of whom would be expected to have chronic medical conditions (DeSisto et al., 2018; Harris et al., 2020). Third, in this study we also controlled for known socioeconomic and clinical risk factors for PTB (as identified in the literature and as occurring among our study population) as well as socioeconomic factors associated with CST in our study population (such as maternal education and health insurance status), strengthening the likelihood that the association between CST IV and PTB is a true association rather than confounding. Finally, the vaginal samples analyzed here were collected within a fairly narrow gestational age window during early pregnancy.

This study is not without limitations, most of which have affected other existing studies of the microbiome. The potential to discover microorganisms whose presence in the vaginal tract is related to the occurrence of sPTB is limited by the inherent low resolution of 16S amplicon sequencing. Despite the known polymicrobial nature of PTB (Payne and Bayatibojakhi, 2014), 16S rRNA gene sequencing cannot identify non-bacterial microbes, such as viruses, fungi, or protozoa, all of which have been linked to PTB risk (Payne and Bayatibojakhi, 2014). Furthermore, 16S rRNA gene approaches are limited in their ability to reliably assign many bacterial genera to the species level and to distinguish strains (Frank et al., 2008; Fettweis et al., 2012; Poretsky et al., 2014). Strains within a species have variability in capacity for virulence due to differences in accessory genes (Tett et al., 2017). Within the vaginal microbiome, the presence of a few highly dominant species common among women experiencing either PTB or term birth (e.g., *L. iners, G. vaginalis*) suggests that crucial differences in the microbiome occur at the *strain level*. Emerging evidence supports that strain-level differences may contribute to PTB risk including that the genome of some *L. iners* strains encode inerolysin, a pore-forming toxin related to vaginolysin of *G. vaginalis*, suggesting clonal variants that in some cases promote a healthy vagina and in others dysbiosis and disease (Petrova et al., 2016); a strain of *G. vaginalis* explains the genus association with PTB in some but not other studies (Callahan et al., 2017a); a strain of *Sneathia amnii* may confer virulence features to BV-associated bacteria and may explain the variable association of BV and the genus *Sneathia* with PTB (Harwich et al., 2012).

In future analyses, we will focus on exploring particular social, environmental, and biobehavioral exposures that may contribute to the high prevalence of CST IV among women in the Atlanta African American cohort as well as employing whole-genome shotgun sequencing to better understand the potential role of particular bacterial strains – especially those present within the CST IV – whose accessory genes might drive risk for sPTB. An understanding of associated exposure and risk factors linked to CST IV in early pregnancy, as well as particular strains within CST IV that are linked with sPTB, may identify modifiable risks that could be targeted through health education and/or public health or clinical interventions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/sra, PRJNA725416.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Emory University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AD and EC conceived the study design, oversaw data collection, and conceptualized and wrote the manuscript. GS advised on methods, supervised the statistical findings, and contributed to the writing of the manuscript. Y-JH performed the statistical modeling and computations. TR and BP contributed to the design of the study and to the manuscript. MW contributed to the interpretation of results and to the manuscript. AK and AS contributed to the conduct of the experiments and to the manuscript. All authors discussed results, provided critical feedback and helped shape the research, analysis, and manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.641005/full#supplementary-material

REFERENCES

Africa, C. W., Nel, J., and Stemmet, M. (2014). Anaerobes and Bacterial Vaginosis in Pregnancy: Virulence Factors Contributing to Vaginal Colonisation. *Int. J. Environ. Res. Public Health* 11, 6979–7000. doi: 10.3390/ijerph110706979

Amabebe, E., and Anumba, D. O. (2018). The Vaginal Microenvironment: The Physiologic Role of Lactobacilli. *Front. Med.* 5, 181. doi: 10.3389/fmed.2018.00181

American College of Obstetricians and Gynecologists (2013). Definition of Term Pregnancy. Committee Opinion No. 579. *Obstet. Gynecol.* 122, 1139–1140. doi: 10.1097/01.AOG.0000437385.88715.4a

American College of Obstetricians and Gynecologists (2014). Method for Estimating Due Date. Committee Opinion No. 611. *Obstet. Gynecol.* 124, 863–866. doi: 10.1097/01.AOG.0000454932.15177.be

Anahtar, M. N., Byrne, E. H., Doherty, K. E., Bowman, B. A., Yamamoto, H. S., Soumillon, M., et al. (2015). Cervicovaginal Bacteria are a Major Modulator of Host Inflammatory Responses in the Female Genital Tract. *Immunity* 42, 965–976. doi: 10.1016/j.immuni.2015.04.019

Ananth, C. V., and Vintzileos, A. M. (2008). Medically Indicated Preterm Birth: Recognizing the Importance of the Problem. *Clinics Perinatol.* 35, 53–67. doi: 10.1016/j.clp.2007.11.001

Behrman, R., and Butler, A. (2007). *Institute of Medicine (US) Committee on Understanding Premature Birth and Assuring Healthy Outcomes. Preterm Birth: Causes, Consequences, and Prevention* (Washington, DC: National Academies Press).

Brown, R., Kindinger, L., Lee, Y. S., Marchesi, J. R., Bennett, P., and MacIntyre, D. (2016). Role of the Vaginal Microbiome in Preterm Prelabour Rupture of the Membranes: An Observational Study. *Lancet* 387, S22. doi: 10.1016/S0140-6736(16)00409-8

Callaghan, W. M., McDorman, M. F., and Rasmussen, S. A. (2006). the Contribution of Preterm Birth to Infant Mortality Rates in the United States. *Pediatrics* 118, 1566–1573. doi: 10.1542/peds.2006-0860

Callahan, B. J., DiGiulio, D. B., Goltsman, D. S. A., Sun, C. L., Costello, E. K., Jeganathan, P., et al. (2017a). Replication and refinement of a vaginal microbial signature of preterm birth in two racially distinct cohorts of US women. *Proc. Natl. Acad. Sci.*, 114 (37), 9966–9971. doi: 10.1073/pnas.1705899114

Callahan, B. J., DiGiulio, D. B., Goltsman, D. S. A., Sun, C. L., Costello, E. K., Jeganathan, P., et al. (2017b). Replication and Refinement of a Vaginal Microbial Signature of Preterm Birth in Two Racially Distinct Cohorts of US Women. *Proc. Natl. Acad. Sci.* 114, 9966–9971. doi: 10.1073/pnas.1705899114

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-Resolution Sample Inference From Illumina Amplicon Data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Cammack, A. L., Buss, C., Entringer, S., Hogue, C. J., Hobel, C. J., and Wadhwa, P. D. (2011). The Association Between Early Life Adversity and Bacterial Vaginosis During Pregnancy. *Am. J. Obstet. Gynecol.* 204, 431. e431–431. doi: 10.1016/j.ajog.2011.01.054

Carey, M. P., Carey, K. B., Maisto, S. A., Gordon, C. M., and Weinhardt, L. S. (2001). Assessing Sexual Risk Behavior With the Timeline Followback (TLFB) Approach: Continued Development and Psychometric Evaluation. *Int. J. STD AIDS* 12, 365–375. doi: 10.1258/0956462011923309

Corwin, E. J., Hogue, C. J., Pearce, B., Hill, C. C., Read, T. D., Mulle, J., et al. (2017). Protocol for the Emory University African American Vaginal, Oral, and Gut Microbiome in Pregnancy Cohort Study. *BMC Pregnancy Childbirth* 17, 161. doi: 10.1186/s12884-017-1357-x

Delnord, M., and Zeitlin, J. (2019). "Epidemiology of Late Preterm and Early Term Births–an International Perspective," in *Seminars in Fetal and Neonatal Medicine*, (WB Saunders) 24 (1), 3–10. doi: 10.1016/j.siny.2018.09.001

DeSisto, C. L., Hirai, A. H., Collins, J. W.Jr, and Rankin, K. M. (2018). Deconstructing a Disparity: Explaining Excess Preterm Birth Among US-Born Black Women. *Ann. Epidemiol.* 28, 225–230. doi: 10.1016/j.annepidem.2018.01.012

DiGiulio, D. B., Callahan, B. J., McMurdie, P. J., Costello, E. K., Lyell, D. J., Robaczewska, A., et al. (2015). Temporal and Spatial Variation of the Human Microbiota During Pregnancy. *Proc. Natl. Acad. Sci.* 112, 11060–11065. doi: 10.1073/pnas.1502875112

Doerflinger, S. Y., Throop, A. L., and Herbst-Kralovetz, M. M. (2014). Bacteria in the Vaginal Microbiome Alter the Innate Immune Response and Barrier Properties of the Human Vaginal Epithelia in a Species-Specific Manner. *J. Infect. Dis.* 209, 1989–1999. doi: 10.1093/infdis/jiu004

Dunlop, A. L., Knight, A. K., Satten, G. A., Cutler, A. J., Wright, M. L., Mitchell, R. M., et al. (2019). Stability of the Vaginal, Oral, and Gut Microbiota Across Pregnancy Among African American Women: The Effect of Socioeconomic Status and Antibiotic Exposure. *PeerJ* 7, e8004. doi: 10.7717/peerj.8004

Elovitz, M. A., Gajer, P., Riis, V., Brown, A. G., Humphrys, M. S., Holm, J. B., et al. (2019). Cervicovaginal Microbiota and Local Immune Response Modulate the Risk of Spontaneous Preterm Delivery. *Nat. Commun.* 10, 1–8. doi: 10.1038/s41467-019-09285-9

Fadrosh, D. W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R. M., et al. (2014). an Improved Dual-Indexing Approach for Multiplexed 16S Rrna Gene Sequencing on the Illumina Miseq Platform. *Microbiome* 2, 6. doi: 10.1186/2049-2618-2-6

Fenollar, F., and Raoult, D. (2016). Does Bacterial Vaginosis Result From Fecal Transplantation? *J. Infect. Dis.* 214, 1784–1784. doi: 10.1093/infdis/jiw472

Fettweis, J. M., Brooks, J. P., Serrano, M. G., Sheth, N. U., Girerd, P. H., Edwards, D. J., et al. (2014). Differences in Vaginal Microbiome in African American Women Versus Women of European Ancestry. *Microbiology* 160, 2272–2282. doi: 10.1099/mic.0.081034-0

Fettweis, J. M., Serrano, M. G., Brooks, J. P., Edwards, D. J., Girerd, P. H., Parikh, H. I., et al. (2019). the Vaginal Microbiome and Preterm Birth. *Nat. Med.* 25, 1012–1021. doi: 10.1038/s41591-019-0450-2

Fettweis, J. M., Serrano, M. G., Sheth, N. U., Mayer, C. M., Glascock, A. L., Brooks, J. P., et al. (2012). Species-Level Classification of the Vaginal Microbiome. *BMC Genomics* 13, S17. doi: 10.1186/1471-2164-13-S8-S17

Forney, L. J., Gajer, P., Williams, C. J., Schneider, G. M., Koenig, S. S. K., McCulle, S. L., et al. (2010). Comparison of Self-Collected and Physician-Collected Vaginal Swabs for Microbiome Analysis. *J. Clin. Microbiol.* 48, 1741–1748. doi: 10.1128/JCM.01710-09

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., and Olsen, G. J. (2008). Critical Evaluation of Two Primers Commonly Used for Amplification of Bacterial 16S Rrna Genes. *Appl. Environ. Microbiol.* 74, 2461–2470. doi: 10.1128/AEM.02272-07

Freitas, A. C., and Hill, J. E. (2017). Quantification, Isolation and Characterization of Bifidobacterium From the Vaginal Microbiomes of Reproductive Aged Women. *Anaerobe* 47, 145–156. doi: 10.1016/j.anaerobe.2017.05.012

Freitas, A. C., and Hill, J. E. (2018). Bifidobacteria Isolated From Vaginal and Gut Microbiomes are Indistinguishable by Comparative Genomics. *PLoS One* 13, e0196290. doi: 10.1371/journal.pone.0196290

Gajer, P., Brotman, R., Bai, G., Sakamoto, J., Schütte, U., Zhong, X., et al. (2012). Temporal Dynamics of the Human Vaginal Microbiota. *Sci. Trans. Med.* 4, 132ra52. doi: 10.1126/scitranslmed.3003605

Georgia Medicaid (2020) *Who Qualifies for Georgia Medicaid: Eligibility Faqs*. Available at: https://medicaid.georgia.gov/eligibility-faqs (Accessed September 1).

Gerson, K. D., Mccarthy, C., Ravel, J., Elovitz, M. A., and Burris, H. H. (2020). Effect of a Nonoptimal Cervicovaginal Microbiota and Psychosocial Stress on Recurrent Spontaneous Preterm Birth. *Am. J. Perinatol.* 38 (5), 407-413. doi: 10.1055/s-0040-1717098

Gill, C., van de Wijgert, J. H., Blow, F., and Darby, A. C. (2016). Evaluation of Lysis Methods for the Extraction of Bacterial DNA for Analysis of the Vaginal Microbiota. *PLoS One* 11, e0163148. doi: 10.1371/journal.pone.0163148

Gohl, D. M., Vangay, P., Garbe, J., MacLean, A., Hauge, A., Becker, A., et al. (2016). Systematic Improvement of Amplicon Marker Gene Methods for Increased Accuracy in Microbiome Studies. *Nat. Biotechnol.* 34, 942–949. doi: 10.1038/nbt.3601

Goldenberg, R. L., Culhane, J. F., Iams, J. D., and Romero, R. (2008). Epidemiology and Causes of Preterm Birth. *Lancet* 371, 75–84. doi: 10.1016/S0140-6736(08)60074-4

Harris, M., Henke, C., Hearst, M., and Campbell, K. (2020). Future Directions: Analyzing Health Disparities Related to Maternal Hypertensive Disorders. *J. Pregnancy* 2020, 7864816. doi: 10.1155/2020/7864816

Harwich, M. D., Serrano, M. G., Fettweis, J. M., Alves, J. M., Reimers, M. A., Buck, G. A., et al. (2012). Genomic Sequence Analysis and Characterization of Sneathia Amnii Sp. nov. *BMC Genomics* 13, S4. doi: 10.1186/1471-2164-13-S8-S4

Holm, J. B., Humphrys, M. S., Robinson, C. K., Settles, M. L., Ott, S., Fu, L., et al. (2019). Ultrahigh-Throughput Multiplexing and Sequencing of> 500-Base-Pair Amplicon Regions on the Illumina Hiseq 2500 Platform. *MSystems* 4 (1), e0029-19. doi: 10.1128/mSystems.00029-19

Hu, Y.-J., and Satten, G. A. (2020). Testing hypotheses about the microbiome using the linear decomposition model (LDM). *Bioinformatics* 36 (14), 4106–4115. doi: 10.1093/bioinformatics/btaa260

Hu, Y. J., Lane, A., and Satten, G. A. (2021). A rarefaction-based extension of the LDM for testing presence–absence associations in the microbiome. *Bioinformatics* btab012. doi: 10.1093/bioinformatics/btab012

Hyman, R. W., Fukushima, M., Jiang, H., Fung, E., Rand, L., Johnson, B., et al. (2014). Diversity of the Vaginal Microbiome Correlates With Preterm Birth. *Reprod. Sci.* 21, 32–40. doi: 10.1177/1933719113488838

Kindinger, L. M., Bennett, P. R., Lee, Y. S., Marchesi, J. R., Smith, A., Cacciatore, S., et al. (2017). the Interaction Between Vaginal Microbiota, Cervical Length, and Vaginal Progesterone Treatment for Preterm Birth Risk. *Microbiome* 5, 6. doi: 10.1186/s40168-016-0223-9

King, G., and Zeng, L. (2001). Logistic Regression in Rare Events Data. *Political Anal.* 9, 137–163. doi: 10.1093/oxfordjournals.pan.a004868

Kramer, M. R., and Hogue, C. R. (2009). What Causes Racial Disparities in Very Preterm Birth? *A biosocial Perspect. Epidemiol. Rev.* 31, 84–98. doi: 10.1093/ajerev/mxp003

Kuczynski, J., Stombaugh, J., Walters, W. A., González, A., Caporaso, J. G., and Knight, R. (2012). Using QIIME to Analyze 16S Rrna Gene Sequences From Microbial Communities. *Curr. Protoc. Microbiol.* 27, 1E. 5.1–1E. 5.20. doi: 10.1002/9780471729259.mc01e05s27

MacIntyre, D. A., Chandiramani, M., Lee, Y. S., Kindinger, L., Smith, A., Angelopoulos, N., et al. (2015). the Vaginal Microbiome During Pregnancy and the Postpartum Period in a European Population. *Sci. Rep.* 5, 8988. doi: 10.1038/srep08988

Manhanzva, M. T., Abrahams, A. G., Gamieldien, H., Froissart, R., Jaspan, H., Jaumdally, S. Z., et al. (2020). Inflammatory and Antimicrobial Properties Differ Between Vaginal Lactobacillus Isolates From South African Women With Non-Optimal Versus Optimal Microbiota. *Sci. Rep.* 10, 1–13. doi: 10.1038/s41598-020-62184-8

Martin, J. A., Hamilton, B. E., Osterman, M. J., and Driscoll, A. K. (2019) *Births: Final Data for 2018* (National Center for Health Statistics Dta Brief) (Accessed September 1, 2020).

Mathews, T. J., and Driscoll, A. K. (2017). "Trends in Infant Mortality in the United State-2014," in *NCHS Data Brief no 279*. (Hyattsville, MD: National Center for Health Statistics).

McGrady, G. A., Sung, J. F., Rowley, D. L., and Hogue, C. J. (1992). Preterm Delivery and Low Birth Weight Among First-Born Infants of Black and White College Graduates. *Am. J. Epidemiol.* 136, 266–276. doi: 10.1093/oxfordjournals.aje.a116492

McInnes, P., and Cutting, M. (2010). *Manual of Procedures for the Human Microbiome Project* (Bethesda, MD: National Institutes of Health).

Nelson, D. B., Shin, H., Wu, J., and Dominguez-Bello, M. G. (2016). the Gestational Vaginal Microbiome and Spontaneous Preterm Birth Among Nulliparous African American Women. *Am. J. Perinatol.* 33, 887–893. doi: 10.1055/s-0036-1581057

Nugent, R. P., Krohn, M. A., and Hillier, S. L. (1991). Reliability of Diagnosing Bacterial Vaginosis is Improved by a Standardized Method of Gram Stain Interpretation. *J. Clin. Microbiol.* 29, 297–301. doi: 10.1128/JCM.29.2.297-301.1991

Payne, M. S., and Bayatibojakhi, S. (2014). Exploring Preterm Birth as a Polymicrobial Disease: An Overview of the Uterine Microbiome. *Front. Immunol.* 5, 595. doi: 10.3389/fimmu.2014.00595

Petrova, M. I., Reid, G., Vaneechoutte, M., and Lebeer, S. (2016). Lactobacillus Iners: Friend or Foe? *Trends Microbiol.* 25 (3), 182–191. doi: 10.1016/j.tim.2016.11.007

Poretsky, R., Rodriguez-R, L. M., Luo, C., Tsementzi, D., and Konstantinidis, K. T. (2014). Strengths and Limitations of 16S Rrna Gene Amplicon Sequencing in Revealing Temporal Microbial Community Dynamics. *PLoS One* 9, e93827. doi: 10.1371/journal.pone.0093827

Ravel, J., Gajera, P., Abdob, Z., Schneiderc, G. M., Koeniga, S. S., McCullea, S. L., et al. (2011). Vaginal Microbiome of Reproductive-Age Women. *Proc. Natl. Acad. Sci.* 108, 4680–4687. doi: 10.1073/pnas.1002611107

Robinson, S. M., Sobell, L. C., Sobell, M. B., and Leo, G. I. (2014). Reliability of the Timeline Followback for Cocaine, Cannabis, and Cigarette Use. *Psychol. Addictive Behav.* 28, 154. doi: 10.1037/a0030992

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Bieda, J., et al. (2014a). The Vaginal Microbiota of Pregnant Women Who Subsequently Have Spontaneous Preterm Labor and Delivery and Those With a Normal Delivery At Term. *Microbiome* 2, 18. doi: 10.1186/2049-2618-2-18

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Nikita, L., et al. (2014b). The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome*, 2 (1), 1-19. doi: 10.1186/2049-2618-2-10

Rowley, D. L., Hogue, C. J., Blackmore, C. A., Ferre, C. D., Hatfield-Timacjchy, K., Branch, P., et al. (1993). Preterm Delivery Among African-American Women: A Research Strategy. *Am. J. Prev. Med.* 9, 1–6. doi: 10.1016/S0749-3797(18)30659-7

Ruxton, G. D., and Neuhäuser, M. (2010). When Should We Use One-Tailed Hypothesis Testing? *Methods Ecol. Evol.* 1, 114–117. doi: 10.1111/j.2041-210X.2010.00014.x

Schmieder, R., Lim, Y. W., Rohwer, F., and Edwards, R. (2010). Tagcleaner: Identification and Removal of Tag Sequences From Genomic and Metagenomic Datasets. *BMC Bioinf.* 11, 341. doi: 10.1186/1471-2105-11-341

Sobell, L. C., and Sobell, M. B. (1996). *Timeline Followback User's Guide: A Calendar Method for Assessing Alcohol and Durg Use* (Toronto, Ontario, Canada: Addiction Research Foundation).

Stackebrandt, E., and Goebel, B. (1994). Taxonomic Note: A Place for DNA-DNA Reassociation and 16S Rrna Sequence Analysis in the Present Species Definition in Bacteriology. *Int. J. System. Evol. Microbiol.* 44, 846–849. doi: 10.1099/00207713-44-4-846

Stout, M. J., Zhou, Y., Wylie, K. M., Tarr, P. I., Macones, G. A., and Tuuli, M. G. (2017). Early Pregnancy Vaginal Microbiome Trends and Preterm Birth. *Am. J. Obstet. Gynecol.* 217 (3), 355–3e1. doi: 10.1016/j.ajog.2017.05.030

Tett, A., Pasolli, E., Farina, S., Truong, D. T., Asnicar, F., Zolfo, M., et al. (2017). Unexplored Diversity and Strain-Level Structure of the Skin Microbiome Associated With Psoriasis. *NPJ Biofilms Microbiomes* 3, 14. doi: 10.1038/s41522-017-0022-5

Torcia, M. G. (2019). Interplay Among Vaginal Microbiome, Immune Response and Sexually Transmitted Viral Infections. *Int. J. Mol. Scienes* 20 (2), 266. doi: 10.3390/ijms20020266

Truong, D. T., Tett, A., Pasolli, E., Huttenhower, C., and Segata, N. (2017). Microbial Strain-Level Population Structure and Genetic Diversity From Metagenomes. *Genome Res.* 27, 626–638. doi: 10.1101/gr.216242.116

Wen, A., Srinivasan, U., Goldberg, D., Owen, J., Marrs, C. F., Misra, D., et al. (2013). Selected Vaginal Bacteria and Risk of Preterm Birth: An Ecological Perspective. *J. Infect. Dis.* 209, 1087–1094. doi: 10.1093/infdis/jit632

Zhou, X., Brown, C. J., Abdo, Z., Davis, C. D., Hansmann, M. A., Joyce, P., et al. (2007). Differences in the Composition of Vaginal Microbial Communities

Found in Healthy Caucasian and Black Women. *ISME J.* 1, 121–133. doi: 10.1038/ismej.2007.12

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The Vaginal Microbial Signatures of Preterm Birth Delivery in Indian Women

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Background: The incidence of preterm birth (PTB) in India is around 13%. Specific bacterial communities or individual taxon living in the vaginal milieu of pregnant women is a potential risk factor for PTB and may play an important role in its pathophysiology. Besides, bacterial taxa associated with PTB vary across populations.

Objective: Conduct a comparative analysis of vaginal microbiome composition and microbial genomic repertoires of women who enrolled in the Interdisciplinary Group for Advanced Research on Birth Outcomes – A DBT India Initiative (GARBH-Ini) pregnancy cohort to identify bacterial taxa associated with term birth (TB) and PTB in Indian women.

Methods: Vaginal swabs were collected during all three trimesters from 38 pregnant Indian women who delivered spontaneous term (n=20) and preterm (n=18) neonates. Paired-end sequencing of V3-V4 region of 16S rRNA gene was performed using the metagenomic DNA isolated from vaginal swabs (n=115). Whole genome sequencing of bacterial species associated with birth outcomes was carried out by shotgun method. *Lactobacillus* species were grown anaerobically in the De Man, Rogosa and Sharpe (MRS) agar culture medium for isolation of genomic DNA and whole genome sequencing.

Results: Vaginal microbiome of both term and preterm samples reveals similar alpha diversity indices. However, significantly higher abundance of *Lactobacillus iners* (p-value $_{All\_Trimesters}$<0.02), *Megasphaera* sp (p-value$_{1st_Trimester}$ <0.05), *Gardnerella vaginalis* (p-value$_{2nd_Trimester}$= 0.01) and *Sneathia sanguinegens* (p-value$_{2nd_Trimester}$ <0.0001) were identified in preterm samples whereas higher abundance of *L. gasseri* (p-value$_{3rd_Trimester}$ =0.010) was observed in term samples by Wilcoxon rank-sum test. The relative abundance of *L. iners*, and *Megasphaera* sp. were found to be significantly different over time between term and preterm mothers. Analyses of the representative genomes of *L. crispatus* and *L. gasseri* indicate presence of secretory transcriptional regulator and several ribosomally synthesized antimicrobial peptides correlated with anti-inflammatory condition in the vagina. These findings indicate protective role of *L. crispatus* and *L. gasseri* in reducing the risk of PTB.

Conclusion: Our findings indicate that the dominance of specific *Lactobacillus* species and few other facultative anaerobes are associated with birth outcomes.

Keywords: vaginal microbiota, microbial ecology, *Lactobacillus*, preterm birth, 16S rRNA gene sequencing

INTRODUCTION

Preterm birth (PTB), defined as birth before 37 completed weeks of gestation, is a major public health problem across the globe. It is one of the leading causes of neonatal mortality and morbidity in developed and developing countries (WHO, 2018). In India, out of 27 million babies born every year, 3.6 million babies are born prematurely (WHO, 2018; Sharma et al., 2018). It accounts for an estimated 40% of neonatal deaths worldwide and affects about 1 in 10 pregnancies every year (WHO, 2018). The consequences of PTB continue from early childhood into adolescence and adulthood (Marret et al., 2007; Wolke et al., 2014). Infants born prematurely also have higher rates of respiratory distress syndrome, cardiovascular disorders, neuro developmental disabilities and learning difficulties as compared to those born at term (TB) (Butler and Behrman, 2007). The underlying etiology that induces PTB may also affect maternal health. Multiple lines of evidence support a role of the vaginal microbial communities in the pathophysiology of PTB delivery (Romero et al., 2014; Klebanoff and Brotman, 2018). Less diverse vaginal microbiota has long been considered the hallmark of reproductive health and associated with TB outcome (Fettweis et al., 2019). In healthy reproductive-aged women, the vaginal microbiome generally shows a predominance of *Lactobacillus* genus. Most women display prevalence of one species among *L. crispatus*, *L. iners*, *L. jensenii* and *L. gasseri* (Ravel et al., 2011; Mehta et al., 2020). These taxa provide protection to the host through various mechanisms, such as lowering of vaginal pH, producing hydrogen peroxide (H_2O_2), synthesis of antimicrobial peptides, competition for nutrients and adhesion sites and modulation of host immune response (Parolin et al., 2015; Calonghi et al., 2017; Younes et al., 2018). However, the composition of the vaginal microbiome can vary depending upon ethnicity and exposure to different environmental factors, such as antimicrobial and non-antimicrobial drugs, diet, microbial load and exposure to different microbes in the living ecosystem (Barrientos-Duran et al., 2020).

Currently, there are several identified risk factors for PTB in which vaginal microbiome contribute substantially to the etiology. Exogenous microorganisms ephemerally colonizing the vagina have been hypothesized as an important contributor of PTB delivery. Many microorganisms isolated from the amniotic fluid or from the amniotic membrane of women who had PTB are also identified in the lower genital tract of the pregnant women (Hillier et al., 1988; Romero et al., 1989; Krohn et al., 1995; Gardella et al., 2004). The composition, diversity and dynamics of the high vaginal microbiome modulate the stability of its ecology and restrain the membership of exogenous microbial species in the vaginal niche. Dominance of non-indigenous microbial species and shift in the vaginal microbiome composition from the dominant *Lactobacillus* to a polymicrobial flora, a dysbiotic state of vaginal microbiome, substantially contribute in the pathophysiology of PTB delivery (Romero et al., 2014; Hyman et al., 2014; DiGiulio et al., 2015; Callahan et al., 2017; Klebanoff and Brotman, 2018). A number of potential microbial species, singly or in combinations, increase the risk of PTB delivery (Fettweis et al., 2019). The list of possible agents continues to expand and includes members of a number of genera, including *Gardnerella*, *Atopobium*, *Prevotella*, *Peptostreptococcus*, *Mobiluncus*, *Sneathia*, *Leptotrichia*, *Mycoplasma*, *Megasphera* and several others (Fettweis et al., 2019; Feehily et al., 2020). Recently, complex microbial assemblage, such as BVAB1, BVAB2, and BVAB3, has also been included in the continuously evolving list, as potential risk factor of PTB delivery (Fredricks et al., 2005). The metabolites or antigens produced by these microbes increases the level of local and systemic inflammatory cytokines and interstitial collagenase synthesis that have been reported to induce the PTB delivery (Stafford et al., 2017; Bukowski et al., 2017). Several molecules produced by *Lactobacillus* are linked with antimicrobial and anti-inflammatory functions and have revealed direct association with PTB risk (Stafford et al., 2017; Anton et al., 2018). However, the influences of such bacterial species and their products in the adverse birth outcomes widely vary (Amabebe and Anumba, 2018).

We recently reported the vaginal microbiome of reproductive age Indian women enrolled in the inter-disciplinary Group for Advanced Research on Birth Outcomes- A DBT India Initiative (GARBH-Ini) cohort (Mehta et al., 2020). However, we currently lack an understanding of the composition, diversity and functional repertoires of vaginal microbiome of pregnant Indian women who deliver a preterm baby. In the present study, we have investigated the differences of vaginal microbiome composition between TB and PTB samples and the genomic repertoires of the dominant *Lactobacillus* species isolated from Indian women. We studied the composition, diversity and dynamics of the vaginal microbiome by targeted sequencing of the V3-V4 hyper-variable region of the 16S rRNA gene. For functional insights, different *Lactobacillus* species associated with birth outcomes were isolated and their whole genome sequences (WGS) were decoded by shotgun sequencing. Findings of the present study enriched our knowledge to understand association of specific microbial species with birth outcomes. The WGS analysis further adds function to such microbes potentially linked with TB and PTB delivery.

MATERIALS AND METHODS

Subject Recruitment

Translational Health Science and Technology Institute human ethics committee have approved this study (Ref.# THS 1.8.1/(30)

dated 11th Feb 2015). Pregnant women who visited the antenatal clinic at Gurugram Civil Hospital (GCH) before completion of 20-weeks period of gestation (POG) and provided written informed consent were enrolled in the GARBH-Ini pregnancy cohort. POG was confirmed based on ultrasonography. Vaginal swab samples were collected from the enrolled women in each trimester of pregnancy i.e., one swab each from 1st (V1: <14 weeks), 2nd (V2: 18-20 weeks) and 3rd (V3: 26-28 weeks) trimester using sterile Catch-All™ sample collection swabs. This study was designed as a case-control study nested into the ongoing GARBH-Ini cohort (Bhatnagar et al., 2019). The cases and controls were derived from a universe of pregnant women without medical complications during pregnancy and who had singleton babies without congenital abnormalities by spontaneous delivery. The cases were women who delivered preterm. Each case was matched with a control (women who delivered at term: at 37 or more completed weeks of gestation) based on month of delivery and parity. Women with history of antibiotic usage in the 7 days prior to sampling and those who used vaginal medications were excluded. The obstetricians measured vaginal pH of the study participants using commercially available pH monitoring strip. A total of 18 preterm (delivered less than 37 completed weeks of gestation) and 20 term (gave birth at 37 or more completed weeks of gestational age) women were selected for the present study.

High Vaginal Swab Collection

The study participants were guided to the procedure room in the GCH and positioned in the lithotomy position. The high vaginal swab samples were collected (n=115) aseptically from the midpoint of the vagina using a Cusco's speculum and four sterile Catch-All™ sample collection swabs. The swabs were gently rubbed for ~20 sec against the mid vaginal wall. One swab placed in a sterile microcentrifuge tube that was pre-filled with 0.5 mL of sterile 50 mM Tris-1 mM EDTA buffer (pH 8.0) supplemented with nuclease inhibitors and protein-denaturing agents (Guanidinium thiocyanate) was used for microbiome study. The tubes containing swabs were vortexed rigorously for detaching microbial cells from its wall. The collected samples were then transported to the Molecular Genetics Laboratory (MGL) at Translational Health Science and Technology Institute (THSTI) within 12 hours of collection in freezing conditions (−192°C). One swab was used for microbial culturing including *Candida* species following standard microbial culture practice reported elsewhere (Pareek et al., 2019).

Extraction of Genomic DNA From HVS Samples

Microbial genomic DNA was extracted from HVS samples using THSTI DNA extraction methods (Bag et al., 2016). Briefly, the collection buffer (Tris-EDTA) containing HVS samples were subjected to chemical, physical, and mechanical lysis procedures for disrupting microbial cells and releasing genomic DNA in the lysis buffer. Mechanical lysis was done by bead beating the samples using 0.1-mm Zirconia beads (Biospec USA) and SpeedMillPLUS bead beater (Analytic Jena, Germany). We used a circulating water bath (LAUDA cooling thermostats Alpha RA, Germany) for heat lysis of the bead beated samples at 75°C for 15 min. A denaturing organic solvent mixture phenol: chloroform and polyvinylpolypyrrolidon (Sigma-Aldrich, USA) was used to remove cellular and extracellular impurities like proteins, lipopolysaccharides and phenolic compounds. Samples were treated with RNase (New England Biolabs, USA) to remove ribonucleic acids (RNAs) from the nucleic acid pools. Finally, genomic DNA was precipitated using 90% ethanol. The precipitated DNA was washed two times with 70% ethanol to remove salt and other contaminants. Heat dried community microbial was resuspended in 100 µl sterile water. Quality and quantity of the DNA isolation from each of the samples were monitored by resolving the sample in 0.8% agarose gel. The 260/230 and 260/280 ratios were used as a secondary measure of genomic DNA purity.

Paired-End Massively Parallel Sequencing of 16S rRNA Gene

The microbial DNA of high vaginal swab samples from 18 PTB and 20 TB mothers at all the three trimesters (V1, V2 and V3) were transported to National Institute of Biomedical Genomics (NIBMG) for amplicon based sequencing of the V3-V4 hyper-variable region of 16S rRNA gene. From the isolated DNA, the V3-V4 hyper-variable region of 16S rRNA gene was amplified using universal barcoded primer pairs: 175F (5′-CCTACGGGN GGCWGCAG-3′) and 512R (5′-GACTACHVGGGTA TCTAATCC-3′) (Klindworth et al., 2013). For each sample, 4µl (>15ng/µl) microbial DNA was mixed with PCR buffer, $MgSO_4$, Platinum Taq DNA Polymerase, PCR grade water, dNTPs and subjected to PCR amplification conditions of 95°C for 5 minutes, then 35 cycles of: (a) 94°C for 30 seconds, (b) 55°C for 30 seconds, (c) 68°C for 1 minute. This was finally followed by 68°C for 1 minute and lastly kept at 10°C until further processing. The negative controls collected during sample collection were processed by the same method as above. Amplified products were purified using Agencourt AMPure-XP (Beckman Coulter) paramagnetic beads and viewed by 1% agarose gel electrophoresis. Sample indexing was done by Nextera XT Index Kit (Illumina) and quantification of DNA library was performed by Qubit Flurometer using Qubit™ dsDNA HS Assay Kit (Invitrogen) and amplicon length was checked using 2100 Bioanalyzer instrument. The final libraries were pooled and sequenced using HiSeq2500 platform following 2x250 paired-end chemistry. The raw data generated was further analyzed for taxonomic classification.

Microbiome Sequence Data Analysis

Demultiplexed FASTQ files for Read 1 (R1.fastq) and Read 2 (R2.fastq) of each sample were subjected to initial quality control based on the FastQC reports generated (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) for each of the paired-end FASTQ files. The key points that were checked from FASTQC reports were whether: (a) the total number of reads in R1.fastq and R2.fastq are similar, (b) the average per base quality value >20 for all the bases in the R1 and R2 reads. The paired-end

reads were then merged and sequencing primers were trimmed. The reads were filtered based on certain criteria viz., (a) average read length – 200 to 1000 bp, (b) average quality score ≥ 25, (c) maximum number of ambiguous bases ≤ 6, (d) maximum number of homopolymers≤ 6. Out of 5 negative control samples collected during sample collection, only 2 could be carried forward for further analysis since the three samples had less than 10 reads per sample. The quality filtered reads for all the samples were further analyzed by using VSEARCH v2.14.0 (Rognes et al., 2016) to generate Operational Taxonomic Units (OTUs) by merging of paired reads and clustering the sequence reads at 97% identity threshold. The singletons (OTUs consisting of only one read) were removed before analysis. Removal of chimeric reads (sequences formed from two or more biological sequences joined together) both by *de novo* and reference-based methods were performed by VSEARCH on the representative sequences obtained from the OTUs. Non-chimeric representative sequences for each of the OTUs were identified and the FASTA file with such sequences were subjected to taxonomic classifications from phyla to genera levels by aligning them to Greengenes (v13_8) database (DeSantis et al., 2006) using QIIME (v1.9.1) (Caporaso et al., 2010) and an OTU table (file consisting of reads for each sample along with taxonomic assignment for each OTU) was formed. For adjusting the negative control samples, the OTUs shared by the negative controls with an average relative abundance of ≥ 1% was removed (Davis et al., 2018). The actual number of reads for each sample was then subsampled (with a bootstrap support of 100) to the minimum number of reads observed among all the samples (Mukherjee et al., 2016; Willis, 2019). Rarefaction plots were generated to confirm if (a) the number of OTUs and (b) the estimated alpha diversity (Shannon, Chao) indices were independent of the inter-individual variation of total number of reads generated for each individual i.e., reached a plateau even with minimum number of reads. Estimations of alpha (Shannon and Chao) (Chao and Shen, 2003) and beta (Jaccard and Bray-Curtis) (Bray and Curtis, 1957) diversities were performed using QIIME (Kuczynski et al., 2012) scripts. For inter-individual comparison, the number of reads that mapped to a particular taxon was normalized by the total number of reads generated for that individual to obtain the relative abundance values for each level of taxonomic hierarchy using QIIME pipeline (Caporaso et al., 2010). Species level classification was performed only on those genera that have relative abundance ≥1% in either Term or Preterm samples in any one of the three trimesters (V1/V2/V3). Those taxa that remained unclassified at the genera level were not included for species level classification. To obtain species level classification, the representative OTUs of the selected genera were aligned to the NCBI's 16S Microbial database by BLCA tool (Gao et al., 2017) which is based on a Bayesian Lowest Common Ancestor (LCA) method. For validation of the species level taxonomy, the representative sequences were also aligned to the NCBI's 16S Microbial database (Johnson et al., 2019) by using BLASTn (Camacho et al., 2009). The species identification was done based on ≥ 80% confidence score in BLCA and ≥ 97%% sequence identity in BLAST.

Statistical Analysis of Vaginal Microbial Taxa Abundances and Diversity Indices Between Term and Preterm Mothers

Differences in POG at delivery and maternal age at conception between term and preterm delivering women were compared by unpaired two-tailed t-test. The microbial taxa identified by analyzing 16S rRNA gene sequencing data were compared between term and preterm samples in each of the three trimesters V1, V2 and V3. The identification of the core microbiome from the total number of taxa was based on fulfilment of the following conditions by any of the two groups of mothers (i.e., delivering term or preterm) and at any of the three trimesters (V1/V2/V3): (a) average relative abundance ≥0.1%, and (b) presence in at least 50% of individuals in the group. Non-parametric Wilcoxon rank-sum test (two-tailed) was performed using the R command (wilcox.test, paired = FALSE) to identify those taxa that are significantly associated with PTB in each of the three trimesters. The alpha and beta diversity indices were also compared between term and preterm samples across all the three trimesters and p-value ≤0.05 was considered significant.

We have carried out Linear Mixed Effects model-based analysis to investigate the fixed effects of Birth Type (preterm/term) and Gestation Time (first/second/third trimesters) on the relative abundance of the predominant genera. We have considered those genera as predominant that have mean relative abundance ≥1% in either TB or PTB group in any of the three trimesters. Species level data were available only for those genera and were also included in this analysis. The current analysis was performed using q2-longitudinal (Bokulich et al., 2018).

Isolation and Identification of *Lactobacillus* Species

For the isolation of *Lactobacillus* species the swab samples were collected from ten term vaginal samples. The swabs were resuspended in the Amies transport medium and transported to the MGL at THSTI in anaerobic condition within 6 hours of sample collection. We used 50 µl of transport medium to isolate discrete colonies onto a de Man-Rogosa-Sharpe (MRS) agar plate (Sigma-Aldrich, Carlsbad, CA). The plates were incubated in an anaerobic workstation (Whitley A95TG, UK) at 37°C for 48 hrs. Distinct colonies were picked up and grown in MRS broth under anaerobic growth conditions at 37°C for 48 hrs. Bacterial isolates grown in the MRS medium were subjected to genomic DNA extraction and amplification of complete 16S rRNA gene followed by DNA sequencing. Bacterial isolates with more than 97% sequence identity of their 16S rRNA gene with the reported *Lactobacillus* genus were selected for further study.

Whole Genome Sequencing Assembly and Annotation

Whole genome sequencing of the confirmed *Lactobacillus* genus was done adopting shotgun sequencing using a high-throughput Illumina MiSeq sequencing platform (Illumina, Inc., USA) at THSTI. Approximately, 100 ng of pure genomic DNA was used

for DNA fragmentation and library preparation and pair-end sequencing using Nextera XT DNA Library preparation kit (Illumina, Inc., USA). FastQC and Trimmomatic programs were used to review the quality of raw reads and remove adapter sequences and low quality reads. An average of 4,03,396 clean quality filtered reads were used to generate the draft genome of 10 *Lactobacillus* isolates belonging to two different species. The average sequencing coverage of the genomes was ~34.63 times. The cleaned pair-end reads were used for genome assembly using Unicycler pipeline (Wick et al., 2017). After assembly, all the contigs of the genome were annotated by Rapid Annotation Subsystem Technology (RAST) automated annotation pipeline (Aziz et al., 2008). Annotated proteins were further confirmed by comparing their sequence homology with the reported proteins publicly available in the Protein database (PDB). Around 98% genes predicted to encode proteins were also available in the PDB. Whole genome sequences of all the *Lactobacillus* strains will be available immediately after acceptance of the article.

Estimation of Core- and Pan-Genome

A total of 42 whole genome sequences of three *Lactobacillus* species viz. *L. crispatus*, *L. iners* and *L. gasseri* were considered for analysis of highly conserved stable core genome and total genomic contents. All orthologous gene clusters were identified by get_homologues (Contreras-Moreira and Vinuesa, 2013) pipeline by applying following parameters for identification and clustering CDS into orthologous groups: (i) -E < 1e-05 for protein searches by protein Basic Local Alignment Search Tool (BLAST) (Camacho et al., 2009) and 40% of sequence identity with 75% coverage in BLAST pairwise alignments. Ortho Markov Cluster algorithm (OMCL) (Enright et al., 2002; Li et al., 2003) with –t 0 was used to find core-genome and the pan-genome.

Phylogenetic Analysis

Comparative genomics were performed by pan- and core-genome based phylogenetic analysis. Based on all the orthologues gene clusters, a present/absent matrix was created to draw a pan-genome based phylogeny. For core genome based phylogeny, only those gene clusters were considered that are present among all the genomes. Some core gene clusters contain inparalogs, and then the longest sequence was chosen for further analysis. Consequently, each core gene cluster contains only single gene from each genome. All gene clusters were aligned by Clustal Omega program (Sievers and Higgins, 2014). Those genes belonging to the same genomes that were concatenated to make a single long sequence. In this way, same numbers of concatenated sequence were created as numbers of genomes were taken in this study. Finally, the aligned sequences were used as input to IQ-TREE (Lam-Tung et al., 2015) program to generate phylogenetic tree based on maximum likelihood method with automatic chosen the best-fit by IQ-TREE server. The branch tree support analysis was performed by 1000 bootstrap and SH-aLRT branch test. Finally, the generated tree was annotated by iTOL server with other metadata (Letunic and Bork, 2019).

DNA Binding Domain and Secretory Signal Motif Analysis

The hypothetical proteins present in the genome of the different lactobacilli were used for finding the DNA binding domain using the Conserved Domain Database (CDD) keeping 0.0001 as the threshold parameter (Lu et al., 2020). 50% bitscore was kept as the criteria for selecting the conserved DNA binding domain. The signal peptide was searched using SecretomeP 2.0 server (Bendtsen et al., 2005). This server produces ab initio predictions of non-classical peptide sequences. For bacterial sequences the SecP value was ≥ 0.5. Based on this criterion the signal peptide sequences were sorted out. Next we used PSORT server (Horton et al., 2007), which is a computer program for the prediction of protein localization sites in cells (Horton et al., 2007). It converts protein amino acid sequences into numerical localization features; based on sorting signals, amino acid composition and functional motifs such as DNA-binding motifs. Finally, it reports the possibility for the input protein to be localized at each candidate site with additional information.

Availability of Nucleotide Sequences

Whole genome sequences of all the 10 *Lactobacillus* strains are deposited in the National Center for Biotechnology Information (NCBI) GenBank (Submission ID is SUB9505031). Accession numbers for all the genome sequences will be communicated shortly. Metadata and 16S rRNA gene sequences are submitted to the European Nucleotide Archive (Study accession number is: PRJEB43005).

RESULTS

Characteristics of Study Participants

The median age of the participants (n=38) included in this study was 22 years (interquartile range (IQR): 21, 25). Significant differences in POG at delivery [TB- (mean ± s.d.) 38.8 ± 1.2 weeks; PTB- (mean ± s.d.) 35 ± 2.4 weeks; p- value= $6.68x10^{-6}$] were observed between term and preterm samples. However, the maternal age at conception (TB- avg. 22.72 ± 3.2 yrs; PTB- avg. 23.5 ± 4.1 yrs; p- value = 0.5) between term and preterm was not significant. Nearly one-third of the women were underweight and about 15% were overweight or obese. Two participants had complaints of vaginal discharge and none had bleeding per vaginum. The median vaginal pH was 5 and the Nugent's score was 4 (IQR: 3,5). Five of the participants had *Candida* species grown in the culture of their vaginal fluid. Further detailed characteristics are provided in **Table 1**.

Diversity Indices of the Core Microbial Taxa in the Vaginal Milieu of Women With Term and Preterm Delivery

A total of 115 high vaginal swab samples collected from three different time points during pregnancy were selected for the 16S rRNA gene sequencing based analysis. The average number of paired-end reads were reduced to 0.86 million from 0.95 million

TABLE 1 | Relevant characteristics of the enrolled study participants (n=38) of the Interdisciplinary Group for Advanced Research on Birth Outcomes-DBT India Initiative (GARBH-Ini) Cohort, Haryana, India.

Characteristics	Term(n=20) n (%) or Median (IQR)	Preterm(n=18) n (%) or Median (IQR)
Clinical and demographic		
Maternal age (year)	23 (21,26)	22 (20,25)
Weight at enrollment (kg)	46.1 (40.9,53.5)	46.0 (41.5,50.2)
BMI at enrollment	19.61 (18.52,22.49)	19.10 (17.48,20.62)
BMI category		
Underweight	5 (25.00%)	8 (44.44%)
Normal	11 (55.00%)	8 (44.44%)
Overweight	2 (10.00%)	0 (0.00%)
Obese	2 (10.00%)	2 (11.11%)
Gravidity		
Primigravida	6 (30.00%)	7 (38.89%)
Multigravida	14 (70.00%)	11 (61.11%)
History of vaginal discharge		
Present	1 (5.00%)	1 (5.56%)
Absent	19 (95.00%)	17 (94.44%)
History of bleeding per vagina		
Present	0 (0.00%)	0 (0.00%)
Absent	20 (100.00%)	18 (100.00%)
History of diarrhea		
Present	0 (0.00%)	1 (5.56%)
Absent	20 (100.00%)	17 (94.44%)
Socioeconomic status		
Upper middle class	2 (10.00%)	3 (16.67%)
Lower middle class	2 (10.00%)	6 (33.33%)
Upper lower class	15 (75.00%)	9 (50.00%)
Lower class	1 (5.00%)	0 (0.00%)
Toilet usage		
Flush/pour flush toilet	19 (95.00%)	17 (94.44%)
Bucket latrine	1 (5.00%)	1 (5.56%)
Laboratory characteristics		
POG at high vaginal		
At enrolment	11w3d (9w2d,12w5d))	10w4d (7w0d,12w5d)
At Visit 2	19w4d (18w1d,19w5d)	19w5d (18w2d,19w6d)
At Visit 3	26w3d (26w2d,26w4d)	26w2d (26w1d,26w4d)
Birth outcomes		
Normal vaginal	18 (90.00%)	17 (94.44%)
Caesarean	2 (10.00%)	1 (5.56%)
Nugent score		
At enrolment	4 (3,5)	4 (3,5)
At Visit 2	4 (3,5)	4 (3,5)
At Visit 3	4 (2,5)	3 (2,4)
Vaginal pH at sampling	5 (5,5)	5 (5,5)
Antibiotic intake at sampling	0 (0.00%)	0 (0.00%)
Microbial culture result for HVS samples (N=37)		
At enrolment		
Candida species	2 (10.53%)	3 (16.67%)
Non-pathogenic microbes	16 (84.21%)	14 (77.77%)
Sterile	1 (5.26%)	1 (5.56%)
At Visit 2		
Candida species	5 (26.31%)	2 (11.11%)
Non-pathogenic microbes	11 (57.89%)	13 (72.22%)
Sterile	2 (10.53%)	3 (16.67%)
Escherichia coli	1 (5.26%)	0 (0.00%)
At Visit 3		
Candida species	3 (15.00%)	0 (0.00%)
Non-pathogenic microbes	13 (65.00%)	12 (70.59%)
Sterile	4 (20.00%)	5 (29.41%)

after initial QA/QC and chimera removal (**Supplementary Table S1**).

Quality filtered non-chimeric reads were clustered into bins based on 97% sequence identity which resulted in 949 OTUs after removal of singletons and OTUs (with relative abundance ≥1%) shared with negative control samples that passed the initial QA/QC step. The sequences were rarefied by subsampling to the minimum number of reads per sample i.e. 262637 sequences per

sample (Rarefaction curve in **Supplementary Figure 1**). Alpha diversity indices such as Shannon (PTB - 1.35 ± 0.65, TB - 1.02 ± 0.63; $p > 0.5$) and Chao1 (PTB - 105.5 ± 93.1, TB - 91.38 ± 24.2; $p > 0.05$) were found to be higher in preterm samples compared to term samples in all the three trimesters of pregnancy but not statistically significant (**Figures 1A, B**).

A total of 16 bacterial phyla and 217 bacterial genera were identified in the term whereas 17 bacterial phyla and 244 bacterial genera were identified in the preterm samples. The core taxa consisted of five phyla namely, *Actinobacteria* (PTB - 6.6%, TB - 4.2%), *Bacteroidetes* (PTB - 0.66%, TB - 0.35%), *Firmicutes* (PTB - 39.02%, TB - 42.5%), *Proteobacteria* (PTB - 53.1%, TB - 52.9%) and *Fusobacteria* (PTB - 0.63%, TB - 0.0009%). Twenty genera were identified as the core genera, which include *Lactobacillus* (PTB - 37.6%, TB - 41.6%), *Enterobacter* (PTB - 36.7%, TB - 40.1%), unclassified genus of *Pseudomonadaceae* family (PTB - 11.6%, TB - 10.4%), *Gardnerella* (PTB - 5.34%, TB - 2.4%) and *Halomonas* (PTB - 2.37%, TB -1.05%) as the top five abundant genera in all the samples. The core vaginal microbiome between term and preterm samples were analyzed and compared. The relative abundances of the core genera in term and preterm samples in all the three trimesters are given in **Table 2** and with species level data in **Supplementary Table S2**.

Differential Abundance of *L. crispatus, L. gasseri and L. iners* in Term and Preterm Mothers

Lactobacillus is the most abundant genus in the vaginal milieu of reproductive age women globally. We have compared relative abundance of a phylum/genus/species between mothers who delivered term or preterm and between trimesters within a group of mothers. These comparisons resulted in a large number of tests. However, we have not performed corrections for multiple testing primarily because these tests are related and cannot be viewed as independent test, which is assumed in the methods used for multiple testing corrections. In view of this, we suggest that our results when declared as significant be considered as tentative. These are discoveries that require validation in future studies to be conducted by us or by other investigators. In the present study, we observed that the abundance of genus *Lactobacillus* is similar in both term (41.6%) and preterm (37.6%) delivering mothers. However, several species of *Lactobacillus* reside in the reproductive tract. A total of 19 species of *Lactobacillus* were identified among term and preterm delivering women with *L. crispatus*, *L. iners*, *L. gasseri*, *L. fornicalis* and *L. delbrueckii* as the top five abundant species. The species level abundance of different lactobacilli between term and preterm mothers reveal distinct patterns. Women delivering at term (23.5%) were found to harbor higher abundance of *L. crispatus* compared to women delivering preterm (9.8), although not statistically significant ($p>0.05$). We have found that in the third trimester abundance of *L. gasseri* is significantly higher in the HVS samples of women who delivered term baby compared to those who delivered preterm (TB: 2.163 ± 6.212, PTB: 0.023 ± 0.074; $p=0.01$, **Figure 2**). *L. iners* was found to be significantly higher in preterm samples in all the three trimesters compared to the term samples ($p \leq 0.02$; **Figure 2**), which gives us an insight to predict the risk of preterm delivery in pregnant women and the type of *Lactobacillus* species predominant in their vaginal microbiome.

Analysis of Microbial Community State Types in the Vaginal Microbiome of Term and Preterm Samples

The vaginal microbiome profiles in term and preterm samples were further assigned to Community State Types (CST) based on the dominant species abundance as reported previously (De Seta et al., 2019; Fettweis et al., 2019). Our analysis revealed four major CSTs in the vaginal microbiota of the Indian women. CST-I, CST-II, and CST-III are dominated by *L. crispatus*, *L. gasseri* and *L. iners*, respectively. CST IV is dominated by non-*Lactobacillus* species. CST-I was predominant in the TB samples (one-tailed p-values were significant only in 2nd trimester for CST-I: 0.02; equality of proportions test between PTB and TB were performed for each trimester) whereas CST-III and CST-IV were predominant in the PTB samples (one-tailed p-values were significant only in 2nd trimester for CST-III: 0.03 and CST-IV: 0.03; equality of proportions test between PTB and TB were performed for each trimester). In addition, we observed

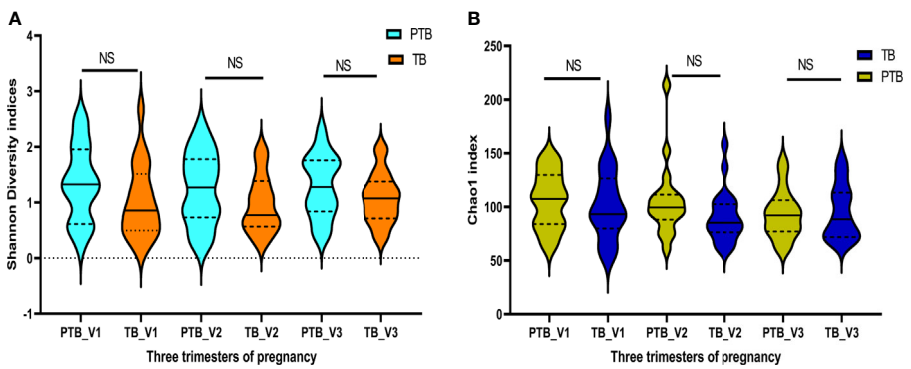

FIGURE 1 | Diversity indices: Intra- individual diversity (alpha diversity) is not significantly different between preterm and term delivering mothers. **(A)** Shannon diversity indices, **(B)** Chao1 indices, at different trimester of pregnancy. ns, non-significant.

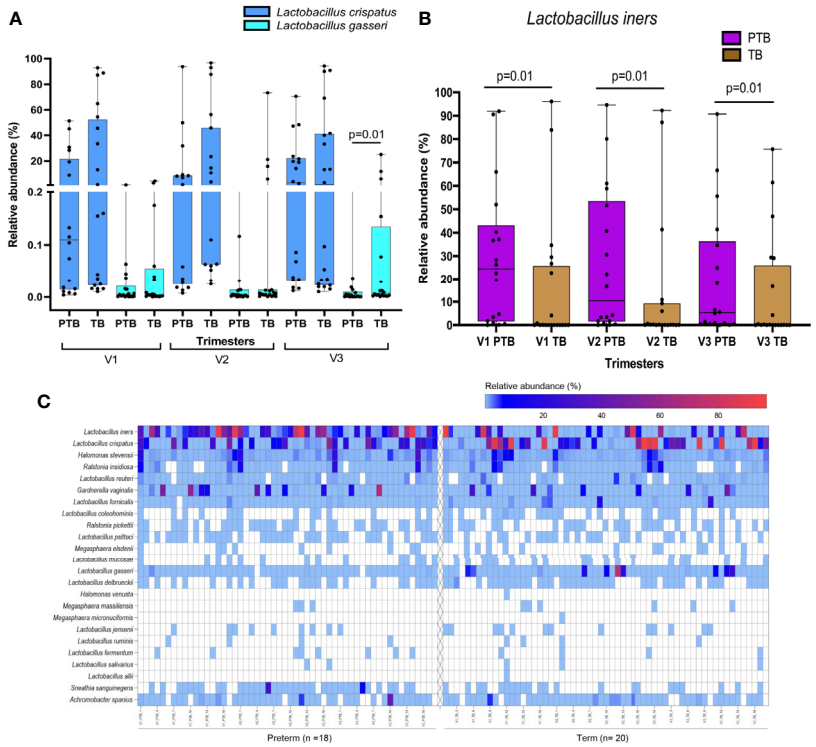

FIGURE 2 | Box plots showing differential abundance of *L. crispatus, L. gasseri* **(A)**, and *L. iners* **(B)** between TB and PTB samples. *L. gasseri* was found to be significantly higher in the TB compared to PTB samples (3rd trimester only). *L. iners* was found to be significantly higher in PTB compared to TB samples in all the trimesters. **(C)** Heatmap representing species level composition of those genera with mean relative abundance >1% in either term or preterm group in any of the three trimesters. Left panel of the heatmap is for the PTB samples while the right side is for the TB samples.

TABLE 2 | Core Vaginal Microbiome in Term and Preterm samples in all the three trimesters.

Core Taxa		**Mean relative abundance of core genera in Term and Preterm samples in all the three trimesters**								
		1st trimester (V1)			**2nd trimester (V2)**			**3rd trimester (V3)**		
Core Phyla	**Core Genera**	**V1_PTB**	**V1_TB**	**P value**	**V2_PTB**	**V2_TB**	**P value**	**V3_PTB**	**V3_TB**	**P value**
Actinobacteria	*Corynebacterium*	0.17	0.06	0.92	0.01	0.01	0.67	0.006	0.01	0.74
	f_Bifidobacteriaceae ;g_unclassified	7.18	3.09	0.23	4.20	0.87	**0.01**	4.65	3.26	0.43
	f_Coriobacteriaceae ;g_unclassified	1.80	2.77	0.68	0.96	1.78	0.07	0.58	0.68	0.53
Bacteroidetes	*Prevotella*	0.22	0.02	0.12	0.06	0.007	0.08	0.45	0.09	0.41
	Elizabethkingia	0.01	0.08	0.44	0.12	0.05	0.51	0.24	0.24	0.55
	Sphingobacterium	0.33	0.35	0.53	0.09	0.07	0.59	0.42	0.09	0.85
Firmicutes	*Aerococcus*	0.09	0.0002	0.09	0.82	$3.6x10^{-05}$	**0.003**	0.03	0.03	0.33
	Lactobacillus	39.71	40.20	0.74	38.22	43.21	0.73	34.95	41.28	0.50
	Streptococcus	0.005	2.21	0.22	0.004	0.005	0.93	0.002	0.004	0.78
	Clostridium	0.07	0.05	0.23	0.10	0.003	0.12	0.07	0.02	0.33
	Megasphaera	1.45	0.001	**0.02**	0.34	0.004	0.12	0.51	0.001	0.11
	Veillonella	0.03	0.004	0.35	0.21	0.003	0.06	0.007	0.03	0.54
Fusobacteria	*Sneathia*	0.01	0.0008	0.82	1.54	0.0004	**0.00001**	0.34	0.0008	**0.04**
Proteobacteria	*Ochrobactrum*	0.05	0.002	0.35	0.001	0.8	0.56	0.009	0.0007	0.65
	Achromobacter	0.10	0.20	1.00	0.20	0.28	0.68	2.32	0.034	0.91
	Ralstonia	0.75	0.61	0.76	1.74	0.58	0.94	1.12	0.59	0.54
	f_Enterobacteriaceae;g_other	35.74	41.79	0.57	43.79	38.73	0.43	30.60	39.73	0.43
	f_Enterobacteriaceae;g_unclassified	0.17	0.20	0.48	0.175	0.178	0.60	0.10	0.16	0.48
	Halomonas	1.80	1.49	0.36	2.91	1.19	0.69	2.40	0.48	0.35
	f_Pseudomonadaceae;g_unclassified	9.92	6.51	0.97	4.07	11.84	0.89	20.72	12.86	0.35

p-values in bold letters are considered to be statistically significant (p<0.05).

that CST-II was present in term samples only but completely absent in the preterm samples (**Table 3**).

Non-*Lactobacillus* Bacterial Taxa Associated With Preterm Birth

Predominance of a mixture of facultative anaerobic bacteria such as *Gardnerella, Sneathia* and *Megasphaera* are inversely correlated with the abundance of *L. crispatus* and some other lactobacilli. Among the 20 core genera (mean relative abundance ≥0.1% and present in 50% samples in any group), species level classification was done for 7 genera (mean relative abundances ≥ 1% in any one group and successfully assigned at the genera level) (**Supplementary Table S2**). We observed that the *Sneathia sanguinegens* (PTB: 1.54%, TB: 0.34%) abundance is significantly (p-value < 0.05) higher in 2nd and 3rd trimesters of preterm delivering women. The prevalence of *Gardnerella vaginalis* (PTB: 4.44%, TB: 0.87%) is also significantly (p- value <0.05) higher in preterm delivering women at 2nd trimester in our study. Abundance of *Megasphaera* sp. (PTB: 1.45%, TB: 0.0007%) is significantly (p- value < 0.05) higher in 1st trimester of preterm delivering mothers (**Table 4**). No particular species of *Megasphaera* sp. was found to be significantly different between TB and PTB samples.

Longitudinal Analysis of Selected Taxa Using q2-Longitudinal

As mentioned in the previous section, species level data were generated for 7 predominant genera among 20 core genera identified in the present study. Statistical analysis of these 7 predominant genera and their species was carried out to identify significance of variation over the time course of pregnancy using q2-longitudinal. For most microbial groups, none of the effects turned out to be significant. The effects of birth type and gestation time were significant for the genera *Lactobacillus* and *Megasphaera*. Within *Lactobacillus*, these effects were also significant for the species *L. iners* and *L. psittaci*. For the two genera (*Lactobacillus* and *Megasphaera*), the trends of change in abundance over gestational time were significantly dissimilar between mothers who gave birth at term and at preterm (interaction effects between birth type and gestational time were significant for these two genera). Detailed results are provided in (**Supplementary Table S3**).

Isolation, Identification and Characterization of Dominant *Lactobacillus* Species Associated With Birth Outcomes

Lactobacilli have long been known as beneficial members of the vaginal microbiota and play an important protective role against microbial infections and reduce the risk of PTB. Different members of lactobacilli are highly diverse and phylogenetically heterogeneous with about more than 170 species being the native members of the vaginal and gastrointestinal tract microbiomes (Goldstein et al., 2015). We observed that the two dominant *Lactobacillus* species (*L. crispatus*, and *L. gasseri*) residing in the vaginal milieu of Indian women are associated with term while *L. iners* in the vaginal milieu of Indian women are associated with PTB outcomes. For functional insights, we have isolated three *Lactobacillus* species from the HVS of the enrolled women in the GARBH-Ini cohort (Bhatnagar et al., 2019). For isolation, we used *Lactobacillus* specific growth medium (MRS) and anaerobic growth conditions as described in the method section. Isolated lactobacilli were further confirmed by complete 16S rRNA gene sequencing using Sangar dideoxy chain termination sequencing technology. We set 97.5% sequence identity and 100% coverage to assign specific taxa for each of the isolates.

Genomic Repertoires of Abundant *Lactobacillus* Species

Since, the relative abundance of *L. crispatus, L. gasseri* and *L. iners* are high compared to the other bacterial species of the vaginal microbiota and these three species are known to play an important role in birth outcomes, we focused to isolate multiple isolates belonging to these three *Lactobacillus* species. We isolated 14 colony-forming units (CFU) and decoded their whole genome sequences by adopting shotgun sequencing to explore genomic repertoires and adding functional insights. Genome sequences of 4 of the 14 isolates were reported in our previous study (Mehta et al., 2020). Genome sequences of all the 14 isolates i.e. *L. crispatus* (n = 7), *L. gasseri* (n = 6) and *L. iners* (n = 1) were deposited in the National Center for Biotechnology Information (NCBI). The genome sequences were subjected to automated annotation using NCBI prokaryotic genome annotation pipeline (Tatusova et al., 2016) or Rapid Annotation using Subsystem Technology (RAST) server (Aziz et al., 2008). Major focus was given to identify and analyze functions that potentially contribute in bacteriocin and lysin productions, which are secretory in nature. Relevant features of the *Lactobacillus* genomes isolated from the GARBH-Ini cohort are mentioned in **Table 5**. The numbers of ORFs in the fourteen genomes differ from 1178 to 2249. All the fourteen genomes harbored several enzyme-encoding genes linked with DNA mobility and site-specific recombination proteins like tyrosine recombinases, transposases and integrases. These functions are often physically linked with mobile genetic elements (MGEs) and play important role in the acquisition

TABLE 3 | Result for CST distribution in term and preterm samples.

Dominant taxa	CST Type	V1_PTB	V1_TB	V2_PTB	V2_TB	V3_PTB	V3_TB
L. crispatus	CST I	6(30%)	9(45%)	3(16.6%)	9(47.3%)	7(38.8%)	10(50%)
L. gasseri	CST II	0(0%)	1(5%)	0(0%)	4(21%)	0(0%)	2(10%)
L. iners	CST III	9(50%)	5(25%)	10(55.5%)	5(26.3%)	8(44.4%)	5(25%)
Non- Lactobacillus	CST IV	3(16.6%)	5(25%)	5(27.7%)	1(5.2%)	3(16.6%)	3(15%)

Total four CSTs were identified in our data set namely CST I, CST II, CST III and CST IV which is dominated by L. crispatus, L.gasseri, L. iners, and *Non*-Lactobacillus *respectively.*

TABLE 4 | Non-Lactobacillus sp. in vaginal environment.

Significant Taxa	Mean Relative abundance of Species in Term and Preterm samples in all the three trimesters								
	V1_PTB (%)	V1_TB (%)	P value	V2_PTB (%)	V2_TB (%)	P value	V3_PTB (%)	V3_TB (%)	P value
Gardnerella vaginalis	7.19	3.09	0.23	4.44	0.87	**0.01**	4.64	3.26	0.43
Megasphaera sp.*	1.45	0.001	**0.02**	0.34	0.004	0.12	0.51	0.001	0.1
Sneathia sanguinegens	0.01	0.0008	0.82	1.54	0.0004	**0.00001**	0.34	0.0008	**0.04**

Sneathia sanguinegens *is significantly (p-value < 0.05) higher in 2nd and 3rd trimesters,* Gardnerella vaginalis *is significantly (p- value <0.05) higher at 2nd trimester and* Megasphaera *sp. is significantly (p-value <0.05) higher in 1st trimester of preterm samples compared to term samples.*

**No particular species of genus Megasphaera was found to be significantly different between TB and PTB samples.*

p-values in bold letters are considered to be statistically significant (p<0.05).

and dissemination of fitness traits, metabolic enzymes, antimicrobial resistance, antimicrobial peptides and other functions that modulates microbial composition and inflammation in the vaginal milieu. Genetic components associated with CRISPR-Cas were also prevalent in the genome of *L. crispatus, L. gasseri* and *L. iners*. Functions conferring resistance to different antibiotics including β-lactamases, multidrug and toxin extrusion (MATE) family efflux pump, multidrug resistance efflux pumps, RND multidrug efflux transporter, ABC transporters and major facilitator superfamily (MFS) multidrug efflux transporter are present in the genome of all three *Lactobacillus* species. Several functions linked with phage replication, integration, and mobility is detected in the *L. crispatus, L. gasseri* and *L. iners* genomes. The genome of *L. crispatus* and *L. gasseri* harbors phage integrase (tyrosine recombinase) that mediate the integration of a bacteriophage into its chromosome. Similar phages are also present in the genome of other *Lactobacillus* (Acc. No. WP_060791041.1, WP_057726712.1). However, we didn't observe any phage integrase in the genome of *L. iners*.

All the three genomes of *Lactobacillus* encode ribosomally synthesized antibacterial peptide–related functions and permease component to protect the vaginal milieu from the invasion of non-indigenous microbiota. A protein that confers tolerance to colicin V is also present in the genome of *L. gasseri*. The genome of *L. crispatus* encodes bacteriocin helveticin and helveticin J, bacteriocin transporters, bacteriocin peptide. These were also observed in the genome of other *L. crispatus* strains (Acc. No. WP_005729773.1, WP_181577227.1, WP_150399102.1). It is known that bacteriocins are antimicrobial peptides and they are mostly active against closely related bacterial species. The genome of *L. crispatus* contains several other lysins, including enterolysin A, autolysin, streptolysin, thermolysin. Phage lysin is present in the genome of both *L. crispatus* and *L. iners* but not in the genome of *L. gasseri*. The bacteriocins produced by the different *Lactobacillus* species help in reducing bacterial diversity in the vaginal milieu and also decrease level of microbial origin inflammatory compounds by inhibiting growth of several Gram-negative bacteria.

Two out of seven genomes of *L. crispatus* are also equipped with several functions including conjugation protein, TraG/TraD that directly facilitate horizontal gene transfer (HGT) and bacterial evolution. The genome of *L. crispatus* harbors type-IIA clustered regularly interspaced short palindromic repeats (CRISPR) and multiple CRISPR spacers. CRISPR-Cas system is reported to be an important bacterial defense mechanism, which provides adaptive immunity to bacteria against invasion of MGEs like phages and plasmids (Bhaya et al., 2011). The type-IIA CRISPR-Cas system is the most dominant among lactobacilli. Interestingly, CRISPR-Cas system is present in *L. crispatus* but not found in the genome of *L. gasseri* and *L. iners*.

Pan- and Core-Genome of *Lactobacillus*

Along with the whole genome sequences of 14 *Lactobacillus* strains isolated from the study subjects enrolled in the GARBH-

TABLE 5 | General genomic features of 14 Lactobacilli assembled genomes.

S.N.	Species name	Genome size (bp)	Contig Number	N50	GC percent	CDS
1	*Lactobacillus crispatus 5.1*	2191964	34	103210	36.7	2249
2	*Lactobacillus crispatus 7.2*	2188668	36	96587	37.7	2248
3	*Lactobacillus crispatus 8.2*	2190096	35	103043	36.7	2248
4	*Lactobacillus crispatus 9.2*	2049433	155	19237	37	2213
5	*Lactobacillus crispatus 10.2*	2069648	165	19122	37	2233
6	*Lactobacillus crispatus Indica1*	1641433	59	33495	37.32	1596
7	*Lactobacillus crispatus Indica2*	2209487	39	153263	36.48	2024
8	*Lactobacillus gasseri 221*	1457943	106	23274	35.1	1462
9	*Lactobacillus gasseri 219*	1542387	64	51720	35	1543
10	*Lactobacillus gasseri 218*	1528392	61	51720	35	1530
11	*Lactobacillus gasseri 217*	1528051	61	51720	35	1531
12	*Lactobacillus gasseri 216*	1561727	50	57861	35	1583
13	*Lactobacillus gasseri Indica1*	2096244	5	1845454	34.92	1547
14	*Lactobacillus iners Indica1*	1331119	1	1331119	33.2	1178

Ini cohort, we also included 28 additional genome sequences of *L. crispatus* (n = 10), *L. iners* (n = 13) and *L. gasseri* (n = 5) publicly available in the NCBI genome database for comparative genome analysis.

Total 8474 orthologous gene clusters were identified from 42 genomes and considered as pan genome (**Figure 3**). Of them, 316 gene clusters were present in all 42 genomes. Therefore, 316 gene clusters were considered as core part of 42 genomes. The core gene clusters usually involved in fundamental and essential cellular processes (Carlos Guimaraes et al., 2015). We observed that 680 gene clusters are present in ≥95% of the genome included in the present analysis and is referred as soft core of 42 genomes. 3339 gene clusters were considered as shell genome as they are present more than 2 genomes but less than 95% of the genomes. The softcore and shell part of pan-genome are collectively considered as accessory or dispensable genome to perform specific functions related to adapt in different niches. It contains virulence factors, antibiotic resistance genes and different metabolic enzymes important for the survival of the microorganism in specific environments (Mira et al., 2010). 4450 gene clusters were identified as cloud genes. This subset of pan-genome is called species or strain specific genes as present in either or less than 2 genomes. These gene clusters are normally acquired by horizontal gene transfer process to get competitive advantage over those strains that do not have (Muzzi et al., 2007; Penn et al., 2009). About 52% orthologues gene clusters belong to cloud genome. It reflects more than half of pan-genome is species specific or acquired genome.

Functional annotation of pan-genome has revealed that fundamental cellular process such as DNA replication, transcription, translation, ribosomal biogenesis (class: J); nucleotide transport & metabolism, recombination and repair (class: F); and several others are most abundant in core and soft-core part of the genomes (**Figure 4**). Several functions associated with cell division, chromosome partitioning (class: D), cell wall/membrane/envelope biogenesis (class: M) are also abundant in the core genome (**Figure 4**). It has also been observed that some of above-mentioned functional classes are present in the shell and cloud genomes. The gene clusters for such additional metabolic functions are possibly acquired through HGT. Functions that provide fitness and growth advantages like carbohydrate transport and metabolism (class: G); amino acid transport and metabolism (E), coenzyme transport and metabolism (class: H); lipid transport and metabolism (class: I), inorganic ion transport and metabolism (class: P); secondary metabolite biosynthesis pathways, transport and catabolism (class: Q) their abundance (H) are also relatively high in the shell and cloud genomes. These functions are part of the shell and cloud genomes and they perform important functions and help *Lactobacillus* to compete with other microorganisms living in the same vaginal milieu.

Pan- and Core-Base Phylogeny

Pan- and core genome based phylogeny have shown similar clustering pattern (**Figure 5**). Both the trees revealed that there are three clades and each clade have made up with same species. Pan-genome based phylogeny reveals that *L. crispatus* clade is more dispersed than other two clades. The branch length of each species is different and the most species has not originated from a single common ancestor. All 7 Indian origin *L. crispatus* are distributed in two groups. It reflects that a significant number of genes may have sporadic distribution or accumulated mutations. This might lead to the significant intra-species heterogeneity in *L. crispatus* living in the same environment. Complete genome sequencing of additional *L. crispatus* isolates can help for a definite conclusion. The phylogenetic clade containing *L. gasseri* is less diverse than *L. crispatus*. All Indian *L. gasseri* strains are very close to each other except *L. gasseri* indica1. The *L. iners* clade is the most conserved among all three *Lactobacillus* species. It reflects that the pan-genome of *L. iners* is much conserved and a similar set of genes may be present in all the

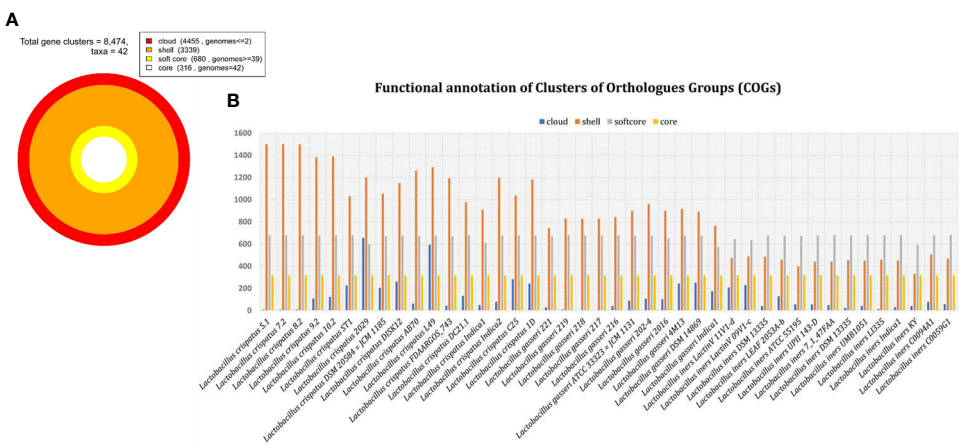

FIGURE 3 | Distribution of orthologous gene clusters. **(A)** Total gene cluster and its distribution in cloud, shell, soft core and core part of genome among 42 genomes. **(B)** The bar graph of individual genome in cloud, shell, soft core and core genome.

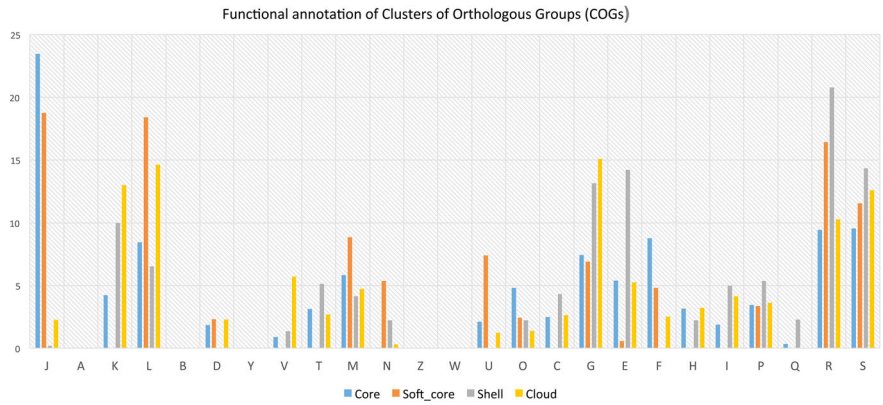

FIGURE 4 | Bar plot showing distribution of COGs in core, softcore, shell and cloud subset of pan-genome J: Translation, ribosomal structure and biogenesis, A: RNA processing and modification, K: Transcription, L: Replication, recombination and repair, B: Chromatin structure and dynamics, D: Cell cycle control, cell division, chromosome partitioning, Y: Nuclear structure, V: Defense mechanisms, T: Signal transduction mechanisms, M: Cell wall/membrane/envelope biogenesis, N: Cell motility, Z: Cytoskeleton, W: Extracellular structures,U: Intracellular trafficking, secretion, and vesicular transport, O: Posttranslational modification, protein turnover, chaperones, C: Energy production and conversion, G: Carbohydrate transport and metabolism, E: Amino acid transport and metabolism, F: Nucleotide transport and metabolism, H: Coenzyme transport and metabolism, I: Lipid transport and metabolism, >P:Inorganic ion transport and metabolism, Q: Secondary metabolites biosynthesis, transport and catabolism, R: General function prediction only, S: Function unknown.

L. iners strains analyzed in the present study. The *L. iners* isolated from the GARBH-Ini cohort is very close to the *L. iners* C0059G1 isolated from Baltimore USA in 2019 (**Supplementary Table S4**).

Our core genome based phylogeny analysis clearly indicated that all the 42 genomes have been distributed in three compact clades and the tree is distinct from the pan-genome based phylogenetic tree (**Figure 5**). Since all the strains of each clade are very close to each other and most are diverged from a single LCA, it reflects that all protein sequences of 316 genes are highly conserved and sequence similarity percentage is very narrow from 69.03 to 100 (**Supplementary Table S5**).

Genome of *L. crispatus and L. gasseri* Enriched With Secretory Proteins With Potential Gene Regulatory and Antimicrobial Functions

Analysis of the representative genomes of *L. crispatus* and *L. gasseri* indicate presence of several secretory transcriptional regulators and several antimicrobial peptides correlated with less diverse microbial composition and also anti-inflammatory condition in the vagina. We have identified 36 and 19 secretory signal peptides containing DNA binding motif in the genome of *L. crispatus* and *L. gasseri* strains, respectively (**Supplementary**

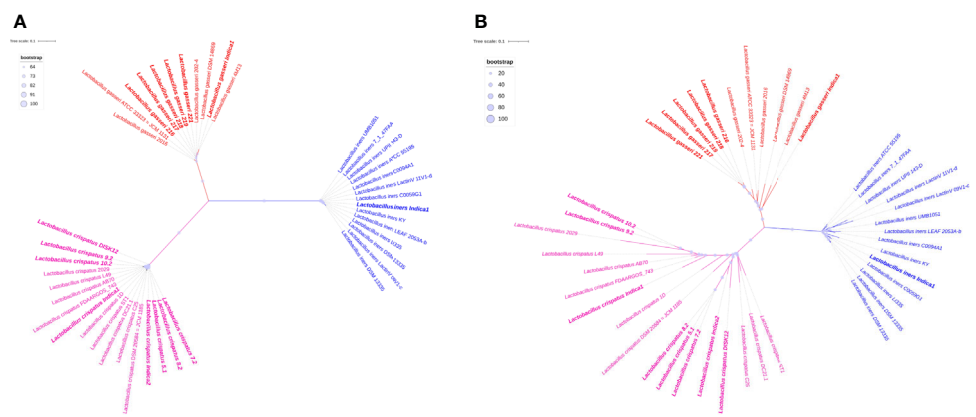

FIGURE 5 | Pan- and core-genome based unrooted phylogenetic trees. **(A)** Core-genome based phylogeny. **(B)** Pan-genome based phylogeny. All three clades of phylogeny have been shown red, green and blue colors. Branch length of each leaf has not shown in number but it is proportional to divergence from last common ancestor. Bootstrap values of pan genome based phylogeny have range to ≥20 to 100. In case of core genome, boot strap value range to ≥64 to 100. It has been shown in light blue circle. The radius of the circle ranges from 5 to 15 pixels in both phylogeny trees. Indian strains of *Lactobacillus* have been shown in bold.

Table S6). The site of localization of these secretory transcriptional regulators have been predicted either the nucleus or mitochondria of host cells. This subcellular localization information gives an important clue to the transcriptional regulatory functions of the secretory proteins. Although localization signals in mRNA appear to play some role (Gonsalvez et al., 2005), but the key determinant of protein localization is the peptide linked with the N-terminal end of the protein molecules. The present finding indicates that these secretory proteins may come out from the *Lactobacillus* species and enter into host cell and act as transcription regulator. Consequentially the host genome may change the expression level of pro- or anti-inflammatory proteins or antimicrobial peptides to modulate the microbial growth and reduce the invasion of pathogenic bacteria.

DISCUSSION

The atypical composition of the vaginal microbiome in genetically predisposed women is a potential environmental risk factor for PTB, which leads to an ascending migration of specific bacterial taxa from the vaginal milieu to amniotic membrane and amniotic fluid. It possibly induces an aberrant immune response and activation of several matrix associated enzymes leading to activation of preterm labor (Alamrani et al., 2017). There has been a recent surge in the information on the role of vaginal microbiome in PTB; In general PTB has been characterized by increased microbial diversity, decline in anti-inflammatory molecules and rise in pro-inflammatory bacteria in the vaginal milieu (Parris et al., 2020). Vaginal microbiota of asymptomatic, otherwise healthy women is mostly dominated by different species of *Lactobacillus* (Ravel et al., 2011; Mehta et al., 2020; Ma et al., 2020). In the present study, *L. crispatus* and *L. gasseri* have been commonly found to be associated with term birth outcome in in accordance with the other previous reports (Kindinger et al., 2017; Stafford et al., 2017). Dominance of *Lactobacillus* species in the vaginal microbiome of white American and Asian women is more common than the Black American and Hispanic women (Ravel et al., 2011). *Lactobacillus* species protect the vaginal milieu from colonization and growth of exogenous and potentially pathogenic bacterial taxa by producing lactic acid, hydrogen peroxide (H_2O_2), maintaining acidic pH of the niche and secreting ribosomally encoded antimicrobial peptides (Amabebe and Anumba, 2018). In addition, lactic acid producing bacteria induce host innate immune system while sensing presence of non-indigenous Gram-negative bacteria in the vaginal milieu (Witkin et al., 2011). *In vitro* colonization studies using vaginal epithelial cell line with *L. crispatus* and other native microbiota demonstrated distinct immunity of epithelial monolayer in a species-specific manner (Lai et al., 2009). In the present study, we characterized the vaginal microbiota of 115 HVS samples collected longitudinally from the 38 pregnant women who were enrolled in the GARBH-Ini pregnancy cohort. The data obtained from our study population indicates that like reports from other populations vaginal pH of the reproductive aged Indian women is also acidic in nature (pH 5.0 ± 0.55). During pregnancy there is a rise in level of progesterone and estrogens, along with some immunological changes which in turn increase the glycogen content of the vaginal epithelial cells and modulate the composition of the vaginal microbiome to a more stable state; this reduces the richness and community diversity by promoting the growth of *Lactobacillus* sp. (Marchesi and Ravel, 2015). Dominance of *Lactobacillus* provides a greater resistance and protective role against genital tract infections (Marchesi and Ravel, 2015). Our high- throughput sequencing reads covering V3-V4 region of 16S rRNA genes allowed us to accurately determine the composition, diversity and dynamics of the vagina microbial ecosystem in asymptomatic Indian women who delivered preterm.

The dominance of non-*Lactobacillus* species in the vaginal milieu has previously been reported as potential risk factor for PTB (Moreno and Franasiak, 2017; Drew et al., 2018). In our study we found that the taxa, which have previously been found to be associated with Bacterial Vaginosis (BV) (Srinivasan et al., 2012) were significantly higher in preterm delivering women compared to term delivering women. When we compared the alpha diversity indices such as Shannon and Chao1 in all the three trimesters of pregnancy between term and preterm samples and observed that the diversity indices are slightly higher in preterm samples but the differences are not statistically significant (**Figure 1**). A total of 217 and 244 bacterial genera were identified in the term and preterm samples, respectively. We have previously reported that the most dominant bacterial taxa in the reproductive age Indian women are *Lactobacillus* (Mehta et al., 2020). Dominance of *Lactobacillus* in the vaginal microbial ecosystem of reproductive age healthy women has also been reported from several other countries and our findings are consistent (Zhou et al., 2007; Drell et al., 2013).

In the present study, we also observed that *L. crispatus*, *L. iners, L. gasseri*, *L. jensenii* and *L. delbrueckii* are the dominant species both in term and preterm samples. However, the relative abundance of *L. iners* is high in preterm samples, while the relative abundance of *L. crispatus* and *L. gasseri* is high in term samples. Similar abundance profile of *L. crispatus*, *L. gasseri* and *L. iners* in term and preterm samples have also been reported in other cohorts studying the role of microbiome in birth outcomes (Tabatabaei et al., 2019).

When we analyzed the relative abundance of other non-*Lactobacillus* bacterial taxa we observed higher abundance of a mixture of facultative anaerobic bacteria such as *Gardnerella*, *Sneathia* and *Megasphaera* in the vaginal milieu. At the species level resolution of 16S rRNA gene sequence reads revealed that the relative abundance of *Sneathia sanguinegens* and *Gardnerella vaginalis* is significantly higher in women delivering preterm compared to the women having term delivery. No claim is made that the significant differences noted in this study are final; this should be viewed as tentative findings that require validation using independent cohorts of mothers. Preterm Premature Rupture of the fetal Membrane (PPROM) is associated with *Sneathia* (Brown et al., 2018). It was previously shown that *G.*

vaginalis acts as a preterm signature in European ancestry (Callahan et al., 2017). Although, similar reports from other cohorts are available (Romero et al., 2014; Seo et al., 2017; Tabatabaei et al., 2019), the genomic content of these abundant bacterial species from the same cohorts have not been explored. For a better and more profound understanding, how the presence or absence of a bacterial species effect the composition of a microbial ecosystem or host physiology, it is important to decode their genome and identify the pertinent functions. In the present study, for functional insights that may link with the birth outcomes we isolated several strains of the three most important *Lactobacillus* species i.e. *L. crispatus*, *L. gasseri* and *L. iners*. A comparative genome analysis of *L. crispatus, L. gasseri* and *L. iners* has revealed that *L. crispatus* isolate genomes are enriched with additional genes related to lactose, galactose, sucrose and fructose fermentation that leads to lactic acid production. Further analysis reveal that different genes linked with defense mechanisms are part of the shell genome and each species has some unique genes to protect themselves in the complex microbial ecosystem. A similar study also supports our findings that a set of unique transposable elements, multidrug resistance protein (MdtG), sensor histidine kinase (RcsC) and phosphate-binding protein (PstS) are prevalent in the genome of *L. crispatus*. Such functions help *L. crispatus* to compete with exogenous microbiota, exclude them from the colonization in the vaginal milieu and keep the environment less diverse and protected from enrichment of pro-inflammatory molecules, mostly produced by the non-indigenous vaginal microbiota. In addition, the genome of *L. iners* harbors thiol-activated cytolysin (TACY). It is an important group of bacterial toxins, of which streptolysin O (SLO) is the prototype of TACY. They are involved in the pathogenesis of a number of Gram-positive species. TACY are pore-forming toxins, their major pathogenic effects may be more delicate than simple lysis of host cells, and may include interference with immune cell function and cytokine induction. This cytolysin was not detected in the genome of *L. crispatus* and *L. gasseri* analyzed in the present study.

Further analysis of the pan- and core genomes of *L. crispatus, L. gasseri* and *L. iners* revealed that each species have distinct genomic contents and they are clearly diverged from each other. We identified 316 core genes in the genomes of all three *Lactobacillus* species (**Supplementary Table S7**). Similar core gene contents among different *Lactobacillus* species have also been reported by the other groups (Inglin et al., 2018; Evanovich et al., 2019; Putonti et al., 2020). The pool of core and soft-core subsets represent highly conserved gene cluster, as these clusters are present in ≥95% of the 42 genomes. The soft-core subset has additional important in comparative genomic analysis as it allows inclusion of draft genomes in which some genes may not be present (Nelson and Stegen, 2015). Therefore functional annotation of core and soft-core gene clusters can provide information about fundamental and essential cellular processes of the *Lactobacillus* genus (Carlos Guimaraes et al., 2015). There are eight classes in which either core or soft-core gene clusters are predominantly present. They are: Nucleotide transport and metabolism (class: F), Coenzyme transport and metabolism (class: H), Translation, ribosomal structure and biogenesis (class: J), Replication, recombination and repair (class: L), Cell wall/membrane/envelope biogenesis (class: M), Cell motility (class: N), Posttranslational modification, protein turnover, chaperones (class: O), Intracellular trafficking, secretion, and vesicular transport (class: U). These classes represent core functions of a prokaryotic organism. Therefore, pool of core and soft-core subsets may call as dispensable genome.

Similarly, there are ten functional classes in which pool of shell and cloud subsets (either shell or cloud or both subset) of pan-genome are predominantly present. They are following: Transcription (class: K), Energy production and conversion (class: C), Amino acid transport and metabolism (class: E), Carbohydrate transport and metabolism (class: G), Lipid transport and metabolism (class: I), Inorganic ion transport and metabolism (class: P), General function prediction only (class: R), Function unknown (class: S), Signal transduction mechanisms (class: T) and Defence mechanisms (class: V). These functions are part of the shell and cloud genomes and they perform important functions and help *Lactobacillus* to compete with other microorganisms living in the same vaginal milieu. Another study shows that shell and cloud genomes contain virulence factors, antibiotic resistance genes and different metabolic enzymes important for the survival of the microorganism in specific environments(Mira et al., 2010; Read and Ussery, 2006). Therefore, gene clusters of the pool of shell and cloud subsets may be called as flexible/accessory genome as they present in ≤95% of the 42 genomes. Analysis of accessory genome may reveal both the evolutionary history of a sub lineage or isolates and their adaptability in different environment (Nelson and Stegen, 2015). These two subsets (i.e. shell and cloud) of pan-genome are thought to have different rates of gene acquisition and deletion through horizontal gene transfer (Collins and Higgs, 2012). It is believed that gene gained and lost slowly happen in shell, whereas comparatively fast in cloud (Collins and Higgs, 2012). Therefore, it is believed that the unique gene cluster comes into cloud subset.

However, pangenome based analysis reveals that *Lactobacillus* species isolated in the present study have several unique genes acquired through horizontal gene transfer (**Supplementary Table S7**). It was observed that the *L. crispatus* clade is more scattered than other two clades. The different branch length of each of the *Lactobacillus* species indicates that the ancestors for these species were also reasonably different. Different functions that are unique to *L. crispatus* genome have potential antimicrobial activity against different opportunistic pathogens like *A. baumanii* and *K. pneumoneae*. Several Gram-negative bacteria associated with microbial dysbiosis in the vaginal milieu are correlated with production of proinflammatory cytokines and induction of labor. Thus the antimicrobial peptides produced by the *L. crispatus* can reduce the abundance of pro-inflammatory molecules in the vaginal milieu by reducing the colonization and growth of pathogenic bacterial taxa. Our findings indicate that *L. crispatus*, *L. gasseri*, *L. iners. S. sanguinigens* and *G. vaginalis*

genome specific signature could be used as the microbial genome signature for predicting birth outcomes. However, this study has certain limitations. Our findings indicate that there is a correlation between some Lactobacilli species and term delivery, but this alone does not explain the protective effect. Sample size of the present study is also not adequate and need validation with larger sample size for a definitive conclusion.

CONCLUSION

The composition and diversity of vaginal microbiota widely vary across populations. Specific microbial taxa contribute substantially in determining term or PTB outcomes. We observed that the higher abundance of *L. crispatus* and *L. gasseri* are associated with TB while the increased abundance of *S. sanguinigens* and *G. vaginalis* are linked with PTB. Prevalence of *L. iners* is also high in mothers having PTB. The genome of *L. crispatus* and *L. gasseri* are enriched with horizontally acquired genetic elements and peptides potentially linked with antimicrobial functions. Such bacterial taxa reduce the microbial diversity in the vaginal milieu and also the microbial origin inflammation inducing antigens. The microbial taxa and their genomic signatures linked with birth outcomes reported in the present study need to be validated with other population. In addition, the present study has limited sample size. Multicenter studies with larger sample size will help to understand whether the observed microbial taxa and their genomic contents associated with TB and PTB births have any link to a specific race or ethnicity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: European Nucleotide Archive (ENA) with identifier PRJEB43005. Genome Accession: JAGSXW000000000, JAGSXV000000000, JAGSXU000000000, JAGSXT000000000, JAGSXS000000000, JAGSXR000000000, JAGSXQ000000000, JAGSXP000000000, JAGSXO000000000, JAGSXN000000000.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Translational Health Science and Technology Institute Human Ethics Committee. The patients/participants provided their written informed consent to participate in this study. [Ref.# THS 1.8.1/(30) dated 11th Feb 2015].

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AUTHOR CONTRIBUTIONS

BD, SB, and GN conceived the idea and designed the experiments. Members of GARBH-Ini conducted the clinical study, collected HVS samples and relevant clinical information. SK, NK, DT, AK, MS, OM, BD, GARBH-Ini study group, SM, and BD performed experiments. BD, SM, SB, PK, RT, NW contributed reagents. BD, SM, SK, NK, DT, and MS performed data analysis. BD and SM wrote the manuscript. SB, NW, TR, and PK edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.622474/full#supplementary-material

Supplementary Figure S1 Rarefaction curve for alpha diversity. **(A)** Chao1 and **(B)** Shannon diversity index.

REFERENCES

Alamrani, A., Mahmoud, S., and Alotaibi, M. (2017). Intrauterine Infection as a Possible Trigger for Labor: The Role of Toll-Like Receptors and Proinflammatory Cytokines. *Asian Biomed.* 9 (6), 727–739. doi: 10.5372/1905-7415.0906.445

Amabebe, E., and Anumba, D. O. (2018). The Vaginal Microenvironment: The Physiologic Role of Lactobacilli. *Front. Med.* 5, 181. doi: 10.3389/fmed.2018.00181

Anton, L., Sierra, L. J., DeVine, A., Barila, G., Heiser, L., Brown, A. G., et al. (2018). Common Cervicovaginal Microbial Supernatants Alter Cervical Epithelial Function: Mechanisms by Which Lactobacillus Crispatus Contributes to Cervical Health. *Front. Microbiol.* 9, 2181. doi: 10.3389/fmicb.2018.02181

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., et al. (2008). The RAST Server: Rapid Annotations Using Subsystems Technology. *BMC Genomics* 9 (1), 1–15. doi: 10.1186/1471-2164-9-75

Bag, S., Saha, B., Mehta, O., Anbumani, D., Kumar, N., Dayal, M., et al. (2016). An Improved Method for High Quality Metagenomics DNA Extraction From Human and Environmental Samples. *Sci. Rep.* 6, 26775. doi: 10.1038/srep26775

Barrientos-Duran, A., Fuentes-López, A., de Salazar, A., Plaza-Díaz, J., and García, F. (2020). Reviewing the Composition of Vaginal Microbiota: Inclusion of Nutrition and Probiotic Factors in the Maintenance of Eubiosis. *Nutrients* 12 (2), 419. doi: 10.3390/nu12020419

Bendtsen, J. D., Kiemer, L., Fausbøll, A., and Brunak, S. (2005). Non-classical Protein Secretion in Bacteria. *BMC Microbiol.* 5 (1), 1–13. doi: 10.1186/1471-2180-5-58

Bhatnagar, S., Majumder, P. P., Salunke, D. M.Interdisciplinary Group for Advanced Research on Birth Outcomes—DBT India Initiative (GARBH-Ini) (2019). A Pregnancy Cohort to Study Multidimensional Correlates of Preterm Birth in India: Study Design, Implementation, and Baseline Characteristics of the Participants. *Am. J. Epidemiol.* 188 (4), 621–631. doi: 10.1093/aje/kwy284

Bhaya, D., Davison, M., and Barrangou, R. (2011). Crispr-Cas Systems in Bacteria and Archaea: Versatile Small RNAs for Adaptive Defense and Regulation. *Annu. Rev. Genet.* 45, 273–297. doi: 10.1146/annurev-genet-110410-132430

Bokulich, N. A., Dillon, M. R., Zhang, Y., Rideout, J. R., Bolyen, E., Li, H., et al. (2018). q2-longitudinal: Longitudinal and Paired-Sample Analyses of Microbiome Data. *mSystems* 3, e00219–e00218. doi: 10.1128/mSystems.00219-18

Bray, J. R., and Curtis, J. T. (1957). An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecol. Monogr.* 27 (4), 326–349. doi: 10.2307/1942268

Brown, R. G., Marchesi, J. R., Lee, Y. S., Smith, A., Lehne, B., Kindinger, L. M., et al. (2018). Vaginal Dysbiosis Increases Risk of Preterm Fetal Membrane Rupture, Neonatal Sepsis and is Exacerbated by Erythromycin. *BMC Med.* 16 (1), 1–15. doi: 10.1186/s12916-017-0999-x

Bukowski, R., Sadovsky, Y., Goodarzi, H., Zhang, H., Biggio, J. R., Varner, M. , et al. (2017). Onset of Human Preterm and Term Birth is Related to Unique Inflammatory Transcriptome Profiles At the Maternal Fetal Interface. *PeerJ* 5, e3685. doi: 10.7717/peerj.3685

Butler, A. S., and Behrman, R. E. (2007). *Preterm Birth: Causes, Consequences, and Prevention* (Washington, DC: National Academies Press).

Callahan, B. J., DiGiulio, D. B., Goltsman, D. S. A., Sun, C. L., Costello, E. K., Jeganathan, P., et al. (2017). Replication and Refinement of a Vaginal Microbial Signature of Preterm Birth in Two Racially Distinct Cohorts of US Women. *Proc. Natl. Acad. Sci. U.S.A* 114 (37), 9966–9971. doi: 10.1073/pnas.1705899114

Calonghi, N., Parolin, C., Sartor, G., Verardi, L., Giordani, B., Frisco, G., et al. (2017). Interaction of Vaginal *Lactobacillus* Strains With HeLa Cells Plasma Membrane. *Benef. Microbes* 8 (4), 625–633. doi: 10.3920/BM2016.0212

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: Architecture and Applications. *BMC Bioinf.* 10 (1), 421. doi: 10.1186/1471-2105-10-421

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME Allows Analysis of High-Throughput Community Sequencing Data. *Nat. Methods* 7 (5), 335–336. doi: 10.1038/nmeth.f.303

Carlos Guimaraes, L., Benevides de Jesus, L., Vinicius Canario Viana, M., Silva, A., Thiago Juca Ramos, R., de Castro Soares, S., et al. (2015). Inside the Pan-Genome-Methods and Software Overview. *Curr. Genomics* 16 (4), 245–252. doi: 10.2174/1389202916666150423002311

Chao, A., and Shen, T. J. (2003). Nonparametric Estimation of Shannon's Index of Diversity When There are Unseen Species in Sample. *Environ. Ecol. Stat.* 10 (4), 429–443. doi: 10.1023/A:1026096204727

Collins, R. E., and Higgs, P. G. (2012). Testing the Infinitely Many Genes Model for the Evolution of the Bacterial Core Genome and Pangenome. *Mol. Biol. Evol.* 29 (11), 3413–3425. doi: 10.1093/molbev/mss163

Contreras-Moreira, B., and Vinuesa, P. (2013). GET_HOMOLOGUES, a Versatile Software Package for Scalable and Robust Microbial Pangenome Analysis. *Appl. Environ. Microbiol.* 79 (24), 7696–7701. doi: 10.1128/AEM.02411-13

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., and Callahan, B. J. (2018). Simple Statistical Identification and Removal of Contaminant Sequences in Marker-Gene and Metagenomics Data. *Microbiome* 6 (1), 226. doi: 10.1186/s40168-018-0605-2

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible With ARB. *Appl. Environ. Microbiol.* 72 (7), 5069–5072. doi: 10.1128/AEM.03006-05

De Seta, F., Campisciano, G., Zanotta, N., Ricci, G., and Comar, M. (2019). The Vaginal Community State Types Microbiome-Immune Network as Key Factor for Bacterial Vaginosis and Aerobic Vaginitis. *Front. Microbiol.* 10, 2451. doi: 10.3389/fmicb.2019.02451

DiGiulio, D. B., Callahan, B. J., McMurdie, P. J., Costello, E. K., Lyell, D. J., Robaczewska, A., et al. (2015). Temporal and Spatial Variation of the Human Microbiota During Pregnancy. *Proc. Natl. Acad. Sci. U.S.A* 112 (35), 11060–11065. doi: 10.1073/pnas.1502875112

Drell, T., Lillsaar, T., Tummeleht, L., Simm, J., Aaspõllu, A., Väin, E., et al. (2013). Characterization of the Vaginal Micro-and Mycobiome in Asymptomatic Reproductive-Age Estonian Women. *PloS One* 8 (1), e54379. doi: 10.1371/journal.pone.0054379

Drew, R. J., Le, B., Kent, E., and Eogan, M. (2018). Relationship Between Absence of Lactobacilli in the Vagina of Pregnant Women and Preterm Birth: A Retrospective Pilot Study. *Obstetr. Gynecol. Rep.* 2 (2), 1–4. doi: 10.15761/OGR.1000128

Enright, A. J., Van Dongen, S., and Ouzounis, C. A. (2002). An Efficient Algorithm for Large-Scale Detection of Protein Families. *Nucleic Acids Res.* 30 (7), 1575–1584. doi: 10.1093/nar/30.7.1575

Evanovich, E., de Souza Mendonça Mattos, P. J., and Guerreiro, J. F. (2019). Comparative Genomic Analysis of *Lactobacillus Plantarum*: An Overview. *Int. J. Genomics* 2019, 4973214. doi: 10.1155/2019/4973214

Feehily, C., Crosby, D., Walsh, C. J., Lawton, E. M., Higgins, S., McAuliffe, F. M., et al. (2020). Shotgun Sequencing of the Vaginal Microbiome Reveals Both a Species and Functional Potential Signature of Preterm Birth. *NPJ Biofilms Microbiomes* 6 (1), 1–9. doi: 10.1038/s41522-020-00162-8

Fettweis, J. M., Serrano, M. G., Brooks, J. P., Edwards, D. J., Girerd, P. H., Parikh, H. I., et al. (2019). The Vaginal Microbiome and Preterm Birth. *Nat. Med.* 25 (6), 1012–1021. doi: 10.1038/s41591-019-0450-2

Fredricks, D. N., Fiedler, T. L., and Marrazzo, J. M. (2005). Molecular Identification of Bacteria Associated With Bacterial Vaginosis. *N. Engl. J. Med.* 353, 1899–1911. doi: 10.1056/NEJMoa043802

Gao, X., Lin, H., Revanna, K., and Dong, Q. (2017). A Bayesian Taxonomic Classification Method for 16S rRNA Gene Sequences With Improved Species-Level Accuracy. *BMC Bioinf.* 18 (1), 247. doi: 10.1186/s12859-017-1670-4

Gardella, C., Riley, D. E., Hitti, J., Agnew, K., Krieger, J. N., and Eschenbach, D. A. (2004). Identification and Sequencing of Bacterial rDNAs in Culture-Negative Amniotic Fluid From Women in Premature Labor. *Am. J. Perinatol.* 21, 319–323. doi: 10.1055/s-2004-831884

Goldstein, E. J., Tyrrell, K. L., and Citron, D. M. (2015). *Lactobacillus* Species: Taxonomic Complexity and Controversial Susceptibilities. *Clin. Infect. Dis.* 60 (2), 98–107. doi: 10.1093/cid/civ072

Gonsalvez, G. B., Urbinati, C. R., and Long, R. M. (2005). RNA Localization in Yeast: Moving Towards a Mechanism. *Biol. Cell* 97 (1), 75–86. doi: 10.1042/BC20040066

Hillier, S. L., Martius, J., Krohn, M., Kiviat, N., Holmes, K. K., and Eschenbach, D. A. (1988). A Case–Control Study of Chorioamnionic Infection and Histologic Chorioamnionitis in Prematurity. *N. Engl. J. Med.* 319 (15), 972–978. doi: 10.1056/NEJM198810133191503

Horton, P., Park, K. J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C. J., et al. (2007). Wolf PSORT: Protein Localization Predictor. *Nucleic Acids Res.* 35 (suppl_2), W585–W587. doi: 10.1093/nar/gkm259

Hyman, R. W., Fukushima, M., Jiang, H., Fung, E., Rand, L., Johnson, B., et al. (2014). Diversity of the Vaginal Microbiome Correlates With Preterm Birth. *Reprod. Sci.* 21 (1), 32–40. doi: 10.1177/1933719113488838

Inglin, R. C., Meile, L., and Stevens, M. J. (2018). Clustering of Pan-and Core-Genome of *Lactobacillus* Provides Novel Evolutionary Insights for Differentiation. *BMC Genomics* 19 (1), 284. doi: 10.1186/s12864-018-4601-5

Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., et al. (2019). Evaluation of 16S rRNA Gene Sequencing for Species and Strain-Level Microbiome Analysis. *Nat. Commun.* 10 (1), 1–11. doi: 10.1038/s41467-019-13036-1

Kindinger, L. M., Bennett, P. R., Lee, Y. S., Marchesi, J. R., Smith, A., Cacciatore, S., et al. (2017). The Interaction Between Vaginal Microbiota, Cervical Length, and Vaginal Progesterone Treatment for Preterm Birth Risk. *Microbiome* 5 (1), 6. doi: 10.1186/s40168-016-0223-9

Klebanoff, M. A., and Brotman, R. M. (2018). Treatment of Bacterial Vaginosis to Prevent Preterm Birth. *Lancet* 392 (10160), 2141. doi: 10.1016/S0140-6736(18)32115-9

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-Based Diversity Studies. *Nucleic Acids Res.* 41 (1), e1–e1. doi: 10.1093/nar/gks808

Krohn, M. A., Hillier, S. L., Nugent, R. P., Cotch, M. F., Carey, J. C., Gibbs, R. S., et al. (1995). The Genital Flora of Women With Intraamniotic Infection. *J. Infect. Dis.* 171 (6), 1475–1480. doi: 10.1093/infdis/171.6.1475

Kuczynski, J., Stombaugh, J., Walters, W. A., González, A., Caporaso, J. G., and Knight, R. (2012). Using QIIME to Analyze 16S rRNA Gene Sequences From Microbial Communities. *Curr. Protoc. Bioinf.* 27 (1), 1E–15. doi: 10.1002/9780471729259.mc01e05s27

Lai, S. K., Hida, K., Shukair, S., Wang, Y. Y., Figueiredo, A., Cone, R., et al. (2009). Human Immunodeficiency Virus Type 1 is Trapped by Acidic But Not by Neutralized Human Cervicovaginal Mucus. *J.Virol.* 83 (21), 11196–11200. doi: 10.1128/JVI.01899-08

Lam-Tung, N., Schmidt, H. A., Von Haeseler, A., and Minh, B. Q. (2015). Iq-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol. Biol. Evol.* 32 (1), 268–274. doi: 10.1093/molbev/msu300

Letunic, I., and Bork, P. (2019). Interactive Tree of Life (iTOL) v4: Recent Updates and New Developments. *Nucleic Acids Res.* 47 (W1), W256–W259. doi: 10.1093/nar/gkz239

Li, L., Stoeckert, C. J., and Roos, D. S. (2003). OrthoMCL: Identification of Ortholog Groups for Eukaryotic Genomes. *Genome Res.* 13 (9), 2178–2189. doi: 10.1101/gr.1224503

Lu, S., Wang, J., Chitsaz, F., Derbyshire, M. K., Geer, R. C., Gonzales, N. R., et al. (2020). CDD/SPARCLE: The Conserved Domain Database in 2020. *Nucleic Acids Res.* 48 (D1), D265–D268. doi: 10.1093/nar/gkz991

Ma, B., France, M. T., Crabtree, J., Holm, J. B., Humphrys, M. S., Brotman, R. M., et al. (2020). A Comprehensive non-Redundant Gene Catalog Reveals Extensive Within-Community Intraspecies Diversity in the Human Vagina. *Nat. Commun.* 11 (1), 1–13. doi: 10.1038/s41467-020-14677-3

Marchesi, J. R., and Ravel, J. (2015). The Vocabulary of Microbiome Research: A Proposal. *Microbiome* 3, 31. doi: 10.1186/s40168-015-0094-5

Marret, S., Ancel, P. Y., Marpeau, L., Marchand, L., Pierrat, V., Larroque, B., et al. (2007). Neonatal and 5-Year Outcomes After Birth At 30–34 Weeks of Gestation. *Obstet. Gynecol.* 110 (1), 72–80. doi: 10.1097/01.AOG.0000267498.95402.bd

Mehta, O., Ghosh, T. S., Kothidar, A., Gowtham, M. R., Mitra, R., Kshetrapal, P., et al. (2020). Vaginal Microbiome of Pregnant Indian Women: Insights Into the Genome of Dominant *Lactobacillus* Species. *Microb. Ecol.* 80, 487–499. doi: 10.1007/s00248-020-01501-0

Mira, A., Martín-Cuadrado, A. B., D'Auria, G., and Rodríguez-Valera, F. (2010). The Bacterial Pan-Genome: A New Paradigm in Microbiology. *Int. Microbiol.* 13 (2), 45–57. doi: 10.2436/20.1501.01.110

Moreno, I., and Franasiak, J. M. (2017). Endometrial Microbiota—New Player in Town. *Fertil. Steril.* 108 (1), 32–39. doi: 10.1016/j.fertnstert.2017.05.034

Mukherjee, S., Mitra, R., Maitra, A., Gupta, S., Kumaran, S., Chakrabortty, A., et al. (2016). Sebum and Hydration Levels in Specific Regions of Human Face Significantly Predict the Nature and Diversity of Facial Skin Microbiome. *Sci. Rep.* 6, 36062. doi: 10.1038/srep36062

Muzzi, A., Masignani, V., and Rappuoli, R. (2007). The Pan-Genome: Towards a Knowledge-Based Discovery of Novel Targets for Vaccines and Antibacterials. *Drug Discovery Today* 12 (11-12), 429–439. doi: 10.1016/j.drudis.2007.04.008

Nelson, W. C., and Stegen, J. C. (2015). The Reduced Genomes of *Parcubacteria* (OD1) Contain Signatures of a Symbiotic Lifestyle. *Front. Microbial.* 6, 713. doi: 10.3389/fmicb.2015.00713

Pareek, S., Kurakawa, T., Das, B., Motooka, D., Nakaya, S., Rongsen-Chandola, T., et al. (2019). Comparison of Japanese and Indian Intestinal Microbiota Shows Diet-Dependent Interaction Between Bacteria and Fungi. *NPJ Biofilms Microbiomes* 5 (1), 37. doi: 10.1038/s41522-019-0110-9

Parolin, C., Marangoni, A., Laghi, L., Foschi, C., Ñahui Palomino, R. A., Calonghi, N., et al. (2015). Isolation of Vaginal Lactobacilli and Characterization of anti-Candida Activity. *PloS One* 10 (6), e0131220. doi: 10.1371/journal.pone.0131220

Parris, K. M., Amabebe, E., Cohen, M. C., and Anumba, D. O. (2020). Placental Microbial–Metabolite Profiles and Inflammatory Mechanisms Associated With Preterm Birth. *J. Clin. Pathol.* 74 (1), 10–18. doi: 10.1136/jclinpath-2020-206536

Penn, K., Jenkins, C., Nett, M., Udwary, D. W., Gontang, E. A., McGlinchey, R. P., et al. (2009). Genomic Islands Link Secondary Metabolism to Functional Adaptation in Marine *Actinobacteria*. *ISME J.* 3 (10), 1193–1203. doi: 10.1038/ismej.2009.58

Putonti, C., Shapiro, J. W., Ene, A., Tsibere, O., and Wolfe, A. J. (2020). Comparative Genomic Study of *Lactobacillus Jensenii* and the Newly

Defined *Lactobacillus Mulieris* Species Identifies Species-Specific Functionality. *Msphere* 5 (4), e00560–20. doi: 10.1128/mSphere.00560-20

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., et al. (2011). Vaginal Microbiome of Reproductive-Age Women. *Proc. Natl. Acad. Sci. U. S. A.* 108 (Supplement 1), 4680–4687. doi: 10.1073/pnas.1002611107

Read, T. D., and Ussery, D. W. (2006). Opening the Pan-Genomics Box. *Curr. Opin. Microbiol.* 5 (9), 496–498. doi: 10.1016/j.mib.2006.08.010

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: A Versatile Open Source Tool for Metagenomics. *PeerJ* 4, e2584. doi: 10.7717/peerj.2584

Romero, R., Dey, S. K., and Fisher, S. J. (2014). Preterm Labor: One Syndrome, Many Causes. *Science* 345 (6198), 760–765. doi: 10.1126/science.1251816

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Bieda, J., et al. (2014). The Vaginal Microbiota of Pregnant Women Who Subsequently Have Spontaneous Preterm Labor and Delivery and Those With a Normal Delivery At Term. *Microbiome* 2 (1), 18. doi: 10.1186/2049-2618-2-18

Romero, R., Sirtori, M., Oyarzun, E., Avila, C., Mazor, M., Callahan, R., et al. (1989). Infection and Labor V. Prevalence, Microbiology, and Clinical Significance of Intraamniotic Infection in Women With Preterm Labor and Intact Membranes. *Am. J. Obstet. Gynecol.* 161 (3), 817–824. doi: 10.1016/0002-9378(89)90409-2

Seo, S. S., Arokiyaraj, S., Kim, M. K., Oh, H. Y., Kwon, M., Kong, J. S., et al. (2017). High Prevalence of *Leptotrichia Amnionii*, *Atopobium Vaginae*, *Sneathia Sanguinegens*, and Factor 1 Microbes and Association of Spontaneous Abortion Among Korean Women. *Biomed. Res. Int.* 2017, 5435089. doi: 10.1155/2017/5435089

Sharma, P., Khan, S., Ghule, M., Shivkumar, V. B., Dargan, R., Seed, P. T., et al. (2018). Rationale & Design of the PROMISES Study: A Prospective Assessment and Validation Study of Salivary Progesterone as a Test for Preterm Birth in Pregnant Women From Rural India. *Reprod. Health* 15 (1), 1–9. doi: 10.1186/s12978-018-0657-6

Sievers, F., and Higgins, D. G. (2014). Clustal Omega. *Curr. Protoc. Bioinf.* 48 (1), 3–13. doi: 10.1002/0471250953.bi0313s48

Srinivasan, S., Hoffman, N. G., Morgan, M. T., Matsen, F. A., Fiedler, T. L., Hall, R. W., et al. (2012). Bacterial Communities in Women With Bacterial Vaginosis: High Resolution Phylogenetic Analyses Reveal Relationships of Microbiota to Clinical Criteria. *PloS One* 7 (6), e37818. doi: 10.1371/journal.pone.0037818

Stafford, G. P., Parker, J. L., Amabebe, E., Kistler, J., Reynolds, S., Stern, V., et al. (2017). Spontaneous Preterm Birth is Associated With Differential Expression of Vaginal Metabolites by Lactobacilli-Dominated Microflora. *Front. Physiol.* 8, 615. doi: 10.3389/fphys.2017.00615

Tabatabaei, N., Eren, A. M., Barreiro, L. B., Yotova, V., Dumaine, A., Allard, C., et al. (2019). Vaginal Microbiome in Early Pregnancy and Subsequent Risk of Spontaneous Preterm Birth: A Case–Control Study. *BJOG* 126 (3), 349–358. doi: 10.1111/1471-0528.15299

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., et al. (2016). NCBI Prokaryotic Genome Annotation Pipeline. *Nucleic Acids Res.* 44 (14), 6614–6624. doi: 10.1093/nar/gkw569

Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017). Unicycler: Resolving Bacterial Genome Assemblies From Short and Long Sequencing Reads. *PloS Comput. Biol.* 13 (6), e1005595. doi: 10.1371/journal.pcbi.1005595

Willis, A. D. (2019). Rarefaction, Alpha Diversity, and Statistics. *Front. Microbiol.* 10, 2407. doi: 10.3389/fmicb.2019.02407

Witkin, S. S., Alvi, S., Bongiovanni, A. M., Linhares, I. M., and Ledger, W. J. (2011). Lactic Acid Stimulates interleukin-23 Production by Peripheral Blood Mononuclear Cells Exposed to Bacterial Lipopolysaccharide. *FEMS Immunol. Med. Microbiol.* 61 (2), 153–158. doi: 10.1111/j.1574-695X.2010.00757.x

Wolke, D., Eryigit-Madzwamuse, S., and Gutbrod, T. (2014). Very Preterm/Very Low Birthweight Infants' Attachment: Infant and Maternal Characteristics. *Arch. Dis. Child Fetal. Neonatal. Ed.* 99 (1), F70–F75. doi: 10.1136/archdischild-2013-303788

World Health Organization (2018) *Preterm-Birth*. Available at: https://www.who.int/news-room/fact-sheets/detail/preterm-birth.

Younes, J. A., Lievens, E., Hummelen, R., van der Westen, R., Reid, G., and Petrova, M. I. (2018). Women and Their Microbes: The Unexpected Friendship. *Trends Microbiol.* 26 (1), 16–32. doi: 10.1016/j.tim.2017.07.008

Zhou, X., Brown, C. J., Abdo, Z., Davis, C. C., Hansmann, M. A., Joyce, P., et al. (2007). Differences in the Composition of Vaginal Microbial Communities Found in Healthy Caucasian and Black Women. *ISME J.* 1 (2), 121–133. doi: 10.1038/ismej.2007.12

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Polyphenol Enriched Diet Administration During Pregnancy and Lactation Prevents Dysbiosis in Ulcerative Colitis Predisposed Littermates

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Neonatal colonization of the gastrointestinal tract depends on mother microbiome, thus mother microbiota dysbiosis is transmitted to the offspring during the delivery and shaped by breastmilk characteristics. Here we used a murine model of UC predisposition (Winnie$^{-/-}$) to evaluate the effects of maternal diet during pregnancy and lactation. Using heterozygous breeders, we obtained both Winnie$^{-/-}$ and C57BL/6 littermates from the same mother and compared their microbiota at weaning and adult age, using a diet enriched with 1% tomato fruit of a line – named Bronze – highly enriched in bioactive polyphenols, or Control tomato. Females received enriched diets two weeks before the beginning of the breeding and never stopped for the following six months. No significant effect was observed in regard to the percentage of Winnie$^{-/-}$ offspring, as with both diets the percentage was about 25% as expected. Winnie littermates from breeders fed with the Bronze-enriched diet showed reduced dysbiosis at 4 weeks of age if compared with Winnie under the Control tomato diet. This effect was then reduced when mice reached adult age. Conversely, the microbiota of C57BL/6 does not change significantly, indicating that fortified mothers-diet significantly contribute to preventing dysbiosis in genetically predisposed offspring, but has mild effects on healthy littermates and adult mice. An overall tendency towards reduced inflammation was underlined by the colon weight and the percentage of Foxp3^{+} cells reduction in Winnie mice fed with Bronze diet. Control diet did not show similar tendency.

Keywords: ulcerative colitis, nutrition, animal model, polyphenols, microbiota

INTRODUCTION

Ulcerative colitis (UC) is a relapsing, chronic and debilitating inflammatory disease involving the large intestine from the caecum to the rectum (Ungaro et al., 2017). UC affects both adults and children and peaks during reproductive years. Multiple risk factors are associated with UC development, including environmental factors, eating disorders, emotional distress, immune disorders, microbiota, and genetic predisposition (Corridoni et al., 2014).

Currently, a complete genetic map explaining disease hereditariness is not available, nonetheless, parents' transmission seems to be the strongest predictor factor for child disease development. Some recent studies suggested that the transmission rate from mothers with Inflammatory Bowel Disease (IBD) is higher than father's transmission, thus, it is intriguing to speculate a primary role for mothers' microbiome in IBD transmission (Akolkar et al., 1997; Zelinkova et al., 2012). Intestinal microbial communities play a non-redundant role in shaping the mucosal immune response both directly and indirectly affecting intestinal barrier functions (Takiishi et al., 2017; Torres et al., 2020).

Dysbiosis in IBD patients is characterized by a reduction in microbial diversity compared with healthy patients, increased concentrations of Gram-negative anaerobes and reduced numbers of colonic *Bifidobacterium species* (Wensinck et al., 1981; Matsuda et al., 2000; Ohkusa et al., 2002; Tamboli et al., 2004).

Modulation of the gut microbiota was recently proposed to be an important strategy to protect newborns from chronic disease development (Houghteling and Walker, 2015; Tanaka and Nakayama, 2017; Liso et al., 2018; Turroni et al., 2020). Intestinal bacteria communities adapt to the intestinal lumen content, including dietary antigens. Thus, nutritional strategies are a privileged line of intervention to prevent dysbiosis. More in general, these strategies can dampen inflammation in distinct pathological conditions, especially those involving the use of foods from the Mediterranean diet characterized by a high content in bioactive compounds (De Santis et al., 2019; Piccinin et al., 2019; Cariello et al., 2020; De Santis et al., 2020a). Furthermore, nutrition may shape mothers' microbiota and breast milk composition, both significantly involved in the development of the offspring intestinal microbial communities (Rautava et al., 2012; Baldassarre et al., 2014; Liu et al., 2014). Thus, nutritional strategies able to promote mothers' "healthy microbial communities", may protect the progeny from IBD development.

Examples of maternal diet affecting neonatal health are reported in literature. Breast milk is a source of a vast spectrum of immune-active and antimicrobial molecules, micronutrients, vitamins, antioxidants, and other nutrients, that, altogether, shape the mucosal immune response (Andreas et al., 2015). Furthermore, a direct axis between breast milk composition and maternal intestinal microbiota has been demonstrated, possibly due to the extraintestinal translocation of intestinal bacteria to the mammary gland during late pregnancy and lactation in mice (Perez et al., 2007).

Different studies reported the positive effects of polyphenol-enriched diets on UC in mice, mostly highlighting the preventive potential of their dietary supplementation, even after a short-term administration (2 weeks of diet) (Martin and Bolling, 2015; Wang et al., 2020). The administration of a polyphenols enriched tomato diet has been previously described as an inducer of positive effects on the pathological symptoms and microbiota composition, compared to a standard rodent diet, in IBD mice models (Liso et al., 2018; Scarano et al., 2018). However, the impact of polyphenols-enriched diets on puppies during lactation and later in adult life, in terms of microbiota modulation, was less characterized.

For this reason, we aimed to verify the impact on the microbiota of puppies fed with custom diets supplemented with 1% of lyophilized wild type (Control) or Bronze tomato lines. The near-isogenic Bronze line and its composition have been previously described in detail, and it is based on different classes of polyphenols, such as flavonols, anthocyanins and stilbenoids (Scarano et al., 2018).

The present study showed that maternal diet enriched with lyophilized fruits of a polyphenol-rich tomato line (Bronze) was protective against newborn dysbiosis development. Our previous data demonstrated that 2 weeks of mice chow enriched with 1% of Bronze lyophilized fruits were able to block the inflammatory pathway in the intestinal tract of DSS (Dextran Sulfate Sodium) treated mice (Scarano et al., 2018) and in the genetically predisposed Winnie mice (Eri et al., 2011; Liso et al., 2019). This mutant mouse strain was characterized by a point mutation in the *Muc2* gene, resulting in accumulation of aberrant MUC2 within the intestinal goblet cells, leading to activation of endoplasmic reticulum (ER) stress. ER stress caused the activation of the innate and adaptive immune response, resulting in a spontaneous and severe mucosal inflammation of the distal colon, resembling UC (Eri et al., 2011). Heterozygote mice do not show UC like phenotype, but present mild dysbiosis transmittable to the offspring (De Santis et al., 2021). The breeding strategy was based on co-housing heterozygote breeders to obtain both Winnie$^{-/-}$ and C57BL/6 littermates from the same mother. Maternal transmission has been indicated as the pivotal event for the onset of colonization of intestinal communities; for this reason, we investigated if the differences observed between the fecal microbiome of C57BL/6 and Winnie littermates could still be observed in offspring from the same parents (Liso et al., 2019).

We previously demonstrated that gut microbiota from Winnie mice, fed with standard rodent diet, was significantly different from their wild type siblings, even if they were generated and weaned from the same mother (Liso et al., 2019).

Data from others (Codoñer-Franch et al., 2013: Wall et al., 2013) demonstrated that supplementation with flavonoids during lactation increases the antioxidant properties of breastmilk and reduces inflammatory cytokine production in fetal membranes. Here we demonstrate that a significant path of dysbiosis was present in the fecal material of 4-week old Winnie born from heterozygote mothers compared to wild type C57BL/6 siblings. Vice versa, a similar bacterial composition was detected in the fecal material of 4-week old C57BL/6 and Winnie born from heterozygote mothers when fed with a Bronze-tomatoes enriched diet.

MATERIAL AND METHODS

Ethical Considerations

Our investigations were performed under the relevant animal protocol which was approved by Institutional Animal Care Committee of National Institute of Gastroenterology "S. de Bellis" (*Organism* engaged for compliance of *Animal Wellbeing*: *OPBA*). All of the animal experiments were carried out according to the national guidelines of Italian Directive n. 26/2014 and approved by

the Italian Animal Ethics Committee of Ministry of Health - General Directorate of Animal Health and Veterinary Drugs (DGSAF- Prot. 768/2015-PR 27/07/2015). All animals were maintained in a controlled environment (20–22°C, 12 h light and 12 h dark cycles, and 45–55% relative humidity).

Generation of Tomato Lines and Diets

The Bronze tomato line (E8::MYB12, E8::Del/Ros, 35S::StSy) was developed as previously described (Scarano et al., 2018), by sequential crossing of two metabolically engineered lines, Indigo and ResTom. The resulting line, named Bronze because of the metallic brown color of the ripe fruit skin, expressed the genes *AmDelila* and *AmRosea1* that induce anthocyanin biosynthesis, *AtMYB12* regulating flavonol biosynthesis and the gene *VvStSy* for the production of resveratrol and stilbenoids (Scarano et al., 2018).

The Control diet (cv. Moneymaker, wild type) has a same basal composition as a standard rodent diet (Nutrient composition_ Amount: Proteins_19.4%; Fats_2.58%; Fibers_5.54%; Ashes_6.76%). The addition of 1% of lyophilized wild-type or Bronze tomato powder was chosen consistently to our previous studies (Liso et al., 2018; Scarano et al., 2018). The addition consists of roughly 0.014 mg of polyphenols/day/mouse incorporated in the Control tomato diet, compared to 0.800 mg polyphenols/day/mouse in the Bronze tomato diet (Scarano et al., 2018).

Murine Models

C56BL/6 mice were purchased from Jackson Laboratories: (C57BL/6, Stock No.: 000664) while Winnie mice were obtained from the University of Tasmania (Eri et al., 2011). Winnie mice were generated by ENU mutagenesis. A missense mutation in the *Muc2* gene (the base change G -> A) caused the substitution of the Cystein in position 52 into a Tyrosine (G9492A, GenBank accession no. AJ511872).

Four couples of heterozygous breeders, subdivided into two groups, were used. Each group of breeders received a different diet. Freeze-dried tomato was supplemented by addition to a standard rodent diet (4RF18) at 1% (tomato-based-diets: wild type (Control) and Bronze). 6-week old females received enriched diets two weeks before the beginning of the breeding and never stopped for the following six months.

New-born pups were weaned at 4 weeks of age, ear-tagged, and then caged based on sex and similar genotype. For fecal material collection mice were single caged for two hours. Genotype was performed from DNA obtained from 5-mm tail tissues. After the weaning, mice were fed with the same tomato supplemented diets used during the breeding until 16 weeks of age. Body weight, stool consistency, and rectal bleeding were assessed every 4 weeks.

Mice were sacrificed at 16 weeks and colon and mesenteric lymph node (MLN) tissues were explanted to evaluate the clinical severity of colitis. Colon length and weight were measured as indicators of colonic inflammation. The colon/body weight indices were calculated as the ratio of the colon wet weight and the total body weight (BW), and as the ratio of the colon length and the total BW of each mouse.

Cytofluorimetric Assay

FoxP3 staining: Mesenteric lymph nodes (MLNs) were isolated from mice fed with tomato (Control or Bronze)-enriched food. MLNs were passed through a 30 μm cell strainer (Miltenyi Biotec, Bergisch Gladbach, Germany) to obtain a single-cell suspension and washed with DPBS (Gibco, Waltham, MA, USA) + 0.5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA).

Single-cell suspensions were stained with CD4-FITC and CD25-PE (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then permeabilized with Foxp3 Fixation/Permeabilization Kit (eBioscience, San Diego, CA, USA) and washed with PERM Buffer (eBioscience, San Diego, CA, USA). Finally, cells were stained with Foxp3-APC (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions.

T cell Intracellular Staining: T cells from MLNs of mice fed with tomato (Control or Bronze)-enriched food were cultured with a 500X Cell Stimulation Cocktail (eBiosceince, San Diego, CA, USA) for 12 h, washed with DPBS + 0.5% BSA and stained with CD4-APC Vio770 (Miltenyi Biotec, Bergisch Gladbach, Germany). After washing, cells were then permeabilized with BD CytoFix/CytoPerm®Fixation/Permeabilization Kit® (BD Biosciences, Franklin Lakes, NJ, USA), washed with PERM Buffer, and stained with TNFα-PE and IFNγ-APC according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

For both stainings, Flow Cytometer acquisition was performed using NAVIOS (Beckman Coulter). At least three experiments were performed. Flow cytometer analysis was performed using Kaluza Software 1.5 (Beckman Coulter, Brea, CA, USA).

RNA Extraction and qPCR Analysis

Total RNA was isolated from the distal colon of mice fed with tomato (Control or Bronze) enriched food. The RNA was extracted using TRIzol® (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using an iScript cDNA Synthesis kit (Biorad, CA, USA) with random primers for cDNA synthesis. Gene expression of Gapdh, Tnf, Ifnγ, Il10, Il17a, Il12b, Il6, Hmox1, Slpi, Slc40a1 was assessed using PrimePCR™ SYBR® Green Assays (Biorad, CA, USA; assay ID: qMmuCED0027497, qMmuCED0004141, qMmuCID0006268, qMmuCID0015452, qMmuCID0026592, qMmuCID0022424, qMmuCID0005613, qMmuCID0040051, qMmuCED0004965, qMmuCID0011775, respectively). Real-time analysis was performed on CFX96 Touch System (Biorad, CA, USA) and for the relative expression, the ΔΔCt method was used. At least three experiments were performed.

DNA Extraction From Stool

Mice were single caged for two hours in a cage without bedding and fecal samples were immediately collected and frozen. Total genomic bacterial DNA was isolated from frozen stool samples of mice using the QIAamp®Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

Bacterial Microbiome Estimated by 16S rRNAs Metagenetics and Statistical Analysis

16S metagenetic analyses were carried out at Genomix4life (spin-off of the University of Salerno, Fisciano, Italy) by using the

Illumina MiSeq platform. The V3-V4 region of the 16S rRNA gene was amplified for analysis of diversity inside the domains of Bacteria (Klindworth et al., 2013). PCR and sequencing analyses were carried out according to the protocol of Genomix4life.

Bioinformatics analyses of sequence data, from processing of raw DNA sequence reads to alpha index estimates were conducted in QIIME2 (https://doi.org/10.1038/s41587-019-0209-9) microbiome platform (version 2020.8). Paired demultiplexed 16S sequences have been denoised by using q2-deblur QIIME plugin (https://github.com/qiime2/q2-deblur). Taxonomy has been inferred by using the SILVA QIIME-compatible classifier (release 138). Alpha diversity metrics including Shannon entropy and Faith's PD were also computed by using QIIME2 platform (Shannon, 1997; Chao and Bunge, 2002). Starting from QIIME2 relative abundances, the q2-emperor plugin was used to compute beta diversity metrics.

Significant taxa among groups were computed by using a two side Welch test corrected by multiple test with Benjamini-Hochberg (**Supplementary Metadata**).

After converting the taxa relative abundances (at the genus level) into a presence/absence matrix, Venn diagram were calculated and graphically rendered in R environment by using the 'VennDiagram' package (https://cran.r-project.org/web/packages/VennDiagram/VennDiagram.pdf).

For colon/body weight indices, gene expression and FACS analyses, a one-way ANOVA test corrected by using Bonferroni within the Graphpad Prism statistical software (release 5.0), was computed. All data were expressed as means ± S.E.M. obtained from at least three independent experiments.

RESULTS

Study Design: Bronze Enriched Diet Administration to UC Predisposed Couples

With the intent to verify the impact of polyphenol enriched diet administration to the offspring of UC predisposed individuals, we created diets supplemented with 1% of lyophilized wild type (Control) or Bronze tomato lines (Scarano et al., 2018).

Figure 1A showed the breeding strategy adopted: C57BL/6 and Winnie puppies were born from the same Winnie heterozygous parents. To study the impact on lactation, Winnie heterozygous breeders were fed with 1% dried fruits of Control tomato variety (cv. Moneymaker) or Bronze tomatoes; the offsprings were fed by lactation until the weaning; afterward, they were fed with the same tomato enriched diets until 16 weeks, when they were sacrificed for further analyses (**Figure 1B**).

Mice were monitored for survival and weight (**Figure 1C** and **Table 1**). **Figure 1C** shows the Family Trees of both experimental groups; siblings have been subdivided by genotype and sex; premature dead mice are also indicated.

Both Control and Bronze tomato enriched diets did not affect mice weight, independently from the genotype. However, in line with our models (De Santis et al., 2017; Liso et al., 2019; De Santis et al., 2020b), Winnie mice showed a reduced body weight as compared to C57BL/6 littermates (**Figure 1D**). As expected, the majority of premature deaths were from mice carrying the Winnie allele (in homo- or heterozygosity), regardless of maternal diet. About 90% of the newborn from each experimental group reached adult age.

The mortality rate in the first weeks of life was near 10% in both groups, significantly lower than what we observed in our animal facility with the same breeding strategy, about 20% with Standard rodent diet (data not shown), and the common mortality rate observed in laboratory mice (Weber et al., 2013; Leidinger et al., 2019). As shown in **Table 2**, the obtained percentage for each genotype of the newborns was similar to the predicted one in the Bronze group, whilst in the Control tomato group we recorded an imbalance between the obtained percentage of C57BL/6 and heterozygote Winnie$^{+/-}$ mice.

Influence of Maternal Diet on Intestinal Microbial Communities of the Offspring

Stools from each mouse were collected at 4 and 16 weeks to carry out a metagenomic analysis of the gut microbiota (**Figure 1B**). We searched for a correspondence in the composition of their microbiota with that of the respective mothers at the weaning.

Both Shannon and phylogenetic Faith's PD indices were concordant in detecting a higher alpha diversity in all mice (mothers, C57BL/6 and Winnie offspring, at 4 and 16 weeks) fed with Bronze diet when compared with Control tomato fed mice (**Supplementary Metadata** and **Supplementary Table 1**).

In estimating beta diversity, unweighted UniFrac metric reveals how Control tomato and Bronze fed mice do not clearly separate into clusters; two different mixed clouds are visible in a tridimensional plot (**Supplementary Figure 1**).

Figure 2 reports the comparison of the main phyla found in the mothers and offspring, C57BL/6 or Winnie at 4 weeks. The Bronze diet induced a general trend to an increase of Firmicutes and to a decrease of Bacteroidota, in mothers and related offspring (**Figure 2A**). Campilobacterota tends to increase in mothers and C57BL/6 offspring fed with Bronze diet, while a decrease was observed in Winnie mice (**Figure 2A**). The Bronze diet also induced a tendency of decrease of Proteobacteria in mothers and related Winnie puppies. As shown in **Figure 2B**, mothers fed with the Bronze diet showed a significant increase of Deferribacterota compared to the Control group and the related Winnie offspring.

At the genus level, the Bronze diet induced in Winnie mice a significant increase of *Prevotellaceae_UCG-001* and *Lachnospiraceae_NK4A136_group* and a decrease of *Mucispirillum*, compared to Winnie mice fed with the Control diet (**Figure 3**). Instead, in C57BL/6 mice fed with the Bronze diet we observed a decrease of *Odoribacter*, *Prevotellaceae*, *Bacteroides*, *Akkermansia*, *Parabacteroides* and *Oscillospiraceae*, but these differences were not statistically significant (**Figure 3** and **Supplementary Figures 2, 3**).

At the species level, we found a significant increase in *Prevotellaceae_UCG-001;s_spp.* and *Rikenellaceae_RC9_gut_group;s_spp*. in Winnie mice fed with the Bronze diet compared to the Control group (**Supplementary Figure 4**). Such modulations were not detected in C57BL/6 mice after the Bronze diet administration, in which we found a significant decrease of *Odoribacter;s_unidentified* and *Oscillospiraceae;g_uncultured;s_spp*. and a significant increase of *Bacteroides sartorii* and *Mucispirillum schaedleri* (**Supplementary Figure 4**).

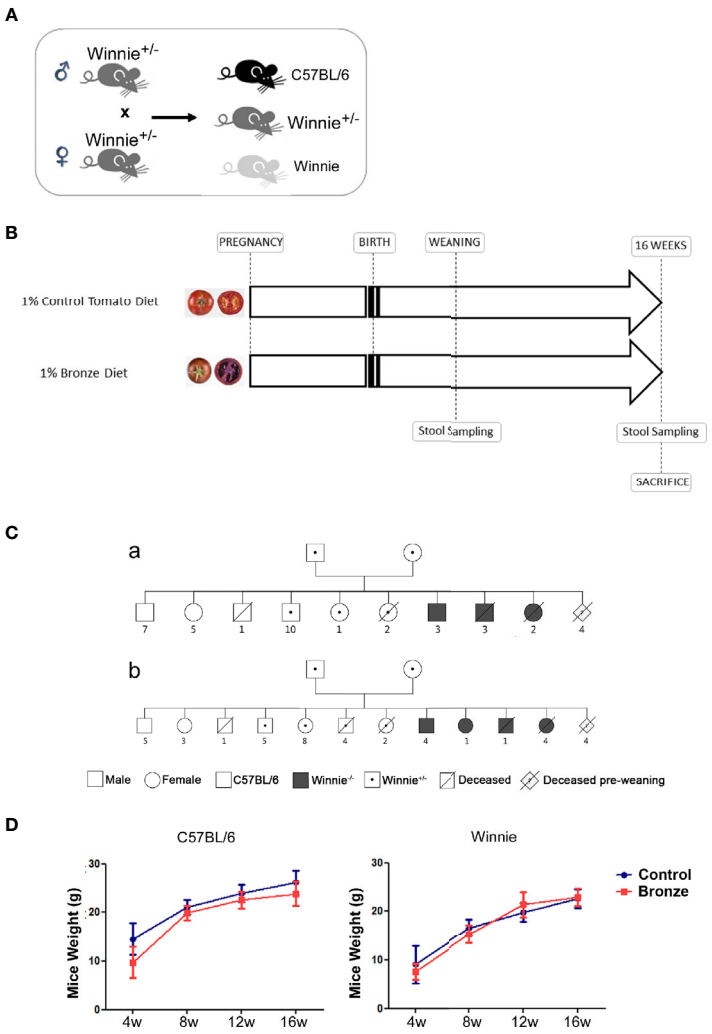

FIGURE 1 | Experimental design. **(A)** Heterozygote Winnie$^{+/-}$ mice were crossed to obtain C57BL/6 and Winnie$^{-/-}$. **(B)** Parental breeders and puppies with the selected genotypes were fed with 1% of Control- or Bronze-enriched diet for up to 16 weeks. **(C)** Family trees of breeding pairs fed with **(a)** Control- or **(b)** Bronze-enriched diet, carried out using the "Pedigree Chart Designer" CeGaT tool. **(D)** Mice weight (male and female) was recorded from weaning at 4 weeks (4 w) to the end of the dietary treatment at 16 weeks (16 w).

Bronze Diet Administration Is Not Sufficient to Prevent Winnie Associated Intestinal Pathology

After the weaning, C57BL/6 and Winnie mice were fed with the same diets used during the breeding (Control or Bronze enriched diet) until 16 weeks of age. **Figure 4** showed that, at a macroscopic level, no differences were observed in C57BL/6 mice fed with both diets in terms of colon length and weight. On the contrary, the Bronze diet tend to reduce the absolute and relative colon weight in Winnie mice (**Figures 4B, D**), even if the

TABLE 1 | Natality and mortality rates for each experimental group.

DIET	N. LITTERS	N. MICE BORN	N. MICE WEANED	F1 GENOTYPE	N. MICE DEAD
CONTROL TOMATO	7	38	34 (89.5%)	C57BL/6	1
				Winnie$^{+/-}$	2
				Winnie	5
BRONZE	6	42	38 (90.5%)	C57BL/6	1
				Winnie$^{+/-}$	6
				Winnie	5

TABLE 2 | Percentages of predicted and obtained genotypes from each breeders couple; total number of siblings for each genotype is shown.

DIET	F1 GENOTYPE	F1% PREDICTED	F1% OBTAINED	N. MICE
CONTROL TOMATO	C57BL/6	25%	38%	13
	Winnie$^{+/-}$	50%	38%	13
	Winnie	25%	24%	8
BRONZE	C57BL/6	25%	24%	9
	Winnie$^{+/-}$	50%	50%	19
	Winnie	25%	26%	10

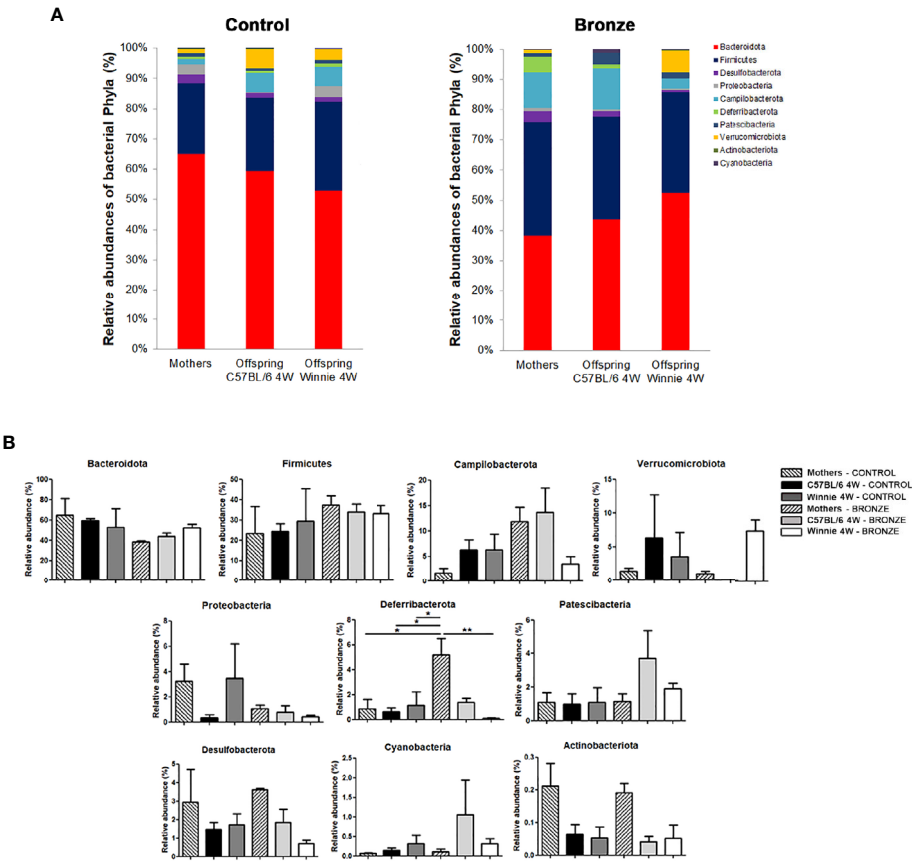

FIGURE 2 | **(A, B)** Relative abundances of the main phyla found at weaning time in the faecal microbiota of mothers and the related offspring (4 weeks), divided in C57BL/6 and Winnie genotypes, fed with Control tomato and Bronze diets. **(B)** *$p < 0.05$, **$p < 0.01$.

typical phenotype of Winnie was maintained, as they showed watery stools and a higher colon weight than their C57BL/6 littermates (**Figures 4A, B, D**).

We then isolated T cells from mesenteric lymph nodes (MLNs) and compare the Treg population and the intracellular cytokines produced by T helper (Th) cells in all the experimental groups, following the protocol described in **Figures 5A, B**, respectively. **Figure 5C** showed that the Bronze diet significantly reduced the percentage of CD4$^+$ Foxp3$^+$ cells in Winnie mice relative to C57BL/6 Bronze-fed and Winnie Control-fed mice. Instead, no significant differences were observed in the intracellular staining performed on Th cells, but we detected an increasing tendency in the CD4$^+$IFNγ^+ cells in C57BL/6 fed with the Bronze diet, as previously observed in adult C57BL/6 mice fed with the Bronze diet for 2 weeks (**Figure 5D**). The Bronze diet also induced a significant increase in total CD4$^+$ cells in Winnie mice (**Figure 5D**).

To investigate the influence of the two diets at the molecular level, we analyzed the relative expression of genes involved in the inflammatory response in the colon of C57BL/6 and Winnie mice (**Supplementary Figure 5**). The Bronze diet induced a significant increase of *Ifnγ* expression in C57BL/6 mice, confirming the intracellular staining data on Th cells isolated from MLNs; on the contrary, a tendency for a reduction was observed for *Il6* (statistically significant), *Il10*, *Il12b* and *Il17a* expression. In Winnie mice fed with the Bronze diet, instead, we

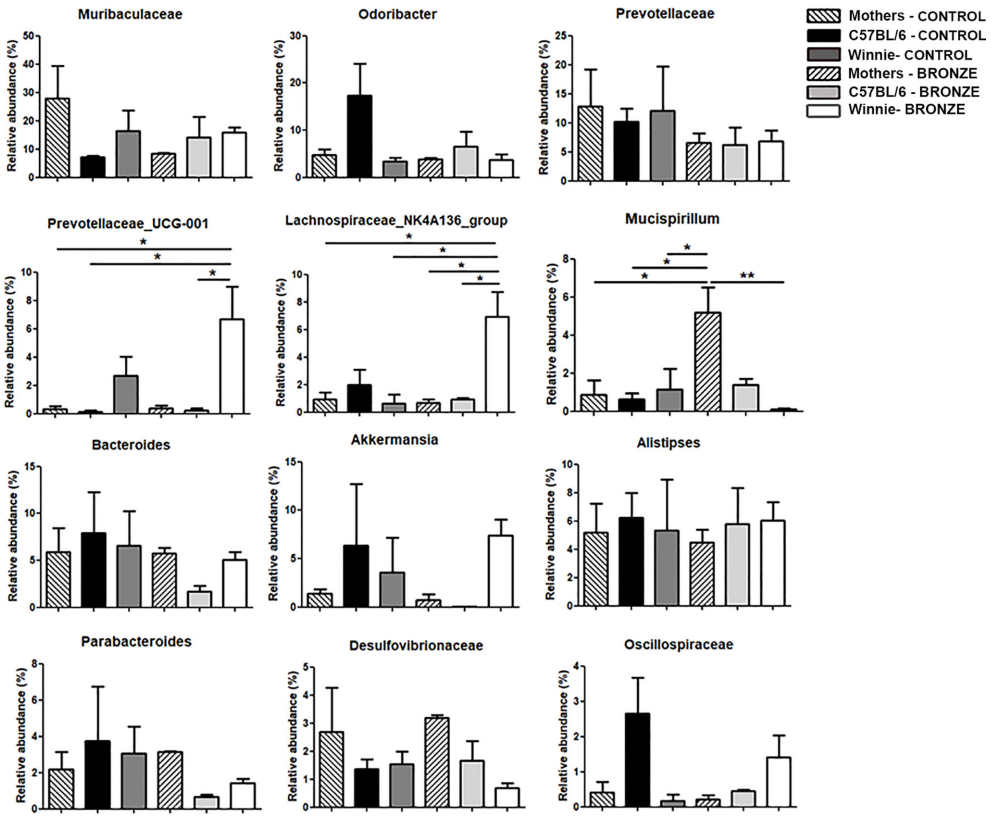

FIGURE 3 | Relative abundances of the bacteria genera found at weaning time in the faecal microbiota of mothers and the related offspring (4 weeks), divided in C57BL/6 and Winnie genotypes, fed with Control tomato and Bronze diets. *p < 0.05, **p < 0.01.

recorded a tendency for a non-significant increase of *Il6*, *Il10* and *Il17a* expression and a tendency for a decrease of *Il12b*. No relevant difference was observed in *Tnf* expression level in C57BL/6 and Winnie mice fed with both diets. We previously demonstrated that polyphenols like quercetin are able to induce the expression of genes involved in iron metabolism. In Winnie mice, the Bronze diet tends to increase for the expression levels of *Slpi* and *Hmox1*, while reduces the expression of *Slc40a1* (encoding Ferroportin). A similar trend was also observed in C57BL/6 mice but with a significant reduction of *Slc40a1* expression (**Supplementary Figure 5**).

Long Term Administration of Bronze Diet Is Not Able to Treat Winnie Associated Dysbiosis

We then studied the long-term effects of the polyphenol enriched diets, by comparing the microbiota of 16-weeks old mice at the end of treatment. **Figure 6** showed that the main differences observed at the weaning were more attenuated in older mice. In fact, within the same genotype, the relative abundance of the main phyla was similar with both diets. In the Control tomato groups, the relative abundances of the major phyla, such as Firmicutes, Bacteroidota and Campilobacterota, were almost comparable to those of the offspring fed with Bronze diet. On the other hand, these latest could be comparable to the relative abundances already observed at 4 weeks. In C57BL/6 mice, the Bronze diet induced a decrease of Patescibacteria and Desulfobacterota (significant) and a concomitant increase in Verrucomicrobiota, Deferribacterota and Proteobacteria (**Figure 6B**). In Winnie mice, instead, the Bronze diet induced an increase of Patescibacteria and a decrease of Proteobacteria, Cyanobacteria and Actinobacteriota (not statistically significant, **Figure 6B**). The attenuation of the differences regarding the phyla relative abundances was mostly reflected in the changes at genus (**Figure 7** and **Supplementary Figure 6**) and species levels at 16 weeks (**Supplementary Figure 7**).

The administration of the Bronze diet induced a significant decrease of *Odoribacter* and an increase of *Prevotellaceae_UCG-001* and *Akkermansia* in both C57BL/6 and Winnie mice, while the *Bacteroides* tends to decrease in Winnie mice fed with the Bronze diet (**Figure 7** and **Supplementary Figure 6**).

Finally, at the species level, the Bronze diet induced in Winnie mice a significant increase of *Prevotellaceae_UCG-001;s_spp.*, as observed at the weaning time; the *Muribaculum;s_spp.* was also increased, while a decrease of *Bacteroides_sartorii*, *Rikenellaceae* spp. and *Odoribacter* spp. was recorded (**Supplementary Figure 7**). In C57BL/6 mice fed with the Bronze diet we observed a significant decrease of *Desulvovibrionaceae* spp.,

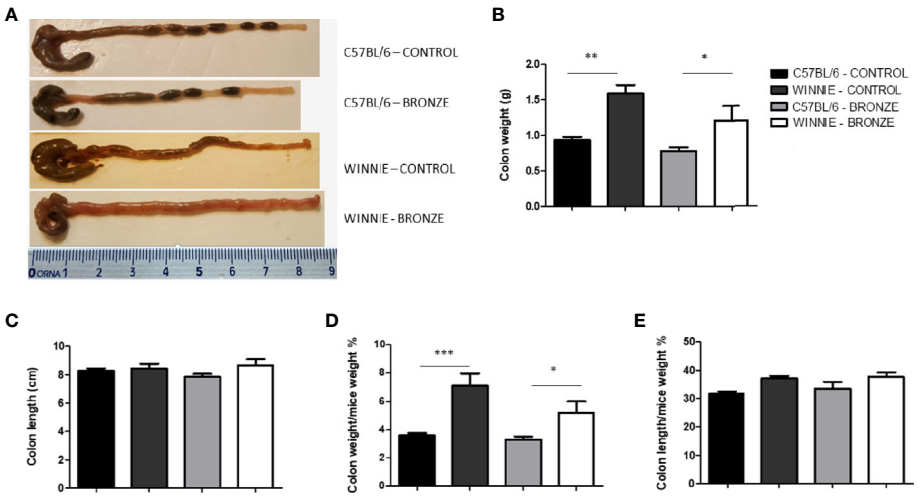

FIGURE 4 | Macroscopic features and measurements of colonic parameters at the end of treatment (16 weeks). **(A)** Representative images of whole colons for each experimental group. Panel **(B, C)** indicate colon weight and length, respectively. Colon weight/body weight and colon length/body weight indices (%) are shown in panel **(D, E)**, respectively. *p < 0.05; **p < 0.01; ***p < 0.001.

Oscillospiraceae;g_uncultured;s_spp. and *Anaerotruncus; s_uncultured_bacterium*, and an increase of *Odoribacter; s_uncultured_bacterium* compared to C57BL/6 mice fed with the Control tomato group (**Supplementary Figure 7**).

DISCUSSION

IBD genetic transmission is a widely accepted concept, together with the idea that IBD-affected mothers have higher risks of complications during pregnancy and a higher rate of premature delivery (Hashash and Kane, 2015). IBD incidence peaks during reproductive years and 25% of patients experience pregnancy after the IBD diagnosis (Loftus, 2004). The gut microbiota has gained attention during the last decade due to the pivotal role in host intestinal homeostasis, indeed, numerous pathological conditions are associated with an "unhealthy" microbiota composition called dysbiosis. The intestinal colonization is believed to start in utero and adapt to numerous host and environmental factors. The host genetics plays a non-redundant role in shaping the intestinal microbiota (Liso et al., 2019), nonetheless, several different factors further influence the composition of the gut microbiota including cesarean or natural birth, feeding types, weaning, birth environment, and mothers' microbiota (Nagpal et al., 2017; Raspini et al., 2020). As IBD patients, including mothers during pregnancy, are characterized by reduced α-diversity, it is not surprising that such reduction is transmitted to the offspring (Torres et al., 2020).

Using a murine model of ulcerative colitis called Winnie, we previously compared the microbiome compositions of homozygous mutant mice (Winnie$^{-/-}$) with their C57BL/6 littermates, after weaning from heterozygote Winnie$^{+/-}$ breeders. Heterozygote breeders transmitted dysbiotic microbiota to APC$^{+/Min}$ offspring, resulting in higher rate of CRC development if compared to C57BL/6 breeders (De Santis et al., 2021). We demonstrated that the Winnie offspring displayed significant dysbiosis as early as 4 weeks of age compared with their C57BL/6 littermates (Liso et al., 2019). Furthermore, we investigated the effects on the microbial composition of the Standard, Control tomato and Bronze diets in C57BL/6 and Winnie mice (Liso et al., 2018; Scarano et al., 2018), observing no significant differences between Standard and Control tomato diet among phyla and genera microbial groups. Significant differences were rather observed following administration of Bronze diet, therefore we compared Bronze with Control, instead of Standard rodent diet. Here we aimed to evaluate the possibility to contrast Winnie offspring dysbiosis by feeding the heterozygote Winnie$^{+/-}$ breeders with chows enriched in 1% dried tomatoes. In the present experimental design, we used the Bronze tomato line, enriched in three distinct classes of polyphenols (flavonols, anthocyanins and stilbenoids), and a Control tomato variety (cv. Moneymaker, (Scarano et al., 2018).

Although dietary intervention has been demonstrated to influence gut related microbial functions, without altering both alpha and beta diversity (Li et al., 2016; De Angelis et al., 2020), a study conducted on female rats fed with obesogenic diet demonstrated that diet can alter the gut microbial alpha diversity, as we observed in our study (Bhagavata Srinivasan et al., 2018). Colonic microflora can be influenced by nutritional polyphenols, either directly due to their ability to influence the intestinal ecology (Kumar Singh et al., 2019; Ansary and Cianciosi, 2020; Ullah et al., 2020) and indirectly due to their effect on the host innate and adaptive immune response or iron sequestrating abilities (Cavalcanti et al., 2014; Delvecchio et al., 2015; Chieppa et al., 2017). Our results indicate that nutritional intervention during pregnancy and lactation may influence neonatal microbiota better than dietary intervention in adults. Future human studies will be required to confirm these preclinical observations.

Using Bronze tomatoes as a dietary supplement for the breeding pairs, we immediately noticed a reduction in the

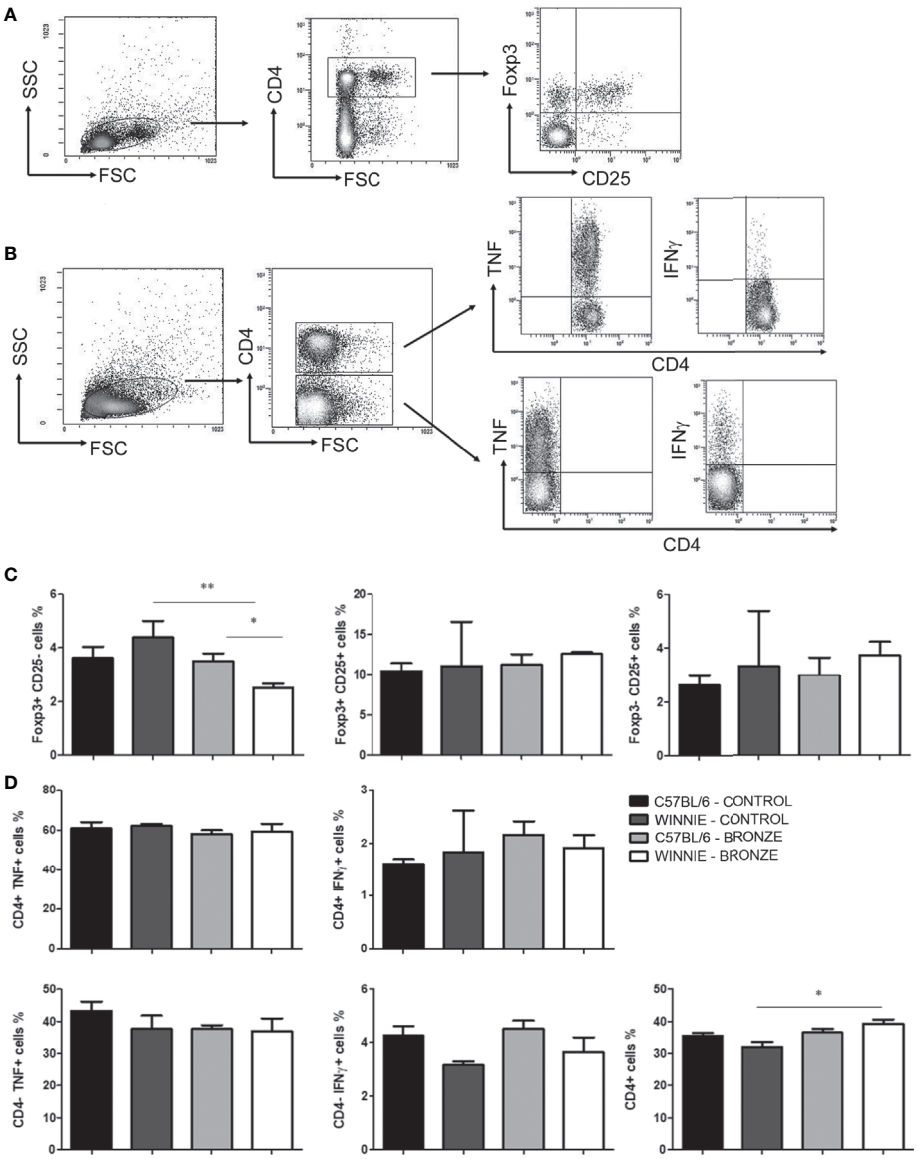

FIGURE 5 | Treg and intracellular cytokine staining of $CD4^+$ and $CD4^-$ cells isolated from MLNs. **(A)** Representative density plot of CD4-CD25-Foxp3 staining on Treg. **(B)** Representative density plot analysis of intracellular staining of TNF and IFNγ from T $CD4^+$ and $CD4^-$ cells. **(C)** Histograms represent the percentages of Foxp3 and CD25 gated on $CD4^+$ cells, in the MLNs of C57BL/6 mice fed with Control or Bronze diet (black and dark grey bars) and Winnie (light grey and white bars, respectively). **(D)** Intracellular staining of TNF and IFNγ gated on $CD4^+$ and $CD4^-$ cells, and percentage of total $CD4^+$ cells isolated from MLNs of C57BL/6 and Winnie mice, fed with Control or Bronze diet. *$p < 0.05$; **$p < 0,01$.

mortality rate and an increase in the number of survived Winnie$^{-/-}$. These data, although extremely interesting, require a specific experimental design to increase the number of breeding pairs and prolong the observation period. In light of these preliminary data, we may speculate that the known beneficial effects of a polyphenol enrich diet contributes to reduce mothers' intestinal and, consequently, systemic inflammation, among the major causes of complication for IBD patients' pregnancy. These results will be important for the design of future human studies.

Offspring microbiota delivered by mothers under Control tomato and Bronze-enriched diet were analyzed before weaning to evaluate if mothers enriched nutrition during breastfeeding could protect genetically predisposed mice from dysbiosis, mainly due to the increase in the relative abundance of Firmicutes and an increase of Proteobacteria. Of notice, the Bronze enriched diet generally affected mothers' microbiota favoring a balanced ratio between Bacteroidota and Firmicutes. During lactation, the mothers' diet has a minor effect on C57BL/6 offspring, while Winnie microbiota is pushed into a "healthier" status. We can't discriminate if Bronze-enriched mother's milk has a direct effect on the intestinal microbiota, or if the increase in bioactive compounds reduces inflammation and consequently the

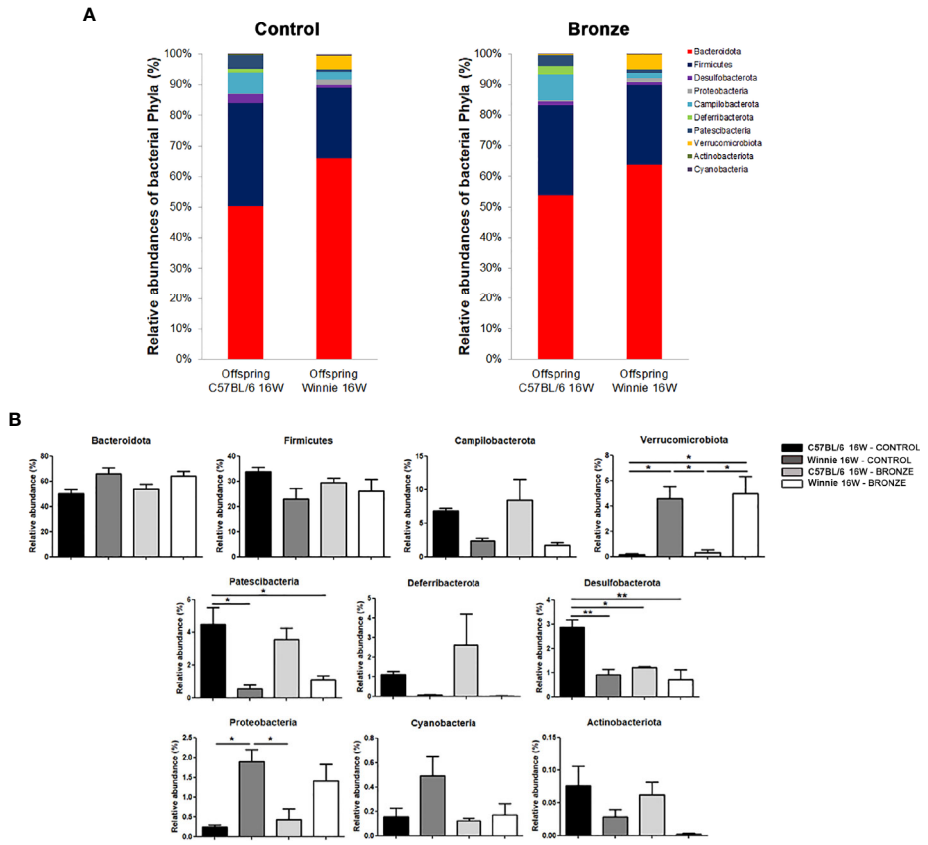

FIGURE 6 | **(A, B)** Relative abundances of the main phyla found in the faecal microbiota of the C57BL/6 and Winnie mice fed with Control tomato and Bronze diets for 16 weeks. **(B)** *p < 0.05; **p < 0.01.

intestinal dysbiosis. Most likely, both effects are true as a polyphenol enriched diet has been already demonstrated to improve breastmilk quality and polyphenol content (Khymenets et al., 2016; Tsopmo, 2018). Metabolomics of mother's milk may help to address this important question. In Winnie offspring, the Bronze diet induced a significant increase of bacterial species belonging to the Lachnospiraceae group and *Prevotellaceae_UCG-001*. The Lachnospiraceae are anaerobic, fermentative bacteria, able to use diet-derived polysaccharides and plant aromatic compounds to produce SCFAs (short-chain fatty acids), like acetate, propionate and butyrate, used from colonocytes as an energetic source, with essential anti-inflammatory properties demonstrated *in vitro* and *in vivo* (Li et al., 2017; Okumura and Takeda, 2018; Vacca et al., 2020). Wang et al. demonstrated an increase in *Lachnospiraceae_NK4A136_group* in a mice model of acute colitis, after administration of probiotics and prebiotics mix (Wang et al., 2019).

An increase of *Prevotellaceae_UCG-001* was associated with a decrease of inflammation and a gut barrier improvement in mice with DSS-induced colitis, treated with a plant-derived decoction (Shen et al., 2020; Zou et al., 2020).

After weaning, mice continued with the same diet of mothers for 12 weeks. The morphological score of the Bronze-enriched diet group had minor improvements if compared to the Control-tomato diet. Similarly, the molecular pathway was similar between mice with the same genetics but different nutritional regimes. However, *Slpi* and *Hmox1* gene expression were increased by the Bronze diet, although not significantly, whilst *Slc40a1* expression was reduced, confirming previous observations on the effects of polyphenols like quercetin on the expression of these markers (De Santis et al., 2016; Galleggiante et al., 2017). At the cellular level, we recorded no significant differences when comparing the cytokines produced from $CD4^+$ and $CD4^-$ T cells in the MLNs. Nevertheless, a significant reduction in the $Foxp3^+$ cells was observed in Winnie mice fed with Bronze diet. Even if $Foxp3^+$ cells are involved in inflammatory suppression, their increased number is a sign of ongoing inflammation as demonstrated by several studies. In particular, this transcriptional factor was observed in the intestinal lamina propria of IBD patients compared to healthy control (Wohlfert and Belkaid, 2008; Iacomino et al., 2020). Foxp3 expression was also increased in the colon mucosa during acute and chronic DSS-induced colitis (Yang and Xu, 2016). In Winnie mice, $CD4^+$ cells are recruited in the peripheral tissue due to the chronic inflammatory response. This effect is partially reverted in Winnie mice fed with Bronze diet. $CD4^+$ cells percentages in the Winnie Bronze MLNs are similar to what

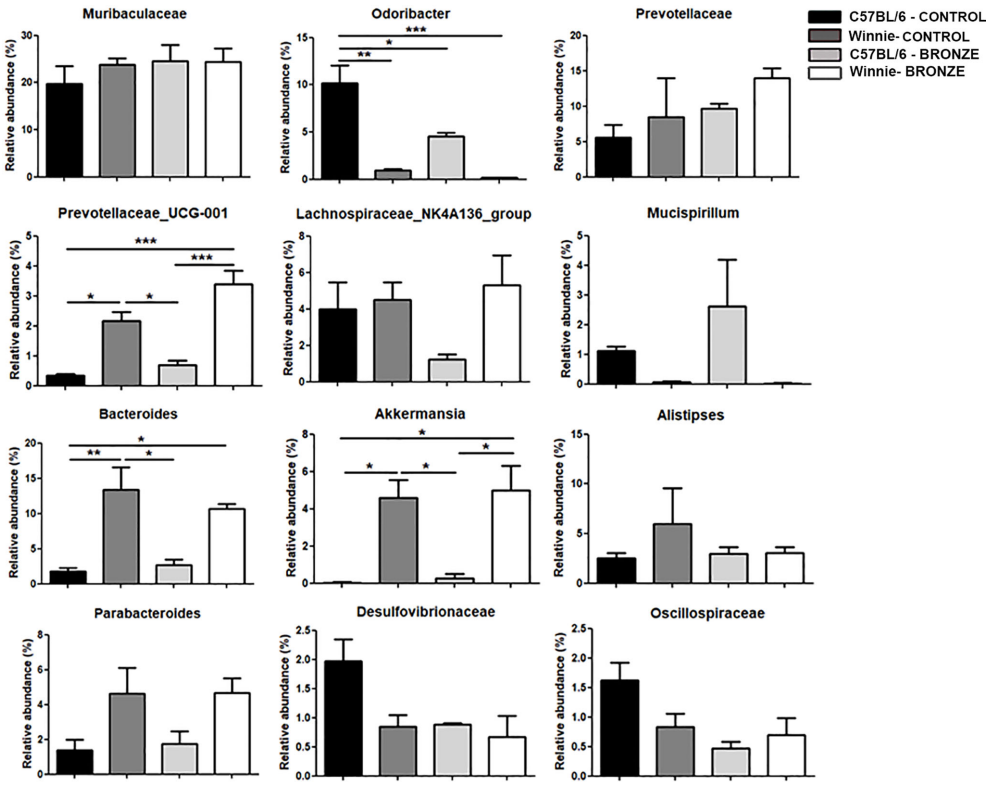

FIGURE 7 | Relative abundances of the main genera found in the faecal microbiota of the C57BL/6 and Winnie mice fed with Control tomato and Bronze diets for 16 weeks. *p < 0.05; **p < 0.01; ***p < 0.001.

observed in the C57BL/6 MLNs, suggesting that intestinal inflammation may be alleviated by the Bronze diet. Furthermore, the microbiota analysis shows a strong correlation between genotype and bacterial content, confirming what we previously observed (Santino et al., 2017; Liso et al., 2018). This genotype-related signature was clearly identifiable at phylum and genus level. These data indicate that long term exposure to polyphenol enriched nutritional regimes may display a reduction in the beneficial effects on microbiota communities selection. On the contrary, this study confirms that short interventions have positive effects on intestinal microbiota of mothers fed with Control or Bronze-enriched tomato diet during pregnancy and lactation and on puppies at 4 weeks.

In conclusion, although our results indicate that prolonged dietary intervention with polyphenol enriched nutritional regimes does not significantly impact the offspring microbiota, we observed significant dysbiosis reduction mainly at weaning time, indicating that the reduction of mothers' dysbiosis may beneficially affect babies' microbiota and help preventing/reducing IBD development. Nutrition should become a common adjuvant therapy for dysbiosis mediated inflammatory disorders that may evolve in intestinal or extraintestinal disorders (Ianiro et al., 2020; De Santis et al., 2021; Xu et al., 2021). Based on these results, the present study can contribute to pave the way for future studies based on nutritional interventions designed for IBD mothers.

DATA AVAILABILITY STATEMENT

Illumina Nextseq550 generated raw sequence reads were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (NCBI). Bioproject accession number: PRJNA718731. Fifthy biosample record IDs: SAMN18559514- SAMN18559563.

ETHICS STATEMENT

The animal study was reviewed and approved by Italian Animal Ethics Committee of Ministry of Health - General Directorate of Animal Health and Veterinary Drugs (DGSAF- Prot. 768/2015-PR 27/07/2015).

AUTHOR CONTRIBUTIONS

SDS, ASc and ML conceived and designed the project. SDS, ASc, ML, GV and EC carried out the experiments. FMC performed bioinformatics analysis. MDA and FMC performed statistical analysis. ASi designed nutritional intervention. AL provided funding acquisition. MC and ASa wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.622327/full#supplementary-material

REFERENCES

Akolkar, P. N., Gulwani-Akolkar, B., Heresbach, D., Lin, X. Y., Fisher, S., Katz, S., et al. (1997). Differences in Risk of Crohn's Disease in Offspring of Mothers and Fathers With Inflammatory Bowel Disease. *Am. J. Gastroenterol.* 92 (12), 2241–2244.

Andreas, N. J., Kampmann, B., and Mehring Le-Doare, K. (2015). Human Breast Milk: A Review on its Composition and Bioactivity. *Early Hum. Dev.* 91 (11), 629–635. doi: 10.1016/j.earlhumdev.2015.08.013

Ansary, J., and Cianciosi, D. (2020). Natural Antioxidants: Is the Research Going in the Right Direction? *Metabolism* 13 (3), 187–191. doi: 10.3233/MNM-200484

Baldassarre, M. E., Bellantuono, L., Mastromarino, P., Miccheli, A., Fanelli, M., and Laforgia, N. (2014). Gut and Breast Milk Microbiota and Their Role in the Development of the Immune Function. *Curr. Pediatr. Rep.* 2 (3), 218–226. doi: 10.1007/s40124-014-0051-y

Bhagavata Srinivasan, S. P., Raipuria, M., Bahari, H., Kaakoush, N. O., and Morris, M. J. (2018). Impacts of Diet and Exercise on Maternal Gut Microbiota Are Transferred to Offspring. *Front. Endocrinol. (Lausanne)* 9, 716. doi: 10.3389/fendo.2018.00716

Cariello, M., Contursi, A., Gadaleta, R. M., Piccinin, E., De Santis, S., Piglionica, M., et al. (2020). Extra-Virgin Olive Oil From Apulian Cultivars and Intestinal Inflammation. *Nutrients* 12 (4). doi: 10.3390/nu12041084

Cavalcanti, E., Vadrucci, E., Delvecchio, F. R., Addabbo, F., Bettini, S., Liou, R., et al. (2014). Administration of Reconstituted Polyphenol Oil Bodies Efficiently Suppresses Dendritic Cell Inflammatory Pathways and Acute Intestinal Inflammation. *PloS One* 9 (2), 1084, e88898. doi: 10.1371/journal.pone.0088898

Chao, A., and Bunge, J. (2002). Estimating the Number of Species in a Stochastic Abundance Model. *Biometrics* 58 (3), 531–539. doi: 10.1111/j.0006-341x.2002.00531.x

Chieppa, M., Galleggiante, V., Serino, G., Massaro, M., and Santino, A. (2017). Iron Chelators Dictate Immune Cells Inflammatory Ability: Potential Adjuvant Therapy for IBD. *Curr. Pharm. Des.* 23 (16), 2289–2298. doi: 10.2174/1381612823666170215143541

Codoñer-Franch, P., Hernández-Aguilar, M. T., Navarro-Ruiz, A., López-Jaén, A. B., Borja-Herrero, C., and Valls-Bellés, V. (2013). Diet Supplementation During Early Lactation With Non-Alcoholic Beer Increases the Antioxidant Properties of Breastmilk and Decreases the Oxidative Damage in Breastfeeding Mothers. *Breastfeed Med.* 8, 164–169. doi: 10.1089/bfm.2012.0059

Corridoni, D., Arseneau, K. O., and Cominelli, F. (2014). Inflammatory Bowel Disease. *Immunol. Lett.* 161 (2), 231–235. doi: 10.1016/j.imlet.2014.04.004

De Angelis, M., Ferrocino, I., Calabrese, F. M., De Filippis, F., Cavallo, N., Siragusa, S., et al. (2020). Diet Influences the Functions of the Human Intestinal Microbiome. *Sci. Rep.* 10 (1), 4247. doi: 10.1038/s41598-020-61192-y

Delvecchio, F. R., Vadrucci, E., Cavalcanti, E., De Santis, S., Kunde, D., Vacca, M., et al. (2015). Polyphenol Administration Impairs T-Cell Proliferation by Imprinting a Distinct Dendritic Cell Maturational Profile. *Eur. J. Immunol.* 45 (9), 2638–2649. doi: 10.1002/eji.201545679

De Santis, S., Cariello, M., Piccinin, E., Sabbà, C., and Moschetta, A. (2019). Extra Virgin Olive Oil: Lesson From Nutrigenomics. *Nutrients* 11 (9), 2085. doi: 10.3390/nu11092085

De Santis, S., Clodoveo, M. L., Cariello, M., D'Amato, G., Franchini, C., Faienza, M. F., et al. (2020a). Polyphenols and Obesity Prevention: Critical Insights on Molecular Regulation, Bioavailability and Dose in Preclinical and Clinical Settings. *Crit. Rev. Food Sci. Nutr.* 61 (11), 1–23. doi: 10.1080/10408398.2020.1765736

De Santis, S., Kunde, D., Galleggiante, V., Liso, M., Scandiffio, L., Serino, G., et al. (2017). TNFα Deficiency Results in Increased IL-1β in an Early Onset of Spontaneous Murine Colitis. *Cell Death Dis.* 8 (8), e2993. doi: 10.1038/cddis.2017.397

De Santis, S., Kunde, D., Serino, G., Galleggiante, V., Caruso, M. L., Mastronardi, M., et al. (2016). Secretory Leukoprotease Inhibitor Is Required for Efficient Quercetin-Mediated Suppression of Tnfβ Secretion. *Oncotarget* 7 (46), 75800–75809. doi: 10.18632/oncotarget.12415

De Santis, S., Liso, M., Vacca, M., Verna, G., Cavalcanti, E., Coletta, S., et al. (2021). Dysbiosis Triggers Acf Development in Genetically Predisposed Subjects. *Cancers (Basel)* 13 (2), 283. doi: 10.3390/cancers13020283

De Santis, S., Verna, G., Serino, G., Armentano, R., Cavalcanti, E., Liso, M., et al. (2020b). Winnie-APCMin/+ Mice: A Spontaneous Model of Colitis-Associated Colorectal Cancer Combining Genetics and Inflammation. *Int. J. Mol. Sci.* 21, 8, 2972. doi: 10.3390/ijms21082972

Eri, R. D., Adams, R. J., Tran, T. V., Tong, H., Das, I., Roche, D. K., et al. (2011). An Intestinal Epithelial Defect Conferring ER Stress Results in Inflammation Involving Both Innate and Adaptive Immunity. *Mucosal Immunol.* 4 (3), 354–364. doi: 10.1038/mi.2010.74

Galleggiante, V., De Santis, S., Cavalcanti, E., Scarano, A., De Benedictis, M., Serino, G., et al. (2017). Dendritic Cells Modulate Iron Homeostasis and Inflammatory Abilities Following Quercetin Exposure. *Curr. Pharm. Des.* 23 (14), 2139–2146. doi: 10.2174/1381612823666170112125355

Hashash, J. G., and Kane, S. (2015). Pregnancy and Inflammatory Bowel Disease. *Gastroenterol. Hepatol. (N. Y.)* 11 (2), 96–102.

Houghteling, P. D., and Walker, W. A. (2015). Why Is Initial Bacterial Colonization of the Intestine Important to Infants' and Children's Health? *J. Pediatr. Gastroenterol. Nutr.* 60 (3), 294–307. doi: 10.1097/MPG.0000000000000597

Iacomino, G., Rotondi Aufiero, V., Iannaccone, N., Melina, R., Giardullo, N., De Chiara, G., et al. (2020). IBD: Role of Intestinal Compartments in the Mucosal Immune Response. *Immunobiology* 225 (1), 151849. doi: 10.1016/j.imbio.2019.09.008

Ianiro, G., Segal, J. P., Mullish, B. H., Quraishi, M. N., Porcari, S., Fabiani, G., et al. (2020). Fecal Microbiota Transplantation in Gastrointestinal and Extraintestinal Disorders. *Future Microbiol.* 15, 1173–1183. doi: 10.2217/fmb-2020-0061

Khymenets, O., Rabassa, M., Rodríguez-Palmero, M., Rivero-Urgell, M., Urpi-Sarda, M., Tulipani, S., et al. (2016). Dietary Epicatechin is Available to Breastfed Infants Through Human Breast Milk in the Form of Host and Microbial Metabolites. *J. Agric. Food Chem.* 64 (26), 5354–5360. doi: 10.1021/acs.jafc.6b01947

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-Based Diversity Studies. *Nucleic Acids Res.* 41 (1), e1. doi: 10.1093/nar/gks808

Kumar Singh, A., Cabral, C., Kumar, R., Ganguly, R., Kumar Rana, H., Gupta, A., et al. (2019). Beneficial Effects of Dietary Polyphenols on Gut Microbiota and Strategies to Improve Delivery Efficiency. *Nutrients* 11 (9), 2216. doi: 10.3390/nu11092216

Leidinger, C. S., Thöne-Reineke, C., Baumgart, N., and Baumgart, J. (2019). Environmental Enrichment Prevents Pup Mortality in Laboratory Mice. *Lab. Anim.* 53 (1), 53–62. doi: 10.1177/0023677218777536

Li, D., Chen, H., Mao, B., Yang, Q., Zhao, J., Gu, Z., et al. (2017). Microbial Biogeography and Core Microbiota of the Rat Digestive Tract. *Sci. Rep.* 7 (1):45840. doi: 10.1038/srep45840

Li, H., Li, T., Beasley, D. E., Heděnec, P., Xiao, Z., Zhang, S., et al. (2016). Diet Diversity Is Associated With Beta But Not Alpha Diversity of Pika Gut Microbiota. *Front. Microbiol.* 7, 1169. doi: 10.3389/fmicb.2016.01169

Liso, M., De Santis, S., Scarano, A., Verna, G., Dicarlo, M., Galleggiante, V., et al. (2018). A Bronze-Tomato Enriched Diet Affects the Intestinal Microbiome Under Homeostatic and Inflammatory Conditions. *Nutrients* 10 (12), 1862. doi: 10.3390/nu10121862

Liso, M., De Santis, S., Verna, G., Dicarlo, M., Calasso, M., Santino, A., et al. (2019). A Specific Mutation in Muc2 Determines Early Dysbiosis in Colitis-Prone Winnie Mice. *Inflammation Bowel Dis.* XX (Xx), 1–11. doi: 10.1093/ibd/izz279

Liu, H., Zhang, J., Zhang, S., Yang, F., Thacker, P. A., Zhang, G., et al. (2014). Oral Administration of Lactobacillus Fermentum I5007 Favors Intestinal Development and Alters the Intestinal Microbiota in Formula-Fed Piglets. *J. Agric. Food Chem.* 62 (4), 860–866. doi: 10.1021/jf403288r

Loftus, E. V.Jr. (2004). Clinical Epidemiology of Inflammatory Bowel Disease: Incidence, Prevalence, and Environmental Influences. *Gastroenterology* 126 (6), 1504–1517. doi: 10.1053/j.gastro.2004.01.063

Martin, D. A., and Bolling, B. W. (2015). A Review of the Efficacy of Dietary Polyphenols in Experimental Models of Inflammatory Bowel Diseases. *Food Funct.* 6 (6), 1773–1786. doi: 10.1039/c5fo00202h

Matsuda, H., Fujiyama, Y., Andoh, A., Ushijima, T., Kajinami, T., and Bamba, T. (2000). Characterization of Antibody Responses Against Rectal Mucosa-Associated Bacterial Flora in Patients With Ulcerative Colitis. *J. Gastroenterol. Hepatol* 15 (1), 61–68. doi: 10.1046/j.1440-1746.2000.02045.x

Nagpal, R., Tsuji, H., Takahashi, T., Nomoto, K., Kawashima, K., Nagata, S., et al. (2017). Ontogenesis of the Gut Microbiota Composition in Healthy, Full-Term, Vaginally Born and Breast-Fed Infants Over the First 3 Years of Life: A Quantitative Bird's-Eye View. *Front. Microbiol.* 8, 1388. doi: 10.3389/fmicb.2017.01388

Ohkusa, T., Sato, N., Ogihara, T., Morita, K., Ogawa, M., and Okayasu, I. (2002). Fusobacterium Varium Localized in the Colonic Mucosa of Patients With Ulcerative Colitis Stimulates Species-Specific Antibody. *J. Gastroenterol. Hepatol* 17 (8), 849–853. doi: 10.1046/j.1440-1746.2002.02834.x

Okumura, R., and Takeda, K. (2018). Maintenance of Intestinal Homeostasis by Mucosal Barriers. *Inflammation Regener.* 38 (1), 5. doi: 10.1186/s41232-018-0063-z

Perez, P. F., Doré, J., Leclerc, M., Levenez, F., Benyacoub, J., Serrant, P., et al. (2007). Bacterial Imprinting of the Neonatal Immune System: Lessons From Maternal Cells? *Pediatrics* 119 (3), e724–e732. doi: 10.1542/peds.2006-1649

Piccinin, E., Cariello, M., De Santis, S., Ducheix, S., Sabbà, C., Ntambi, J. M., et al. (2019). Role of Oleic Acid in the Gut-Liver Axis: From Diet to the Regulation of Its Synthesis Via Stearoyl-CoA Desaturase 1 (SCD1). *Nutrients* 11 (10), 2283. doi: 10.3390/nu11102283

Raspini, B., Porri, D., De Giuseppe, R., Chieppa, M., Liso, M., Cerbo, R. M., et al. (2020). Prenatal and Postnatal Determinants in Shaping Offspring's Microbiome in the First 1000 Days: Study Protocol and Preliminary Results At One Month of Life. *Ital J. Pediatr.* 46 (1), 45. doi: 10.1186/s13052-020-0794-8

Rautava, S., Luoto, R., Salminen, S., and Isolauri, E. (2012). Microbial Contact During Pregnancy, Intestinal Colonization and Human Disease. *Nat. Rev. Gastroenterol. Hepatol* 9 (10), 565–576. doi: 10.1038/nrgastro.2012.144

Santino, A., Scarano, A., De Santis, S., De Benedictis, M., Giovinazzo, G., and Chieppa, M. (2017). Gut Microbiota Modulation and Anti-Inflammatory Properties of Dietary Polyphenols in IBD: New and Consolidated Perspectives. *Curr. Pharm. Des.* 23 (16), 2344–2351. doi: 10.2174/1381612823666170207145420

Scarano, A., Butelli, E., De Santis, S., Cavalcanti, E., Hill, L., De Angelis, M., et al. (2018). Combined Dietary Anthocyanins, Flavonols, and Stilbenoids Alleviate Inflammatory Bowel Disease Symptoms in Mice. *Front. Nutr.* 4, 75. doi: 10.3389/fnut.2017.00075

Shannon, C. E. (1997). The Mathematical Theory of Communication. 1963. *MD Comput.* 14 (4), 306–317.

Shen, Y., Zou, J., Chen, M., Zhang, Z., Liu, C., Jiang, S., et al. (2020). Protective Effects of Lizhong Decoction on Ulcerative Colitis in Mice by Suppressing Inflammation and Ameliorating Gut Barrier. *J. Ethnopharmacol* 259, 112919. doi: 10.1016/j.jep.2020.112919

Takiishi, T., Fenero, C. I. M., and Câmara, N. O. S. (2017). Intestinal Barrier and Gut Microbiota: Shaping Our Immune Responses Throughout Life. *Tissue Barriers* 5 (4), e1373208–e. doi: 10.1080/21688370.2017.1373208

Tamboli, C. P., Neut, C., Desreumaux, P., and Colombel, J. F. (2004). Dysbiosis in Inflammatory Bowel Disease. *Gut* 53 (1), 1–4. doi: 10.1136/gut.53.1.1

Tanaka, M., and Nakayama, J. (2017). Development of the Gut Microbiota in Infancy and Its Impact on Health in Later Life. *Allergol Int. Off. J. Japanese Soc. Allergol.* 66 (4), 515–522. doi: 10.1016/j.alit.2017.07.010

Torres, J., Hu, J., Seki, A., Eisele, C., Nair, N., Huang, R., et al. (2020). Infants Born to Mothers With IBD Present With Altered Gut Microbiome That Transfers Abnormalities of the Adaptive Immune System to Germ-Free Mice. *Gut* 69 (1), 42. doi: 10.1136/gutjnl-2018-317855

Tsopmo, A. (2018). Phytochemicals in Human Milk and Their Potential Antioxidative Protection. *Antioxid (Basel Switzerland)* 7 (2), 32. doi: 10.3390/antiox7020032

Turroni, F., Milani, C., Duranti, S., Lugli, G. A., Bernasconi, S., Margolles, A., et al. (2020). The Infant Gut Microbiome as a Microbial Organ Influencing Host Well-Being. *Ital J. Pediatr.* 46 (1), 16. doi: 10.1186/s13052-020-0781-0

Ullah, H., De Filippis, A., Santarcangelo, C., and Daglia, M. (2020). Epigenetic Regulation by Polyphenols in Diabetes and Related Complications. *Med. J. Nutr. Metab.* 13, 289–310. doi: 10.3233/MNM-200489

Ungaro, R., Mehandru, S., Allen, P. B., Peyrin-Biroulet, L., and Colombel, J.-F. (2017). Ulcerative Colitis. *Lancet (London Eng.)* 389 (10080), 1756–1770. doi: 10.1016/S0140-6736(16)32126-2

Vacca, M., Celano, G., Calabrese, F. M., Portincasa, P., Gobbetti, M., and De Angelis, M. (2020). The Controversial Role of Human Gut Lachnospiraceae. *Microorganisms* 8 (4), 573. doi: 10.3390/microorganisms8040573

Wall, C., Lim, R., Poljak, M., and Lappas, M. (2013). Dietary Flavonoids as Therapeutics for Preterm Birth: Luteolin and Kaempferol Suppress Inflammation in Human Gestational Tissues In Vitro. *Oxid. Med. Cell Longev* 2013, 485201. doi: 10.1155/2013/485201

Wang, Y. N., Meng, X. C., Dong, Y. F., Zhao, X. H., Qian, J. M., Wang, H. Y., et al. (2019). Effects of Probiotics and Prebiotics on Intestinal Microbiota in Mice With Acute Colitis Based on 16S rRNA Gene Sequencing. *Chin. Med. J. (Engl)* 132 (15), 1833–1842. doi: 10.1097/cm9.0000000000000308

Wang, Y., Wang, Y., Shen, W., Wang, Y., Cao, Y., Nuerbulati, N., et al. (2020). Grape Seed Polyphenols Ameliorated Dextran Sulfate Sodium-Induced Colitis Via Suppression of Inflammation and Apoptosis. *Pharmacology* 105 (1-2), 9–18. doi: 10.1159/000501897

Weber, E. M., Algers, B., Würbel, H., Hultgren, J., and Olsson, I. A. (2013). Influence of Strain and Parity on the Risk of Litter Loss in Laboratory Mice. *Reprod. Domest. Anim. = Zuchthygiene* 48 (2), 292–296. doi: 10.1111/j.1439-0531.2012.02147.x

Wensinck, F., Custers-van, L., Poppelaars-Kustermans, P. A., and Schröder, A. M. (1981). The Faecal Flora of Patients With Crohn's Disease. *J. Hygiene* 87 (1), 1–12. doi: 10.1017/s0022172400069187

Wohlfert, E., and Belkaid, Y. (2008). Role of Endogenous and Induced Regulatory T Cells During Infections. *J. Clin. Immunol.* 28 (6), 707–715. doi: 10.1007/s10875-008-9248-6

Xu, H. M., Huang, H. L., Zhou, Y. L., Zhao, H. L., Xu, J., Shou, D. W., et al. (2021). Fecal Microbiota Transplantation: A New Therapeutic Attempt From the Gut to the Brain. *Gastroenterol. Res. Pract.* 2021, 6699268. doi: 10.1155/2021/6699268

Yang, J., and Xu, L. (2016). Elevated IL-23R Expression and Foxp3+Rorgt+ Cells in Intestinal Mucosa During Acute and Chronic Colitis. *Med. Sci. Monit* 22, 2785–2792. doi: 10.12659/msm.896827

Zelinkova, Z., Stokkers, P. C., van der Linde, K., Kuipers, E. J., Peppelenbosch, M. P., and van der Woude, C. P. (2012). Maternal Imprinting and Female Predominance in Familial Crohn's Disease. *J. Crohns Colitis* 6 (7), 771–776. doi: 10.1016/j.crohns.2012.01.002

Zou, J., Shen, Y., Chen, M., Zhang, Z., Xiao, S., Liu, C., et al. (2020). Lizhong Decoction Ameliorates Ulcerative Colitis in Mice Via Modulating Gut Microbiota and Its Metabolites. *Appl. Microbiol. Biotechnol.* 104 (13), 5999–6012. doi: 10.1007/s00253-020-10665-1

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Preterm Birth Is Correlated With Increased Oral Originated Microbiome in the Gut

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Background: Preterm birth is one of the leading causes of perinatal morbidity and mortality. Gut microbiome dysbiosis is closely related to adverse pregnancy outcomes. However, the role of the gut microbiome in the pathogenesis of preterm birth remains poorly studied.

Method: We collected fecal samples from 41 women (cases presenting with threatened preterm labor =19, 11 of which delivered preterm; gestational age-matched no-labor controls, all of which delivered at term = 22) were recruited for the study. We performed 16S rRNA amplicon sequencing to compare the composition of the gut microbiome in threatened preterm labor cases and controls and among women who delivered preterm and at term. By annotating taxonomic biomarkers with the Human Oral Microbiome Database, we observed an increased abundance of potential oral-to-gut bacteria in preterm patients.

Results: Patients with preterm birth showed a distinct gut microbiome dysbiosis compared with those who delivered at term. Opportunistic pathogens, particularly *Porphyromonas*, *Streptococcus*, *Fusobacterium*, and *Veillonella*, were enriched, whereas *Coprococcus* and *Gemmiger* were markedly depleted in the preterm group. Most of the enriched bacteria were annotated oral bacteria using the Human Oral Microbiome Database. These potential oral-to-gut bacteria were correlated with clinical parameters that reflected maternal and fetal status.

Conclusions: This study suggests that patients who deliver preterm demonstrate altered gut microbiome that may contain higher common oral bacteria.

Keywords: preterm birth, preterm birth subtypes, gut microbiome, maternal gut microbiome, bacteria translocation

INTRODUCTION

Preterm birth, defined as birth before 37 weeks, is one of the leading causes of global neonatal morbidity. An estimated 15 million infants are born preterm every year (Blencowe et al., 2012; Liu et al., 2016). Preterm birth complications can increase the risk of metabolic abnormalities, respiratory distress, and poor neurobehavioral development in mothers and newborns (Mwaniki et al., 2012; Platt, 2014). Although infectious and sterile inflammation, oxidative stress and maternal hormone imbalance are recognized as important factors for preterm birth (Romero et al., 2005; Keelan, 2018), the etiology remains controversial.

In the recent decade, growing evidence has shown that the human microbiome plays a critical role in the development of many diseases. While the changes in the maternal vaginal microbiome and the development of the infant microbiome across different body sites have been well studied, the relationship between the maternal gut microbiome and preterm birth is rarely reported (Mychaliska, 2014). The dysbiosis of the maternal gut microbiome could result in severe gestational disease, including preeclampsia, gestational diabetes mellitus and metabolic syndrome (Koren et al., 2012; Wang et al., 2018; Chen et al., 2020). The gut microbiome may deliver new insights into understanding mechanisms and potential clinical strategies to detect the risk of preterm delivery and prevent preterm birth.

In the present study, we recruited patients experiencing threatened preterm labor and healthy pregnant women not in labor to provide fecal samples at similar gestational age to investigate the association of intestinal microbes with the outcome of preterm birth. We performed 16S rRNA gene amplicon sequencing to identify gut bacteria exhibiting altered abundance in women who experienced early/late preterm birth.

METHODS

Research Participants and Sample Collection

Healthy pregnant women and those with threatened preterm labor were recruited in the Department of Obstetrics, The First Affiliated Hospital of Nanchang University, Jiangxi Province, China. Ethical approval of the study was granted by the Ethics Committee of the First Affiliated Hospital of Nanchang University (2019-061). All participants have been informed of the purpose, background, process, risks, and benefits of the study, and signed the informed consent to participate in this study. The Department of Gynecology and Obstetrics, the First affiliated Hospital of Nanchang University was responsible for the study.

The inclusion criteria of patients: (1) The patients delivered between 28 weeks and 37 weeks of gestation, which were classified into preterm group. (2) The patients at 28 weeks and less than 37 weeks gestation suffered regular or irregular contractions accompanied by progressive dilation or shortening of the cervical canal, which were classified into Sym.preterm group in this study.

The exclusion criteria of patients: (1) Administration of any antibiotic or probiotic treatment one month before sample collection. (2) Diseases that may affect microbiome composition such as thyroid disorders, asthma, lipid metabolic disorders, inflammatory bowel disease, irritable bowel syndrome, and celiac disease. (3) Other obstetric conditions complicating pregnancy, such as gestational hypertension, gestational diabetes, twin or multiple pregnancies, placenta previa. (4) Other chronic diseases, such as chronic hypertension, chronic kidney disease. (5) Termination of pregnancy due to fetal or maternal factors. Fecal samples were collected from all enrolled subjects at the hospital, while samples from patients with threatened preterm labor were taken at symptom initiation. The fecal samples were collected at admission and the average sampling time of preterm group was 25.9 gestational weeks and 26.5 gestational weeks for the control group. All fecal samples were stored at −80°C until further processing.

Bacterial Genomic DNA Extraction, Sample Processing, and Sequencing

Bacterial genomic DNA was extracted using a MinkaGene Stool DNA kit (Magigene, Guangdong, China) according to the manufacturer's instructions. After extraction, the 16S rRNA V4 region was amplified by quantitative real-time PCR with the following barcoded primers (shown from 5′ to 3′): V4F, GTGYCAGCMGCCGCGGTAA and V4R, GGACTAC NVGGGTWTCTAAT. Sun and colleagues (Sun et al., 2013) showed that the V4 and V5 regions of bacteria 16S rRNA concentrate the least intragenomic heterogeneity. Further works verified its efficiency by revision to the region primer and avoided its bias to particular taxa like Proteobacteria (Caporaso et al., 2011; Caporaso et al., 2012; Parada et al., 2016; Walters et al., 2016). Therefore, in this study, 16S rRNA V4 region was selected for sequencing. Briefly, amplifications were performed using a step cycling protocol consisting of 98°C for 30 s, 35 cycles at 98°C for 10 s, 54°C for 30 s, and 72°C for 45 s, ending with the final elongation at 72°C for 10 min. PCR products were purified using an AxyPrep PCR Cleanup Kit (Axygen, California, U.S.A.). Sequencing libraries were generated using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations, and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The MiSeq platform (Illumina, 2 × 250 bp paired-end, CA, USA) was employed for the 16S rRNA sequencing.

Bioinformatics Processing

QIIME2 was used for controlling the sequencing data quality and clustering the 16S rRNA gene reads into Amplicon Sequence Variants [ASVs, based on DADA2 pipeline (Bolyen et al., 2019)], taxonomic assignment (based on the Greengenes Database V.13_8), and performing alpha diversity (Observed ASVs, Shannon Index and Phylogenetic Diversity Whole Tree Index),

beta diversity, and PCoA analyses. All samples were rarefied to 3,000 sequences, and three samples were filtered. Permutational multivariate analysis of variance (PERMANOVA) was performed to determine if the microbiota composition differed between groups and generate the explained variation based on distance metrics. Linear discriminant analysis effect size (LEfSe) was performed to identify the bacterial biomarkers between groups (Segata et al., 2011). Distance-based redundancy analysis and variance projection were employed to show the tendency of variables. Oral bacteria were annotated according to the Human Oral Microbiome Database (HOMD, www.homd.org) (Chen et al., 2010). Sequencing reads had been uploaded on European Nucleotide Archive (access number: PRJEB39133).

Statistical Analysis

The significance of differences between the two groups was determined by the Wilcoxon rank-sum test. The Kruskal–Wallis test was used for multi-groups. P values less than or equal to 0.05 were considered significant. The Benjamini and Hochberg method was used to adjust the P-value for multiple hypotheses (Benjamini and Yekutieli, 2001). Correlation analyses were performed based on the Pearson's product–moment correlation. Statistical analyses and data visualization were performed using R V.3.5.0 (under RStudio V.1.1.453), with the vegan, ggplot2, pheatmap and corrplot packages.

RESULTS

Gut Microbiome Profiles Differ in Preterm Patients

After rarefied to 3,000 sequences, a total of 19 women with threatened preterm labor and 22 healthy pregnant women were included in the present study. In the threatened preterm group, eight patients with preterm symptoms delivered (Sym.PTB) at term, and 11 patients delivered preterm. The preterm-delivered group was further divided into an early week preterm group (seven women, Early PTB) and a late-week preterm group (four women, Late-PTB), depending on whether the condition was detected before 34 weeks of gestation. The clinical parameters are summarized in **Table 1**.

The fecal samples of these participants were collected for DNA extraction and 16S rRNA sequencing. By employing QIIME2 bioinformatic software, we showed that alpha-diversity indices, including observed ASVs, Shannon Index, and PD Whole Tree Index, were increased in the preterm group, indicating a higher richness and evenness in preterm patients than in healthy controls. We did not observe significant differences in alpha diversity within the preterm subgroups (**Figure 1A**, **Supplementary Figures S1A, B**).

Beta diversity was calculated using the Bray–Curtis distance metrics to measure the extent of similarity in fecal microbial communities. According to the principal co-ordinate analysis, the gut microbiome of the preterm group significantly differed from that of the healthy group (PERMANOVA, $P = 0.004$, $R^2 = 0.051$, **Figure 1B**), indicating that the structure of the microbiota of preterm patients differed from that of healthy controls. Interestingly, we observed that the preterm subgroups showed a gradually drifting tendency away from the healthy groups with increasing severity (PERMANOVA, $P = 0.019$, $R^2 = 0.107$, **Figures 1C, D**), which suggests there are distinct gut microbiome profiles among laboring women who experience term birth, late preterm birth, or early preterm birth.

Subsequently, we analyzed the phylum-level profiles for the gut microbiota between preterm patients and healthy controls, which were fairly similar. The dominant phyla of both healthy and preterm groups were Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia, and Fusobacteria (**Figure 1E**).

Taken together, these results demonstrated the presence of gut microbiota dysbiosis in preterm patients compared with healthy controls.

Clinical Parameters Associated With Gut Microbiota Dysbiosis

Given that the gut microbiota was significantly different between preterm patients and healthy controls, we next investigated whether the gut microbiota was associated with the clinical parameters of patients.

To measure the extent of clinical parameters associated with the gut microbiome between preterm patients and healthy controls, we employed permutational multivariate analysis of variance to calculate the explained variation in host parameters based on the Bray–Curtis distance metric. Among 28 parameters with less than 20% missing data, five were significantly associated with gut microbial variations between preterm patients and healthy controls, namely, gestational age (recorded in weeks), neonatal weight, and Apgar scores (at 1, 5, and 10 min) (**Supplementary Figure S2A**). For the preterm subgroups, eight parameters were significantly associated with the subgroup microbiome variation, namely, C-reactive protein (CRP), tocolytic therapy, BMI at delivery, maternal age,

TABLE 1 | Characteristics of the study cohort.

	Health (N = 22)	Preterm (N = 19)	Preterm subgroups		
			P.sym (N = 8)	P.late (N = 4)	P.early (N = 7)
Age	34.0 (5.30)	32.6 (4.54)	30.5 (3.63)	33.8 (5.50)	34.4 (4.54)
Gestational weeks	38.7 (1.19)	32.9 (7.45)	39.1 (1.37)	34.9 (0.443)	24.6 (5.43)
Weight	71.5 (7.32)	59.6 (8.91)	52.4 (7.14)	62.8 (1.89)	65.0 (8.47)
BMI at delivery	28.6 (2.25)	24.4 (3.98)	21.4 (3.21)	25.8 (1.49)	26.6 (3.97)
Neonatal weight	3,140 (368)	1,860 (1120)	3,000 (418)	2,360 (218)	756 (558)

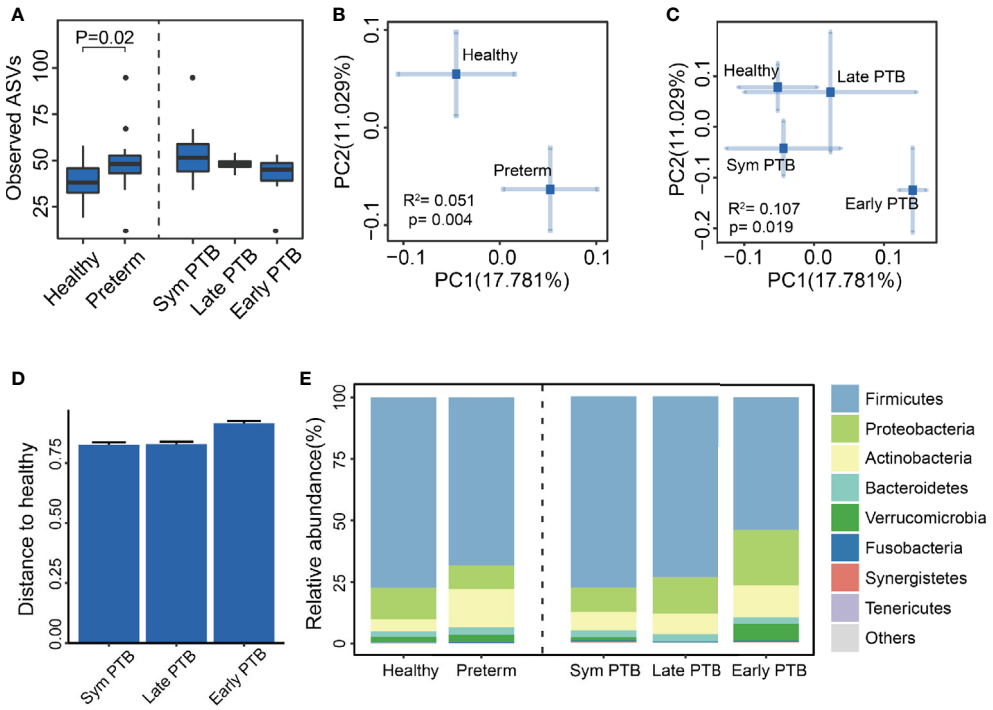

FIGURE 1 | The diversity and composition of the gut microbiome. **(A)** Observed ASVs of all groups. Observed ASVs between the healthy group and the preterm group (Wilcoxon rank-sum test), along with the preterm symptoms delivered group (Sym PTB), late-week preterm group (Late PTB) and early week preterm group (Early PTB). (Subgroups are compared with healthy group using Wilcoxon rank-sum test and adjusted by the Benjamini and Hochberg method). **(B, C)** Bray–Curtis distances PCoA of all groups. PCoA of Bray–Curtis distances for the bacterial community structure of the gut microbiome between the healthy group and the preterm group **(B)**, the healthy group and the Sym PTB group and the late PTB group and the early PTB group. **(C)** The eigenvalues of axe PC1 and PC2 were 0.29 (17.781%) and 0.46 (11.029%), respectively. The eigenvalues of axe PC1 and PC2 were 0.60 (17.781%) and 0.97 (11.029%), respectively. PERMANOVA was employed. **(D)** Preterm subgroup distances to healthy group. The distance of the preterm birth subgroups to the healthy group, based on Bray–Curtis distances. **(E)** Relative abundance of all groups. Comparison of the relative abundance of the dominant phylum in the healthy group, the preterm group, and the preterm birth subgroups.

gestational age, and Apgar scores (at 1, 5, and 10 min) (**Supplementary Figure S2B**). CRP is an inflammatory marker, while other parameters correlate with maternal–fetus prenatal and postpartum status, indicating that gut microbiome profiles are distinguished by gestational age at delivery and other clinical characteristics, many of which are expected to vary with gestational age at delivery.

We used distance-based redundancy analysis (db-RDA) to show the relationship of continuous parameters across samples by using R package vegan based on the Bray–Curtis distance metric. The projection of parameters demonstrated that gestational age (in weeks) and Apgar scores extended along axis-1, which distinguished between preterm patients and healthy controls (**Figures 2A–C**). While in the projection of preterm subgroup samples, CRP, BMI at delivery and maternal age were almost in the opposite direction to gestational age, neonatal weight, and Apgar scores, reflecting disease severity (**Figures 2D–J**). In the subgroup analysis, we included 13 preterm patients without missing value of all the included host parameters to avoid the projection change. Thus, the limited sample size could only provide us suggested indications, and further study on larger population is required. Altogether, these results again indicated that the gut microbiome profiles of preterm patients may be linked with other host parameters known to correlate with gestational age at birth.

Potential Oral–Gut Bacterial Translocation in Preterm Patients

To further identify the unique bacterial biomarkers between preterm and healthy groups, we performed LEfSe analysis based on the genus level and identified fifteen genera showing significant differences. Preterm patients exhibited a significant increase in the relative abundance of the genus *Fusobacterium, Streptococcus, Neisseria, Haemophilus, Lautropia, Porphyromonas, Clostridium, Prevotella, Rothia, Oscillospira, Granuliccatella, Actinomyces, and Bilophila*. On the contrary, genus Coprococcus and Gemmiger were depleted in preterm patients compared to those in healthy controls (**Figure 3A**).

Of note, several genera enriched in preterm groups were commonly considered oral pathogens, for example, Porphyromonas, Streptococcus, and *Fusobacterium*. As oral symptoms are common during pregnancy, we further examined the changes in the common resident oral bacteria in feces. We clustered and annotated bacteria to resident oral

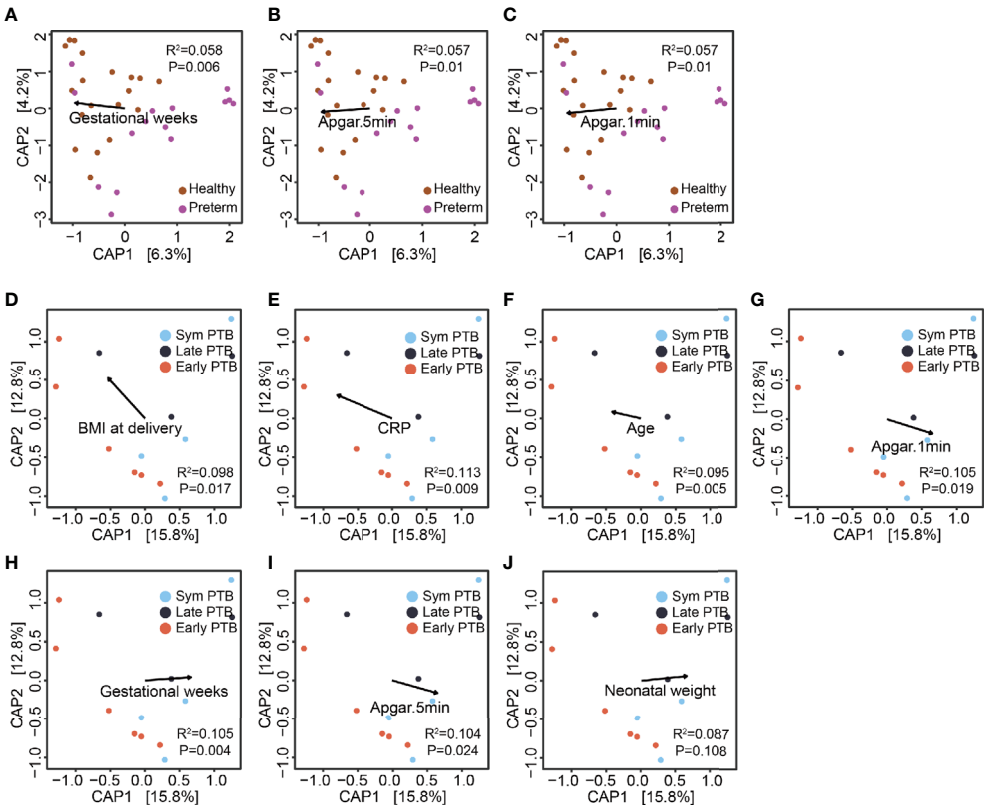

FIGURE 2 | The association of the gut microbiome and host parameters. **(A–C)** Clinical features' projection on all samples. The projection of continuous parameters in the healthy group and the preterm group samples, based on the Bray–Curtis distance metric, gestational weeks **(A)**, Apgar score at 5 min **(B)** and Apgar score at 1 min **(C)**, respectively. **(D–J)** Clinical features projection on preterm birth subgroups. The projection of continuous parameters in the preterm subgroup samples, based on the Bray–Curtis distance metric, BMI at delivery **(D)**, CRP **(E)**, age **(F)**; Apgar score at 1 min **(G)** gestational weeks (H); Apgar score at 5 min **(I)** and neonatal weight **(J)**, respectively.

microbes according to the HOMD database. The relative abundance of common oral bacteria was markedly higher in the preterm group than in the healthy group ($P < 0.001$), as in the three subgroups (**Figure 3B**). We clustered the identified oral bacteria to examine their distribution among all individuals. Based on the heatmap, there was one cluster with thirteen genera enriched in preterm patients. Of these, nine were identified by LEfSe, and three were reported potential pathogens, indicating the possible higher intensity of bacterial oral–gut-translocation in preterm patients (**Figure 3C**). Among those enriched bacteria, Fusobacterium, Porphyromonas, and Streptococcus were common oral opportunistic pathogens.

Collectively, these results demonstrated that the gut microbiota of preterm patients differed from that of healthy individuals. Furthermore, a high proportion of genera enriched in preterm patients coincided with oral opportunistic pathogens.

Potential Oral–Gut Translocated Bacteria for Distinguishing Women in Preterm From Healthy Control

Given that the gut microbiome profile was associated with the status of preterm patients and potential oral–gut translocated bacteria were enriched in preterm patients, we next investigated the association between potential oral–gut translocated bacteria and host parameters. We used Pearson's product–moment correlation to evaluate the link between the relative abundance of oral-to-gut bacteria and host parameters. Results showed that oral-to-gut bacteria were negatively correlated with gestational age (in weeks), neonatal weight and Apgar scores (**Figure 4**). Altogether, the oral bacteria were correlated with clinical parameters reflecting the maternal and fetal status.

DISCUSSION

In the last few decades, a lack of effective methods for the prediction and prevention of preterm birth has placed a burden on the medical system and millions of families (Blencowe et al., 2012; Liu et al., 2016). The present study aimed to seek results from the microbiome. We found that maternal gut microbiome profiles were distinct in women with early *vs.* late preterm birth. The profile of the gut microbiome in preterm patients revealed that a group of common resident oral bacteria was enriched and associated with clinical parameters that reflected gestational and

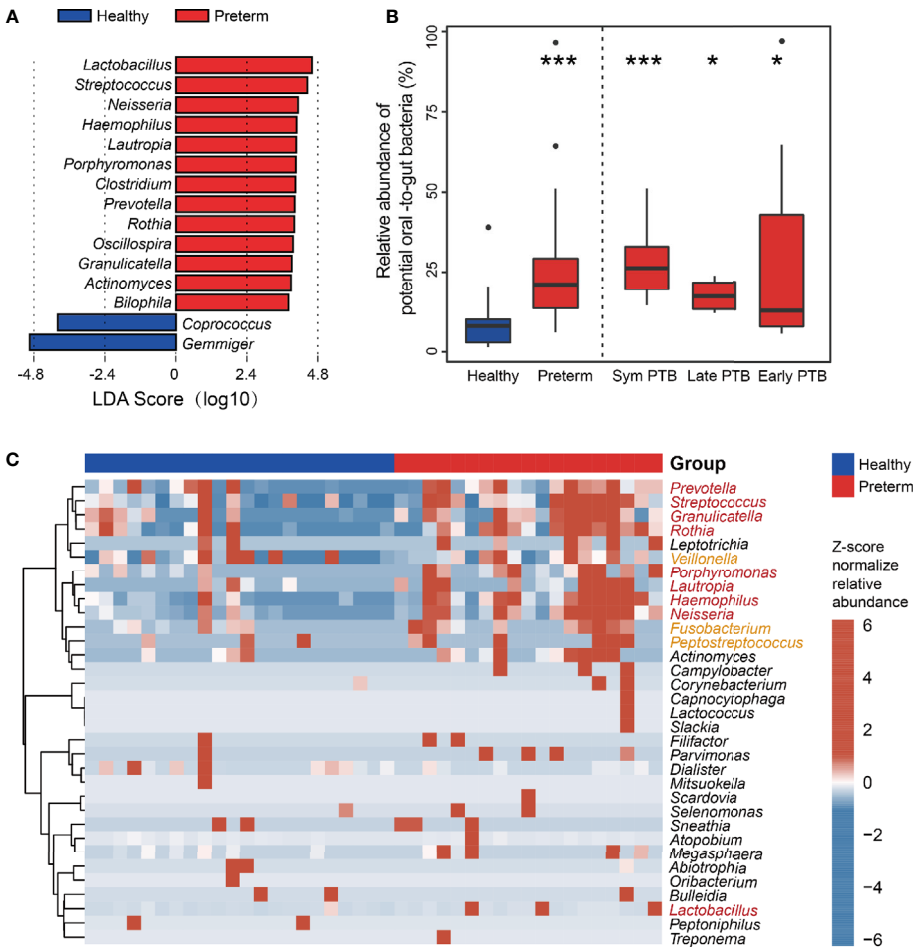

FIGURE 3 | Identification of the gut microbial biomarkers between the healthy and preterm groups. **(A)** LEfSe analyses of healthy and preterm groups. Linear discriminant analysis effect size identified the genus between the healthy and preterm groups. Preterm-enriched taxa are indicated with a positive LDA score, and taxa enriched in healthy controls have a negative score. Only taxa meeting an LDA significant threshold of >3 are shown. **(B)** Total relative abundance of the common oral bacteria. The relative abundance of the common oral bacteria in the healthy group, the preterm group, and the preterm birth subgroups (healthy group and preterm group are compared using Wilcoxon rank-sum test; subgroups are compared with healthy group using Wilcoxon rank-sum test and adjusted by the Benjamini and Hochberg method). **(C)** Genus comparison between healthy and preterm group. Bacteria identified by the Wilcoxon rank-sum test with $p < 0.05$ are shown in the heatmap. The bar on the top indicates the group information of each sample. Red genus represents the common oral bacteria that overlapped with LEfSe results. Yellow genera represent the common oral bacteria but not overlapped with LEfSe results. *$p < 0.05$; ***$p < 0.001$.

infantile status. These potential oral-to-gut bacteria, evaluated by the random forest model, would provide us another aspect to understand the relationship of gut bacteria and preterm birth.

The gut dysbiosis in preterm patients observed in our study echoed the findings of previous reports. Shiozaki et al. found compositional changes in the gut microbiome using terminal restriction fragment length polymorphism (Shiozaki et al., 2014). Interestingly, the gut dysbiosis pattern showed the enrichment of *Porphyromonas*, *Fusobacterium*, *Veillonella*, *Streptococcus*, *Bilophila*, and *Haemophilus* in preterm patients. Similar to the gut bacterial profile of pregnancy adverse outcome patients, *Fusobacterium*, *Streptococcus*, and *Veillonella* are associated with chronic inflammatory conditions, gut barrier damage, upregulate inflammation and led to adverse pregnancy outcomes (Chen et al., 2020). While in the present study, we found that alpha diversity increased in the preterm group. Although alpha diversity had been linked with gut health, it has also been reported to increase in different kinds of diseases, including gynecology and obstetrics disease (Prehn-Kristensen et al., 2018; Riquelme et al., 2019; Heshiki et al., 2020; Mrozinska et al., 2021). Thus, the dysbiosis pattern reported in this study is reliable.

Periodontitis is reported as a potential risk factor for preterm labor (Cobb et al., 2017; Chopra et al., 2020). *Porphyromonas*, commonly associated with periodontitis, can induce systemic inflammation, tissue damage, and possible maternal and fetal gut dysbiosis (Chopra et al., 2020). *Fusobacterium*, one of the opportunistic pathogens identified in the oral cavity and gut, can disrupt epithelial integrity and lead to tissue breakdown (Han, 2015; Yan et al., 2017). Recent studies showed that *Fusobacterium* was enriched in patients with adverse pregnancy outcomes and played a

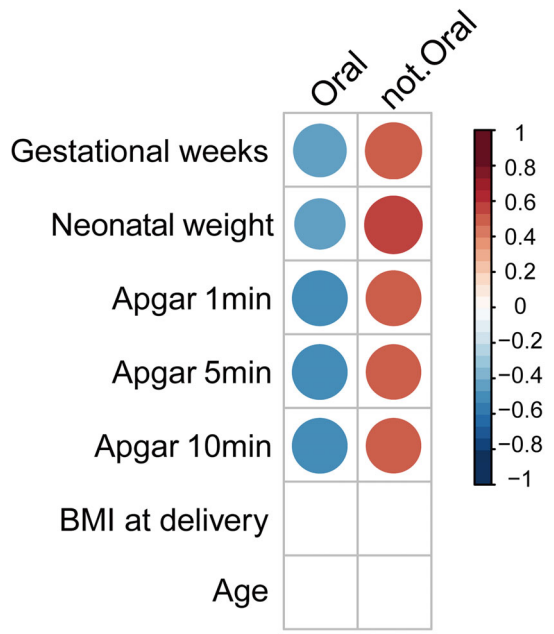

FIGURE 4 | Correlations of parameters and abundance of oral-to-gut bacteria. Coefficient of correlation between clinical features and total potential oral bacteria. The relationship between the relative abundance of oral and non-oral bacteria and host parameters (gestational weeks; neonatal weight; Apgar scores in 1, 5, and 10 min; BMI at delivery).

potential causal role in preeclampsia (Lv et al., 2019; Wang et al., 2019; Chen et al., 2020). In our study, *Porphyromonas* and *Fusobacterium*, which were common oral opportunistic pathogens, were significantly increased in the fecal samples of preterm patients. Though in the present study, we could not confirm whether these bacteria were originated in gut or translocated from oral cavity, the increase in relative abundance of these bacteria could lead to impairment of gut barrier (Chen et al., 2020)or oral epithelial barrier dysfunction (Fardini et al., 2011; Mahtout et al., 2011; Takeuchi et al., 2019). Yet the bacterial translocation from oral niche to the intestine is considered rare and aberrant. By studying salivary and fecal microbiome from several nations, researchers from Bork lab suggested that oral cavity could be an endogenous reservoir for gut microbiome, and oral–fecal transmission could play an important role in shaping the gut microbiome in health and disease (Schmidt et al., 2019). In our study, the enrichment of resident oral bacteria in the fecal samples of preterm patients could be the result of bacterial migration by swallowing through the digestive tract. Moreover, multiple studies indicated that dysbiotic gut bacteria could translocate to the placenta through impaired gut barrier (Chen et al., 2020). Although the existence of a placental microbiome is still debatable, there is a possibility that translocated pathogens could deteriorate placental structure and eventually lead to adverse outcomes (Aagaard et al., 2014; Seferovic et al., 2019). Our findings provide some evidence for the oral cavity being an endogenous reservoir for gut microbiome, potentially seeding it with oral microbes linked to preterm birth.

We also observed correlations between the oral bacteria and clinical parameters, such as gestational age, neonatal weight, and Apgar scores, indicating that the bacteria might reflect the disease status and outcomes. Our results suggested that the gut microbiota might have a potential to becoming a biomarker for distinguished preterm birth with further research on a larger population with rigorous control on variates like region and ethnicity (Deschasaux et al., 2018; He et al., 2018). The sample size in our study was relatively small and a larger population from multiple countries and races are required in future studies.

The present study has several limitations. First, for the sample and metadata collection, we only collected human fecal samples but missed oral sample like saliva or gem swab and the oral status of participants. Therefore, without clinical metadata on oral health and the oral microbiome sequencing data, we were unable to compare the similarity of gut and oral microbiome. As oral bacteria colonized in the gut are an unavoidable consequence and periodontitis is known etiologically linked to preterm birth through gut independent mechanisms, there are two ways to solve this problem in the future studies. One is employing meta-transcriptomics and shotgun metagenomics to evaluate the active transcription and cellular replication of oral strains in the fecal sample (Franzosa et al., 2014; Brown et al., 2016). And the second is performing animal experiment by constructing fluorescent opportunistic pathogen. Second, as the sample size was relatively small, our findings should be tested on repeated and larger population studies. Third, in the present study, the fecal samples from two out of nineteen patients in the preterm group were collected in labor. We could not rule out the effect of labor itself as a co-factor in this study. In future studies, collecting samples before labor or including a term labor control group would alleviate the effect of labor.

In summary, increased maternal oral bacteria in the guts of women experiencing preterm birth provided clues to further understanding the relationship between gut microbiome and preterm birth. However, the key role of microbiota in preterm birth pathogenesis and prospective mechanistic studies needs to be further investigated. Cohort studies that follow up from early pregnancy to *postpartum* and observe maternal gut microbiome dynamics along with fetal development will provide more comprehensive views on the effect of the gut microbiome on preterm birth. In conclusion, our study indicates that the gut microbiome in preterm birth women was significantly shifted compared with term women, which contained higher common oral bacteria.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The data presented in the study are deposited in the EBI repository (https://www.ebi.ac.uk/), accession number PRJEB39133.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Committee of the First Affiliated Hospital of

Nanchang University (2019-061). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CY, JC and Y-EH designed the ideas and methods of this study; XW, JC and QH collected the data and processed the samples; JC, YL and YC analyzed the data; SL guided, supervised and supported the study. CY, JC and SL drafted and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.579766/full#supplementary-material

REFERENCES

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., Versalovic, J., et al. (2014). The Placenta Harbors a Unique Microbiome. *Sci. Transl. Med.* 6, 237r–265r. doi: 10.1126/scitranslmed.3008599

Benjamini, Y., and Yekutieli, D. (2001). The Control of the False Discovery Rate in Multiple Testing Under Dependency. *Ann. Stat.* 29.4, 1165–1188. doi: 10.1214/aos/101369998

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A. B., Narwal, R., et al. (2012). National, Regional, and Worldwide Estimates of Preterm Birth Rates in the Year 2010 With Time Trends Since 1990 for Selected Countries: A Systematic Analysis and Implications. *Lancet* 379, 2162–2172. doi: 10.1016/S0140-6736(12)60820-4

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Brown, C. T., Olm, M. R., Thomas, B. C., and Banfield, J. F. (2016). Measurement of Bacterial Replication Rates in Microbial Communities. *Nat. Biotechnol.* 34, 1256–1263. doi: 10.1038/nbt.3704

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global Patterns of 16S rRNA Diversity at a Depth of Millions of Sequences Per Sample. *Proc. Natl. Acad. Sci. U.S.A.* 108 Suppl 1, 4516–4522. doi: 10.1073/pnas.1000080107

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Chen, T., Yu, W. H., Izard, J., Baranova, O. V., Lakshmanan, A., Dewhirst, F. E., et al. (2010). The Human Oral Microbiome Database: A Web Accessible Resource for Investigating Oral Microbe Taxonomic and Genomic Information. *Database (Oxford)* 2010, q13. doi: 10.1093/database/baq013

Chen, X., Li, P., Liu, M., Zheng, H., He, Y., Chen, M. X., et al. (2020). Gut Dysbiosis Induces the Development of Pre-Eclampsia Through Bacterial Translocation. *Gut* 69, 513–522. doi: 10.1136/gutjnl-2019-319101

Chopra, A., Radhakrishnan, R., and Sharma, M. (2020). Porphyromonas Gingivalis and Adverse Pregnancy Outcomes: A Review on its Intricate Pathogenic Mechanisms. *Crit. Rev. Microbiol.* 46, 213–236. doi: 10.1080/1040841X.2020.1747392

Cobb, C. M., Kelly, P. J., Williams, K. B., Babbar, S., Angolkar, M., and Derman, R. (2017). *The Oral Microbiome and Adverse Pregnancy Outcomes* (New Zealand: Dove Medical Press Limited), 551–559.

Deschasaux, M., Bouter, K. E., Prodan, A., Levin, E., Groen, A. K., Herrema, H, et al. (2018). Depicting the Composition of Gut Microbiota in a Population With Varied Ethnic Origins But Shared Geography. *Nat. Med.* 24, 1526–1531. doi: 10.1038/s41591-018-0160-1

Fardini, Y., Wang, X., Temoin, S., Nithianantham, S., Lee, D., Shoham, M., et al. (2011). Fusobacterium Nucleatum Adhesin FadA Binds Vascular Endothelial Cadherin and Alters Endothelial Integrity. *Mol. Microbiol.* 82, 1468–1480. doi: 10.1111/j.1365-2958.2011.07905.x

Franzosa, E. A., Morgan, X. C., Segata, N., Waldron, L., Reyes, J., Earl, A. M., et al. (2014). Relating the Metatranscriptome and Metagenome of the Human Gut. *Proc. Natl. Acad. Sci. U.S.A.* 111, E2329–E2338. doi: 10.1073/pnas.1319284111

Han, Y. W. (2015). Fusobacterium Nucleatum: A Commensal-Turned Pathogen. *Curr. Opin. Microbiol.* 23, 141–147. doi: 10.1016/j.mib.2014.11.013

Heshiki, Y., Vazquez-Uribe, R., Li, J., Ni, Y., Quainoo, S., Imamovic, L., et al. (2020). Predictable Modulation of Cancer Treatment Outcomes by the Gut Microbiota. *Microbiome* 8, 28. doi: 10.1186/s40168-020-00811-2

He, Y., Wu, W., Zheng, H. M., Li, P., McDonald, D., Sheng, H. F., et al. (2018). Regional Variation Limits Applications of Healthy Gut Microbiome Reference Ranges and Disease Models. *Nat. Med.* 24, 1532–1535. doi: 10.1038/s41591-018-0164-x

Keelan, J. A. (2018). Intrauterine Inflammatory Activation, Functional Progesterone Withdrawal, and the Timing of Term and Preterm Birth. *J. Reprod. Immunol.* 125, 89–99. doi: 10.1016/j.jri.2017.12.004

Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Backhed, H. K., et al. (2012). Host Remodeling of the Gut Microbiome and Metabolic Changes During Pregnancy. *Cell* 150, 470–480. doi: 10.1016/j.cell.2012.07.008

Liu, L., Oza, S., Hogan, D., Chu, Y., Perin, J., Zhu, J., et al. (2016). Global, Regional, and National Causes of Under-5 Mortality in 2000-15: An Updated Systematic Analysis With Implications for the Sustainable Development Goals. *Lancet* 388, 3027–3035. doi: 10.1016/S0140-6736(16)31593-8

Lv, L. J., Li, S. H., Li, S. C., Zhong, Z. C., Duan, H. L., Tian, C., et al. (2019). Early-Onset Preeclampsia Is Associated With Gut Microbial Alterations in Antepartum and Postpartum Women. *Front. Cell Infect. Microbiol.* 9, 224. doi: 10.3389/fcimb.2019.00224

Mahtout, H., Chandad, F., Rojo, J. M., and Grenier, D. (2011). Fusobacterium Nucleatum Binding to Complement Regulatory Protein CD46 Modulates the Expression and Secretion of Cytokines and Matrix Metalloproteinases by Oral Epithelial Cells. *J. Periodontol.* 82, 311–319. doi: 10.1902/jop.2010.100458

Mrozinska, S., Kapusta, P., Gosiewski, T., Sroka-Oleksiak, A., Ludwig-Slomczynska, A. H., Matejko, B., et al. (2021). The Gut Microbiota Profile According to Glycemic Control in Type 1 Diabetes Patients Treated With Personal Insulin Pumps. *Microorganisms* 9. doi: 10.3390/microorganisms9010155

Mwaniki, M. K., Atieno, M., Lawn, J. E., and Newton, C. R. (2012). Long-Term Neurodevelopmental Outcomes After Intrauterine and Neonatal Insults: A Systematic Review. *Lancet* 379, 445–452. doi: 10.1016/S0140-6736(11)61577-8

Mychaliska, G. (2014). Seminars in Fetal & Neonatal Medicine. Update on Congenital Diaphragmatic Hernia. Introduction. *Semin. Fetal Neonatal Med.* 19, 323. doi: 10.1016/j.siny.2014.10.002

Parada, A. E., Needham, D. M., and Fuhrman, J. A. (2016). Every Base Matters: Assessing Small Subunit rRNA Primers for Marine Microbiomes With Mock Communities, Time Series and Global Field Samples. *Environ. Microbiol.* 18, 1403–1414. doi: 10.1111/1462-2920.13023

Platt, M. J. (2014). Outcomes in Preterm Infants. *Public Health* 128, 399–403. doi: 10.1016/j.puhe.2014.03.010

Prehn-Kristensen, A., Zimmermann, A., Tittmann, L., Lieb, W., Schreiber, S., Baving, L., et al. (2018). Reduced Microbiome Alpha Diversity in Young Patients With ADHD. *PloS One* 13, e200728. doi: 10.1371/journal.pone.0200728

Riquelme, E., Zhang, Y., Zhang, L., Montiel, M., Zoltan, M., Dong, W., et al. (2019). Tumor Microbiome Diversity and Composition Influence Pancreatic Cancer Outcomes. *Cell* 178, 795–806. doi: 10.1016/j.cell.2019.07.008

Romero, R., Erez, O., and Espinoza, J. (2005). Intrauterine Infection, Preterm Labor, and Cytokines. *J. Soc. Gynecol. Investig.* 12, 463–465. doi: 10.1016/j.jsgi.2005.09.001

Schmidt, T. S., Hayward, M. R., Coelho, L. P., Li, S. S., Costea, P. I., Voigt, A. Y., et al. (2019). Extensive Transmission of Microbes Along the Gastrointestinal Tract. *Elife* 8. doi: 10.7554/eLife.42693

Seferovic, M. D., Pace, R. M., Carroll, M., Belfort, B., Major, A. M., Chu, D. M., et al. (2019). Visualization of Microbes by 16S in Situ Hybridization in Term and Preterm Placentas Without Intraamniotic Infection. *Am. J. Obstet. Gynecol.* 221, 141–146. doi: 10.1016/j.ajog.2019.04.036

Segata, N., Izard, J., Waldron, L., Li, S. S., Costea, P. I., Voigt, A. Y., et al. (2011). Metagenomic Biomarker Discovery and Explanation. *Genome Biol.* 12, R60. doi: 10.1186/gb-2011-12-6-r60

Shiozaki, A., Yoneda, S., Yoneda, N., Yonezawa, R., Matsubayashi, T., Seo, G., et al. (2014). Intestinal Microbiota Is Different in Women With Preterm Birth: Results From Terminal Restriction Fragment Length Polymorphism Analysis. *PloS One* 9, e111374. doi: 10.1371/journal.pone.0111374

Sun, D. L., Jiang, X., Wu, Q. L., and Zhou, N. Y. (2013). Intragenomic Heterogeneity of 16S rRNA Genes Causes Overestimation of Prokaryotic Diversity. *Appl. Environ. Microbiol.* 79, 5962–5969. doi: 10.1128/AEM.01282-13

Takeuchi, H., Sasaki, N., Yamaga, S., Kuboniwa, M., Matsusaki, M., Amano, A., et al. (2019). Porphyromonas Gingivalis Induces Penetration of Lipopolysaccharide and Peptidoglycan Through the Gingival Epithelium Via Degradation of Junctional Adhesion Molecule 1. *PloS Pathog.* 15, e1008124. doi: 10.1371/journal.ppat.1008124

Walters, W., Hyde, E. R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., et al. (2016). Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. *Msystems* 1. doi: 10.1128/mSystems.00009-15

Wang, J., Gu, X., Yang, J., Wei, Y., and Zhao, Y. (2019). Gut Microbiota Dysbiosis and Increased Plasma LPS and TMAO Levels in Patients With Preeclampsia. *Front. Cell Infect. Microbiol.* 9, 409. doi: 10.3389/fcimb.2019.00409

Wang, J., Zheng, J., Shi, W., Du, N., Xu, X., Zhang, Y., et al. (2018). Dysbiosis of Maternal and Neonatal Microbiota Associated With Gestational Diabetes Mellitus. *Gut* 67, 1614–1625. doi: 10.1136/gutjnl-2018-315988

Yan, Q., Gu, Y., Li, X., Yang, W., Jia, L., Chen, C., et al. (2017). Alterations of the Gut Microbiome in Hypertension. *Front. Cell Infect. Microbiol.* 7, 381. doi: 10.3389/fcimb.2017.00381

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The P4 Study: Postpartum Maternal and Infant Faecal Microbiome 6 Months After Hypertensive Versus Normotensive Pregnancy

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Objective/Hypothesis: To explore potential differences in faecal microbiome between women, and their infants, who had normotensive pregnancies (NP) and those who had a hypertensive pregnancy (HP), either gestational hypertension (GH) or preeclampsia (PE).

Methods: This is a sub study of P4 (Postpartum Physiology, Psychology, and Paediatrics Study) and includes 18 mother-infant pairs: 10 NP and 8 HP (HP as defined by blood pressure > 140/90mmHg; of which 6 had PE, and 2 GH), six months postpartum. The participating mothers collected stool samples from themselves and their infants. 16S rRNA V3-V4 amplicons were used to study the faecal microbiome.

Results: The sample of women and their infants were mostly primiparous (n =16) with vaginal birth (n = 14). At the time of faecal sampling 8 women were using hormonal contraception, and one HP woman remained on an antihypertensive. All women had blood pressure < 130/80mmHg, and 10 had high BMI (> 30). All infants had started solids, 8 were exclusively breastfed, 1 exclusively formula fed and 9 both. Three infants had been exposed to a course of antibiotics. Six months postpartum, there were no significant differences in alpha or beta diversity between the gut microbiota of HP and NP women ($P > 0.05$). However, a statistically significant difference was detected in alpha diversity between infants following HP and NP, with lower diversity levels in HP infants ($P < 0.05$). It was also found that at a genus and species level, the gut microbiota of HP women was enriched with *Bifidobacterium* and *Bifidobacterium* sp. and depleted in *Barnesiella* and *Barnesiella intestinihominis* when compared to NP women ($P < 0.05$). Similarly, the gut microbiota of infants born from HP was enriched in *Streptococcus infantis* and depleted in *Sutterella*, *Sutterella* sp., *Bacteroides* sp. and *Clostridium aldenense* compared to infants born from NP ($P < 0.05$).

Discussion: While our findings are at best preliminary, due to the very small sample size, they do suggest that the presence of hypertension in pregnancy may adversely affect the maternal microbiota postpartum, and that of their infants. Further analysis of postpartum microbiome data from future studies will be important to validate these early findings and provide further evidence about the changes in the microbiota in the offspring of women following hypertensive disorders of pregnancy (HDP), including possible links to the causes of long-term cardiovascular disease, the prevalence of which is increased in women who have experienced HDP.

Keywords: pregnancy, infancy, microbiome, preeclampsia, hypertensive pregnancy, postpartum

INTRODUCTION

There is mounting evidence of the impact of the human microbiome on health, including during pregnancy. In pregnancy, gut dysbiosis has been associated with several important pregnancy complications including gestational diabetes (GDM) (Crusell et al., 2018; Wang et al., 2018; Tenenbaum-Gavish et al., 2020) and hypertensive disorders of pregnancy (HDP) (Chen et al., 2020). HDP affect 5-10% of pregnant women globally (Duley, 2009), with the most common HDP being preeclampsia (PE) and gestational hypertension (GH). PE is a multisystem disorder associated with both severe maternal effects (including renal, neurological, hepatic, haemotologic), and fetal complications including fetal growth restriction, placental abruption and prematurity (Brown Mark et al., 2018). GH (hypertension in pregnancy without the multisystem features characterising PE) has minimal short-term pregnancy impacts but progresses to PE in 25-50% of cases and in the long term, women with PE or GH are at increased risk of cardiovascular disease related morbidity and mortality, with this risk apparent within 10 years of an affected pregnancy and continuing lifelong (Theilen et al., 2016; Wu et al., 2017; Arnott et al., 2020).

The aetiology of HDP, in particular preeclampsia, remains unclear, though it appears to be multifactorial, which raises the possibility of under-studied factors such as the maternal microbiome. These include *Faecalibacterium* sp. which is involved in the metabolism of dietary fibre and providing anti-inflammatory effects, and *Akkermansia* sp. which have actions in strengthening intestinal barrier functions in the gut. Chen et al. found *Faecalibacterium* and *Akkermansia* species to be depleted in the faecal microbiome of women with PE (Chen et al., 2020). Lv et al. demonstrated that disruption in gut microbiota in women with PE persisted at 6 weeks postpartum (Lv et al., 2019).

Another field of uncertainty is the long-term effects of HDP on the fetus and child development. Children of pregnancies complicated by preeclampsia have increased blood pressure and body mass index (Davis et al., 2012). Several studies have examined the gut microbiota during pregnancy in recent years (Koren et al., 2012; Aagaard et al., 2012; Aagaard et al., 2014; Nuriel-Ohayon et al., 2019; van der Giessen et al., 2020), but few have examined the postpartum stage in both mother and infant. Further analysis of the infant microbiota may help to inform our understanding of how maternal factors can influence infant health. As such, it is pertinent to clarify the pathophysiological basis of long-term cardiovascular effects of HDP and establish biomarkers which may aid in the prediction of poor outcomes.

The aim of this study was to explore possible differences in faecal microbiota six months postpartum in women and their infants who had normotensive pregnancies (NP) versus those who had gestational hypertension or preeclampsia (HP). We hypothesised that the faecal microbiota of infants born to mothers with HP are altered from those born to NP mothers.

MATERIALS AND METHODS

This is a sub-study of a large prospective cohort study known as P4 (Postpartum Physiology, Psychology and Paediatrics) being conducted at St George Hospital which is a metropolitan teaching hospital that serves a diverse sociodemographic patient population in Sydney. The P4 Study aims to investigate maternal health (physical and psychological) and child health (physical and developmental) in the first 5 years after HP (PE and GH) compared to NP. A detailed P4 study protocol has been published (Davis et al., 2016) as has six months postpartum maternal P4 data which found higher blood pressure and more adverse cardiometabolic health markers in women after PE versus NP (Brown Mark et al., 2020).

Following an ethics amendment (ethical approval from South-Eastern Sydney Local Health District Human Research Ethics Committee, reference number: 12/195), mothers from the P4 study were invited to participate in this microbiome sub-study at 6 months postpartum. Women who were due for their six-month P4 follow up visit were contacted with information about the sub-study and provided their written informed consent to participate. Inclusion criteria for this sub-study, in addition to P4 inclusion criteria, were: no maternal antibiotic usage for the three months prior to the visit; no maternal probiotic usage within one month prior to their 6 month P4 follow up visit; and no history of an acute diarrheal illness for the month prior to and at the time of visit.

Sample Collection and DNA Extraction

Stool samples were collected from the participating mothers and infants through self-collection. Samples were aliquoted and stored at -80°C and extracted using the PSP Spin Stool DNA extraction kit (Invitek) with additional mechanical lysis for 5 minutes at 30 Hz, using the Tissuelyzer II (Qiagen) to ensure complete lysis of bacterial cells. DNA concentrations were then

quantified (Qubit) and the DNA subjected to 16S rRNA V3-V4 amplicon sequencing on the Illumina Miseq platform. QIIME 2 was used to process and perform quality control on sequencing data. Chimeric and primer sequences were removed using DADA2. Sequence alignment and taxonomic classification was performed as per previously published methodology (Bokulich et al., 2018). The dataset used for analysis is available in full at NCBI repository under BioProject ID PRJNA701500.

Bioinformatic Analysis

The 16S rRNA gene forward and reverse reads were imported into Qiime2 (Bolyen et al., 2019). The DADA2 pipeline (Callahan et al., 2016) was used for detecting and correcting Illumina amplicon sequences, removal of primers and chimeric reads, and assembly into sequence variants (SV)/operational taxonomic units (OTUs) (Callahan et al., 2017). Taxonomy was assigned using a naïve Bayes classifier trained on the Greengenes database13_8. Alpha-diversity metrics investigated included Faith's phylogenetic diversity (PD), Pielou's evenness, Observed operational taxonomy units (OTUs) and Shannon's diversity index which were calculated using qiime2-q2-diversity. Beta-diversity metrics was calculated using qiime2 and distance metrics were quantified using Bray–Curtis dissimilarity index. Statistical analysis was conducted using R v3.6.3 (R Core Team, 2020) in RStudio v1.3.959 (RStudio Team, 2020). Data was visualized using principal coordinates analysis (PCoA) plots and alpha diversity plots generated within RStudio using ggplot2 (Wickham, 2016) and phyloseq (McMurdie and Holmes, 2013). Other packages used included dplyr (Wickham et al., 2019) and qiime2R (Bisanz et al., 2018).

The Wilcoxon rank sum test was used for alpha diversity comparisons between two groups, and the Kruskal–Wallis test, with Dunn's *post-hoc* test, was used for alpha diversity comparisons between three groups. Distance based permutation multivariate analysis of variance (PERMANOVA) (Koleva et al., 2015) was performed to test the null hypothesis that there were no differences in microbial community structure across treatments at a significance level of $P = 0.05$ based on 999 permutations. P values for alpha and beta diversity measures were false discovery rate (FDR) corrected using the Benjamini-Hochberg procedure, with 0.05 as the significance threshold.

Linear discriminant analysis Effect Size (LEfSe) (Segata et al., 2011) was used to detect differences in taxonomic abundance between groups. Taxa were identified as differentially abundant through Kruskal-Wallis testing on classes and Wilcoxon rank-sum pairwise testing on subclasses. This was combined with a linear discriminant analysis (LDA) model to evaluate effect size. Using LEfSe, taxa were considered significantly differentially abundant if their P value was < 0.05 and their LDA log score was > 2. FDR corrections were additionally performed using the Benjamini-Hochberg procedure, with 0.05 as the significance threshold.

RESULTS

Maternal and Infant Characteristics

Ten NP and 8 HP (6 preeclampsia, 2 gestational hypertension) mother-infant pairs participated in the sub study. Maternal characteristics are shown in **Table 1**, with women predominantly primiparous ($n = 16$), with vaginal birth ($n = 14$). One HP woman had a history of beta-thalassaemia minor and another HP woman developed cholestasis of pregnancy in addition to PE. Two HP women had high BMI (> 30 kg/m^2). None of the women in either group had pre-existing or gestational diabetes, thyroid disease, or a history of gastrointestinal disease. Eight women reported pre-pregnancy alcohol consumption, all ceased during pregnancy and 5 recommenced postpartum (average of 3-5 standard drinks/week at time of six-month postpartum sampling). One HP woman was vegetarian, otherwise all other women reported no special dietary requirements. A statistically significant difference in diastolic blood pressure and mode of birth was found between the two groups of mothers involved in the sub-study.

Infant characteristics are shown in **Table 2**. All infants born to normotensive mothers (NP) were born vaginally ($n = 10$). Of the hypertensive group, all infants of women in the GH group were born by caesarean delivery ($n = 2$) and two of the six mothers in the PE group had caesarean deliveries. All but one infant (from the NP group) had commenced solid food intake. Three infants had received antibiotics in their lifetime, two at the time of delivery: one, born at 32 weeks and 6 days in the PE group who also used a paediatric multivitamin; the second a term baby born at 39 weeks and 6 days gestation, for suspected chorioamnionitis; and the third had daily cephalexin exposure since birth for a duplex kidney. A statistically significant difference was found in gestational age at birth between the two groups of infants involved in the sub-study.

Differences in the Gut Microbiota of Mothers at 6 Months Postpartum and Infants at 6 Months of Age

Figures 1A, B illustrate the relative abundance of the top 15 bacterial phyla and genera in the maternal and infant gut microbiota at 6 months postpartum and six months of age, respectively. The maternal and infant gut microbiota were primarily composed of phyla Firmicutes, Bacteroidetes and Proteobacteria. *Bacteroides, Escherichia* and *Veillonella* were the main genera composing the infant gut microbiota whilst *Bacteroides* and *Oscillospira* were the primary genera in the maternal gut microbiota. Overall, mothers had a significantly greater alpha diversity compared to that of infants, when measured by Shannon's diversity index, Pielou's evenness, Faith's phylogenetic diversity (PD) and observed operational taxonomic units (OTUs) ($P < 0.001$) (**Figure 2A–D** and **Supplementary Table 1**). Furthermore, analysis revealed significant differences in beta diversity between mothers and infants, showing a fundamental variation in microbial community composition ($P = 0.001$) (**Supplementary Table 2**). This is illustrated by the separate clustering of maternal and infant samples in the PCoA graph (**Figure 2E**).

Changes in Gut Microbiota Composition Between Women After HP and NP

No significant differences in alpha or beta diversity were detected at 6 months postpartum between HP and NP women ($P > 0.05$)

TABLE 1 | Maternal characteristics.

	Total Cohort (*n* = 18)	Normotensive Pregnancy (NP) (*n* = 10)	HP = Preeclampsia (PE) or Gestational Hypertension (GH) (*n* = 8)	*p* value NP vs HP
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	
Age (years) at time of birth	30.8 ± 4.4	30.7 ± 3.9	30.9 ± 5.1	*0.64*
Systolic blood pressure (mmHg)	101.8 ± 8.6	101.5 ± 10.7	102.3 ± 5.7	*0.96*
Diastolic blood pressure	69.8 ± 5.4	67.3 ± 4.8	72.9 ± 4.6	***0.049***
Height (cm)	165.1 ± 8.0	164.1 ± 8.6	166.3 ± 7.6	*0.76*
Weight 6 months postpartum (kg)	70.5 ± 17.3	64.7 ± 10.0	77.8 ± 22.2	*0.22*
Gestational weight gain (kg)	11.1 ± 6.0	11.1 ± 4.9	11.2 ± 8.0	*0.51*
Body Mass Index (BMI) (kg/m2)	25.7 ± 4.9	24.1 ± 3.6	27.8 ± 5.7	*0.19*
	***n* (%)**	***n* (%)**	***n* (%)**	
Mode of birth:				
Vaginal birth	14 (77)	10 (100)	4 (50)	***0.029***
Caesarean delivery	4 (22)	0 (0)	4 (50)	
Smoking:				*0.32*
Pre-pregnancy	4 (22)	2 (20)	4 (50)	*Pre-pregnancy use*
During pregnancy	0 (0)	0 (0)	0 (0)	
Postpartum	1 (6)	0 (0)	1 (13)	
Alcohol:				
Pre-pregnancy	8 (44)	2 (20)	6 (75)	*0.054*
During pregnancy	0 (0)	0 (0)	0 (0)	*Pre-pregnancy use*
Postpartum	5 (28)	2 (20)	3 (38)	
	(mean ± SD)	(mean ± SD)	(mean ± SD)	
Postpartum standard drink per week	3.4 ± 2.3	2 ± 1.4	3.8 ± 2.4	
Medication use:				
Multivitamin	6 (33)	4 (40)	2 (25)	*0.64*
Antidepressant	1 (6)	0 (0)	1 (16)	*0.45*
Antihypertensive	1 (6)	0 (0)	1 (16)	*0.45*
Hormonal contraceptive	8 (44)	4 (40)	4 (50)	*1.00*

NP, normotensive pregnancy; HP, hypertensive pregnancy; PE, preeclampsia; GH, gestational hypertension; mmHg, milimetres of mercury; cm, centimetres; kg, kilograms. Bold refers to those p values that are < 0.05.

TABLE 2 | Infant characteristics.

	Total Cohort (*n* = 18)	Normotensive Pregnancy (NP) (*n* = 10)	Preeclampsia (PE) Gestational Hypertension (GH) (*n* = 8)	*p* value
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	
Birthweight (kg)	3.3 ± 0.5	3.4 ± 0.4	3.2 ± 0.7	*0.20*
Gestation at birth (weeks)	38.6 ± 2.3	39.6 ± 1.3	37.4 ± 2.4	***0.016***
Weight at 6 months (kg)	7.95 ± 1.10	7.76 ± 0.84	8.19 ± 1.38	*0.85*
	***n* (%)**	***n* (%)**	**n (%)**	
Feeding method:				
Breastfed	8 (44)	4 (40)	4 (50)	*1.00*
Formula	1 (6)	0 (0)	1 (12)	*0.45*
Mixed	9 (50)	6 (60)	3 (38)	*0.64*
Commenced solids	17 (94)	9 (90)	8 (100)	*1.00*
Antibiotic treatment	3 (17)	1 (10)	2 (25)	*0.56*

Bold refers to those p values that are < 0.05.

(**Figures 3A–E** and **Supplementary Tables 3, 4**). However, LEfSe analysis revealed differentially abundant taxa at 6 months postpartum between HP and NP women (**Figure 3F** and **Supplementary Table 5**). The gut microbiota of HP women was enriched in phylum Actinobacteria, order Bifidobacteriales, family Bifidobacteriaceae, genus *Bifidobacterium* and species *Bifidobacterium* sp. compared to NP women (LDA > 2, $P < 0.05$). Conversely, the gut microbiota of HP women was depleted in genus *Barnesiella* and species *Barnesiella intestinihominis* when compared to NP women (LDA > 2, $P < 0.05$). These differences in gut microbiota taxonomic composition between HP and NP women did not remain significant after FDR correction ($P > 0.05$). However, taxa identified as significant prior to FDR correction were considered as potential biomarkers for future investigation. This is due to their identification through LEfSe's rigorous multistep process of biomarker discovery and the acknowledgement that future investigation with larger sample sizes may detect significance.

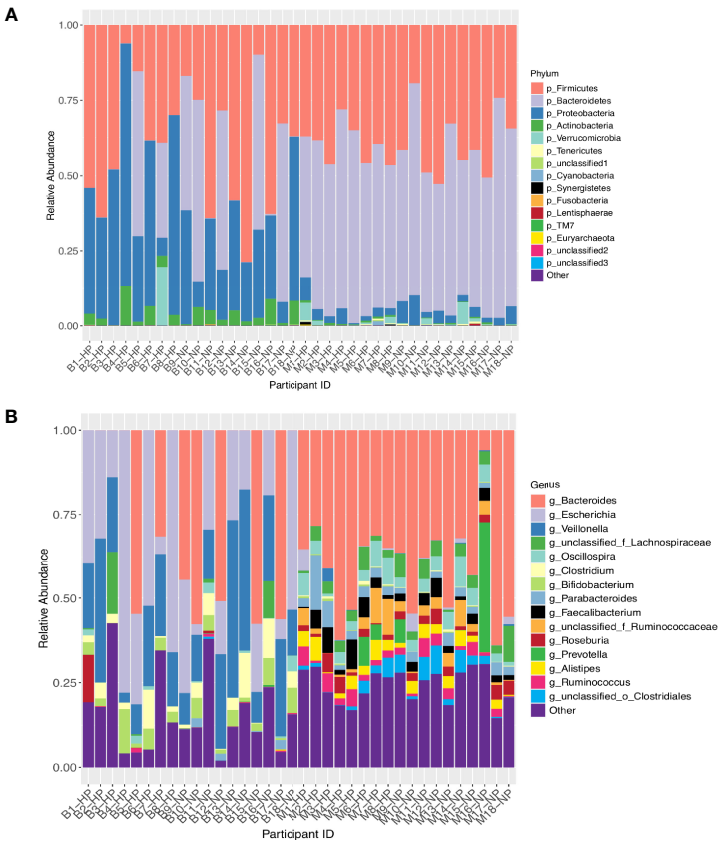

FIGURE 1 | **(A)** Relative abundance of the top 15 bacterial phyla. **(B)** Relative abundance of the top 15 bacterial genera. The X axis represents participants in the study. The Y axis is a scale of relative abundance out of 1. M, mother; B, baby; NP, normotensive pregnancy; HP, hypertensive pregnancy. Taxon levels are abbreviated with p, phylum; c, class; o, order; f, family; g, genus and s, species.

Impact of Clinical Factors on the Maternal Gut Microbiota

Several other maternal characteristics with the potential to influence microbial composition were analyzed. Pre-pregnancy and postpartum smoking status and alcohol consumption were found to not be associated with differences in microbial alpha and beta diversity in mothers ($P > 0.05$) (**Supplementary Tables 6, 7, 9**). However, pregnancy and postpartum multivitamin use was associated with elevated alpha diversity as measured by observed OTUs in the maternal gut microbiota ($P = 0.040$) (**Figure 4A**, **Supplementary Table 8**). Mean Shannon's index, Pielou's evenness and Faith's PD measures were higher in mothers who took multivitamins, although not significantly ($P > 0.05$) (**Figures 4B–D** and **Supplementary Table 8**). However, significant differences in beta diversity were found between women grouped according to multivitamin intake ($P = 0.035$) (**Figure 4E** and **Supplementary Table 9**). Additionally, LEfSe analysis revealed that multivitamin intake had a significant impact on gut microbiota taxonomic composition in maternal cohorts at the genus (**Figure 4F** and **Supplementary Table 10**) and species level (LDA > 2, $P < 0.05$) (**Figure 4G** and **Supplementary Table 11**). These differences in microbiota composition based on multivitamin intake did not remain significant following FDR correction ($P > 0.05$).

Changes in Gut Microbiota Diversity and Composition Between Infants Born From HP and NP

Significant differences in alpha diversity were found between HP and NP infants. Both Shannon's diversity ($P = 0.031$) and Pielou's evenness ($P = 0.031$) indices were significantly lower in infants born to HP women compared to those born to NP women (**Figures 5A, B** and **Supplementary Table 12**). Similarly, Faith's PD and the mean number of observed OTU's were lower, although not significantly, in infants whose mothers had HP compared to those who had NP (**Figures 5C, D** and **Supplementary Table 12**). However, there were no significant difference in beta diversity between infants born from HP or NP women ($P > 0.05$) (**Figure 5E** and **Supplementary Table 13**). LEfSe analysis revealed taxonomic differences in the gut microbiota of HP versus NP infants (**Figure 5F** and **Supplementary Table 14**). The gut microbiota of HP infants was depleted in phylum Bacteroidetes, classes Betaproteobacteria, Coriobacteriia and Bacteroidia, orders

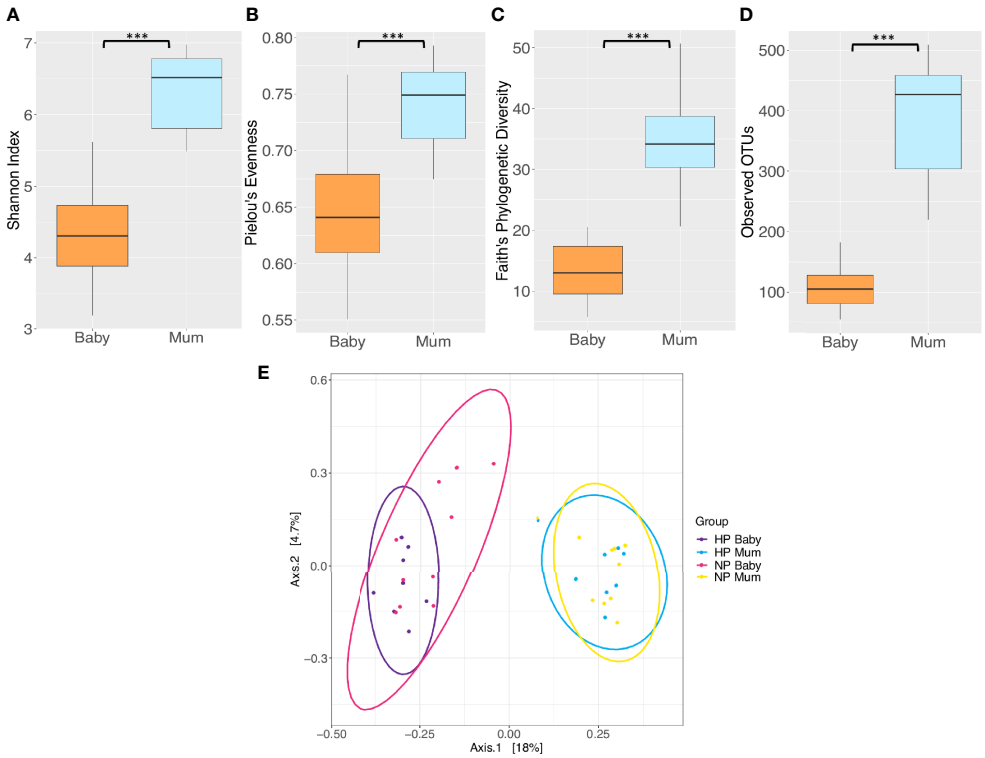

FIGURE 2 | Comparison of microbiota diversity between mothers (n = 18) and their babies (n = 18) based on faecal samples collected at 6 months postpartum and at six months of age respectively. Alpha diversity measured by **(A)** Shannon index **(B)** Pielou's evenness **(C)** Faith's phylogenetic diversity **(D)** Observed operational taxonomic units (OTUs). Boxes represent the interquartile range (IQR), the line inside the box indicates the median and whiskers represent values 1.5 × IQR from the first and third quartiles respectively. Differences between mothers and babies were tested using the Wilcoxon sum rank test and false discovery rate (FDR) corrected. ***P < 0.001. **(E)** Principal Coordinates Analysis (PCoA) of microbiota community structure in mothers and babies. NP, normotensive pregnancy; HP, hypertensive pregnancy. The points represent individual samples from mothers and their babies, and the ellipses illustrates the 95% confidence intervals of multivariate normal distribution.

Bacteroidales, Burkholderiales and Coriobacteriales, families Alcaligenaceae and Coriobacteriaceae, genus *Sutterella* and species *Clostridium aldenense, Sutterella* sp. and *Bacteroides* sp., compared NP infants (LDA > 2, P < 0.05). Conversely the species *Streptococcus infantis* was enriched in HP compared to NP infants (LDA > 2, P < 0.05). Differences in taxonomic composition between NP and HP infants did not remain significant after FDR correction (P > 0.05). However, in this preliminary study, taxa identified as significant by LEfSe prior to FDR correction, were explored as potential biomarkers for further investigation in larger future studies.

Impact of Clinical Factors on the Infant Gut Microbiota

In infants, maternal pre-pregnancy smoking status, pre-pregnancy alcohol consumption, birthing methods (caesarian or vaginal delivery), feeding methods (breast, bottle and mixed feeding) and pregnancy multivitamin intake had no significant effect on alpha and beta diversity indices (P > 0.05) (**Supplementary Figure 1** and **Supplementary Tables 15–20**). However, alpha diversity measures were higher, albeit non-significantly so, in babies whose mothers took multivitamins during pregnancy. LEfSe analysis identified differentially abundant taxa in the gut microbiota of babies when grouped according to maternal multivitamin intake at both genus (**Figure 6A** and **Supplementary Table 21**) and species level (LDA > 2, P < 0.05) (**Figure 6B** and **Supplementary Table 22**). These differences in infant microbiota composition based on maternal multivitamin intake did not remain significant following FDR correction, and this may be in part due to the small sample size of the study (P > 0.05).

DISCUSSION

This paper uniquely compares the microbiome six months postpartum of mothers and their infants with and without a hypertensive disorder of pregnancy. Understanding the long-term impacts of hypertensive disorders of pregnancy on mothers and infants is crucial, especially as early interventions to improve cardiovascular and metabolic health outcomes are being made possible through epidemiological, physiological and biochemical

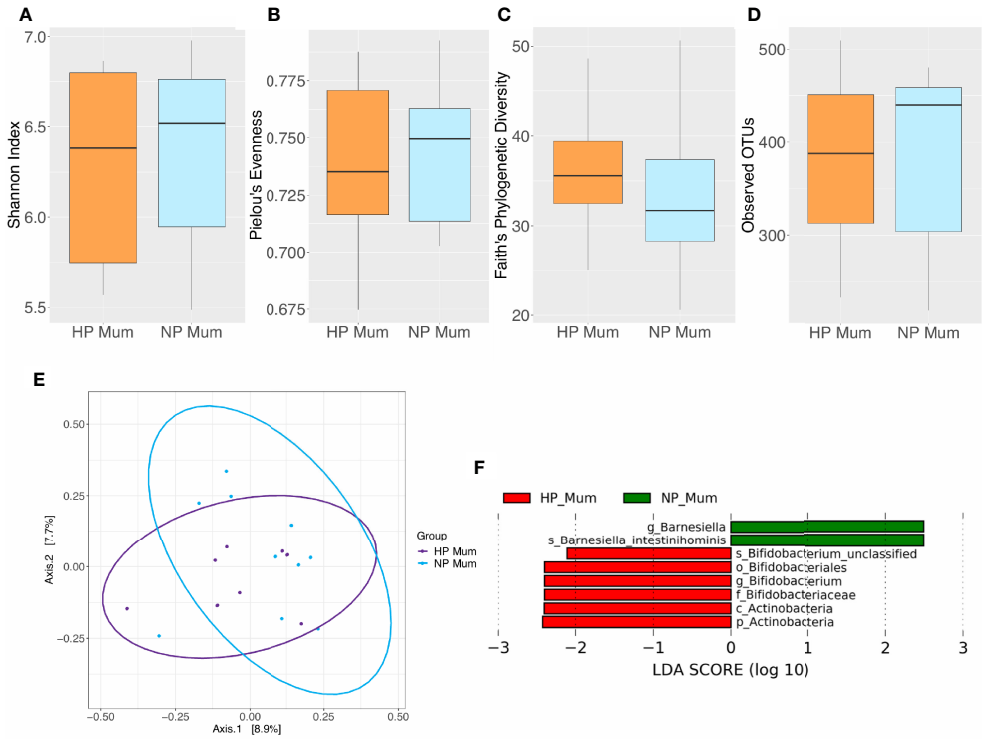

FIGURE 3 | Comparison of microbiota composition and diversity between hypertensive pregnancy (HP) mothers (n = 8) and normotensive pregnancy (NP) mothers (n = 10) based on faecal samples. Alpha diversity measured by **(A)** Shannon index **(B)** Pielou's evenness **(C)** Faith's phylogenetic diversity **(D)** Observed operational taxonomic units (OTUs). Boxes represent the interquartile range (IQR), the line inside the box indicates the median and whiskers represent values 1.5 × IQR from the first and third quartiles respectively. Differences between groups were tested using the Wilcoxon sum rank test and false discovery rate (FDR) corrected. Overall, no significant differences were observed in alpha diversity between HP and NP groups. **(E)** Principal Coordinates Analysis (PCoA) of microbiota community structure in HP and NP mothers. The points represent individual samples from mothers and the ellipse illustrates the 95% confidence intervals of multivariate normal distribution. **(F)** Differentially abundant bacterial taxa as identified by Linear discriminant analysis Effect Size (LEfSe). Bacterial taxa were classified as differentially abundant if their P value was < 0.05 and their linear discriminant analysis (LDA) log score was > 2. Taxa enriched in HP mothers are indicated by red, and taxa enriched in NP mothers are indicated by green. Taxon levels are abbreviated with p_ = phylum, c_ = class, o_ = order, f_ = family, g_ = genus and s_ = species. LDA effects sizes and significant P values are in detailed in **Supplementary Table 5**.

studies (Theilen et al., 2016; Wu et al., 2017; Brown Mark et al., 2018; Lui et al., 2019).

This study forms part of the larger P4 study investigating the long term impacts of HDP. The main findings to date have been (a) in mothers, women six months after HP versus NP show more markers of cardiovascular and metabolic disease susceptibility including; higher blood pressure, higher fat mass, more tendency to insulin resistance, and higher rates of micronutrient insufficiency (b) in infants, more infants were born small for gestational age (SGA) after HP, and SGA infants, regardless of hypertensive pregnancy status, were more likely to experience rapid weight gain 0-6 months (rapid catch-up growth being itself associated with future cardiovascular disease) (Brown Mark et al., 2020; Gow et al., 2021; McLennan et al., 2021). One potential mechanism for these observations could be microbiota related. The findings of our study when viewed mechanistically allow for further evaluation of the potential physiological reasons behind our findings and illuminate further areas for scientific exploration.

When conducting the analysis for this paper, using 16S rRNA sequencing we looked into the individual bacteria that resulted to try and postulate links and associations with the underlying maternal and infant physiology following both NP and HP.

Differences were observed in mothers and infants based on whether the pregnancy was normotensive or hypertensive. Maternally, in NP there was an increase in *Barnesiella* and *Barnesiella intestinihominis* compared to HP. Fielding et al. found that the passage of *Barnesiella intestinihominis* sp. via faecal microbial transplantation (FMT) into germ-free mice led to increased muscle mass and strength (Fielding et al., 2019). By extrapolation to humans, it could be argued that these bacterial species are beneficial in normotensive women.

The faecal samples of HP mothers were found to be enriched with six types of Actinobacteria, sharing down to the genus *Bifidobacterium*. This finding is biologically plausible given that mother to infant transmission of intestinal bacteria has been observed (Makino et al., 2013), and the presence of *Bifidobacterium*

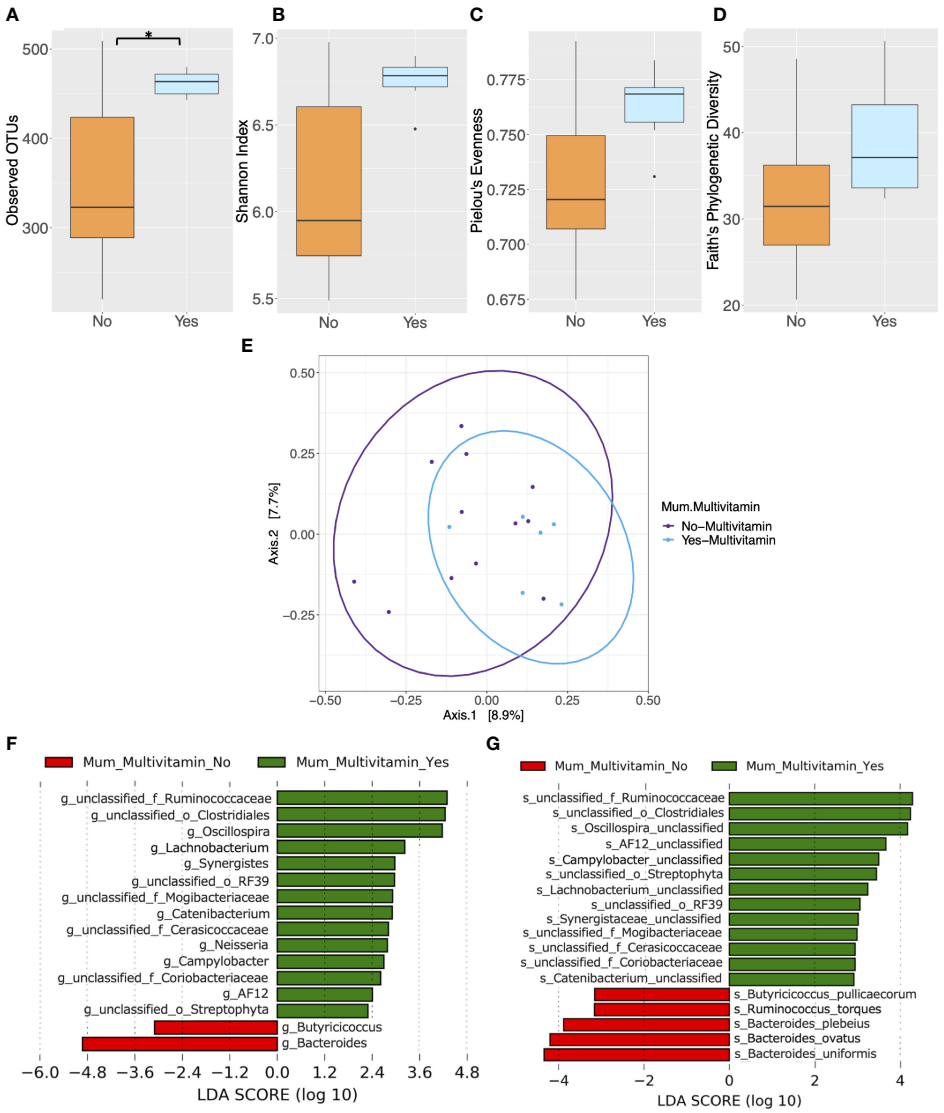

FIGURE 4 | Comparison of microbiota composition and diversity between mothers who took multivitamins (n = 6) and those who did not (n = 12) based on faecal samples collected at 6 months postpartum. Alpha diversity measured by **(A)** Observed operational taxonomic units (OTUs) **(B)** Shannon index **(C)** Pielou's evenness **(D)** Faith's phylogenetic diversity. Yes = mother took multivitamins, No = mother did not take multivitamins. Boxes represent the interquartile range (IQR), the line inside the box indicates the median and whiskers represent values 1.5 × IQR from the first and third quartiles respectively. Differences between groups were tested using the Wilcoxon sum rank test and false discovery rate (FDR) corrected. *P < 0.05 **(E)** Principal Coordinates Analysis (PCoA) of microbiota community structure based on maternal multivitamin intake. The points represent individual samples from mothers and the ellipse illustrates the 95% confidence intervals of multivariate normal distribution. **(F)** Differentially abundant bacterial genera as identified by Linear discriminant analysis Effect Size (LEfSe). **(G)** Differentially abundant bacterial species as identified by LEfSe. Bacterial taxa were classified as differentially abundant if their P value was < 0.05 and their linear discriminant analysis (LDA) log score was > 2. Taxa enriched in mothers who did not take multivitamins are indicated by red, and taxa enriched in those who did take multivitamins are indicated by green. Taxon levels are abbreviated with p, phylum; c, class; o, order; f, family; g, genus and s, species. LDA effects sizes and significant P values are in **Supplementary Tables 10–11**.

sp. in the gut of infants aids the digestion of human milk oligosaccharides (HMOs) (Marcobal and Sonnenburg, 2012; Underwood et al., 2017). Considering that HDP, particularly preeclampsia and its associated placental dysfunction can lead to fetal growth restriction and the birth of small neonates, an increase in *Bifidobacterium*, would seemingly only aid the energy and nutrient extraction from maternal breastmilk in order to reach maximal infant growth velocities once born. Venagas et al. (Parada Venegas et al., 2019) suggest that the presence of butyrate producing Actinobacteria, and acetate and propionate producing *Bifidobacterium*, may also play a role in hypertension. In the current study, the enrichment of phylum Actinobacteria and order Bifidobacteriales found in HP women, and the statistically significant difference in maternal diastolic blood pressure between

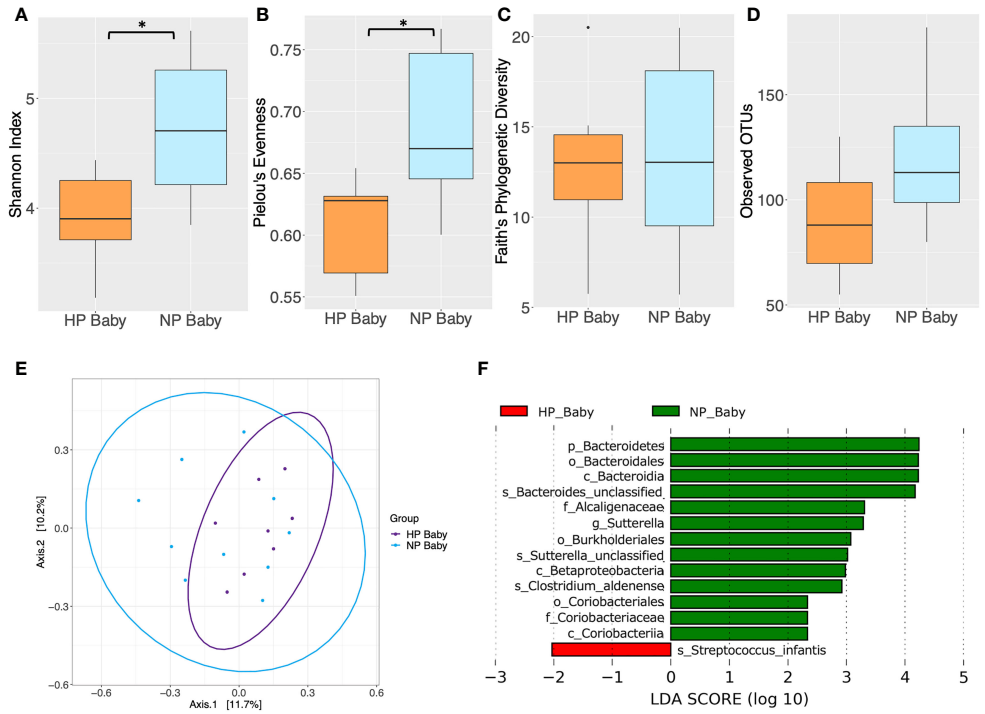

FIGURE 5 | Comparison of diversity indices between hypertensive pregnancy (HP) infants (n = 8) and normotensive pregnancy (NP) infants (n = 10) based on faecal samples collected at 6 months of age. Alpha diversity measured by **(A)** Shannon index **(B)** Pielou's evenness **(C)** Faith's phylogenetic diversity **(D)** Observed operational taxonomic units (OTUs). Boxes represent the interquartile range (IQR), the line inside the box indicates the median and whiskers represent values 1.5 × IQR from the first and third quartiles respectively. Differences between mothers and babies were tested using the Wilcoxon sum rank test and false discovery rate (FDR) corrected. *P < 0.05. **(E)** Principal Coordinates Analysis (PCoA) of microbiota community structure in HP babies and NP babies. The points represent individual samples from babies and the ellipse illustrates the 95% confidence intervals of multivariate normal distribution. **(F)** Differentially abundant bacterial taxa as identified by Linear discriminant analysis Effect Size (LEfSe). Bacterial taxa were classified as differentially abundant if their P value was < 0.05 and their linear discriminant analysis (LDA) log score was > 2. Taxa enriched in the HP babies are indicated by red, and taxa enriched in the NP babies are indicated by green. Taxon levels are abbreviated with p_ = phylum, c_ = class, o_ = order, f_ = family, g_ = genus and s_ = species. LDA effects sizes and significant P values are in **Supplementary Table 14**.

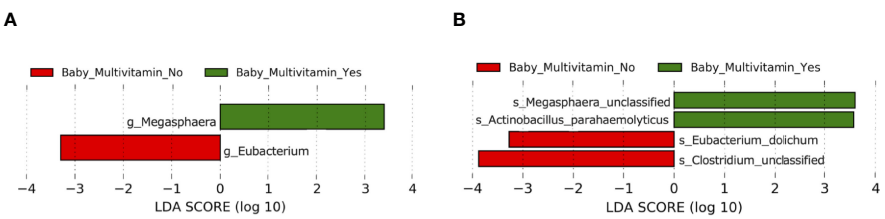

FIGURE 6 | Comparison of taxonomic composition between babies with mothers who took multivitamins in gestation (n = 6) and babies with mothers who did not take multivitamins in gestation (n = 12). **(A)** Differentially abundant bacterial genera as identified by Linear discriminant analysis Effect Size (LEfSe). **(B)** Differentially abundant bacterial species as identified by LEfSe. Bacterial taxa were classified as differentially abundant if their P value was < 0.05 and their linear discriminant analysis (LDA) log score was > 2. Taxa enriched in babies whose mothers did not take multivitamins in gestation are indicated by red, and taxa enriched in babies whose mothers did take multivitamins in gestation are indicated by green. Taxon levels are abbreviated with p_ = phylum, c_ = class, o_ = order, f_ = family, g_ = genus and s_ = species. LDA effects sizes and significant P values are in **Supplementary Tables 21–22**.

NP and HP at 6 months postpartum, suggest that the butyrate and proportionate producing microbiota is potentially correlated to elevated blood pressure (Gomez-Arango et al., 2016; Chen et al., 2020).

In this study, one enriched species of bacteria from the Firmicutes phylum, *Streptococcus infantis* was found in the infants born from HP mothers. This bacterium is identified in scientific literature under the Mitis group of commensals

recovered from upper respiratory tract specimens (Pimenta et al., 2019), however, its role in infant faeces remains unclear. The current study found a reduction in *Sutterella* sp. in the infants born from HP mothers. Wang et al. found increased *Sutterella* species in children who went on to develop autism spectrum disorder (Wang et al., 2013) and a meta-analysis performed by Maher et al. showed a 35% increase in the odds of having a child with autism in hypertensive disorder exposed pregnancies (Maher et al., 2018). These studies results differ from the current findings, which only serve to demonstrate the multifactorial nature of complex diagnoses, such as autism spectrum disorder, and the importance of long term follow up of the infants born from pregnancies with complications.

Infants born to HP mothers were found to be depleted in phylum Bacteroidetes and Bacteroides sp. which are generally viewed as gastrointestinal tract commensals beneficial to human functioning. There are a number of perinatal and postnatal factors associated with reduced Bacteroidetes or *Bacteroides* abundance in infant stool including caesarean delivery (Bäckhed et al., 2015; Vatanen et al., 2016; Bokulich et al., 2016), exclusive breastfeeding (Penders et al., 2006; Fallani et al., 2010) and maternal high fat diet (Chu et al., 2016). HP infants in this study were more likely to be born by caesarean, so their Bacteroidetes and *Bacteroides* sp. depletion may not be solely attributable to their HP exposure, nonetheless, the findings indicates a less beneficial infant microbial ecosystem after HP. Additionally, whilst the overall relative abundance of phylum Bacteroidetes was lower in HP compared to NP infants, at an individual level several NP infants also exhibited low levels of Bacteroidetes, suggesting the potential of multifactorial influences upon its abundance.

Whilst multivitamin intake in the mother did not change infant faecal microbiota beta diversity, there was a trend towards alpha diversity measures being higher, albeit non significantly so, in babies whose mothers took multivitamins during pregnancy. Of note, differentially abundant taxa at both genus and species level, were present prior to FDR adjustment, in infants grouped by maternal multivitamin intake. Of note, the larger P4 study showed that HP and non-breastfeeding status were associated with maternal micronutrient insufficiency (Siritharan et al., 2021).

Chu et al. performed the largest study of whole metagenomic sequencing on neonatal and infant stool to date, and demonstrated that by six weeks of age the infant microbial community structure and function had significantly expanded and diversified (Chu et al., 2016). The design of the current study, testing at a single time point of 6 months postpartum, was not able to detect initial fluctuations of the microbiota in infants included. In HP infants faeces, Coriobacteriia were also reduced. It is postulated that Coriobacteriaceae carry out important functions such as the conversion of bile salts and steroids, as well as the activation of dietary polyphenols (Clavel et al., 2014), whilst also playing a role in vitamin K2 production (Clavel et al., 2014). Liu et al. also demonstrated that an increase in Coriobacteriaceae within Actinobacteria might contribute to improved glucose tolerance and insulin sensitivity in diabetic animal models (Liu et al., 2018). Extrapolating this, it is biologically plausible that the offspring born to NP mothers have an increased abundance of this bacterium. Infants born to HP mothers do not, and this could potentially be related to longer term health outcomes, given the known risk of obesity and elevated body mass index in offspring born to mothers with preeclampsia (Davis et al., 2012).

Strengths and Limitations

Study limitations include the small sample size of this pilot/proof of concept study, and diverse characteristics within the sample, as well as any bias inherent in the group of women who chose to participate in this sub-study. Another limitation is the lack of microbiome samples from mothers in late pregnancy or at the time of birth. Strengths include that this is the first Australian examination of mothers and children after HP versus NP, and, as a pilot study, provides proof of acceptability and feasibility for the larger cohort of the Microbiome in Maternity Study (MUMS) (Susic et al., 2020).

A comprehensive Australian based maternity microbiome study (MUMS) will delve more deeply into the questions raised, including impacts on the infants born from hypertensive pregnancies. These questions and scientific exploration will then naturally lead to proposing whether supplementation of specific microbial taxa could play a role in ameliorating the long-term impact of hypertensive disorders during pregnancy on the next generation.

CONCLUSION

The pathophysiology of hypertensive pregnancies and the impact on the next generation is a complex and difficult subject to study. There are multiple confounding factors that impact the interpretation of results from emerging microbiome data. We have shown that microbes may indeed be involved in the transgenerational impact of hypertensive pregnancies, through observing changes in the gut microbiota of both women who experienced hypertension during pregnancy and their offspring compared to normotensive groups. Further longitudinal studies delving deeper into this area are warranted given the potential for future preventative and therapeutic considerations.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: BioProject ID PRJNA701500.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by South-Eastern Sydney Local Health District Human Research Ethics Committee, reference number: 12/195. Written

informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

DS, LR, GD, EE-O, and AH contributed to conception and design of the sub-study. AG contributed to the laboratory processing of samples. LW, EM, and XJ have contributed to the bioinformatic analysis. DS, LW, LR, MB, AG, EM, XJ, GD, EE-O and AH have contributed to the editing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.646165/full#supplementary-material

REFERENCES

Aagaard, K., Riehle, K., Ma, J., Segata, N., Mistretta, T-A., Coarfa, C., et al. (2012). A Metagenomic Approach to Characterization of the Vaginal Microbiome Signature in Pregnancy. *PloS One* 7 (6), e36466. doi: 10.1371/journal.pone.0036466

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., Versalovic, J., et al. (2014). The Placenta Harbors a Unique Microbiome. *Sci. Trans. Med.* 6 (237), 237ra65–237ra65. doi: 10.1126/scitranslmed.3008599

Arnott, C., Nelson, M., Ramirez, M. A., Hyett, J., Gale, M., Henry, A., et al. (2020). Maternal Cardiovascular Risk After Hypertensive Disorder of Pregnancy. *Heart* 106 (24), 1927. doi: 10.1136/heartjnl-2020-316541

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and Stabilization of the Human Gut Microbiome During the First Year of Life. *Cell Host Microbe* 17 (5), 690–703. doi: 10.1016/j.chom.2015.04.004

Bisanz, J. E., Soto-Perez, P., Lam, K. N., Bess, E. N., Haiser, J. J., Allen-Vecoe, E., et al. (2018). Illuminating the Microbiome's Dark Matter: A Functional Genomic Toolkit for the Study of Human Gut Actinobacteria. *bioRxiv* 304840. doi: 10.1101/304840

Bokulich, N. A., Chung, J., Battaglia, T., Henderson, N., Jay, M., Li, H., et al. (2016). Antibiotics, Birth Mode, and Diet Shape Microbiome Maturation During Early Life. *Sci. Trans. Med.* 8 (343), 343ra82. doi: 10.1126/scitranslmed.aad7121

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., et al. (2018). Optimizing Taxonomic Classification of Marker-Gene Amplicon Sequences With QIIME 2's Q2-Feature-Classifier Plugin. *Microbiome* 6 (1), 90. doi: 10.1186/s40168-018-0470-z

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat. Biotechnol.* 37 (8), 852–857. doi: 10.1038/s41587-019-0209-9

Brown, M. A., Magee, L. A., Kenny, L. C., Karumanchi, S. A., McCarthy, F. P., Saito, S., et al. (2018). Hypertensive Disorders of Pregnancy. *Hypertension* 72 (1), 24–43. doi: 10.1161/HYPERTENSIONAHA.117.10803

Brown, M. A., Roberts, L., Hoffman, A., Henry, A., Mangos, G., O'Sullivan, A., et al. (2020). Recognizing Cardiovascular Risk After Preeclampsia: The P4 Study. *J. Am. Heart Assoc.* 9 (22), e018604. doi: 10.1161/JAHA.120.018604

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P.. (2016). DADA2: High-Resolution Sample Inference From Illumina Amplicon Data. *Nat. Methods* 13 (7), 581–583. doi: 10.1038/nmeth.3869

Callahan, B. J., McMurdie, P. J., and Holmes, S. P. (2017). Exact Sequence Variants Should Replace Operational Taxonomic Units in Marker-Gene Data Analysis. *ISME J.* 11 (12), 2639–2643. doi: 10.1038/ismej.2017.119

Chen, X., Li, P., Liu, M., Zheng, H., He, Y., Chen, M.-X., et al. (2020). Gut Dysbiosis Induces the Development of Pre-Eclampsia Through Bacterial Translocation. *Gut* 69 (3), 513–522. doi: 10.1136/gutjnl-2019-319101

Chu, D. M., Antony, K. M., Ma, J., Prince, A. L., Showalter, L., Moller, M., et al. (2016). The Early Infant Gut Microbiome Varies in Association With a Maternal High-Fat Diet. *Genome Med.* 8 (1), 77. doi: 10.1186/s13073-016-0330-z

Clavel, T., Lepage, P., and Charrier, C. (2014). "The Family Coriobacteriaceae," in *The Prokaryotes*. Eds. E Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt and F. Thompson (Berlin, Heidelberg: Springer). doi: 10.1007/978-3-642-30138-4_343

Crusell, M. K. W., Hansen, T. H., Nielsen, T., Allin, K. H., Rühlemann, M. C., Damm, P., et al. (2018). Gestational Diabetes Is Associated With Change in the Gut Microbiota Composition in Third Trimester of Pregnancy and Postpartum. *Microbiome* 6 (1), 89–89. doi: 10.1186/s40168-018-0472-x

Davis, E. F., Lazdam, M., Lewandowski, A. J., Worton, S. A., Kelly, B., Kenworthy, Y., et al. (2012). Cardiovascular Risk Factors in Children and Young Adults Born to Preeclamptic Pregnancies: A Systematic Review. *Pediatrics* 129 (6), e1552. doi: 10.1542/peds.2011-3093

Davis, G. K., Roberts, L., Mangos, G., Henry, A., Pettit, F., O'Sullivan, A., et al. (2016). Postpartum Physiology, Psychology and Paediatric Follow Up Study (P4 Study) - Study Protocol. *Pregnancy Hypertens.* 6 (4), 374–379. doi: 10.1016/j.preghy.2016.08.241

Duley, L. (2009). The Global Impact of Pre-Eclampsia and Eclampsia. *Semin. Perinatol.* 33 (3), 130–137. doi: 10.1053/j.semperi.2009.02.010

Fallani, M., Young, D., Scott, J., Norin, E., Amarri, S., Adam, R., et al. (2010). Intestinal Microbiota of 6-Week-Old Infants Across Europe: Geographic Influence Beyond Delivery Mode, Breast-Feeding, and Antibiotics. *J. Pediatr. Gastroenterol. Nutr.* 51 (1), 77–84. doi: 10.1097/MPG.0b013e3181d1b11e

Fielding, R. A., Reeves, A. R., Jasuja, R., Liu, C., Barrett, B. B., and Lustgarten, M. S.. (2019). Muscle Strength Is Increased in Mice That Are Colonized With Microbiota From High-Functioning Older Adults. *Exp. Gerontol.* 127, 110722–110722. doi: 10.1016/j.exger.2019.110722

Gomez-Arango, L. F., Barrett, H. L., McIntyre, H.D., Callaway, L. K., Morrison, M., Nitert, M. D., et al. (2016). Increased Systolic and Diastolic Blood Pressure Is Associated With Altered Gut Microbiota Composition and Butyrate Production in Early Pregnancy. *Hypertension* 68 (4), 974–981. doi: 10.1161/HYPERTENSIONAHA.116.07910

Gow, M. L., Roberts, L. M., Henry, A., Davis, G., Mangos, G., Pettit, F., et al. (2021). Growth From Birth to 6-Months of Infants With and Without Intrauterine Preeclampsia Exposure. *J. Dev. Orig. Health Dis.* doi: 10.1017/S2040174421000167

Koleva, P. T., Kim, J.-S., Scott, J. A., and Kozyrskyj, A. L.. (2015). Microbial Programming of Health and Disease Starts During Fetal Life. *Birth Defects Res. C Embryo Today* 105 (4), 265–277. doi: 10.1002/bdrc.21117

Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Bäckhed, H. K., et al. (2012). Host Remodeling of the Gut Microbiome and Metabolic Changes During Pregnancy. *Cell* 150 (3), 470–480. doi: 10.1016/j.cell.2012.07.008

Liu, H., et al. (2018). The Family Coriobacteriaceae Is a Potential Contributor to the Beneficial Effects of Roux-En-Y Gastric Bypass on Type 2 Diabetes. *Surg. Obes. Related Dis: Off. J. Am. Soc. Bariatric Surg.* 14 (5), 584–593. doi: 10.1016/j.soard.2018.01.012

Lui, N. A., Jeyaram, G., and Henry, A. (2019). Postpartum Interventions to Reduce Long-Term Cardiovascular Disease Risk in Women After Hypertensive Disorders of Pregnancy: A Systematic Review. *Front. Cardiovasc. Med.* 6, 160–160. doi: 10.3389/fcvm.2019.00160

Lv, L.-J., Sheng-Hui, L., Shao-Chuan, L., Zhi-Cheng, Z., Hong-Li, D., Tian, C., et al. (2019). Early-Onset Preeclampsia Is Associated With Gut Microbial Alterations in Antepartum and Postpartum Women. *Front. Cell. Infect. Microbiol.* 9, 224–224. doi: 10.3389/fcimb.2019.00224

Maher, G. M., O'Keeffe, G. W., Kearney, P. M., Kenny, L. C., Dinan, T. G., Mattsson, M., et al. (2018). Association of Hypertensive Disorders of Pregnancy With Risk of Neurodevelopmental Disorders in Offspring: A

Systematic Review and Meta-Analysis. *JAMA Psychiatry* 75, (8), 809–819. doi: 10.1001/jamapsychiatry.2018.0854

Makino, H., Kushiro, A., Ishikawa, E., Kubota, H., Gawad, A., Sakai, T., et al. (2013). Mother-to-Infant Transmission of Intestinal Bifidobacterial Strains Has an Impact on the Early Development of Vaginally Delivered Infant's Microbiota. *PLoS ONE* 8, (11), e78331. doi: 10.1371/journal.pone.0078331

Marcobal, A., and Sonnenburg, J. L. (2012). Human Milk Oligosaccharide Consumption by Intestinal Microbiota. *Clin. Microbiol. Infection: Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* 18 Suppl 4 (0 4), 12–15. doi: 10.1111/j.1469-0691.2012.03863.x

McLennan, S. L., Henry, A., Roberts, L. M., Siritharan, S. S., Ojurovic, M., Yao, A., et al. (2021). Maternal Adiposity and Energy Balance After Normotensive and Preeclamptic Pregnancies. *J. Clin. Endocrinol. Metab*. 106 (8), e2941–e2952. doi: 10.1210/clinem/dgab223

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PloS One* 8 (4), e61217. doi: 10.1371/journal.pone.0061217

Nuriel-Ohayon, M., Neuman, H., Ziv, O., Belogolovski, A., Barsheshet, Y., Bloch, N., et al. (2019). Progesterone Increases Bifidobacterium Relative Abundance During Late Pregnancy. *Cell Rep*. 27 (3), 730–736.e3. doi: 10.1016/j.celrep.2019.03.075

Parada Venegas, D., et al. (2019). Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. *Front. Immunol.* 10, 277. doi: 10.3389/fimmu.2019.00277

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., et al. (2006). Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy. *Pediatrics* 118 (2), 511–521. doi: 10.1542/peds.2005-2824

Pimenta, F., Gertz, R. E.Jr., Park, S. H., Kim, E., Moura, I., Milucky, J., et al. (2019). Streptococcus Infantis, Streptococcus Mitis, and Streptococcus Oralis Strains With Highly Similar Cps5 Loci and Antigenic Relatedness to Serotype 5 Pneumococci. *Front. Microbiol.* 9, 3199–3199. doi: 10.3389/fmicb.2018.03199

R Core Team. (2020). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, (Vienna, Austria). Available at: https://www.R-project.org/.

RStudio Team. (2020). *RStudio. Integrated Development for R* (Boston, MA: RStudio, PBC). Available at: http://www.rstudio.com/.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic Biomarker Discovery and Explanation. *Genome Biol.* 12, R60. doi: 10.1186/gb-2011-12-6-r60

Siritharan, S. S., Henry, A., Gow, M. L., Roberts, L. M., Yao, A., Ojurovic, M., et al. (2021). Maternal Macro- and Micronutrient Intake Six Months After Hypertensive Versus Normotensive Pregnancy: Is Poor Diet Quality Contributing to Future Cardiometabolic Disease Risk? *Pregnancy Hypertension* 23, 196–204. doi: 10.1016/j.preghy.2020.11.002

Susic, D., Davis, G., O' Sullivan, A. J., McGovern, E., Harris, K., Roberts, L. M., et al. (2020). Microbiome Understanding in Maternity Study (MUMS), an Australian Prospective Longitudinal Cohort Study of Maternal and Infant Microbiota: Study Protocol. *BMJ Open* 10 (9), e040189. doi: 10.1136/bmjopen-2020-040189

Tenenbaum-Gavish, K., Sharabi-Nov, A., Binyamin, D., Møller, H. J., Danon, D., Rothman, L., et al. (2020). First Trimester Biomarkers for Prediction of Gestational Diabetes Mellitus. *Placenta* 101, 80–89. doi: 10.1016/j.placenta.2020.08.020

Theilen, L. H., Fraser, A., Hollingshaus, M. S., Schliep, K. C., Varner, M. W., Smith, K. R., et al. (2016). All-Cause and Cause-Specific Mortality After Hypertensive Disease of Pregnancy. *Obstet. Gynecol.* 128 (2), 238–244. doi: 10.1097/AOG.0000000000001534

Underwood, M. A., Davis, J. C.C., Kalanetra, K. M., Gehlot, S., Patole, S., Tancredi, D. J., et al. (2017). Digestion of Human Milk Oligosaccharides by Bifidobacterium Breve in the Premature Infant. *J. Pediatr. Gastroenterol. Nutr.* 65 (4), 449–455. doi: 10.1097/MPG.0000000000001590

van der Giessen, J., Binyamin, D., Belogolovski, A., Frishman, S., Tenenbaum-Gavish, K., Hadar, E., et al. (2020). Modulation of Cytokine Patterns and Microbiome During Pregnancy in IBD. *Gut* 69 (3), 473. doi: 10.1136/gutjnl-2019-318263

Vatanen, T., Kostic, A. D., d'Hennezel, E., Siljander, H., Franzosa, E. A., Yassour, M., et al. (2016). Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. *Cell* 165 (4), 842–853. doi: 10.1016/j.cell.2016.04.007

Wang, L., Christophersen, C. T., Sorich, M. J., Gerber, J. P., Angley, M. T., and Conlon, M. A.. (2013). Increased Abundance of Sutterella Spp. And Ruminococcus Torques in Feces of Children With Autism Spectrum Disorder. *Mol. Autism* 4 (1), 42. doi: 10.1186/2040-2392-4-42

Wang, J., Zheng, J., Shi, W., Du, N., Xu, X., Zhang, Y., et al. (2018). Dysbiosis of Maternal and Neonatal Microbiota Associated With Gestational Diabetes Mellitus. *Gut* 67 (9), 1614–1625. doi: 10.1136/gutjnl-2018-315988

Wickham, H. (2016). "Elegant Graphics for Data Analysis," in *Ggplot2, 2 ed*, vol. XVI. (Houston, TX: Springer International Publishing), 260.

Wickham, H., François, R., Henry, L., and Müller, K. (2019) *Dplyr: A Grammar of Data Manipulation, R.F.* Available at: https://CRAN.R-project.org/package=dplyr.

Wu, P., Haththotuwa, R., Kwok, C. S., Babu, A., Kotronias, R. A., Rushton, C., et al. (2017). Preeclampsia and Future Cardiovascular Health: A Systematic Review and Meta-Analysis. *Circ. Cardiovasc. Qual. Outcomes* 10 (2), e003497. doi: 10.1161/CIRCOUTCOMES.116.003497

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Influence of Geographical Location on Maternal-Infant Microbiota: Study in Two Populations From Asia and Europe

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Early gut microbial colonization is driven by many factors, including mode of birth, breastfeeding, and other environmental conditions. Characters of maternal-neonatal microbiota were analyzed from two distinct populations in similar latitude but different continents (Oriental Asia and Europe). A total number of 120 healthy families from China (n=60) and Spain (n=60) were included. Maternal and neonatal microbiota profiles were obtained at birth by 16S rRNA gene profiling. Clinical records were collected. Geographical location influenced maternal-neonatal microbiota. Indeed, neonatal and maternal cores composed by nine genera each one were found independently of location. Geographical location was the most important variable that impact the overall structure of maternal and neoantal microbiota. For neonates, delivery mode effect on neonatal microbial community could modulate how the other perinatal factors, as geographical location or maternal BMI, impact the neoantal initial seeding. Furthermore, lower maternal pre-pregnancy BMI was associated with higher abundance of *Faecalibacterium* in maternal microbiota and members from *Lachnospiraceae* family in both mothers and infants. At genus-level, Chinese maternal-neonate dyads possessed higher number of phylogenetic shared microbiota than that of Spanish dyads. *Bifidobacterium* and *Escherichia/Shigella* were the genera most shared between dyads in the two groups highlighting their importance in neonatal colonization and mother-infant transmission. Our data showed that early gut microbiota establishment and development is affected by interaction of complex variables, where environment would be a critical factor.

Keywords: maternal, neonate, gut microbiota, geographical location, *Bifidobacterium*

1 INTRODUCTION

An adequate early microbial colonization is crucial for proper immunological and metabolic development (Clemente et al., 2012). Shifts during this process have been linked to an increased risk of non-communicable diseases (NCDs) such as allergies, metabolic disorders, as well as other long-lasting effects (Milani et al., 2017).

Neonatal gut colonization represents the *de novo* assembly of a complex microbial community, a process that is influenced by several environmental and also, host factors (Koleva et al., 2015). This complex process, still not well understood, and follows a time frame sequence depending on primary events at birth, such as delivery mode and feeding type, and subsequently undergoes a dynamic and non-random process during the development and maturation of infant gut microbiota (Sprockett et al., 2018). In this scenario, maternal microbiota represents one of the major determinants in the assembly of the offspring's microbial profile (Dominguez-Bello et al., 2010; Ferretti et al., 2018). Maternal gut microbiota would reflect the impact of specific factors and other environmental exposures, such as diet, lifestyle and antibiotic exposure, which could be transferred to the neonate at birth and later, during lactation (Dominguez-Bello et al., 2010; Koleva et al., 2015). Thus, mode of delivery and type of feeding have been proposed as the main drivers and contributors that would shape the neonatal gut microbiota (Dominguez-Bello et al., 2010). Several studies have explored the impact of perinatal factors on early gut microbial colonization, but available information about the potential impact of geographical location, including diet, lifestyle and climate on maternal-neonatal microbiota composition, is still scarce. Previous studies have been shown a differential impact of specific factors on milk microbiota according to geographical location (Kumar et al., 2016), while the data on maternal-neonatal gut microbiota was not provided.

In this sense, geographical location has been highlighted as an important factor shaping the microbiota composition in the adult population due to differences in dietary patterns, cultural practices, and religion (Singh and Mittal, 2020). Thus, the current study aims to assess the impact of the maternal microbiota on the distribution of infant gut microbial communities at birth from two distinct populations.

2 MATERIAL AND METHODS

2.1 Study Design and Participants

The study comprised 194 mother-neonate pairs from two independent cohorts from China and Spain, corresponding to 87 dyads from each location.

2.1.1 Chinese Cohort

Mother-infant pairs were recruited from a community-based randomized controlled trial conducted in Shaanxi Province, China (Latitude 34.34 and Longitude 108.940). All mother-infant pairs lived in Bin county, Shaanxi Province, China. Mothers were recruited after admission to the hospital for delivery. The eligibility criteria for the present study included: healthy pregnant women without complications such as hypertension, diabetes, or any other diagnosed disease, singleton pregnancies and healthy full-term neonates. All the participants were informed about the study and gave written consent. The study protocol was approved by the Ethic Committee of Xi'an Jiaotong University Health Science Center, China. Furthermore, the study was registered on the ClinicalTrials.gov platform, with the registration number NCT02537392.

2.1.2 Spanish Cohort

Mother-infant pairs were randomly selected as a subset from a prospective and observational MAMI birth cohort recruited from 2015-2017 in the Spanish-Mediterranean area (Latitude 39.46 and Longitude 0.375), as detailed elsewhere (García-Mantrana et al., 2019). Clinical parameters of the mother and the newborn were obtained from medical staff's clinical records at the hospital. The inclusion criteria included healthy pregnancies without diagnosed disease, and mothers older than 18 years of age. The exclusion criteria were the non-compliance with any of the inclusion criteria and, pro- and prebiotic treatment or the use of any other medication and drugs. Women were enrolled at the end of gestation and with follow-up participation during the first year of life. All participants received oral and written information about the study and written informed consent was obtained from all the families. The study protocol was approved by the Hospital Ethics Committees (Hospital Universitario y Politécnico La Fe and Hospital Clinico Universitario de Valencia). The study is registered on the ClinicalTrial.gov platform, with the registration number NCT03552939.

2.2 Biological Samples

In both cohorts, maternal fecal samples were collected by trained personnel prior birth. In the newborns, the first-pass fecal samples were collected at birth and within the first 24 hours. Both samples were stored in pre-numbered sterile tubes and immediately stored at -80°C until further analysis.

2.3 DNA Extraction and 16S rRNA Amplicon Sequencing

2.3.1 Chinese Cohort

Total DNA was extracted from approximately 200 mg of fecal sample by using the QIAamp Fast DNA Stool Mini Kit (51504, QIAGEN, Germany), according to the specifications from the manufacturer. DNA concentration and purity were detected on NanoDrop2000 (ThermoFisher, USA) and 1.0% agarose gel electrophoresis.

2.3.2 Spanish Cohort

Total DNA was extracted from the fecal material (approx. 50-100 mg) using the Master-Pure DNA extraction Kit (Epicentre, Madison, WI, US) following the manufacturer's instructions with the following modifications: samples were treated with lysozyme (20 mg/mL) and mutanolysin (5U/mL) for 60 min at 37°C and a preliminary step of cell disruption with 3-μm diameter glass beads for 1 min at 6 m/s by a bead beater FastPrep 24-5G Homogenizer (MP Biomedicals) as described elsewhere (García-Mantrana et al., 2019). Purification of the DNA was performed using DNA Purification Kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions. DNA concentration was measured using Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, US) for further analysis.

2.3.3 Sequencing

The V3-V4 region of the 16S rRNA sequence was amplified using a specific primer (Klindworth et al., 2013; Michelsen et al., 2014). DNA libraries were performed with the amplification of the V3-V4 variable region of the 16S rRNA gene. A multiplexing step was conducted by the NextEra XT Index Kit (FC-131-2001) (Illumina, San Diego, CA, United States). Amplicons were checked with a

Bioanalyzer DNA 1000 system (Agilent Technologies, Santa Clara, CA, United States) and libraries were sequenced using a 2x300bp paired-end run (MiSeq Reagent kit v3) on an Illumina MiSeq platform according to manufacturer instructions. Controls during DNA extraction and PCR amplification were also included and sequenced. The sequenced data were submitted to SRA with the accession number of PRJNA637167 and PRJNA614975.

2.4 Bioinformatic and Statistical Analyses

Raw sequences from both locations were processed in the same manner but independently. The resulting taxonomical tables were then merged at genus level to avoid the potential bias at amplicon sequence variant level (ASV). The paired-ends reads were merged using FLASH v1.2.7 (Magoč and Salzberg, 2011). Then, Deblur method of Quantitative Insights into Microbial Ecology2 (QIIME2) software (v2020.1) (Bolyen et al., 2019) was used to extract taxonomical composition from the sequencing reads with the standard recommended options for the filtering and denoising process as well as in the chimeral identification and removal. Taxonomic assignment was conducted using the Silva v138 database (Yarza et al., 2008) and the available pretrained naive Bayes classifier (Bokulich et al., 2018; Kaehler et al., 2019). Final QIIME2 objects were imported to Rstudio environment for further quality filtering and statistical analysis. To manage the potential contaminants, all sequences from the negative controls from both, Spain and China data sets, were obtained and included in the pipeline. Furthermore, the *decontam* package (Davis et al., 2017) in R environment (R Core Team, 2019) was used to determine the presence of potential contaminants-related sequence and they were removed from the final table. Additionally, samples with less than 1000 sequences were removed from the final data set (n=0).

Alpha diversity indices, including those for determination of richness (Chao1), diversity (Shannon) was obtained through the phyloseq package (McMurdie and Holmes, 2013) after the rarefaction of the tables to the minimum reads per sample (3947 reads). Differences in alpha-diversity index were performed using Mann-Whitney test considering a $p<0.05$ as significant. Beta diversity analysis were performed based on Bray-Curtis distance using also phyloseq package. Adonis test from vegan package (Oksanen et al., 2019) was used to assess the association between gut microbial community composition and studied variables (Bäckhed et al., 2015). The analysis included the following variables for the study of maternal microbiota: geographical location, maternal age, maternal body mass index (BMI) and delivery mode. Principal Coordinate Analysis (PCoA) and Discriminant analysis of Principal components (DAPC) were performed to the visualization of the β-diversity similarities according to geographical location through the adegenet (Jombart and Ahmed, 2011), ggplot2 (Wickham, 2016) and vegan package.

Maternal and neoantal core genera were obtained through the microbiome package. A threshold of 0.01% in 95% of the samples and 85% was considered for the identification of maternal and neonatal microbial core, respectively. The core taxa were plotted using ggplot2 with a after log transformation of the data to facilitate the visualization. For the core genera the co-occurrence OTUs were verified. Spearman's rank correlation was calculated and employed to identify the co-occurrence patterns among the relative abundance of core genera. R package "corrplot" (Wei and Simko, 2021) was used to plot the correlations matrix. For the analysis of the compositional differences between the studied variables two approaches were used. For the initial exploration, differences in the phyla and core genera were assessed by Mann-Whitney analysis on the centered log ration (CLR) normalization through the microbiome package (Lahti and Shetty). To control for multiple testing, false discovery rate (FDR) values were estimated by the Benjamini-Yekutieli method (Benjamini and Yekutieli, 2001) (referred as q-value). Besides this, Maaslin2 package (Mallick et al., 2021) was used to performed multivariate analysis for the effect of geographical location in the relative abundance of microbial genera in both maternal and infant microbiota including the potential influential covariables (maternal BMI, delivery mode and age). For multivariate analysis in Maaslin2 those genera that appear less than 3 times in at least 10% of the samples were removed (remaining 181 genera). Mann-Whitney test was also used for testing the differences between the bacterial counts, for total bacteria and *Bifidobacterium* genus, in infant samples measured by quantitative PCR using GraphPad prism software (GraphPad Prism version 8.4.3 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com).

2.5 Bacterial Quantification by Quantitative PCR Analysis

A small subset of infant samples (n=67) according to DNA availability (China n=36; Spain n=31) were used for the specific bacterial count determination by the qPCR. Total bacterial and *Bifidobacterium* genus counts were measured by quantitative system based on the amplification of specific 16S rRNA gene region by use of Light Cycler 480 Real-Time PCR System (Roche, Basilea, Switzerland). Primers used were for total bacteria (Fwd: 5'-CGTGCCAGCAGCCGCGG-3', Rv: 5'-TGGACTACCAGGGTATCTAATCCTG-3') and for *Bifidobacterium* genus (5'-GATTCTGGCTCAGGATGAACGC-3'; Rv: 5'-CTGATAGGACGCGACCCCAT-3') (Gueimonde et al., 2004; Farhana et al., 2018). Reaction mixture consisted in SYBR Green I master mix (Roche, Basilea, Switzerland), 0.25 μM of each specific primer set and 1 μl of DNA. Melting curves were also assessed to test the specificity of the reaction. Standard curves for the specific targeted bacterial group were generated using Ct values and the calculated gene copy numbers were determined based on the fragment amplification length.

3 RESULTS

3.1 Study Population

A total of 194 pregnant women from China and Spain were included in the study, of which 74 dropped out. Reasons for the drop-out of participants included the following: no longer fulfilling the inclusion and exclusion criteria (n=31), withdrawal of informed consent (n=25), missing data of subjects (n=11) and other reasons (n=7). Finally, 120 mother-neonate dyads (240 fecal samples) were suitable for analysis. The characteristics of the subjects participating in the study are listed in **Table 1**. Chinese mothers were younger than the Spanish ones (a median of 27 vs 31.6 years) ($p=0.001$). Similarly, a slight

TABLE 1 | The demographic and birth characteristics of subjects from China and Spain.

	China (n=60)	Spain (n=60)	*P*
Maternal characteristics			
Maternal age (y)	27.0 (21.3-37.3)	31.6 (22.3-40.1)	*0.001**
BMI	21.3 (16.6-28.3)	22.5 (16.4-29.7)	0.110
Infant characteristics			
Gestational age	38.8 ± 1.1	39.6 ± 1.1	*>0.001**
Male	39/65%	35/58.3%	0.453
Female	21/35%	25/41.7%	
Vaginal delivery	45/75%	50/83.3%	0.261
Non-vaginal delivery	15/25%	10/16.7%	
Birth weight (g)	3205.7 ± 399.5	3347.5 ± 440.3	0.067

*$P < 0.05$.

difference was observed in gestational age showing lower gestational age in Chinese population than in Spain, although both were term deliveries (38.8 vs 39.6 weeks) ($p<0.001$). No differences were observed in the other recoded clinical characteristics.

3.2 Sequencing Summary and Gut Microbiota Characteristic in Maternal-Neonatal Dyads

After data filtration and chimera removal, the 120 mother-neonate dyads dataset contained 7,218,087 reads (min-max reads: 3947- 93206). The resulted phyloseq object consist in a total of 944 different genera catalogued in the Silver138 database (**Figure 1**). Furthermore, the retrieved genera were distributed among 37 phyla and 364 families. Among genera, 171 were shared between Chinese and Spanish population (18.11%) (**Figure 1A**). The microbial core at genus level of maternal and neonatal microbiota were composed by 9 genera in both populations (**Figures 1B, C** and **Supplementary Data**, **Table S1**).

For the brief characterization of the maternal-neonatal gut microbiota from China and Spain, the most abundant bacterial phyla are presented in **Figure 1**. In 120 pregnant women, Firmicutes (average 63.47%) and Bacteroidetes (average 13.45%) were the most dominant phyla, and *Bacteroides* (8.33%), *Subdoligranulum* (8.50%) and *Bifidobacterium* (8.62%) were the most abundant genera on average (**Supplementary Data**, **Table S1**). Regarding neonatal microbiota, Proteobacteria (41.24%) and Firmicutes (37.73%) were the dominant phyla, and the main genera were *Escherichia/Shigella* (18.35%) and *Bifidobacterium* (9.41%) (**Figure 1D**).

3.3 Impact of Location on Maternal Gut Microbiota

There were nine core genera present in 120 mothers which is composed by *Subdoligranulum, Bifidobacterium, Bacteroides, Blautia, Lachnospiraceae spp., Roseburia, Streptococcus, Ruminococcus torques group and Ruminococcaeae* unclassified spp. (**Figure 1B; Supplementary Data**, **Table S1**). The collective core was overwhelmingly dominant (abundance >30%) in almost two thirds of the subjects (64%) but showed dramatic variations in the relative abundance of each genus in different cohorts, regardless of geography, lifestyle, and ethnicity (**Supplementary Data**, **Figure S1**). Spearman's rank correlation test was performed to identify the co-occurrence patterns among the 9 core genera (**Figure 1E**). In general, correlations were found among the core genera components from related genera such as *Lachnospiraceae*

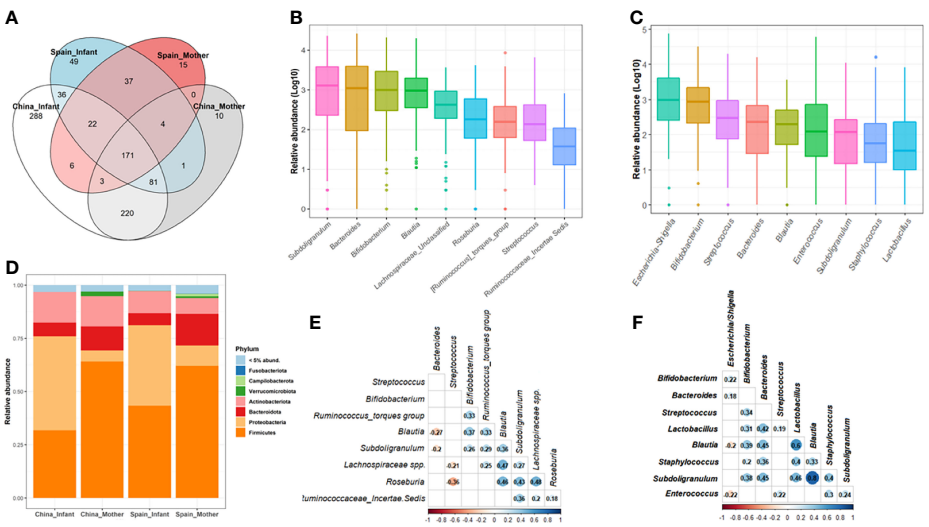

FIGURE 1 | Microbial composition of maternal-neonatal microbiota in both populations, China and Spain. **(A)** Venn diagram of the shared genera between mothers and neonates as well as both populations. **(B, C)** The relative abundance distribution of the core genera in the whole maternal **(B)** and neonatal group **(C)**. Microbiota data at genus level was transformed to log10-values for plotting [log (x+1)]. **(D)** The microbial composition of the whole population at phylum level according to both geographical location and mother/infant category. Phyla with a relative abundance lower than 5% were groups as "Others". **(E, F)** Co-occurrence patterns among the core genera across the 120 maternal **(E)** and neonatal **(F)** samples determined as Spearman's correlation. Data were transformed to relative abundance for correlations analysis. Only the significant relations are colored.

and *Ruminococacceae* groups and *Subdoligranulum*, *Blautia* and *Roseburia* with a rho ranged 0.2-0.48. A negative association was found between some of these genera such as *Blautia* (rho=-0.27, p=0.003) and *Suboligranulum* (rho=-0.20, p=0.031) with *Bacteroides* genus.

Despite the common core, significant differences were found in maternal microbiota between China and Spain (Adonis, F.model = 19.30; R2=0.138, *p=0.001*) which were visualized by PCoA (**Figure 2A**). In terms of alpha-diversity, spanish mothers (M-ES) showed higher richness based on Shannon (*p<0.001*) index than chinesse mothers (M-CN) group, but no differences were found in microbial diversity (*p=0.730*) (**Figure 2B**).

Compositional analysis revealed that China mothers harbored a microbiota enriched in Actinobacteriota (*p=0.003*, *q=0.008*) and Verrucomicrobiota (*p=0.006*, *q=0.019*) phyla while mothers from Spain showed higher relative abundance of Proteobacteria (*p=<0.001*, *q<0.001*) and Bacteroidota (*p=0.008*, *q=0.019*). Among the nine core genera present in 120 mothers, the maternal microbiota core had significant difference between the two countries, for *Bifidobacterium (p<0.001, q<0.001), Blautia (p=0.006, q=0.012), Subdoligranulum (p=0.014, q=0.021), Roseburia (p=0.006, q=0.012)* and those groups from *Ruminococcaceae* family (**Figure 2C**). Indeed, the adjusted multivariate analysis revealed that Chinese mother harbored higher relative abundance of *Faecalibacterium* (*q<0.001*), *Ruminococcus gnavus* group (*q<0.001*), *Eubacterium_coprostanoligenes*_group (*q<0.001*) or *Enterococcus* (*q<0.001*), among others compared to Spanish mothers (**Figure 2D**). However, *Finegoldia* (*q<0.001*), *Ezakiella* (*q<0.001*), *Prevotella (q<0.001)* or *Campylobacter* genera had significantly lower relative abundance in the M-CN group than in the M-ES group.

Furthermore, the multivariate analysis revealed that maternal BMI was significantly negative associated to *Eubacterium eligens* group (Coef=-0.84, *q=0.020*), *Lachnospira* (Coef=-0.78, *q=0.021*) and *Oscillonospiraceae* UCG005 (Coef=-0.93, *q=0.023*) after adjustment by covariates (**Supplementary Data**, **Table S2**).

3.4 Impact of Location and Delivery Mode on Neonatal Gut Microbiota

There were nine core/predominant genera present in 120 neonatal microbiota including *Escherichia/Shigella*, *Bifidobacterium*, *Streptococcus*, *Bacteroides* and *Blautia* as the most abundant/prevalent (**Figure 1C** and **Supplementary Data**, **Table S1**). Indeed, these nine collective cores were dominant (abundance >30%) in more than 73% of the subjects (**Supplementary Data**, **Figure S2**). Results of Spearman's rank correlation test showed that except for the *Eschericha/Shigella* genus, most of the other core genera were positively correlated with each other (**Figure 1F**).

Differences in neonatal microbiota between countries were revealed by PCoA (**Figure 3A**) (Adonis, F.model=4.36, R^2=0.034, *p=0.001*). In terms of alpha-diversity, higher microbial richness was observed in Spanish infants compared to those born in China (*p=0.022*) while no differences were found in diversity measured as Chao index (*p=0.590*) (**Figure 3B**). Among nine core genera present in 120 neonates, Mann-Whitney test on the

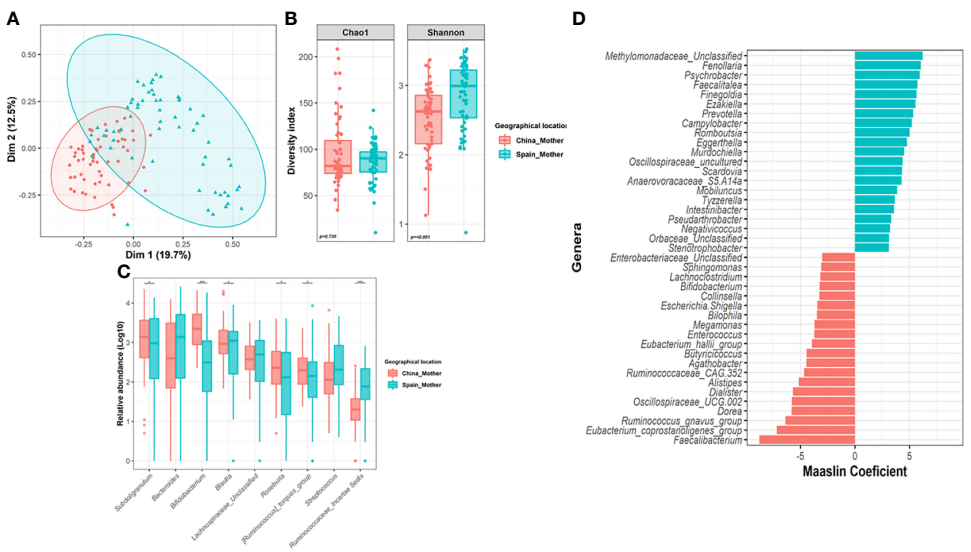

FIGURE 2 | Impact of geographical location on the maternal gut microbiota. **(A)** Principal Co-ordinates Analysis (PCoA) analysis based on Bray-Curtis distance at genus level according to location. **(B)** Differences in alpha-diversity of the maternal microbiota (Shannon and Chao1 index) according to location. Significance of the differences were assessed by Mann-Whitney test. **(C)** Comparison of core genera relative abundance between mothers from both locations. Significance was assessed by Mann-Whitney test after centered log ration transformation (CLR). Microbiota data were transformed to log10-values for plotting. **(D)** Results from the multivariate Maaslin2 analysis showing the differences in terms of microbial composition according to location adjusted by maternal age and body mass index. Only those genera that appeared more than 3 times in at least 10% of samples were included in the analysis. To facilitate the visualization, only those genera with a coefficient higher than 3 was plotted (complete list in **Table S2**). (*P < 0.05; ***P < 0.001).

CLR data, revealed that *Subdoligranulum* (*p<0.001*, *q=0.001*), *Staphylococcus* (*p=0.001*, *q=0.001*) and *Lactobacillus* (*p<0.001*, *q=0.001*), showed a significant difference in terms of relative abundance between the two countries (**Figures 3C, D**). However, in the multivariate analysis, the relative abundance of *Ruminococcus gnavus* group (*q=0.001*), *Enterobacter (q<0.001)* and *Corynebacterium* (*q<0.001*) among others, were observed higher in Chinese neonates compared to Spanish ones, while *Eggerthella* (*q<0.001*), *Finegoldia* (*q<0.001*) or *Prevotella* (*q=0.007*) were all significantly lower in Chinese neonates compared to Spanish ones **(Figures 3C, D; Supplementary Data**, **Table S3**). Furthermore, Chinese neonates group showed higher total bacterial counts (*p=0.001*) than those born from Spain group (**Figure 3E**). Indeed, while no differences in terms of total counts of *Bifidobacterium* were observed according to country (*p=0.542*); higher number of infants from Spanish group showed a *Bifidobacterium* counts lower than the detection values (100% positive samples in China group compared to 77.4% of Spanish samples) (**Figure 3E**).

Delivery mode also showed an effect in the overall β-diversity of the neonatal microbiota at delivery (F.model=3.36, R^2=0.026, *p=0.002*). The multivariate analysis adjusted by nationality demonstrated that vaginally delivered neonates were associated with an increased relative abundance of *Escherichia/shigella* genus (*q=0.001*) (**Supplementary Figure S2** and **Table S2**). However, C-section neonates showed a higher abundance of *Veillonella* (*q=0.001*) genus (**Supplementary Data** and **Figure S3** and **Table S3**). Due to the potential impact of geographical location on specific genera, the microbiota similarity among Chinese neonates (N-CN) and Spanish neonates (N-ES) groups in vaginal and cesarean delivery was assessed separately (**Supplementary Data**, **Figure S3**). Among 120 neonates, within the same delivery mode, the gut microbiota composition showed a significant difference between the two countries (N-CN *vs.* N-ES, Adonis, vaginal delivery, F=2.71, R^2=0.028, *p=0.006*; cesarean delivery, F=3.87, R^2=0.14, *p=0.001)*. Within the same country, while there was no difference observed according to delivery mode in children born in Spain (vaginal *vs.* cesarean, Adonis, N-ES; F.model=1.71, R^2=0.029, *p=0.062*), delivery mode showed an impact in the overall microbial b-diversity in samples from children born in China (vaginal *vs.* cesarean, Adonis, N-CN; F=3.83, R^2=0.062, *p=0.001)*.

The multivariate analysis adjusted by the previously stated covariables also revealed that some genera from neonatal microbiota were negatively associated with maternal BMI, including *Lactobacillus* (*q=0.035*), *Lachnospiraceae_* NK4A136_group (*q=0.003*) and *Ruminococcus* (*q=0.023*), among others (**Supplementary Data**, **Table S3**).

3.5 A Genus-Level Phylogenetic Shared Microbiota in Maternal-Neonate Dyads

For the shared genera between maternal-neonate dyads, simultaneously detected genus at the same maternal-neonate dyads was regarded as a positive shared genus (**Figure 4**). Three

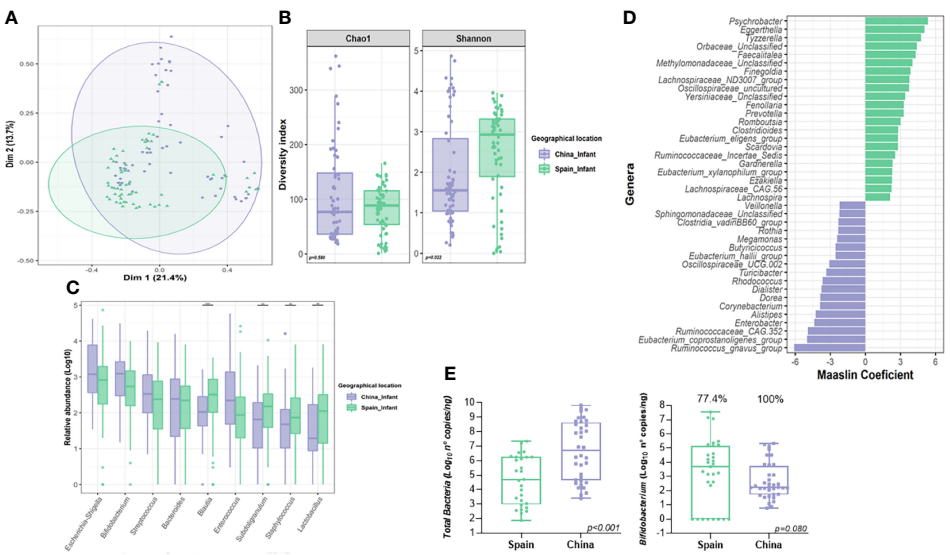

FIGURE 3 | Influence of geographical location on the neonatal gut microbiota. **(A)** Principal Co-ordinates Analysis (PCoA) analysis based on Bray-Curtis distance at genus level according to location. **(B)** Differences in alpha-diversity of the neonatal gut microbiota (Shannon and Chao1 index) according to location. Significance of the differences were assessed by Mann-Whitney test. **(C)** Comparison of core genera relative abundance between neonates from both locations. Significance was assessed by Mann-Whitney test after centered log ration transformation. Microbiota data was transformed to log10-values for plotting. **(D)** Results from the multivariate Maaslin2 analysis showing the differences in terms of microbial composition according to location adjusted by maternal, body mass index and delivery mode. Only those genera that appeared more than 3 times in at least 10% of samples were included in the analysis. To facilitate the visualization, only those genera with a coefficient higher than 2 was plotted (complete list in **Table S3**). **(E)** Comparison of the quantitative analysis of total bacterial and *Bifidobacterium* counts expressed as $\log_{10}$ (number of copies of 16S rRNA gene for each group/ng of DNA). Significance of the differences were assessed by Mann-Whitney test on the log-transformed data. (**P < 0.01; ***P < 0.001).

shared modes were established, M+N+ (genus exists both in the mother and her neonate); M+N- (genus exists only in the mother, not in her neonate); M-N+ (genus does not exist in the mother, only in her neonate).

When the maternal and infant core genera was analyzed, *Bifidobacterium*, *Escherichia/Shigella* or *Streptococcus* were observed in the M+N+ mode in more than 95% of the dyads indicating stable and important functions of those two genera in the gut microbiota for the maternal-neonatal microbial relation. Some genera from the maternal-neonatal core were observed only in mothers in both populations and were rarely observed in an infant without being in the mother including *Blautia*, *Subdoligranulum* or *Lachnospiraceae* spp. and unclassified genera from *Ruminococcaceae* family suggesting a potential maternal transference to the offspring microbiota during the delivery. On the contrary, *Staphylococcus*, specially in Spanish population, showed higher presence of the M-N+ pattern.

4 DISCUSSION

Human gut microbiota co-evolved with the host and participates in maintaining local and distant physiological homeostasis. During the birth process and immediately after, newborns experience vast contact with maternal and surrounding environmental microorganisms, which serve as early inoculation sources affecting short and long-term health outcomes (Bäckhed et al., 2015). Certain features of the infant gut microbiota, such as reduced diversity or atypical composition, have been linked to diseased states in the following childhood or adulthood, including asthma, inflammatory bowel disease or metabolic disorders (Sevelsted et al., 2015; Milani et al., 2017).

Bacterial transference from the mother at birth is one of the main contributors to neonates' first contact with bacterial communities. Multiple factors can affect the "seeding" process and the maternal transference of microorganisms to the newborn, such as mode of delivery, gestational age at birth, antibiotic exposure, maternal diet, neonate feeding mode, environment (family lifestyle and geographical location) or host genetics (Milani et al., 2017). The birth mode is one of the most studied parameters affecting neonatal microbiota acquisition, but population or region-specific factors influencing the microbiota composition of newborns are largely unexplored. Our study aims to assess the impact of the maternal microbiota on the distribution of infant gut microbial communities at birth from two distinct populations, and we proposed a hypothesis that differential microbiota patterns associated with distant geographical locations are also transferable to the newborns.

Different geographical locations are related to specific dietary, behavioral, climatic and economic factors that shape the human microbiota (Benezra et al., 2012). Previous research has shown significant microbiota composition variations in healthy individuals from different races and ethnicities belonging to different or proximal geographical areas (Gupta et al., 2017). The microbiota of children (age from 1-6) of Europe and rural Africa (De Filippo et al., 2010), children (age from 9-14) of urban Bangladesh and suburban United States (Lin et al., 2013), infants (1 year of age) of Caucasian and South Asian descent (Stearns et al., 2017) significantly differ. We observed an effect of geographical location in microbiota profiles of first-pass neonatal and maternal fecal samples from China and Spain.

In our study, nine core genera present in 120 mothers constituting a genus-level phylogenetic core where location (country) is the main contributor of the microbiota variations. Among the core genera, Chinese women had a higher abundance of *Bifidobacterium* and *Subdoligranulum* than their Spanish counterparts, likely promoting maternal-infant transmission at birth and a primary ethnographic impact. The role of birth mode on bacterial transmission has been widely reported, however, recent studies described stable meconium microbiota structure regardless of mode of delivery (Chu et al., 2017). Liu reported three distinct types of meconium samples not influenced by the

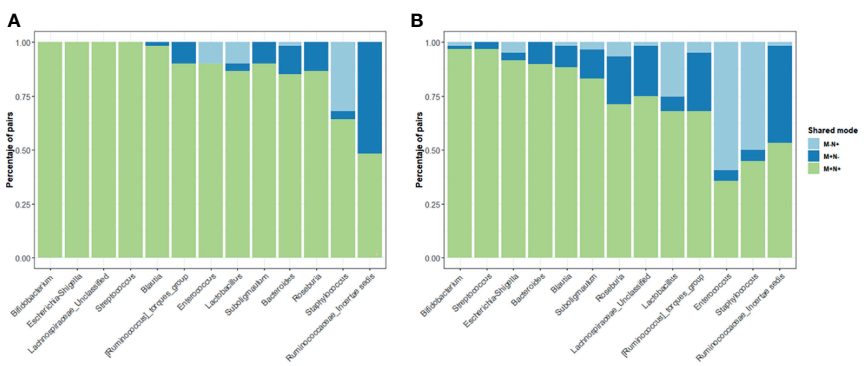

FIGURE 4 | Distribution of the shared genera between mother-neonate dyads Only those genera from both maternal and neonatal core were included in the study of shared genera in China **(A)** and Spain **(B)** populations. Three modes of genera presences/absence were established: M+N+ (genera present in both mother and infant of each dyad), M+N- and M-N+ (genera present in only mother or only neonate, respectively). The percentage of dyads in each share mode were calculated for each core genera.

delivery mode (Liu et al., 2019). In our results, regarding the diversity of neonates' gut microbiota, it seems geographical location has a higher influence than delivery mode. Thus, the first neonatal microbiota acquisition is a multifactorial process that needs to consider multiple factors such as birth mode, geographical location, maternal diet, environmental parameters, drugs/antibiotic exposure, parental contact, and many others still unknown. These results point out the need for further research on the maternal transmission of microbiota to the newborn.

Different studies have determined the microbiota composition of the first pass feces in neonates, showing that *Bifidobacterium*, *Enterobacteriaceae*, *Enterococcaceae*, and *Bacteroides-Prevotella* were prevalent genera (Hansen et al., 2015). Our microbiota in first-pass fecal sample results from 120 neonates are in line with published data, with a core microbiota composed mainly by *Escherichia/Shigella*, *Bifidobacterium*, *Enterococcus*, *Streptococcus* and *Bacteroides* in both geographical locations, however with specific differences in relative abundances between the Spanish and Chinese groups. Differences in first-pass neonatal fecal microbiota would be related to the sampling time as samples from Spain were collected immediately after birth in the delivery room while samples from China were collected during first 24h where the neonate would be exposed to other bacteria. In both cases, the microbial alpha-diversity indexes were comparable to those from the maternal gut and potentially higher than infant gut microbiota during first days, as reported previously (Mueller et al., 2017; Selma-Royo et al., 2020).

In our study, *Bifidobacterium* is the only bacteria shared in almost 100% of the maternal-neonate dyads at birth with no ethno-geographical difference. *Bifidobacterium* is established in the neonatal gut within the first days after delivery, but antibiotic exposure or reduced maternal transference can lag in engrafting on neonatal gut communities. In addition, it has been reported that delayed or disturbed colonization of *Bifidobacterium* during infancy increases the risk of suffering childhood diseases such as asthma or allergies but could also modulate the host health in adulthood by affecting the programming and development of the immune system and the protective functions of epithelial cells (Bailey et al., 2014). Previously, *B. breve*, *B. infantis* and *B. longum* were the most found species in an infant's gut (Grönlund et al., 2011; Mikami et al., 2012; Matsuki et al., 2016). Toda et al. reported 11 isolates of *B. pseudocatenulatum* from a total of 48 isolates of feces and oral fluids of Japanese vaginally born neonates (Toda et al., 2019); in particular, the authors reported a *B. pseudocatenulatum* with high DNA homology in fecal samples of mothers, and oral cavity and fecal samples of the newborns, indicating possible fecal/oral route of transmission during birth. Thus, in vaginally delivered infants, acquisition of *Bifidobacterium* seems to occur *via* an oral route, which would explain the lower prevalence of *Bifidobacterium* species in neonates from cesarean section births.

Previously, Mikami et al., found a significant association between gut maternal colonization by *B. bifidum* and *B. breve* and increased numbers of *Bifidobacterium* species in the infant gut at 1 and 6 months (Mikami et al., 2012). Likely, observed changes in our study could be maintained during a critical window of an infant's immune development (Milani et al., 2017). Recently, Yang et al., studied the fecal microbiota and bifidobacterial communities of 111 healthy Chinese volunteers of varying age profiles, from childhood (1-5 years) to long-lived individuals (≥90 years) (Yang et al., 2020). *Bifidobacterium* species have been isolated from human milk samples, therefore maternal transmission through breastfeeding may be a primary source for the infant gut (Turroni et al., 2019). In our study, the presence of *Bifidobacterium* in first-pass feces may derive from another source than breastmilk. Remarkably, Chinese neonates showed a higher number of positive samples for *Bifidobacterium* genus than Spanish samples. The first-pass fecal samples were obtained during the first 24 hours of life in Chinese population; therefore, some influence of early breastfeeding could be present in the sample. Similarly, Chinese infants showed higher total bacterial counts than the infants form Spain, which could also indicate a potential impact of the first 24h.

On the other hand, the genus only found in neonatal samples are likely acquired by different sources than the mother (M-N+), such as *Staphylococcus* or *Enterococcus* in Spanish neonates. The increase of *Enterococcus* in children has been related with food sensitization (Chen et al., 2016), however, the consequences of differential gut microbiota acquisition and temporal patterns of microbiota evolution in our cohorts would require longitudinal studies assessing specific health outcomes. Compared to the Chinese cohort, the Spanish cohort has more M-N+ samples, likely due to different birth practices (e.g., wiping or aspiring the neonates' mouth, time from birth until breastfeeding or disinfecting products.) or differential environmental microbiota. Adams et al., showed that geography and building type shape indoor environmental microbes (Adams et al., 2015). Lax et al., showed that bacterial communities on patients' skin and hospital room surfaces became similar throughout a patient's stay (Lax et al., 2017), which involves a two-way sharing process between humans and the indoor environment. Indoor hospital microbiota would likely be different between China and Spain, influencing the microorganisms found in both populations. The presence of more shared genera (M+N+) between Chinese mothers and newborns indicates a more efficient vertical transmission of the microbiota, which can be associated to external/environmental influences (e.g., diet, birth practices) or inherent factors from both host (e.g., genetics) or microbiota (e.g., structure, resilience) and deserves further attention.

The acquisition and early maturation of infant microbiota is not well understood despite its likely influence on later health. In our study, the microbiota overlap between the maternal fecal sample and neonatal first-pass feces was minimal, while the similarity between paired maternal-neonate gut microbiota was more pronounced. The sources of a large proportion of infant microbiota could not be identified in maternal microbiota, the sources and function of seeding of infant gut microbiota remain to be elucidated.

This study has important strengths and also, some limitations. We had a bigger sample size compared to the prior

studies of maternal-neonatal gut microbiota at birth time, and it includes gut microbiota data directly from maternal-neonatal dyads in two geographical locations, where the ethnicity, geographical location, maternal diet, and environmental parameters were all in consideration. Other limitation would be associated with the low-biomass present in the samples from first pass neonatal gut microbiota and the potential contaminant removal that would introduce some bias in the data and interpretation. It is difficult to completely control for certain effects, particularly different birth practices for the first-pass fecal samples. Despite those limitations, our main aim was to show the impact of the environment on the maternal-neonatal microbiota. In our study, geographical location would imply different diet, lifestyle, and genetic background, among other parameters. Therefore, it is so difficult to distinguish the main contributors to the maternal-neonatal variation and specific information on diet and lifestyle was not fully completed and not included in the study. Thus, further studies targeted to identify the main contributors are needed.

In all those potential influences, we verified that differential microbiota patterns associated with distant geographical locations are also transferable to newborns. *Bifidobacterium* is the only bacteria shared in almost 100% maternal-neonate dyads. The sources, function and shaping of seeding of neonate gut microbiota need, require more follow-up data and warrants further studies focused on microbial strain-level transference as well as on the potential microbiome functionality. Furthermore, more studies are needed to clarify how geographical location and differences in lifestyles could modify the effect of other perinatal factors on the initial microbial seeding. The consequences of differential gut microbiota acquisition and temporal patterns of microbiota evolution in our cohorts would require longitudinal studies assessing specific health outcomes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA637167, https://www.ncbi.nlm.nih.gov/genbank/, PRJNA614975.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Health Science Center, Xi'an Jiaotong University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The Spanish samples are from a MAMI birth cohort study which was conducted according to the guidelines of the Declaration of Helsinki, and it was approved by the Hospital Clínico Universitario de Valencia and the Bioethics Committee of CSIC (Consejo Superior de Investigaciones Científicas).

AUTHOR CONTRIBUTIONS

Conceptualization, BH and MCC. Methodology, YC and MS-R. Software, XC. Validation, QQ and JZ. Formal analysis, MC, MS-R, and YC. Investigation, QQ and XC. Resources, LZ. Data curation, YC and IG-M. Writing-original draft preparation, BH, MS-R and XC. Writing—review and editing, BH and MCC. Visualization, YC and MS-R. Supervision, BH and MCC. Project administration, LZ, BH, and MCC. Funding acquisition, BH and MCC. All authors agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.663513/full#supplementary-material

REFERENCES

Adams, R. I., Bateman, A. C., Bik, H. M., and Meadow, J. F. (2015). Microbiota of the Indoor Environment: A Meta-Analysis. *Microbiome* 3, 49. doi: 10.1186/s40168-015-0108-3

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and Stabilization of the Human Gut Microbiome During the First Year of Life. *Cell Host Microbe* 17, 690–703. doi: 10.1016/j.chom.2015.05.012

Bailey, L. C., Forrest, C. B., Zhang, P., Richards, T. M., Livshits, A., and DeRusso, P. A. (2014). Association of Antibiotics in Infancy With Early Childhood Obesity. *JAMA Pediatr.* 168, 1063–1069. doi: 10.1001/jamapediatrics.2014.1539

Benezra, A., DeStefano, J., and Gordon, J. I. (2012). Anthropology of Microbes. *Proc. Natl. Acad. Sci. U. S. A* 109, 6378–6381. doi: 10.1073/pnas.1200515109

Benjamini, Y., and Yekutieli, D. (2001). The Control of the False Discovery Rate in Multiple Testing Under Dependency. *Ann. Statist.* 29, 1165–1188. doi: 10.1214/aos/1013699998

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., et al. (2018). Optimizing Taxonomic Classification of Marker Gene Sequences. *Microbiome* 6 (1), 90. doi: 10.1186/s40168-018-0470-z

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat. Biotechnol.* 37, 1091. doi: 10.1038/s41587-019-0252-6

Chen, C. C., Chen, K. J., Kong, M. S., Chang, H. J., and Huang, J. L. (2016). Alterations in the Gut Microbiotas of Children With Food Sensitization in Early Life. *Pediatr. Allergy Immunol.* 27, 254–262. doi: 10.1111/pai.12522

Chu, D. M., Ma, J., Prince, A. L., Antony, K. M., Seferovic, M. D., and Aagaard, K. M. (2017). Maturation of the Infant Microbiome Community Structure and Function Across Multiple Body Sites and in Relation to Mode of Delivery. *Nat. Med.* 23, 314–326. doi: 10.1038/nm.4272

Clemente, J. C., Ursell, L. K., Parfrey, L. W., and Knight, R. (2012). The Impact of the Gut Microbiota on Human Health: An Integrative View. *Cell* 148, 1258–1270. doi: 10.1016/j.cell.2012.01.035

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., and Callahan, B. J. (2018). Simple Statistical Identification and Removal of Contaminant Sequences in Marker-Gene and Metagenomics Data. *Microbiome* 6 (1), 226. doi: 10.1186/s40168-018-0605-2

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., et al. (2010). Impact of Diet in Shaping Gut Microbiota Revealed by a Comparative Study in Children From Europe and Rural Africa. *Proc. Natl. Acad. Sci. U. S. A.* 107, 14691–14696. doi: 10.1073/pnas.1005963107

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., et al. (2010). Delivery Mode Shapes the Acquisition and Structure of the Initial Microbiota Across Multiple Body Habitats in Newborns. *Proc. Natl. Acad. Sci. U. S. A.* 107, 11971–11975. doi: 10.1073/pnas.1002601107

Farhana, L., Antaki, F., Murshed, F., Mahmud, H., Judd, S. L., Nangia-Makker, P., et al. (2018). Gut Microbiome Profiling and Colorectal Cancer in African Americans and Caucasian Americans. *World J. Gastrointest. Pathophysiol.* 9 (2), 47–58. doi: 10.4291/wjgp.v9.i2.47

Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., et al. (2018). Mother-To-Infant Microbial Transmission From Different Body Sites Shapes the Developing Infant Gut Microbiome. *Cell Host Microbe* 24, 133–145. doi: 10.1016/j.chom.2018.06.005

García-Mantrana, I., Alcántara, C., Selma-Royo, M., Boix-Amorós, A., Dzidic, M., Gimeno-Alcañiz, J., et al. (2019). MAMI: A Birth Cohort Focused on Maternal-Infant Microbiota During Early Life. *BMC Pediatr.* 19, 140. doi: 10.1186/s12887-019-1502-y

Grönlund, M. M., Grześkowiak, Ł., Isolauri, E., and Salminen, S. (2011). Influence of Mother's Intestinal Microbiota on Gut Colonization in the Infant. *Gut Microbes* 2, 227–233. doi: 10.4161/gmic.2.4.16799

Gueimonde, M., Tölkkö, S., Korpimäki, T., and Salminen, S. (2004). New Real-Time Quantitative PCR Procedure for Quantification of Bifidobacteria in Human Fecal Samples. *Appl. Environ. Microbiol.* 70 (7), 4165–4169. doi: 10.1128/AEM.70.7.4165-4169.2004

Gupta, V. K., Paul, S., and Dutta, C. (2017). Geography, Ethnicity or Subsistence-Specific Variations in Human Microbiome Composition and Diversity. *Front. Microbiol.* 8, 1162. doi: 10.3389/fmicb.2017.01162

Hansen, R., Scott, K. P., Khan, S., Martin, J. C., Berry, S. H., Stevenson, M., et al. (2015). First-Pass Meconium Samples From Healthy Term Vaginally-Delivered Neonates: An Analysis of the Microbiota. *PloS One* 10, e0133320. doi: 10.1371/journal.pone.0133320

Jombart, T., and Ahmed, I. (2011). Adegenet 1.3-1: New Tools for the Analysis of Genome-Wide SNP Data. *Bioinformatics* 27 (21), 3070–3071. doi: 10.1093/bioinformatics/btr521

Kaehler, B. D., Bokulich, N. A., McDonald, D., Knight, R., Caporaso, J. G., and Huttley, G.A. (2019). Species Abundance Information Improves Sequence Taxonomy Classification Accuracy. *Nat. Commun.* 10, 4643. doi: 10.1038/s41467-019-12669-6

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-Based Diversity Studies. *Nucleic Acids Res.* 41, e1. doi: 10.1093/nar/gks808

Koleva, P. T., Kim, J. S., Scott, J. A., and Kozyrskyj, A. L. (2015). Microbial Programming of Health and Disease Starts During Fetal Life. *Birth Defects Res. C. Embryo. Today* 105, 265–277. doi: 10.1002/bdrc.21117

Kumar, H., du Toit, E., Kulkarni, A., Aakko, J., Linderborg, K. M., Zhang, Y., et al. (2016). Distinct Patterns in Human Milk Microbiota and Fatty Acid Profiles Across Specific Geographic Locations. *Front. Microbiol.* 7, 1619. doi: 10.3389/fmicb.2016.01619

Lahti, L., and Shetty, S. *Microbiome R Package*. Available at: http://microbiome.github.io.

Lax, S., Sangwan, N., Smith, D., Larsen, P., Handley, K. M., Richardson, M., et al. (2017). Bacterial Colonization and Succession in a Newly Opened Hospital. *Sci. Transl. Med.* 9, eaah6500. doi: 10.1126/scitranslmed.aah6500

Lin, A., Bik, E. M., Costello, E. K., Dethlefsen, L., Haque, R., Relman, D. A., et al. (2013). Distinct Distal Gut Microbiome Diversity and Composition in Healthy Children From Bangladesh and the United States. *PloS One* 8, e53838. doi: 10.1371/journal.pone.0053838

Liu, C. J., Liang, X., Niu, Z. Y., Jin, Q., Zeng, X. Q., Wang, W. X., et al. (2019). Is the Delivery Mode a Critical Factor for the Microbial Communities in the Meconium? *EBioMedicine* 49, 354–363. doi: 10.1016/j.ebiom.2019.10.045

Magoč, T., and Salzberg, S. L. (2011). FLASH: Fast Length Adjustment of Short Reads to Improve Genome Assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Mallick, H., Rahnavard, A., McIver, L. J., Ma, S., Zhang, Y., Nguyen, L. H., et al. (2021). Multivariable Association Discovery in Population-Scale Meta-Omics Studies. *PloS Comput. Biol.* 17 (11), e1009442. doi: 10.1371/journal.pcbi.1009442

Matsuki, T., Yahagi, K., Mori, H., Matsumoto, H., Hara, T., Tajima, S., et al. (2016). A Key Genetic Factor for Fucosyllactose Utilization Affects Infant Gut Microbiota Development. *Nat. Commun.* 7, 11939. doi: 10.1038/ncomms11939

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PloS One* 8 (4), e61217. doi: 10.1371/journal.pone.0061217

Michelsen, C. F., Pedas, P., Glaring, M. A., Schjoerring, J. K., and Stougaard, P. (2014). Bacterial Diversity in Greenlandic Soils as Affected by Potato Cropping and Inorganic Versus Organic Fertilization. *Polar. Biol.* 37, 61–71. doi: 10.1007/s00300-013-1410-9

Mikami, K., Kimura, M., and Takahashi, H. (2012). Influence of Maternal Bifidobacteria on the Development of Gut Bifidobacteria in Infants. *Pharm. (Basel)* 5, 629–642. doi: 10.3390/ph5060629

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., et al. (2017). The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiol. Mol. Biol. Rev.* 81, e00036–e00017. doi: 10.1128/MMBR.00036-17

Mueller, N. T., Shin, H., Pizoni, A., Werlang, I. C., Matte, U., Goldani, M. Z., et al. (2017). Delivery Mode and the Transition of Pioneering Gut-Microbiota Structure, Composition and Predicted Metabolic Function. *Genes (Basel)* 8, 364. doi: 10.3390/genes8120364

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2019). "Vegan: Community Ecology Package," in *R Package Version 2*, 5–5.

R Core Team. (2019). "R: A Language and Environment for Statistical Computing," in *R Foundation for Statistical Computing* (Vienna, Austria).

Selma-Royo, M., Calatayud, A. M., García-Mantrana, I., Parra-Llorca, A., Escuriet, R., Martínez-Costa, C., et al. (2020). Perinatal Environment Shapes Microbiota Colonization and Infant Growth: Impact on Host Response and Intestinal Function. *Microbiome* 8, 167. doi: 10.1186/s40168-020-00940-8

Sevelsted, A., Stokholm, J., Bønnelykke, K., and Bisgaard, H. (2015). Cesarean Section and Chronic Immune Disorders. *Pediatrics* 135, e92–e98. doi: 10.1542/peds.2014-0596

Singh, A., and Mittal, M. (2020). Neonatal Microbiome - A Brief Review. *J. Matern. Fetal. Neonatal. Med.* 33, 3841–3848. doi: 10.1080/14767058.2019.1583738

Sprockett, D., Fukami, T., and Relman, D. A. (2018). Role of Priority Effects in the Early-Life Assembly of the Gut Microbiota. *Nat. Rev. Gastroenterol. Hepatol.* 15, 197–205. doi: 10.1038/nrgastro.2017.173

Stearns, J. C., Zulyniak, M. A., de Souza, R. J., Campbell, N. C., Fontes, M., Shaikh, M., et al. (2017). Ethnic and Diet-Related Differences in the Healthy Infant Microbiome. *Genome Med.* 9, 32. doi: 10.1186/s13073-017-0421-5

Toda, K., Hisata, K., Satoh, T., Katsumata, N., Odamaki, T., Mitsuyama, E., et al. (2019). Neonatal Oral Fluid as a Transmission Route for Bifidobacteria to

the Infant Gut Immediately After Birth. *Sci. Rep.* 9, 8692. doi: 10.1038/s41598-019-45198-9

Turroni, F., Duranti, S., Milani, C., Lugli, G. A., van Sinderen, D., and Ventura, M. (2019). *Bifidobacterium Bifidum*: A Key Member of the Early Human Gut Microbiota. *Microorganisms* 7, 544. doi: 10.3390/microorganisms7110544

Wei, T., and Simko, W. (2021). *R Package 'Corrplot': Visualization of a Correlation Matrix (Version 0.92).* Available at: https://github.com/taiyun/corrplot.

Wickham, H. (2016). "Ggplot2: Elegant Graphics for Data Analysis," in *Mastering the Grammar* (Springer-Verlag New York), 27–40.

Yang, B., Yan, S., Chen, Y., Ross, R. P., Stanton, C., Zhao, J., et al. (2020). Diversity of Gut Microbiota and Bifidobacterial Community of Chinese Subjects of Different Ages and From Different Regions. *Microorganisms* 8, 1108. doi: 10.3390/microorganisms8081108

Yarza, P., Richter, M., Peplies, J., Euzeby, J., Amann, R., Schleifer, K. H., et al. (2008). The All-Species Living Tree Project: A 16S rRNA-Based Phylogenetic Tree of All Sequenced Type Strains. *Syst. Appl. Microbiol.* 31, 241–250. doi: 10.1016/j.syapm.2008.07.001

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