Prenatal vitamin D3 supplementation: pharmacology and offspring health outcomes

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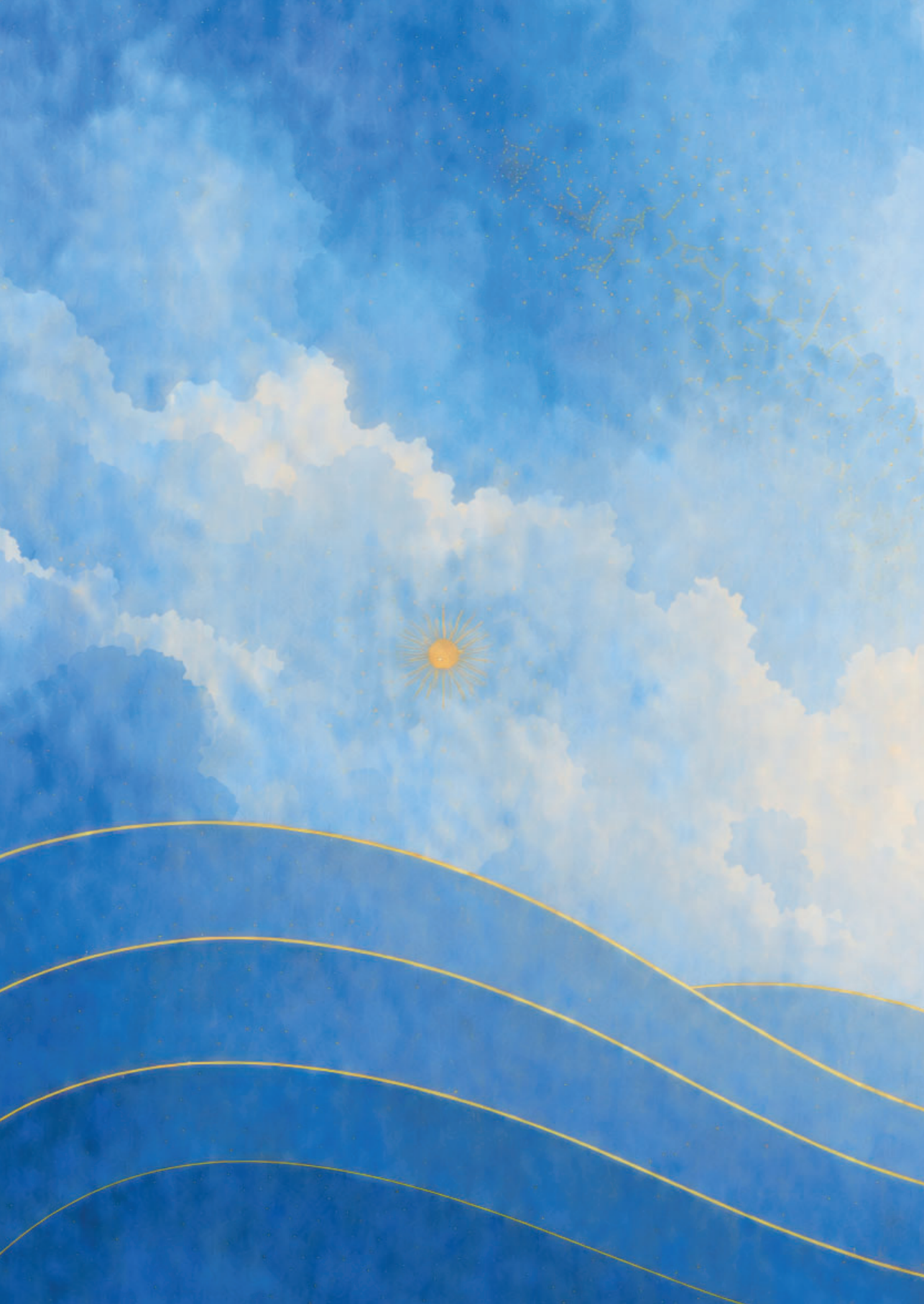

Chapter 9

Early life gut microbiome in children following spontaneous preterm birth and maternal preeclampsia

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Abstract

The early life microbiome plays an important role in developmental and long-term health outcomes. However, it is unknown whether adverse pregnancy complications affect the offspring's gut microbiome postnatally and in early years. In a longitudinal cohort with a 5-year follow-up of mother-child pairs affected by preeclampsia (PE) or spontaneous preterm birth (sPTB), we evaluated offspring gut alpha and beta diversity as well as taxa abundances considering factors like breastfeeding and mode of delivery. Our study highlights a trend where microbiome diversity exhibits comparable development across adverse and normal pregnancies. However, specific taxa at genus level emerge with distinctive abundances, showing enrichment and/or depletion over time in relation to PE or sPTB. These findings underscore the potential for certain adverse pregnancy complications to induce alterations in the offspring's microbiome during early life. The implications of these findings on the immediate and long-term health of offspring should be investigated in future studies.

Introduction

The gut microbiome plays several essential local and systemic roles in maintaining physiological homeostasis.[1,2] The human gut microbiome has been established to produce beneficial effects locally within the human gastrointestinal tract by providing crosstalk between microbes and the intestinal epithelium.[3,4] Additionally, the gut microbiome influences human health during life through different microbial-derived metabolites that could affect the immune system and its pro-inflammatory responses.[5] Stimulation by surface antigens on micro-organisms influences both innate and adaptive immune system maturation through tolerogenic dendritic cell activation, Th1 cell differentiation, and the generation and expansion of T regulatory cells in peripheral tissues.[4,6,7] Alterations in gut microbes have been linked to disorders of both immune and non-immune origins such as asthma, allergy, obesity, chronic cardiometabolic diseases, and cognitive disorders with life-long consequences.[2,8-10]

Colonization of the infant's gut is postulated to begin before or during birth,[7,11,12] suggesting that perinatal factors could impact the infant microbiome, although in utero colonization remains a matter of debate.[13] Recent studies show that to sustain normal physiological processes and developmental programming, a healthy maternal-fetal interface is dependent on microbial interaction.[13] As such, the initial colonizing microbiota could later translate into risks for a variety of human diseases.[14] Therefore, fetal life and an infant's first few months of life could provide a critical window for the development of a healthy infant microbiome profile.[13,15,16] Factors that influence the formation of the gut microbiome during infancy and childhood may have a significant impact on the development of immune system dysfunctions and disorders that are also non-immune related.[11,17,18]

In early life, the microbiota in the human gut quantitatively provides the most important post-natal source of microbial stimulation of the immune system.[4,6] Therefore, the development of a normal infant gut and the immune system requires interaction between intestinal epithelial cells, lymphoid tissue, and the thriving microbiota. These interactions enable the infant gut to quickly develop tolerance to food antigens and the microbiota itself. This phenomenon further provides a barrier against the penetration of pathogenic microbes into the mucosa and submucosa.[4,6] Furthermore, the early microbiota produces active metabolites such as folate, butyrate, and acetate. These products could epigenetically alter gut epithelium and hepatic and immune cells, affecting developmental programming which might later translate into risks for a variety of human diseases.[19] The early life microbiome is mainly established by the age of 3 years in healthy full-term infants and is a determinant

of the assemblage direction of the adult and life-long microbiome signature.[14,20] However, several early life and perinatal factors could affect microbial colonization and establishment.[21] A neonate may procure its microbiota from the environment during delivery and from its mother via breastfeeding.[21,22] Prenatal complications such as preterm birth (PTB) or preeclampsia (PE) might pose a special risk to the development of the early life microbiome. PTB may be spontaneous preterm birth (sPTB, i.e., following preterm labor, preterm premature rupture of membranes [PPROM], or cervical insufficiency) or it may be indicated by a specific maternal or fetal complication such as PE or placental insufficiency.[23] PPROM defined as rupture of fetal membranes prior to 37 weeks of gestation, complicates approximately 2–4% of all pregnancies and is responsible for 40% of all spontaneous preterm births, while spontaneous preterm labor with intact membranes represents the remaining 60% of sPTB. [24]

Prior studies investigating factors associated with microbial composition and diversity in preterm infants mostly rely on small sizes and restricted groups of infants.[25] No comprehensive studies using prospectively collected data and samples have compared the effect of PE and sPTB on the fetal and infant microbiome. In this investigation, we hypothesized that there would be quantitative differences in early life offspring's microbiome in those born with sPTB and from pregnancies with PE in comparison to those from normal pregnancies. We further investigated whether there would be dissimilarities between subtypes of PE (i.e., with and without preterm birth) and sPTB with or without PPROM. Finally, we examined whether breastfeeding and vaginal delivery were associated with taxa that may be affected by PE or sPTB. For these purposes, we assessed the abundance of bacterial taxa by 16S rRNA gene sequencing in stool samples from infants at ages 3–6 months, 1 year, 3 years, 4 and 5 years, born to pregnant participants in the Vitamin D Antenatal Asthma Reduction Trial (VDAART). VDAART was a clinical trial of prenatal Vitamin D supplementation to assess the adverse outcomes in pregnant participants during pregnancy and their offspring at age of six years.[26]

Results

Study population

Eight hundred and six mother-child pairs comprised the ITT cohort of VDAART, 671 of whom provided stool samples with suitable quality for 16S rRNA gene sequencing (**Figure 9.1**). Samples were collected between ages 3–6 months, at year 1, year 3, year 4 and year 5 (**Online Figure 1**). Overall, the subjects who were included in this ancillary study

had similar characteristics to the VDAART ITT cohort (Online Table 1), except for a lower percentage of study subjects at the Boston and San Diego clinical sites and a higher percentage of subjects at the Seattle site in the group included in the current study. Of the included mothers (N=671), 63 (9.4%) had PE during pregnancy of which 17 with PTB, 36 (5.4%) had sPTB of which 12 with PPROM, and 572 (85.2%) had a normal pregnancy. Comparisons of characteristics across categories showed that infants born to mothers with PE or sPTB were more likely to have received perinatal antibiotics and have low birth weight (Chi square $p<0.001$, **Table 9.1**).

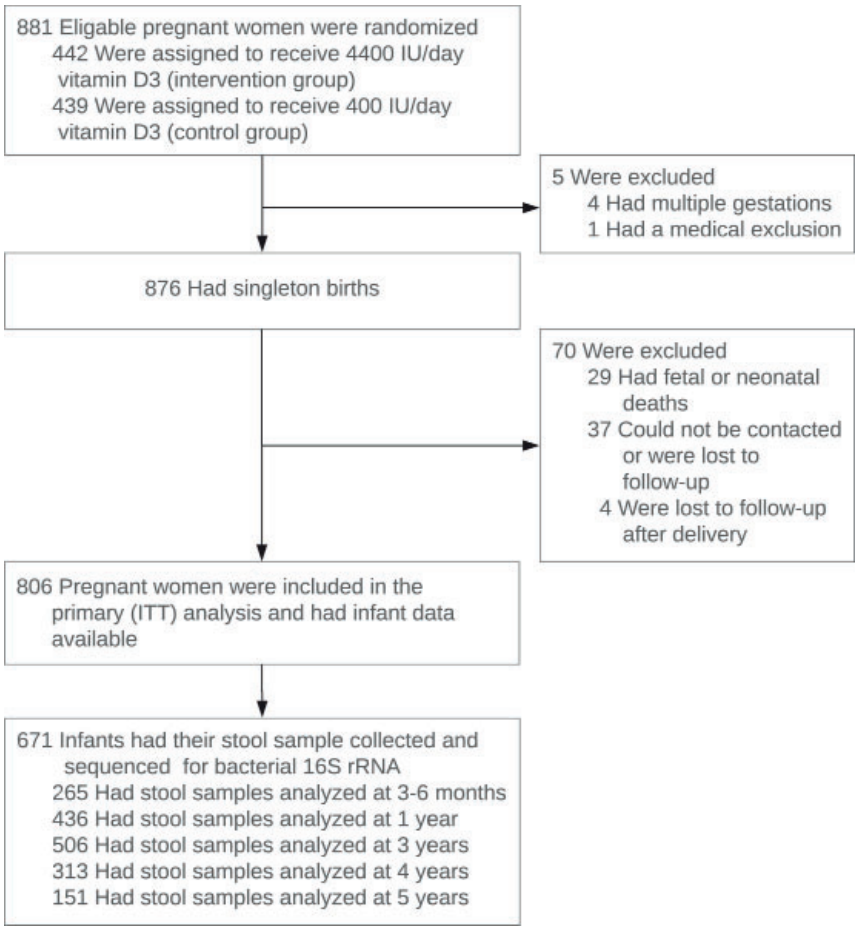


Figure 9.1: VDAART and the Ancillary study participant flow.

Table 9.1: Characteristics and comparisons of subgroups included in the study

Characteristics	Preeclampsia N=63	sPTB[3] N=36	Normal pregnancy N=572	p-value[2]
Maternal age – years[1]	25.9 (5.1)	27.6 (5.8)	27.6 (5.5)	0.07
Study site, n (%)				0.06
Boston	11 (18)	7 (19)	167 (29)	
San Diego	17 (27)	12 (33)	188 (33)	
Seattle	35 (56)	17 (47)	217 (38)	
Child race, n (%)				0.31
White	17 (27)	13 (36)	194 (34)	
African American	38 (61)	18 (50)	270 (48)	
Other	7 (11)	5 (14)	103 (18)	
Missing data	1 (1.6)	0 (0.0)	5 (0.9)	0.49
Infant antibiotic administered, n (%)	14 (22)	15 (42)	35 (6.2)	<0.001
Missing data	0 (0.0)	0 (0.0)	3 (1)	0.77
Fetal sex - male, n (%)	37 (59)	19 (53)	303 (53)	0.68
Treatment group, n (%)				0.82
4,400 IU vitamin D/daily	31 (49)	20 (56)	289 (51)	
400 IU vitamin D/daily	32 (51)	16 (44)	283 (50)	
Gestational age at delivery – weeks[1]	37.9 (2.9)	33.9 (3.3)	39.3 (1.2)	<0.001
34–37 weeks, n (%)	11 (18)	24 (67)	7 (1.2)	<0.001
<34 weeks, n (%)	6 (10)	12 (33)	1 (0.2)	<0.001
C-section pregnancy, n (%)	29 (46)	7 (19)	166 (29)	0.07
Breastfed, n (%)	23 (38)	18 (53)	311 (58)	0.02
Missing data	3 (4.8)	2 (5.6)	33 (5.8)	0.95
Low birth weight – <2,500 grams, n (%)	16 (25)	17 (47)	25 (4)	<0.001
Stool samples				
Months 3–6	26 (41)	15 (42)	224 (39)	0.91
Year 1	43 (68)	26 (72)	367 (64)	0.52
Year 3	48 (76)	33 (92)	424 (74)	0.06
Year 4	32 (51)	17 (47)	264 (46)	0.78
Year 5	14 (22)	8 (22)	129 (23)	0.99

[1] Mean (Standard Deviation).
[2] Welch Two Sample t-test; Pearson's Chi-squared test; Fisher's exact test.
[3] Spontaneous preterm birth.

Preeclampsia, spontaneous preterm birth, and early life fecal microbiome diversity

We used alpha and beta diversity measures to compare overall microbial richness and evenness between study groups (phenotypes). Temporal changes in alpha diversity assessed by the Shannon index in children's stool showed the lowest measure at 3–6 months and the alpha diversity measure gradually increased by the age of 3 years. Between the ages of 3 and 5 years, the diversity metrics reached a plateau. Overall, similar patterns by the age of 5 years were observed among children with sPTB and those born to mothers with PE, such that the diversity metrics were not different from those of children born to normal pregnancies (**Figure 9.2, A&B**). However, a moderately higher alpha diversity was observed at 3–6 months and the 5th year among children born to mothers with PE as compared to those with uncomplicated

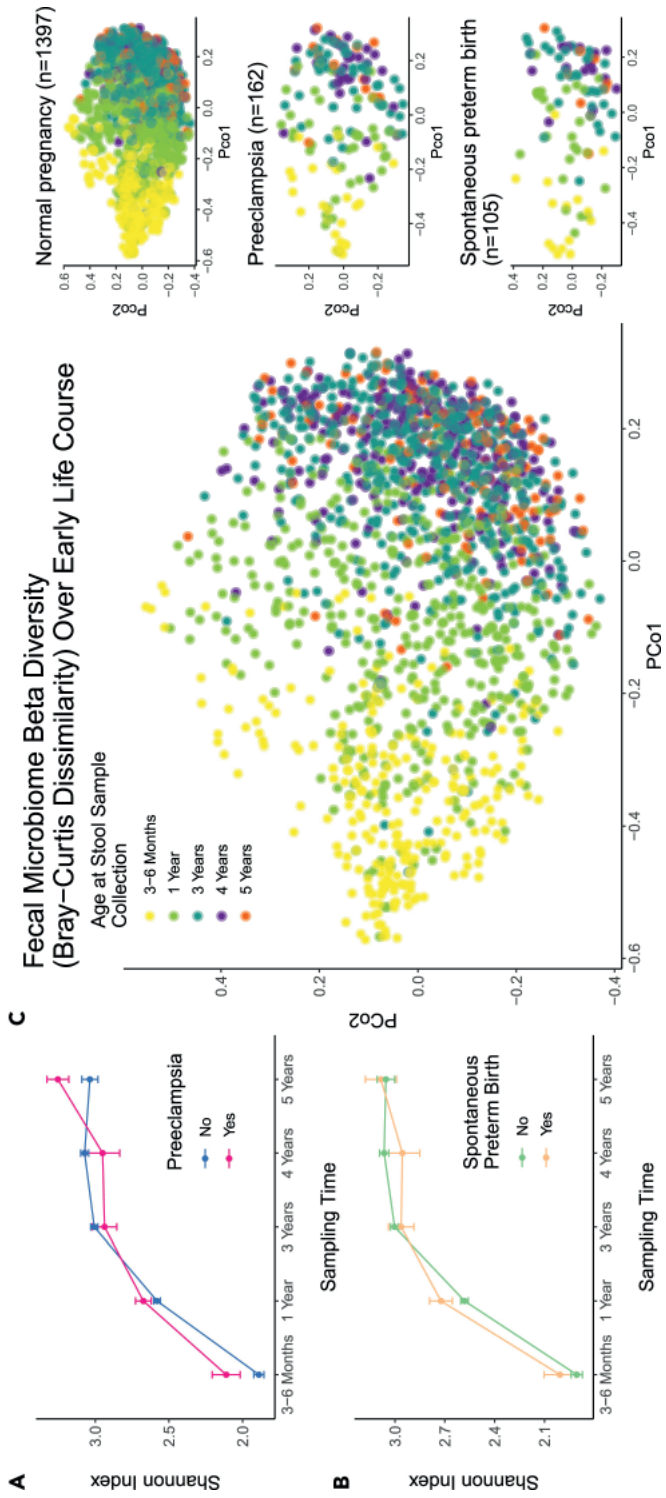


Figure 9.2: Infant gut microbiome diversity trajectories from birth to age 5 years. (A) Line plot showing the mean alpha diversity (Shannon Index) with standard error bars at each time point by preeclampsia (PE) phenotype. **(B)** Line plot showing the mean alpha diversity (Shannon Index) with standard error bars at each time point by spontaneous preterm birth (sPTB) phenotype. **(C)** Principal Coordinates Analysis (PCoA) plots with Bray-Curtis dissimilarity, showing infant gut microbiome compositions of all subjects, controls, and by PE and sPTB phenotypes, respectively. Data points are colored by age at stool sample collection.

pregnancy and normal delivery (controls). After adjustment for potential confounders, alpha diversity at the age of 3-6 months was still higher in infants born to pregnancies complicated with PE (adjusted β=0.24; 95% CI: 0.03, 0.46; p=0.028, **Online Table 2**). No difference in alpha diversity was observed between children with sPTB and those with normal pregnancy and delivery in all study time points (adjusted p's>0.05, **Online Table 2**).

The quantification of the compositional dissimilarity (beta diversity) over early life, assessed by Bray-Curtis dissimilarity index, showed temporal shifts from infancy to age 3 years, being largely established between 3–5 years (**Figure 9.2C**). No temporal differences in beta diversity were observed in the stool of children born with sPTB or born to pregnancies complicated with PE as compared to those of children born to healthy pregnancies with normal deliveries in both unadjusted and adjusted models (PERMANOVA F-statistic range = 0.46–1.26; p's>0.05, **Online Table 3** and **Online Figure 2**).

Preeclampsia and spontaneous preterm birth, and early life fecal microbial taxa

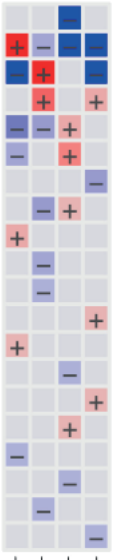
The results of longitudinal analysis using the full linear mixed model are presented in **Table 9.2**, **Table 9.3**, **Online Table 4** and **Online Table 5**, and the univariable mixed model in Online Table 6. We identified seven and eight fecal microbiome taxa at the genus level associated with PE and sPTB, respectively, in comparison to controls from normal pregnancies (q's<0.05, **Table 9.2** and **Figure 9.3**). The dominant phyla with altered abundance were similar in both phenotypes and included Firmicutes, Actinobacteria, and Fusobacteriota. At the genus level, the altered taxa in association with PE included lower abundance of lachnospiraceae *UCG-003*, *Leptotrichia*, *Lachnospira* and *Christensenella*, and higher abundance of *Lawsonella*, *Caproiciproducens* and *Robinsoniella*. Fecal genera associated with sPTB showed a lower abundance of *Moryella*, *Lawsonella*, *Negativicoccus* and *Terrisporobacter*, as well as a higher abundance of *Lachnospira*, *Leptotrichia*, *Enterococcaceae* and *Enterorhabdus*.

In the sub-phenotype analyses, we identified taxonomical differences between PE with and without PTB, and sPTB with and without PPROM in comparison with children born following healthy pregnancies (q's<0.05, **Table 9.3** and **Figure 9.4**). Bacteria of the phyla Firmicutes and Proteobacteria were altered in PE cases with PTB, whereas the phyla Firmicutes and Actinobateriota were dominant in the associations with PE without PTB. Six identified associations with PE and PTB included lower abundance of *Paludicola*, *Eubacterium Ruminantium*, *Sarcina*, *Tuzzerella*, *Mitochondria* and *Dorea*. PE without PTB was associated with a lower abundance of *Lachnospira*, Lachnospiraceae *GCA-900066575* and *Eggerthellaceae*, and a higher microbial gut abundance of *Robinsoniella*.

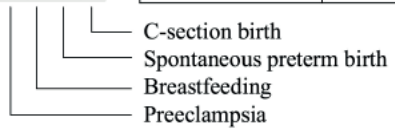
Table 9.2: Adjusted analysis of altered early life gut microbiota in offspring of main phenotype, including main explanatory variables, in comparison to offspring with normal pregnancies (controls) at q<0.05 level

Feature	Phylum	Class	Order	Family	Genus	Coef. [CI]*	p-value	q-value
Preeclampsia (with PTB or without PTB)	Actinobacteriota	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Lawsonella*	11.7 [10.1, 13.3]	<0.001	<0.001
	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*UCG-003*	-0.9 [-1.1, -0.6]	<0.001	<0.001
	Fusobacteriota	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	*Leptotrichia*	-9.9 [-14.2, -5.6]	<0.001	<0.001
	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Lachnospira*	-3.1 [-4.8, -1.3]	<0.001	0.008
	Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	*Caproiciproducens*	7.3 [2.9, 11.7]	0.001	0.01
	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Robinsoniella*	5.4 [2.0, 8.7]	0.002	0.02
	Firmicutes	Clostridia	Christensenellales	Christensenellaceae	*Christensenella*	-6.3 [-10.6, -2.1]	0.003	0.04
Spontaneous preterm birth (with PPROM or without PPROM)	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Moryella*	-0.2 [-0.3, -0.2]	<0.001	<0.001
	Actinobacteriota	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Lawsonella*	-20.7 [-23.5, 17.9]	<0.001	<0.001
	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Lachnospira*	1.7 [0.9, 2.4]	<0.001	<0.001
	Fusobacteriota	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	*Leptotrichia*	12.1 [5.0, 19.2]	<0.001	0.01
	Firmicutes	Negativicutes	Veillonellales-Selenomonadales	Veillonellaceae	*Negativicoccus*	-1.9 [-3.1, -0.7]	0.002	0.02
	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	-	4.5 [1.5, 7.5]	0.003	0.04
	Actinobacteriota	Coriobacteriia	Coriobacteriales	Eggerthellaceae	*Enterorhabdus*	0.9 [0.3, 1.4]	0.003	0.04
	Firmicutes	Clostridia	Peptostreptococcales-Tissierellales	Peptostreptococcaceae	*Terrisporobacter*	-0.4 [-0.6, -0.1]	0.005	0.047
Breastfeeding	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*UCG-003*	3.6 [2.8, 4.4]	<0.001	<0.001
	Firmicutes	Clostridia	Oscillospirales	Oscillospiraceae	*Pseudoflavonifractor*	1.5 [0.9, 2.0]	<0.001	<0.001
	Fusobacteriota	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	*Leptotrichia*	-12.8 [-19.8, -5.8]	<0.001	0.005
	Actinobacteriota	Coriobacteriia	Coriobacteriales	Eggerthellaceae	*Enterorhabdus*	-0.7 [-1.2, -0.3]	<0.001	0.005
	Actinobacteriota	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Lawsonella*	-0.6 [-0.9, -0.3]	<0.001	0.007
	Firmicutes	Clostridia	Eubacteriales	Anaerofustaceae	*Anaerofustis*	-0.7 [-1.1, -0.3]	0.001	0.02
	Actinobacteriota	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	*Collinsella*	-0.2 [-0.2, -0.1]	0.001	0.02
	Firmicutes	Clostridia	Peptostreptococcales- Tissierellales	Peptostreptococcaceae	-	-0.2 [-0.3, -0.1]	0.004	0.047
C-section birth	Actinobacteriota	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Lawsonella*	-12.4 [-14.2,-10.7]	<0.001	<0.001
	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*UCG-003*	-1.4 [-1.7, -1.0]	<0.001	<0.001
	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium sensu stricto 2*	-2.0 [-3.0, -0.9]	<0.001	0.004
	Firmicutes	Clostridia	Oscillospirales	Oscillospiraceae	*Pseudoflavonifractor*	0.6 [0.3, 1.0]	0.001	0.01
	Bacteroidota	Bacteroidia	Bacteroidales	Dysgonomonadaceae	*Dysgonomonas*	2.7 [1.0, 4.4]	0.001	0.02
	Actinobacteriota	Coriobacteriia	Coriobacteriales	Eggerthellaceae	*Senegalimassilia*	0.4 [0.2, 0.7]	0.003	0.03
	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Marvinbryantia*	-0.3 [-0.6, -0.1]	0.004	0.049

Coefficients indicate the enrichment or depletion, and the effect size, of the specific taxa associated with the exposure phenotypes as compared to healthy pregnancies. Results are from mixed effect models adjusted for age at stool sample collection, breastfeeding in the first six months, antibiotic administration, Cesarean section, sex, and race of the child as fixed variables, with subject identifier and clinical center as random effects. * 95% confidence interval.



Phylum	Class	Order	Family	Genus
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Moryella*
Actinobacteriota	Actinobacteria	Corynebacteriales	Corynebacteriales	*Lawsonella*
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*UCG-003*
Firmicutes	Clostridia	Oscillospirales	Oscillospiraceae	*Pseudoflavonifractor*
Fusobacteriota	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	*Leptotrichia*
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Lachnospira*
Firmicutes	Clostridia	Clostridiales	Clostridiidiaceae	*Clostridium sensu stricto 2*
Actinobacteriota	Coriobacteriia	Coriobacteriales	Eggerthellaceae	*Enterorhabdus*
Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	*Caproiciproducens*
Firmicutes	Clostridia	Eubacteriales	Anaerofustaceae	*Anaerofustis*
Actinobacteriota	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	*Collinsella*
Bacteroidota	Bacteroidia	Bacteroidales	Dysgonomonadaceae	*Dysgonomonas*
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Robinsoniella*
Firmicutes	Negativicutes	Veillonellales-Selenomonadales	Veillonellaceae	*Negativicoccus*
Actinobacteriota	Coriobacteriia	Coriobacteriales	Eggerthellaceae	*Senegalimassilia*
Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	-
Firmicutes	Clostridia	Christensenellales	Christensenellaceae	*Christensenella*
Firmicutes	Clostridia	Peptrostreptococcales-Tissierellales	Peptrostreptococcaceae	*Terrisporobacter*
Firmicutes	Clostridia	Peptrostreptococcales-Tissierellales	Peptrostreptococcaceae	-
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Marvinbryantia*



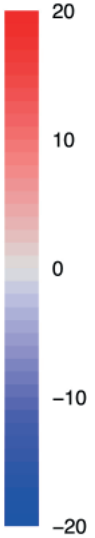


Figure 9.3: Heat map depicting the coefficients of associations between the variables of interest in the primary analysis and the significantly associated microbes (q<0.05). Coefficients were calculated as (−log(qval)*sign(coeff)) and show enrichment (+) or depletion (-) in red and blue respectively.

Table 9.3: Adjusted analysis of the altered early life gut microbiome in the offspring of the subphenotypes in comparison to offspring with normal pregnancies (controls) at q<0.05 level

Feature	Phylum	Class	Order	Family	Genus	Coef. [CI]*	p-value	q-value
Preeclampsia with PTB	Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	*Paludicola*	-10.6 [-14.0, -7.2]	<0.001	<0.001
	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Eubacterium Ruminantium*	-0.2 [-0.3, -0.1]	<0.001	<0.001
	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Sarcina*	-4.4 [-6.6, -2.3]	<0.001	<0.001
	Firmicutes	Clostridia	Lactobacillales	Lachnospiraceae	*Tuzzerella*	-4.1 [-6.3, -1.9]	<0.001	0.004
	Proteobacteria	Alphaproteobacteria	Rickettsiales	Mitochondria	*Mitochondria*	-6.6 [-10.8, -2.3]	0.002	0.03
	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Dorea*	-0.4 [-0.7, -0.2]	0.003	0.03
without PTB	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Lachnospira*	-1.2 [-1.6, -0.8]	<0.001	<0.001
	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*GCA-900066575*	-1.4 [-2.2, -0.7]	<0.001	0.004
	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Robinsoniella*	5.7 [2.3, 9.1]	0.001	0.02
	Actinobateriota	Coriobacteriia	Coriobacteriales	Eggerthellaceae	-	-8.8 [-14.6, -3.0]	0.003	0.04
Spontaneous preterm birth with PPROM	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Lachnospira*	1.7 [1.5, 1.8]	<0.001	<0.001
	Firmicutes	Clostridia	Oscillospirales	Butyricicoccaceae	-	-17.9 [-19.8, 16.0]	<0.001	<0.001
	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Eubacterium ruminantium*	-0.2 [-0.3, -0.1]	<0.001	<0.001
	Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	*Subdoligranulum*	-0.5 [-0.8, -0.2]	0.001	0.02
without PPROM	Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	*DTU089*	3.1 [2.5, 3.7]	<0.001	<0.001
	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Weisella*	10.6 [6.5, 14.6]	<0.001	<0.001
	Firmicutes	Bacteroidia	Peptostreptococcales-Tissierellales	Peptostreptococcaceae	*Terrisporobacter*	-0.5 [-0.8, -0.2]	0.002	0.03

Coefficients indicate the enrichment or depletion, and the effect size, of the specific taxa associated with the exposure phenotypes as compared to healthy pregnancies. Results are from mixed effect models adjusted for age at stool sample collection, breastfeeding in the first six months, antibiotic administration, Cesarean section, sex, and race of the child as fixed variables, with subject identifier and clinical center as random effects.
* 95% confidence interval.

Children in both subcategories of sPTB showed differentially abundant genera within the phylum Firmicutes. Of the four offspring taxa associated with sPTB due to PPROM, *Lachnospira* was found to have a higher abundance, while the genera *Butyricicoccaceae*, *Eubacterium Ruminantium* and *Subdoligranulum* had lower gut abundance. Conversely, Ruminococcaceae *DTU089* and *Weisella* were increased in sPTB without PPROM and *Terrisporobacter* was decreased.

Mode of delivery, breastfeeding and altered microbiome taxa in associations with preeclampsia and spontaneous preterm birth

The primary analysis showed independent associations of breastfeeding and mode of delivery with children's early life gut microbiome compositional taxa (**Table 9.2** and **Figure 9.3**) and alpha diversity over time (**Online Figure 3**).

Early life breastfeeding was associated with higher abundance of two taxa (Lachnospiraceae *UCG-003* and *Pseudoflavonifractor*) and lower abundance of six taxa (*Leptotrichia*, *Enterorhabdus*, *Lawsonella*, *Anaerofustis*, *Collinsella* and *Peptostreptococcaceae*). The enrichment of Lachnospiraceae *UCG-003* and depletion of *Lawsonella* associated with breastfeeding were opposite to the respective underabundance and overabundance observed in association with birth after PE. Moreover, breastfeeding was also associated with lower and higher abundance of *Leptotrichia* and *Enterorhabdus*, respectively, which had opposite associated abundances in the case of birth after sPTB.

C-sectional birth was associated with decreased gut abundance of four taxa (*Lawsonella*, Lachnospiraceae *UCG-003*, *Clostridum sensu stricto* II and *Marvinbryantia*) and increased abundance of three taxa (*Pseudoflavonifractor*, *Dysgonomonas*, *Senegalimassilia*). In the case of Lachnospiraceae *UCG-003*, vaginal delivery had an associated abundance in the opposite direction of PE cases.

Sensitivity analysis

The sensitivity analysis, using the full model on the dataset split at the three-year mark, revealed that the taxa previously identified in the primary analysis (e.g., Lachnospiraceae *UCG-003* for PE and *Terrisporobacter* for sPTB) exhibited associations with PE or sPTB across distinct time frames (**Online Figure 4**). Furthermore, this supplementary analysis unveiled previously unreported taxa linked to the phenotypes of interest during specific stages of offspring microbiome development.

Discussion

We conducted an analysis of pregnancies complicated with PE and sPTB and the impact on the longitudinal microbiome in children from birth to 5 years of age. Overall, these children's gut microbiota showed similar diversity and maturation trajectories compared to their peers with normal delivery. However, infants born to PE pregnancies had a slightly higher number of species and inequality between species abundances (alpha diversity) at 3–6 months of life. Despite the similarities in overall diversity, we observed several imbalances in the relative abundance of offspring gut taxa in relation to PE, sPTB and the subphenotypes as compared to healthy pregnancies during the offspring's early life. Furthermore, we observed that breastfeeding during first six months and vaginal delivery shifted the abundance of genera related to the pregnancy complications. Our results highlight the possibility that while offspring born to pregnancies with adverse outcomes may develop a similar early life microbiome diversity and maturity to their peers born from normal pregnancies, they might have alterations in abundances of specific gut microbial taxa, which may have potential short- and long-term health implications.[27]

Prior studies have mainly explored maternal gut microbiome in a normal pregnancy or in association with PE.[15,28-32] Offspring gut microbiota is expected to show similarities to their mother's microbiota that increase with the age of the children, most probably due to the effect of a shared diet and environment on shaping the microbiota.[29] PE occurrence might disrupt these gut microbial patterns in pregnant participants.[30,33] However, data on the effect of PE, and sPTB, on offspring gut microbiome remains limited. We found that PE was associated with increased alpha diversity at 3–6 months of age compared to controls, which reached to measures comparable to controls at age 1–5 years. This observation might be of importance since the first year of life is crucial for healthy growth as well as being a critical time window for gut colonization.[16,34] While higher alpha diversity is commonly associated with healthier phenotypes,[27,35] it should not be used as an exclusive indicator of gut health, as dysbiosis might manifest as both excessive and insufficient diversity. Notably, mothers diagnosed with PE may exhibit increased alpha diversity,[28] potentially due to immune system dysregulation or antibiotic use.[36] Consequently, these microbial perturbations could be transmitted to their offspring through the birthing process or breastfeeding.[21,22] Finally, it is worth noting that studies establishing higher alpha diversity as more beneficial are primarily in adult and children might exhibit different dynamics.

We did not observe a significant difference in alpha and beta diversity between children born after sPTB compared to term controls at any study time point. A prior investigation on

the neonate's first week of life observed a lower gut microbiota alpha diversity and distinct beta diversity clustering in preterm neonates compared to term neonates.[37] Along with our observations, this could suggest that the influence of sPTB on gut microbiome diversity might be limited to the neonatal period. While our data suggest the impact of PE or sPTB on the global structure of children's gut microbiome might wear off as children grow, their core microbiota might still be affected.[25,35,38] For example in preterm infants, Firmicutes and Actinobacteriota have been shown to be respectively more and less abundant in early life compared to term infants.[39] Accordingly, we identified several altered abundance patterns in the taxa across groups over the course of early life (**Table 9.2** and **Table 9.3**). Our results showed that *Lachnospira* (Firmicutes) and *Leptorichia* (Actinobacteriota) were enriched in offspring born to pregnancies with PE. Of note, an inverse relationship between *Lachnospira* and maternal blood pressure and proteinuria during PE has been observed previously. Occurrence of *Leptotrichia* in the amniotic fluid has been associated with PE and inflammatory pathways in pregnant participants.[40] sPTB was associated with remarkably similar microbial dysbiosis of the phyla Firmicutes, Actinobacteriota and Fusobacteriota, but differences emerged at the genus level. *Lawsonella* was substantially depleted in association with sPTB, in line with reports of an overall reduction of Actinobacteriota as a marker of preterm birth.[37] NICU-associated core microbiota has been demonstrated to compose of Enterococcaceae families in longitudinal analyses of preterm children,[35,38] which also showed increased abundances in our analysis.

Our subphenotype analysis revealed novel taxa associated with different phenotypes of PE and sPTB, likely reflective of their unique underlying mechanisms of disease. On the other hand, expected consistencies with the main phenotypes were observed. For example, the genus *Robinsoniella* was enriched in PE cases and specifically in PE cases without PTB, indicating an exclusive contribution to this clinical presentation of the general pathology. Moreover, in case of the PTB subphenotype of PE, there was an increased similarity at the genus level between PE and sPTB that was previously not observed in the main phenotype analysis, most notably in the decreased abundances of the common gut inhabitant *Eubacterium Ruminantium*. In the same vein, the depletion of the genus *Terrisporobacter* that appeared in children born after sPTB, was pinpointed to instances without PPROM. The neonatal microbiome comes to more closely resemble the maternal microbiome after spontaneous delivery with PPROM in comparison to iatrogenic delivery,[37] and this may drive the distinct patterns between sPTB phenotypes.

Early life breastfeeding and mode of delivery (vaginal or C-section) are commonly indicated as important influencers of microbial composition.[21,22,41,42] While our results also showed

such an independent effect it also indicated that breastfeeding and mode of delivery might change the abundances of the infant's gut microbiota that were also associated with PE or sPTB. However, further research is necessary to determine whether these perinatal outcomes could modify and effectively mitigate the gut microbiome dysbiosis in offspring exposed to PE and sPTB.

As previously observed in healthy children,[43] we found that the impact of C-section and exclusive breastfeeding on the gut microbiota of offspring born from pregnancies complicated by PE or sPTB was normalized over time. Finally, we observed that vaginally delivered and/or breastfed infants exhibited gut microbiota that displayed greater similarity in alpha diversity between case and control groups at age 3–6 months (**Online Figure 3**).

Considering the rapidly increasing knowledge on the connection between microbial dysbiosis and our health, perturbations may pose short- or long-term health risks to children. The ability to assess these risks starts by identifying which early life complications affect the infant microbiome. Then, mitigating factors can be evaluated while leveraging the malleability of the microbiome through targeted interventions to reduce health risks. Based on this study, it may be reasonable to assume that pregnancy complications can impact the infant microbiome long after birth. Moreover, breastfeeding and mode of delivery should be part of the clinical equation when the infant's microbiome is suspected to be compromised in early life.

Future work should further explore transferred microbial alterations from the mother to child in pregnancies with adverse outcomes. Such a future study should try to identify shared species and strain occurrences in affected children over the course of their early life, ideally be means of whole genome sequencing. Additionally, advances in the rapidly evolving field of microbiome research (e.g., replication, standardization, databases and bioinformatic pipeline improvements) could create new insights into public data and previous works.

Limitations of the study

Our study included a diverse and well-characterized cohort of mother-child pairs who were longitudinally followed until the offspring's age of 6 years for the primary outcome of a clinical trial, i.e., VDAART. Pregnancy complications were closely monitored for adverse maternal or fetal events and were a secondary outcome of the trial.

Despite the large sample size of our cohort and its validated phenotypes of sPTB and PE, inclusion of a higher number of cases and deeper metagenomic sequencing will enable a more precise comparison of sub-phenotypes and improve estimated abundances.

Taxonomic resolution of 16S rRNA gene sequencing of the stool samples allowed for sensitive identification down to the genus level, but thereby excluded species information.

In conclusion, we assessed whether offspring born to pregnancies complicated with PE or sPTB demonstrated dysbiosis in their gut microbiome using a longitudinal analysis from birth to 5 years of age. We found that the overall trajectory of microbial diversity observed in affected children may be like those of their peers born to healthy pregnancies. However, perturbations of specific taxa were associated with PE and sPTB, thus highlighting the importance of taking microbiota dynamics in children born with adverse pregnancy outcomes into account.

Ethics approval and consent to participate

All included patients were enrolled in the VDAART study (ClinicalTrials.gov ID NCT00902621), approved by the institutional review boards at each participating institution. All participants provided written informed consent prior to study inclusion. All analyses were performed in accordance with local and international regulations for research ethics in human subject research. This study conformed to the principles of the Helsinki Declaration.

Authors' contributions

Funding: VC, AL, STW. Concept and design: IS, KLS, GOC, RSZ, LB, VC, AL, STW, HM. Data collection and verification: IS, KLS, HM. Data analysis: IS, KLS, HM. Statistical support: Input from all authors. Manuscript writing and editing: IS and HM with input from all authors. Manuscript approval: All authors read and approved the final manuscript. Agree to be accountable for all aspects of the work: All authors.

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Declaration of interests

Dr. Zeiger reports grants from the NHLBI for the present work. Other grants and personal fees from MERCK & Co; MedImmune/AstraZeneca, Genentech/Novartis, and GSK. Personal fees from Regeneron Pharmaceuticals, UpToDate, and DBV Technologies. Grants from ALK Pharma. TEVA, and Quest. The other authors report no conflicts.

Inclusion and diversity

We support inclusive, diverse, and equitable conduct of research.

STAR Methods

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Microbiome sequencing data used in this study	Gillman et al. [44]	https://dash.nichd.nih.gov/study/417122
99% SILVA 138 database	Quast et al.[45]	http://www.arb-silva.de
Software and algorithms		
R working scripts	This paper	https://github.com/IskanderShadid/Microbiome/blob/main/OffspringMicrobiome_PE_SPTB.Rmd
Vegan v2.6–4 (R package)	Oksanen et al.[46]	https://cran.r-project.org/web/packages/vegan/index.html
Phyloseq v1.40 (R package)	McMurdie and Holmes[47]	https://www.bioconductor.org/packages/release/bioc/html/phyloseq.html
MaAsLin2 (R package)	Mallick et al.[48]	https://www.bioconductor.org/packages/release/bioc/html/Maaslin2.html
Qiime (R package)	Caporaso et al.[49]	http://qiime.org/index-qiime1.html
Qiime2 (R package)	Bolyen et al.[50]	https://qiime2.org/
DADA2 (R package)	Callahan et al.[51]	https://benjjneb.github.io/dada2/

Resource availability

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Prof. Hooman Mirzakhani (hoomi@post.harvard.edu).

Materials availability

This study did not develop new unique reagents or materials.

Data and code availability

- Microbiome sequencing data from VDAART are integrated into the Environmental influences on Child Health Outcomes (ECHO) program, a program sponsored by the National Institutes of Health (NIH). Deidentified data from the ECHO Program are available through NICHD's Data and Specimen Hub (DASH), https://dash.nichd.nih.gov/study/417122. The 99% SILVA 138 database containing representative sequences specific to the 515F/806R region for bacterial data, which was used for taxonomy assignment, can be found at http://www.arb-silva.de.
- All code for analysis and generating individual figure panels are available on GitHub (https://github.com/IskanderShadid/Microbiome/blob/main/OffspringMicrobiome_PE_SPTB.Rmd).
- All relevant data is also available from the Lead Contact and authors upon request.

Experimental model and study participants details

This study is an ancillary analysis of VDAART (NCT00920621), a randomized, double-blind, placebo-controlled trial of pregnant participants randomized to treatment (4,400 IU vitamin D daily) or placebo (multivitamin containing 400 IU vitamin D daily) for the prevention of adverse pregnancy outcomes and offspring asthma (see **Figure 9.1** for participant flow and allocation). Participants were recruited from three sites in the United States: Boston, Massachusetts; Washington University at St Louis, St Louis, Missouri; and Kaiser Permanente Southern California Region, San Diego. Eligible participants were between ages 18–39 years, presenting between gestational ages 10–18 weeks, who themselves, or the other biological parent, had a history of asthma, eczema or allergic rhinitis. Racial/ethnical characteristics of the participants were Asian (4.5%), American Indian or Alaskan Native (1.2%), Black or African American (44.6%), Native Hawaiian or Pacific Islanders (1%), White (40.1%), or other (8.6%), from different socioeconomic backgrounds. The VDAART protocol and the VDAART flora ancillary study were approved by the institutional review

boards (IRB) at each participating institution and at the Brigham and Women's Hospital. All participants provided written consent to participate in the trial and supplementary studies. More details about the parent trial VDAART are previously described.[26]

Method details

Assessment of the exposure variables of interest and co-variates

PE diagnosis during pregnancy and sPTB delivery were the main exposures of interest. After delivery, medical records were abstracted, and a committee of 4 board-certified obstetricians conducted a blinded review of abstracted charts of subjects with a noted diagnosis of hypertension, proteinuria, or preeclampsia to determine PE status. The diagnosis of PE used in VDAART[52] was based on the definition established by the American College of Obstetricians and Gynecologists (ACOG) 2013 Task Force Report on Hypertension in Pregnancy,[53] which included the identification of high blood pressure (BP; ≥140 mm Hg systolic and/or ≥90 mm Hg diastolic at two occasions at least 4 hours apart) and either proteinuria (≥300 mg per 24-hour collection or ≥1+ on a urine dipstick) or the presence of elevated liver enzymes, thrombocytopenia, cerebral or visual disturbances after 20 weeks of gestation. Gestational age at birth was calculated based on the estimated conception date (using ultrasonography) and date of delivery. PTB was defined as the presence of a live birth before 37 ($37^{0/7}$) weeks of gestation among the pregnant participants in VDAART. For this study, participants were excluded if they had PTB due to gestational hypertension during pregnancy, a provider-initiated (induced) delivery, a positive lab test for chorioamnionitis, placental insufficiency or abruption. Therefore, infants born preterm included in this study were those with sPTB, i.e., delivered with the commencement of labor with intact or pre-labor rupture of membrane (idiopathic PTB in the absence or presence of PPROM) at less than 37 weeks of gestation. PPROM, a subset of those with sPTB, refers to those infants born with rupture of the fetal membranes prior to the onset of regular uterine contractions.

Healthy pregnancies were identified among the VDAART participants, ensuring the absence of any adverse events or pregnancy complications throughout the duration of the trial. Offspring born from the pregnancies included in the study also exhibited no adverse health outcomes at the time of stool sample collection.

Additional study variables for this analysis were selected as a priori determinants or potential confounders in the relationship between the infant gut microbiome and sPTB and PE.[11] These variables included age at stool sample collection, clinical site, mode of

delivery (vaginal or Caesarean), exclusive breastfeeding in the first six months of life as assessed by a questionnaire, offspring race, offspring sex, and antibiotic administration in the first week of life were abstracted from medical records.[54,55] Intervention arm of the participants was not included in the model as it was previously shown to have no influence on offspring microbiome at any study timepoints in VDAART.[55]

Stool sample collection and 16S rRNA gene sequencing

Participants were asked to collect ½ teaspoon of their infant's stool using a tongue depressor and store the sample in their home freezer. Frozen stool samples were then brought to the clinical center by the infant's mother within 1–2 days or picked up at participants' homes within 24 hours of collection and stored at -80°C. Stool samples were not collected if the infant had taken antibiotics within the prior 7 days. Samples were analyzed in 671 offspring at ages 3 to 6 months (n=265), 1 year (n=436), 3 years (n=506), 4 years (n=313) and 5 years (n=151). Microbiome profiling was performed by amplifying the bacterial 16S rRNA hypervariable region 4 (V4 515F/806R region)[49] using the Illumina MiSeq platform at Partners Personalized Medicine in Boston, MA.[55]

In detail, approximately 200 mg of stool per sample was resuspended in 1 mL InhibitEX Buffer from Qiagen QIAamp Fast DNA Stool Mini Kit (Qiagen, Catalogue # 51604) in tubes containing 0.1 mm silicon beads (Genesee Scientific, Catalogue # 31-212S1). Samples were disrupted using a Mini-Beadbeater 24 from Biospec Products for 3 minutes. Extraction continued on the contents of the entire tube using the Qiagen QIAamp Fast DNA Stool Mini Kit automated on a Qiagen QIAcube. Resulting DNA quality control was evaluated using a PicoGreen assay (Quant-iT dsDNA assay kit, Thermofisher, catalogue number P7589) on a Gemini XP spectrophotometer from Molecular Devices.

Input into library construction was 15 ng DNA using NEXTflex 16S V4 Amplicon-Seq kit 2.0 (Bioo Scientific, Catalogue # 4203-04). Finished libraries were normalized to 10 nM using PicoGreen quantitation and sizing information gathered using a Tapestation D100 screen tapes and reagents (Agilent, Catalogue # 5067-5582 and 5067-5583). Final quality control was carried out to establish the amount of library containing ligated Illumina adaptors using KAPA Library Quantification Kits (KAPA biosystems, Catalogue # KK4824). Up to 288 libraries were pooled to provide equimolar amounts of each library in the pool, which was then run on Illumina MiSeq using MiSeq Reagent Kit v3 600 cycle kit (MS-102-3003). Each pool contained a 20% spike-in of PhiX Control library v3 (Illumina, Catalogue # FC-110-3001) to increase diversity of the library sequence. FASTQ files were generated on the MiSeq instrument.

Primer and adapter trimming was performed using Skewer. Chimera checking and filtering were performed using Qiime2.[50] Reads were denoised using DADA2 as implemented in Qiime2.[51] Processing of microbiome data was performed using Qiime and Phyloseq package for R.[47] Taxonomy was assigned to representative sequences using a naive Bayes classifier pre-built from the 99% SILVA 138 database specific to the 515F/806R region for bacterial data.[45,46] A total of 14,488 amplicon sequencing variants (ASVs) were detected, corresponding to 402 genera.

Quantification and statistical analysis

All statistical tests were conducted using R version 4.1.1 (R Foundation for Statistical Computing), within RStudio 2022.12.0+353.[54] We compared the study groups based on the exposure of interests (PE, sPTB and normal pregnancies) using Student's t-test and Chi-square or Fisher's exact test, as appropriate.

Preeclampsia, spontaneous preterm birth, and fecal microbial diversity

The microbial diversity of samples at genus level was examined by the diversity within one sample (alpha diversity) and by changes in species abundances across different samples (beta diversity),[57] using the R packages 'Vegan v 2.6-4' and 'Phyloseq v 1.40'.[46,47] Alpha diversity was calculated using the Shannon index. Beta diversity was estimated using the Bray-Curtis dissimilarity. The association of the alpha diversity index with PE and sPTB was examined using linear regression models. Then, we tested the associations of our study exposures with the beta diversity Bray–Curtis distances using the Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA; 1,000 permutations) from Vegan. The models of the associations of alpha and beta diversities with PE and sPTB were adjusted for the VDAART clinical center, mode of delivery, early life antibiotic usage, breastfeeding, and child's race and sex. All tests were two-sided and a p-value less than a pre-specified alpha of 0.05 was considered statistically significant.

Preeclampsia and spontaneous preterm birth, and early life fecal microbial taxa

First, we examined whether the phenotypes of interest (PE or sPTB), independent of other factors and exposures, influence the abundance of offspring's early life microbial taxa using MaAsLin2 (Microbiome Multivariate Association with Linear Models). The analysis was longitudinally performed at the genus level using the R package "MaAsLin2", a comprehensive package for efficiently determining multivariable associations between phenotypes, exposures, covariates, and microbial features. MaAsLin2 relies on general linear models to accommodate most modern epidemiological study designs, including longitudinal data and

offers data normalization and transformation methods.[48] Then, we examined the altered taxa between PE with and without PTB, and sPTB with and without PPROM in comparison with infants with normal delivery. We further examined whether early life breastfeeding and vaginal delivery could affect altered taxa associated with PE or sPTB.

To conservatively address the issue of zero-inflation in longitudinal microbiome data and ensure robust associations even for less common taxa, a minimum abundance threshold was set to test only microbiome features reaching at least a 0.1% relative abundance.[48] Additionally, we filtered the lowest quartile of taxa prevalence in our data (<0.2%). Ultimately, this approach resulted in 1,658 samples and 229 taxa. We used a zero-inflated negative binomial model with trimmed mean of M values (TMM) normalization.[59,60] Diversity in the microbiome can arise depending on the longitudinal changes, microbiome study site and sampling differences per study participant across study time points in a longitudinal study. Therefore, we used linear mixed model analyses to address the potential difference in the microbiome by random variables affecting the observed association with the exposure variables. In the models, the subject identifier and clinical center were added in as random-effect terms along with fixed terms for the age at stool sample collection, mode of delivery, antibiotics, breastfeeding, and the child's race and sex.

Finally, we conducted a sensitivity analysis to pinpoint possible associations between PE or sPTB and infant taxa to specific stages of microbiome development. To retain power and because the infant microbiome is mainly established around age 3 years,[14,20,55] we split the data at the 3 year mark (age at stool sample collection <3 years or ≥3 years) and ran the full MaAsLin2 model in both datasets.

All tests were two-sided and considered significant with p-values adjusted for false discovery rate with the Benjamini-Hochberg method (q-value) <0.05.

Additional resources

Additional information on the trial design of VDAART can be found on ClinicalTrials.gov (https://www.clinicaltrials.gov/study/NCT00920621).

Trial registration

This study is an ancillary analysis from the VDAART, which is registered with ClinicalTrials.gov (NCT00902621).

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