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Research Paper

Oral administration of maternal vaginal microbes at birth to restore gut microbiome development in infants born by caesarean section: A pilot randomised placebo-controlled trial



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ABSTRACT

Background: Birth by caesarean section (CS) is associated with aberrant gut microbiome development and greater disease susceptibility later in life. We investigated whether oral administration of maternal vaginal microbiota to infants born by CS could restore their gut microbiome development in a pilot single-blinded, randomised placebo-controlled trial (Australian New Zealand Clinical Trials Registry, ACTRN12618000339257).

Methods: Pregnant women scheduled for a CS underwent comprehensive antenatal pathogen screening. At birth, healthy neonates were randomised to receive a 3 ml solution of either maternal vaginal microbes (CS-seeded, $n = 12$) or sterile water (CS-placebo, $n = 13$). Vaginally-born neonates were used as the reference control (VB, $n = 22$). Clinical assessments occurred within the first 2 h of birth, and at 1 month and 3 months of age. Infant stool samples and maternal vaginal extracts from CS women underwent shotgun metagenomic sequencing. The primary outcome was gut microbiome composition at 1 month of age. Secondary outcomes included maternal strain engraftment, functional potential of the gut microbiome, anthropometry, body composition, and adverse events.

Findings: Despite the presence of viable microbial cells within transplant solutions, there were no observed differences in gut microbiome composition or functional potential between CS-seeded and CS-placebo infants at 1 month or 3 months of age. Both CS groups displayed the characteristic signature of low *Bacteroides* abundance, which contributed to a number of biosynthesis pathways being underrepresented when compared with VB microbiomes. Maternal vaginal strain engraftment was rare. Vaginal seeding had no observed effects on anthropometry or body composition. There were no serious adverse events associated with treatment.

Interpretation: Our pilot findings question the value of vaginal seeding given that oral administration of maternal vaginal microbiota did not alter early gut microbiome development in CS-born infants. The limited colonisation of maternal vaginal strains suggest that other maternal sources, such as the perianal area, may play a larger role in seeding the neonatal gut microbiome.

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Research in context

Evidence before this study

A previous pilot trial reported partial restoration of the neonatal microbiome in four neonates born by caesarean section that received topical application of maternal vaginal fluids immediately after birth. Microbiome restoration was greater for oral and skin microbiome samples, compared with anal skin samples, suggesting the gut microbiome remained relatively unaffected by vaginal seeding.

Added value of this study

This is the first study to (1) assess oral administration of maternal vaginal microbes as a strategy to restore gut microbiome development in infants born by CS, (2) assess microbial viability of the vaginal seeding inoculum, and (3) report on anthropometric measures and body composition in response to vaginal seeding. We show that oral administration of maternal vaginal microbiota did not alter development of the early gut microbiome of CS infants, nor did it influence infant anthropometric measures or body composition up to 3 months of age.

Implications of all the available evidence

Collectively our results suggest vaginal seeding has negligible impact on infant gut microbiome development, and supports recent findings showing that maternal vaginal strains rarely colonise the infant gut.

1. Introduction

Development of the gut microbiome during early life plays an influential role in facilitating immune development and milk digestion [1,2]. While the debate as to whether microbial colonisation begins *in utero* is ongoing [3–6], the birthing process represents a critical period of microbial exposure. Neonates born by caesarean-section (CS) have consistently been found to harbour distinct gut microbiome profiles compared to vaginally-born (VB) neonates [7–15]. Notably, *Bacteroides* spp. and *Bifidobacterium* spp. are often underrepresented in the microbiomes of CS infants, while species associated with the hospital environment (often possessing virulence and antibiotic resistance genes) are overrepresented [7–10,16,17]. Compared to VB infants, the gut microbiome of CS infants also exhibits a higher degree of strain turnover in early life, with fewer maternally-derived strains [9,10,18], leading to functional differences in the immunostimulatory potential of the gut community [10]. While these effects seem to diminish with time, microbiome differences are still detectable at 1 year of age [9].

Collectively, these alterations in the microbiome of infants born by CS are thought to impair the development of the infant immune system, contributing towards their higher susceptibility to various metabolic and immune disorders later in life [19,20]. However, it should be noted that this hypothesis is mostly based on association studies and is not yet supported by mechanistic evidence. One of the largest association studies to date assessed chronic immune disorders in 1.9 million Danish children, finding modestly higher rates of asthma, systemic connective tissue disorders, juvenile arthritis, inflammatory bowel disease, immune deficiencies, and leukaemia in those born by CS [21]. While subsequent studies have also reported associations between birth by CS and disease risk [22–32], findings have not always been consistent, particularly with regards to obesity [33–35]. Nonetheless, recent research has shown that infants born by CS tend to grow more rapidly during their first year of life compared with their VB counterparts [36] and differences in body mass

index (BMI) have been reported as early as six months of age [35]. Yet whether these growth differences persist into childhood is still unclear [29–35].

If early gut microbiome development does play a critical role in altering disease risk trajectories, microbial interventions may be beneficial for neonates born by CS. Neonatal exposure to maternal vaginal fluids immediately after CS birth (more commonly known as ‘vaginal seeding’) is a potential mechanism to foster the development of the gut microbiome. Vaginal seeding has gained traction in recent years [37,38], despite warnings from some healthcare professionals that this procedure poses considerable infection risks to neonates [39,40]. Notably, evidence for the efficacy of this procedure has not yet been established. One very small pilot trial reported partial microbiota restoration in four neonates who received vaginal seeding following CS [41]. However in their pilot study, vaginal microbiota were only applied to the outer surfaces of the neonates’ skin, anal swabs were used instead of stool samples to profile the gut microbiome, and the researchers did not report on clinical outcomes. We hypothesised that greater restoration might have been achieved had the vaginal microbiota been administered orally [42], particularly given neonatal stomachs remain pH neutral for a few hours after birth [43]. Therefore, we conducted a pilot randomised controlled trial to evaluate the efficacy of vaginal seeding by oral administration at birth to restore gut microbiome development in infants born by CS.

2. Methods

2.1. Ethics

Ethics approval was granted by the Northern A Health and Disability Ethics Committee on 31st May 2018 (18/NTA/49). Participants provided verbal and written informed consent. All procedures in this study were conducted according to the ethical principles and guidelines laid down in the Declaration of Helsinki [44].

2.2. Study design

The Early Colonisation with Bacteria After Birth (ECOBABe) trial was a pilot single-blinded, randomised placebo-controlled trial. Trial registration was with the Australian New Zealand Clinical Trials Registry (ACTRN12618000339257). A detailed account of the study design and methodology can be found in the published trial protocol [42]. In brief, healthy pregnant women (aged ≥ 18 years) carrying singletons and planning either a CS (intervention groups) or a vaginal birth (VB, reference group) were recruited from three hospitals in the Auckland region, New Zealand (Auckland City Hospital, Middlemore Hospital, and North Shore Hospital) between May 2019 and March 2020. Eligible women in the CS groups underwent pathogen screening approximately one week prior to their planned (elective) CS to ensure they did not harbour any transmissible pathogens that could potentially be passed on to the newborn. Women were subsequently excluded if they tested positive for any of the following: Group B *Streptococcus*; hepatitis A, B, or C viruses; human immunodeficiency virus; herpes simplex viruses; human papilloma virus; *Chlamydia trachomatis*; *Neisseria gonorrhoeae*; *Trichomonas* spp.; and *Treponema pallidum* (syphilis). Women were also screened for *Candida albicans* (thrush) and bacterial vaginosis (assessed using Nugent’s criteria) if they were symptomatic and were subsequently excluded if these tests were positive. Women in the VB group did not undergo additional pathogen screening prior to birth, but were excluded if any of their routine antenatal screens tested positive for transmissible pathogens. Women in the CS group were excluded if they had spontaneous rupture of membranes or laboured. For both CS and VB groups, women were excluded if they delivered preterm (<37 weeks of gestation), had an emergency CS, had taken antibiotics or

probiotics within the last two weeks of pregnancy (excluding antibiotics administered during CS), had an intrapartum fever ≥ 38 °C, or their neonate exhibited congenital abnormalities and/or respiratory distress at birth (5 min Apgar score < 7).

Maternal vaginal microbiota were obtained from women in the CS groups prior to birth and mixed with 5 ml of sterile water. Healthy neonates born by planned CS were randomised 1:1 to receive either vaginal seeding (3 ml of vaginal microbiota solution; CS-seeded) or placebo (3 ml of sterile water; CS-placebo). The randomisation procedure was based on computer generated randomisation and was independent of site of birth. Treatment was administered orally to neonates by a clinical member of the research team (not blinded) within the hospital theatre shortly after birth. Neonates were subsequently monitored for 2 h. Parents were blinded to the neonate's treatment allocation. Neonates born vaginally did not receive any intervention and served as a reference group.

Clinical assessments and microbiome sampling were performed within the first 24 h of birth, and at 1 month and 3 months of age.

2.3. Vaginal seeding procedure

Collection of vaginal microbiota from women in the CS groups was performed in the hospital using similar methods to those described previously [41]. Briefly, a sterile 25 x 300 mm porous ribbon gauze with x-ray strip (Propax[®], BSN medical, New Zealand, #2908521) was inserted into the vagina and incubated for approximately 30 min prior to surgery. Upon removal, the gauze was cut in half using sterile procedure, with one half placed in a sterile 10 ml syringe. To extract the vaginal microbiota from the gauze, 5 ml of sterile water was aspirated and passed through the gauze 20 times. Subsequently, 3 ml of the resulting solution was transferred to a 3 ml syringe and kept at room temperature for a mean of 35 ± 8 min prior to administration (range 15–60 min). The remaining 2 ml solution was dispensed into a tube and transferred to the laboratory along with the other half of the gauze swab for microbiome assessment.

Following neonatal examination in theatre after birth, the allocated treatment was administered orally to neonates in the CS groups while they were in a reclining position. The solution was gently squeezed out of the 3 ml administration syringe into the neonate's mouth, triggering the swallowing reflex. The procedure was performed by a clinical member of the research team and took approximately 10 s. Neonates were monitored by a clinical member of the research team for two hours following treatment in case of adverse events.

2.4. Validation of method for isolation of maternal vaginal microbiota

Before commencing the trial, preparation of the vaginal microbiota solution was conducted on three pregnant women at term. The resulting solutions were analysed by flow cytometry to confirm the presence of viable microbial cells and were not administered to neonates. Microbiota solutions were individually dispensed into 1 ml aliquots and incubated for 5 min in the dark with $5 \mu\text{M}$ SYTO BC Green Fluorescent Nucleic Acid Stain (Thermo Fisher Scientific, Massachusetts, USA, #S34855) and $10 \mu\text{g/ml}$ propidium iodide (PI, Thermo Fisher Scientific, Massachusetts, USA, #P3566). Controls included an unstained fraction, separate incubations of SYTO BC and PI (for compensation), and a dead cell fraction (thermal shock at 70 °C for 30 min, followed by PI staining). Samples were analysed on a LSR II flow cytometer (BD, New Jersey, USA). Microbial cells were filtered by size and gated into three fluorescently-distinct populations: viable cells (SYTO BC positive), dead cells (PI positive), and damaged cells (SYTO BC positive, PI positive). The proportion of each subpopulation was calculated with reference to the total cell count.

2.5. Clinical assessments

Clinical assessments were performed within the first 2 h of birth, and at 1 month and 3 months of age. Neonates born by CS were assessed by research staff within the hospital during the treatment monitoring phase. Research staff did not attend the birth of VB neonates and obtained neonatal anthropometric data from medical records. Medical records were also used to access relevant clinical data on the mother and newborn, as well as feeding mode and medications.

Clinical assessments at 1 month and 3 months of age took place at the Maurice and Agnes Paykel Clinical Research Unit, Liggins Institute, University of Auckland. Anthropometric measurements included weight, length, and circumferences of the head, chest, and abdomen. Body composition was assessed using whole body dual-energy x-ray absorptiometry (DXA) scans at 3 months of age. Ponderal index (g/cm^3) was calculated as per Röhler's formula: $(100 \times \text{weight})/(\text{length}^3)$. Birth weight, length, and BMI were transformed into z-scores as per Niklasson et al. [45]. At 1 and 3 months of age, weight and length z-scores were derived as per Tanner & Whitehouse [46], and BMI as per Cole et al. [47].

2.6. Sample collection and processing

The remaining vaginal microbiota solution (2 ml) from CS women and the other half of their gauze swab were kept on ice and transferred to the laboratory shortly after the planned CS for long-term storage at -80 °C.

Meconium and infant stool samples were collected by the parents from fresh nappies within the first 24 h of life, and at 1 month and 3 months of age. Approximately one gram of meconium/stool was collected in a sterile specimen tube (Sarstedt, Nümbrecht, Germany, #SARS80.623) pre-filled with 5 ml of DNA/RNA Shield[™] solution (Zymo Research, California, USA, #R1100). After shaking the tube to mix its contents, meconium/stool samples were kept at room temperature until transfer to the laboratory where they were split into 1 ml aliquots and stored at -80 °C (usually within 3 days of collection). All samples were processed by a researcher blinded to group allocation.

DNA extraction was performed using the ZymoBIOMICS[™] 96 MagBead DNA kit (Zymo Research, California, USA, #D4308). For meconium and stool samples, 500 μl of stool solution was used as input and combined with 500 μl of lysis buffer within the bead bashing tube. For vaginal microbiota solutions, microbiota were pelleted by centrifugation (16,000 g, 15 min) and 1.75 ml of the supernatant was removed. The pellet was resuspended in the remaining 250 μl of supernatant and subsequently mixed with 750 μl lysis buffer before transferring to the bead bashing tube. For vaginal gauze samples, a section of approximately 1 cm^2 was cut using sterile scissors, and placed within a bead bashing tube with 1 ml of lysis buffer. A blank DNA extraction control (1 ml lysis buffer) was also run in parallel. All samples were subsequently processed in the same manner according to the manufacturer's protocol. DNA was eluted in 50 μl DNase/RNase-free water, and quantified using the Qubit[®] dsDNA high-sensitivity assay (Thermo Fisher Scientific, Massachusetts, USA, #Q32854).

DNA was sent to Novogene (Beijing, China) for library preparation and shotgun metagenomic sequencing. DNA libraries were prepared using the NEBNext[®] Ultra[™] DNA Library Prep Kit for Illumina[®] (NEB, Massachusetts, USA, #E7370), and sequenced on an Illumina[®] NovaSeq6000 platform, generating an average of 23 million read pairs per sample (150 bp paired-end reads).

2.7. Microbiome data analyses

Raw sequencing files were processed using BioBakery workflows [48]. Firstly, adapter sequences were removed using Trim Galore!

[49], while contaminating human sequences and low-quality reads were removed using KneadData [50]. Quality filtered reads were then mapped against a collection of species-specific marker genes using MetaPhlan2 [51] with default parameters. This generated relative abundance profiles of identified taxa within each metagenome sample. Species identified by MetaPhlan2 were fed into StrainPhlan [52] to characterise strain diversity among metagenome samples using the most lenient setting “–relaxed_parameters3”. For each species present with a minimum read depth of ≥ 5 reads, genomic variation within the species-specific marker genes was used to generate a single nucleotide polymorphism (SNP) haplotype representing the dominant strain within the metagenome sample. Characterisation of the species-stratified gene content and metabolic potential was performed by HUMAnN2, utilising the UniRef90 gene family and MetaCyc pathway databases [53]. For each sample, default abundances of UniRef90 gene families expressed as reads per kilobase (RPK), were normalised by the total number of read counts and multiplied by a million to give copies per million (CPM).

Microbiome data analyses were performed in R (v3.6.1). Diversity metrics and ordinations were performed using the vegan package (v2.5-6) [54]. Shannon diversity index was used to estimate species diversity within individual metagenomic samples (alpha diversity). The Bray-Curtis dissimilarity index was used to estimate between-sample diversity (beta diversity) based on genus-level relative abundance profiles. Variations in microbiome composition between infant groups were visualised by non-metric multi-dimensional scaling.

Maternal strain transmission was assessed by comparing the genetic similarity of SNP haplotypes for species that were present in both the maternal vaginal and infant stool samples (using methods described by Ferretti et al. [69]). The Jukes and Cantor (JC69) model within the phangorn package (v2.5.5) [55] was used to calculate genetic similarity (DNA distances) between conspecific strains from different metagenome samples. Due to variations in strain diversity between species, DNA distances were normalised by the median DNA distance across all strain comparisons of a given species. A normalised DNA distance < 0.06 was considered a strain-match (i.e., the two strains were considered to be genetically identical). To visualise strain diversity among metagenome samples, phylogenetic trees were constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) hierarchical clustering, with optimisation by maximum likelihood estimation using the Kimura model (K80). Phylogenetic trees were visualised using the ggtree package (v1.16.6) [56].

Due to ethical limitations, the raw sequencing files cannot be shared publicly. However, the quality-filtered sequencing reads (with human sequences removed) and accompanying metadata have been deposited in the NCBI's Sequence Read Archive (BioProject PRJNA701480). The BioBakery workflow script is available at <https://github.com/brookewilson/ecobabe>. The BioBakery output files are available at Figshare (doi: <https://doi.org/10.17608/k6.auckland.14390939.v1>).

2.8. Statistical analysis

The primary outcome was a difference in gut microbiome composition between CS groups at 1 month of age. As specified in the trial protocol [42], the primary outcome was powered to detect a moderate effect size difference (0.25 standard deviations) in microbiome composition with 15 infants per CS group. Due to the COVID-19 pandemic, the trial was halted prematurely resulting in reduced participant numbers (i.e., 12 CS-seeded vs. 13 CS-placebo). This reduced study power from 85% to 77%.

Statistical analyses of microbiome data were performed in R (v3.6.1) by a researcher blinded to group allocation. Primary outcome analysis was conducted on the basis of intention-to-treat using genus-level taxonomic profiles derived from all 25 randomised CS

infants at 1 month of age. Statistical significance was assessed by PERMANOVA (999 permutations) using the adonis2 function in the vegan package (v2.5-6) [54] with marginal adjustments for feeding mode and sex. Secondary microbiome outcomes included differences in gut microbiome composition (i.e., alpha diversity, beta diversity, taxa relative abundances) and functional potential (i.e., MetaCyc pathway abundances) based on intervention group and birth mode. The non-parametric Kruskal–Wallis test was used to assess differences in alpha diversity between infant groups at each time point. PERMANOVA tests were performed cross sectionally at each timepoint as described above to assess differences in beta diversity between infant groups. General linear models as implemented in the MaAsLin2 package (v0.99.18) [57] were used to examine associations between individual microbiome features (e.g., specific taxa/metabolic pathways) and infant groups. Taxa profiles were tested at the species, genus, and phylum levels; counts were log-transformed, and rare taxa that were present in $< 10\%$ of samples were excluded. Feeding mode was included in all models as a fixed effect. Nominal p -values were adjusted for multiple testing using Benjamini-Hochberg procedure, with FDR-adjusted q values < 0.2 considered statistically significant.

Statistical analysis of growth data were performed in SAS (v9.4) (SAS Institute, Cary, NC, USA) and Minitab (v16) (Pennsylvania State University, State College, Pennsylvania, USA) by an unblinded researcher. All statistical tests were two-tailed, with significance maintained at 5% level and without adjustment for multiple comparisons. There was no imputation of missing values. Secondary growth outcomes included differences in anthropometry (i.e., BMI z-score) and body composition (i.e., total body fat percentage). Generalised linear regression models based on repeated measures were used to assess treatment effects on anthropometry, including an interaction term between visit and group, while adjusting for baseline value of the outcome and sex. The average monthly change (Δ) in z-score between birth and 3-month assessment was calculated for weight and BMI. Group differences in Δ were assessed using generalised linear regression models, adjusting for the baseline outcome value and sex. Data on body composition were analysed using the same latter models, except that BMI z-score at birth was used as the baseline covariate because DXA scans were not performed at birth. Model-adjusted estimates and group differences were calculated and tested.

2.9. Role of the funding source

This study was funded by the Health Research Council of New Zealand and A Better Start – National Science Challenge. The funders were not involved in study design, data collection, analysis, interpretation or writing.

3. Results

3.1. Study participants

From a pool of 171 interested participants, 80 pregnant women passed initial eligibility assessments and were subsequently screened. The 80 pregnant women included 45 planning a CS and 35 a vaginal birth (Fig. 1). Nine women planning a CS were excluded following pathogen screening, eight of whom returned a positive Group B *Streptococcus* test. A further 11 women in the group planning a CS were excluded during the perinatal period, six of whom required an emergency CS (Fig. 1). Similarly, 11 women in the VB group were excluded due to perinatal complications, while a further 2 withdrew prior to data collection. Thus, 47 women were included in the trial: 22 in the VB group and 25 women whose neonates were randomised at birth into CS-seeded ($n = 12$) or CS-placebo ($n = 13$) groups (Fig. 1).

The number of recruited participants was short of our recruitment target of 30 women planning a CS (i.e., 15 per intervention group). This exception occurred because our trial had to end prematurely, as

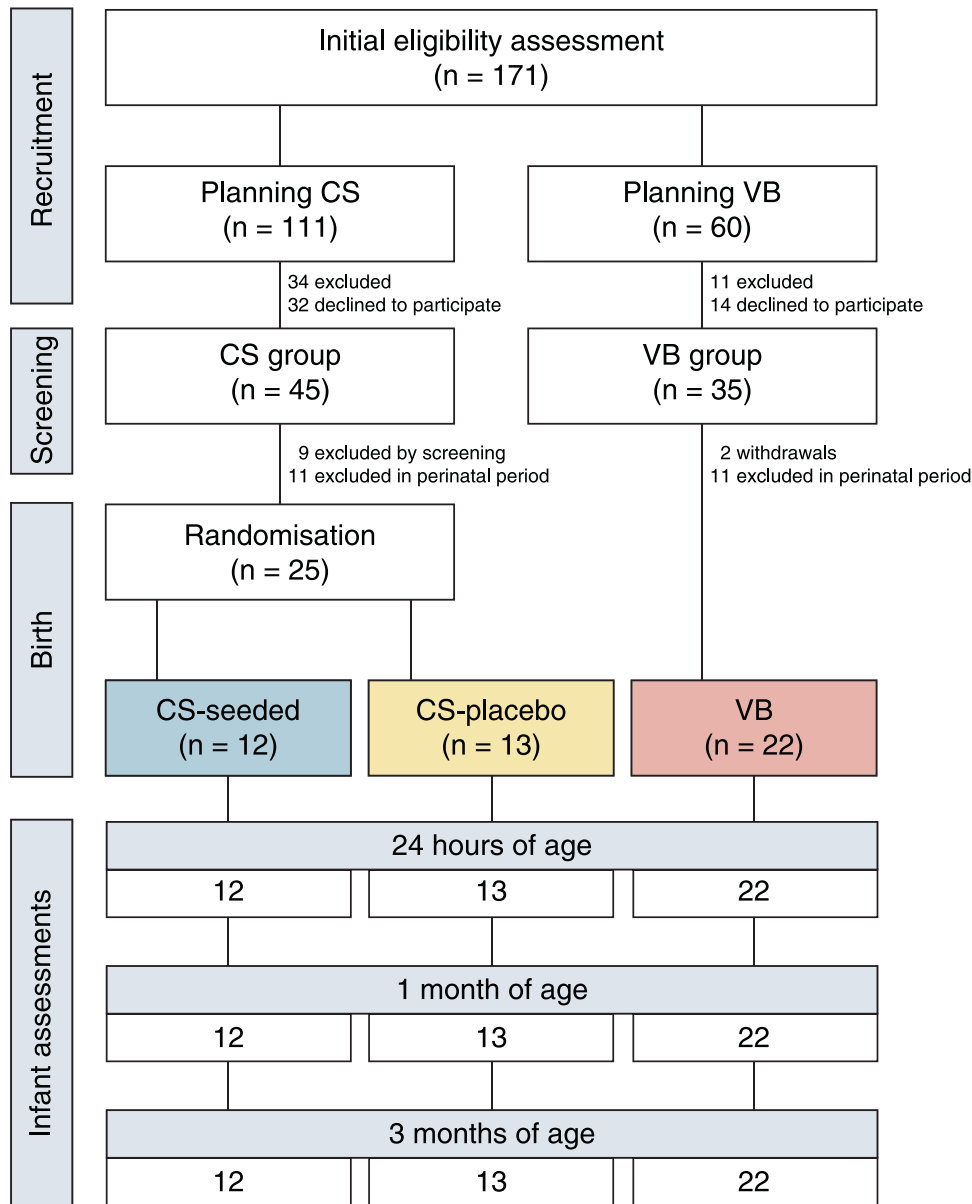


Fig. 1. CONSORT diagram showing the flow of participants through the ECOBABe trial, including infants born by caesarean section (CS) or vaginally (VB).

screening laboratories in the Auckland region during the study period were forced to prioritise testing for SARS-CoV-2 over research-based requests due to the COVID-19 pandemic. Therefore, we were unable to continue pathogen screening potential participants.

Overall, participants were mostly of European ethnicity, university-educated, and of a similar pre-pregnancy BMI (Table 1). Participants in the CS groups (combined) were slightly older than participants in the VB group, and their neonates were born slightly earlier in gestation (Supplementary Table 1). The majority of participants (85%) gave birth at Auckland City Hospital. The total CS rate at this hospital in 2019 was 38.6% (20.4% planned/elective, 18.2% unplanned/emergency) [58]. As per our inclusion criteria, all neonates were born healthy with a 5 min APGAR score ≥ 7 .

3.2. Metagenomic sequencing

The meconium samples did not meet the standard DNA yield requirements for library preparation, preventing these samples from being sequenced (mean \pm standard deviation of DNA yield: 21 ± 53 ng). The maternal vaginal solutions also had a

relatively low DNA yield (148 ± 186 ng) and were subsequently combined with their respective gauze extract ($1266 \text{ ng} \pm 927 \text{ ng}$) to obtain a composite maternal vaginal sample. Thus, metagenomic sequencing was performed on 116 samples including 25 maternal vaginal samples, 45 infant 1-month stool samples, and 46 infant 3-month stool samples. Removal of contaminating human sequences dramatically reduced the read count of maternal vaginal samples from a mean of 21.2 ± 1.6 million read pairs/sample after QC filtering to a post-processed mean of 1.9 ± 1.4 million read pairs/sample. By contrast, quality filtering had a limited effect on infant stool samples, with post-processed read counts of 20.6 ± 3.5 and 19.9 ± 4.9 million read pairs/sample for 1-month and 3-month samples, respectively.

3.3. Vaginal seeding did not alter the gut microbiome of CS-born infants

Prior to trial commencement, we confirmed that over a quarter of vaginal microbiota individually extracted from three pregnant women remained viable in solution (mean viability $26.5\% \pm 6.5\%$; Supplementary Table 2). To assess the impact of oral administration of maternal vaginal microbes on gut microbiome development, we

Table 1

Maternal demographic characteristics, infant characteristics at birth, and early feeding practices in the three groups in the ECOBAbE trial.

	CS-seeded	CS-placebo	VB
n	12	13	22
Maternal characteristics			
Age (years)	38.5 [34.6, 40.3]	34.9 [33.1, 37.0]	33.3 [31.7, 35.3]
Pre-pregnancy BMI (kg/m ²)	24.3 ± 2.5	23.6 ± 3.1	23.2 ± 3.3
Ethnicity			
European	10 (83%)	11 (85%)	16 (73%)
Māori	1 (8%)	1 (8%)	3 (14%)
Asian	nil	1 (8%)	2 (9%)
Other	1 (8%)	nil	1 (5%)
Education			
High-school or lesser	nil	1 (8%)	1 (5%)
Vocational	2 (17%)	3 (23%)	2 (9%)
University	10 (83%)	9 (69%)	19 (86%)
Intrapartum antibiotic prophylaxis [†]	12 (100%)	13 (100%)	nil
Time before birth (minutes)	14 [11, 16]	18 [9, 30]	-
Infant characteristics at birth			
Female sex	7 (58%)	7 (54%)	9 (41%)
Gestational age (weeks)	39.0 [39.0, 39.0]	39.0 [38.0, 39.5]*	40.0 [39.0, 41.0]
Weight (g)	3658 ± 382	3612 ± 571	3660 ± 455
Weight z-score	0.71 ± 0.84	0.64 ± 1.14	0.46 ± 0.85
Length z-score	1.02 ± 0.57	1.28 ± 1.06	1.35 ± 1.13
BMI z-score	0.44 ± 0.94	0.06 ± 1.23	-0.07 ± 1.04
Ponderal index (g/cm ³)	2.67 ± 0.22	2.56 ± 0.27	2.51 ± 0.27
Infant feeding			
At 1 month			
Exclusive breastfeeding	9 (75%)	9 (69%)	17 (77%)
Partial breastfeeding	3 (25%)	3 (23%)	5 (23%)
Formula feeding	nil	1 (8%)	nil
At 3 months			
Exclusive breastfeeding	5 (42%)	7 (54%)	16 (73%)
Partial breastfeeding	5 (42%)	5 (39%)	6 (27%)
Formula feeding	2 (17%)	1 (8%)	nil

Data are n (%), mean ± standard deviation, or median [quartile 1, quartile 3], as appropriate.

BMI, body mass index; CS-placebo, babies born by caesarean section who received placebo; CS-seeded, babies born by caesarean section who received vaginal seeding; VB, babies born from vaginal births.

[†] All but one women who gave birth by caesarean section received 2 g of cefazolin prior to surgery; the exception was one women from the CS-placebo group who received 600 mg of clindamycin instead.

* $P = 0.027$ for a pairwise comparison to the VB group; there were no other observed differences between groups on birth characteristics or infant feeding. Comparisons between CS (group as a whole) and VB group are presented in Supplementary Table 1.

profiled the taxonomic composition and gene repertoire of microbes present within infants' stool samples at 1 month and 3 months of age. Differences in microbial composition between infant groups were visualised by multi-dimensional scaling (Fig. 2b) and tested by PERMANOVA (Supplementary Table 3). While the composition of the infants stool microbiota was clearly distinct from the maternal vaginal microbiota (Fig. 2a), we did not observe any difference in the microbial composition of stools collected from CS infants who received vaginal seeding compared to those receiving the placebo at either 1 month (primary outcome, $p = 0.90$) or 3 months of age ($p = 0.18$) (Supplementary Table 3). Similarly, there were no differences between CS groups in the relative abundance of any particular phyla, genera, or species within the infant stool microbiota (linear models, $q > 0.2$; Supplementary Table 4). With respect to functional potential, vaginal seeding of neonates born by CS did not alter the abundance of any microbial metabolic pathways compared to placebo (linear models, $q > 0.2$; Supplementary Table 5). Microbial alpha diversity and gene richness were similar between the three infant groups (Kruskal–Wallis test, $p > 0.05$, Fig. 2c,d).

Consistent with published observations [7–14], compositional differences were detected between CS and VB infants at both 1 month ($p = 0.022$) and 3 months of age ($p = 0.001$) explaining 5.7% and 8.8% of the variance, respectively (Supplementary Table 3). At the bacterial family level, VB infants harboured proportionally more *Bacteroidaceae* (Fig. 3a), reflecting higher relative abundances of genus *Bacteroides* (Fig. 3b). *Bacteroides* was detected in 28% of stool samples from CS infants at 1 month of age, compared to 80% of stool samples from VB infants. While the study was underpowered to investigate the cause of this low *Bacteroides* profile, having low *Bacteroides* did not appear to be related to feeding mode or antibiotic

exposure after birth (Supplementary Fig. 1). At the species-level, VB infants had higher relative abundances of *Bacteroides vulgatus* at 1 month of age, and *B. vulgatus*, *B. dorei*, and *B. fragilis* at 3 months of age compared with CS infants (linear model, $q < 0.2$; Supplementary Table 4). Conversely, CS infants had proportionally more *Atopobium* spp. (in particular, *Atopobium parvulum*), *Clostridium* spp., *Haemophilus* spp., and *Streptococcus* group *mitis/oralis/pneumoniae* at 3 months of age (linear model, $q < 0.2$; Supplementary Table 4). There were no differences in the relative abundances of *Bifidobacterium* or *Lactobacillus* genera between infant groups (Fig. 3b).

3.4. Functional differences in the gut microbiome of CS and VB infants

The higher abundance of *Bacteroides* spp. in the gut microbiomes of VB infants contributed towards functional differences in the microbiome's metabolic potential (Fig. 4). In total, 20 microbial pathways were more abundant in VB infants when compared to CS infants at either 1 month or 3 months of age (linear model, $q < 0.2$; Supplementary Table 5). At a broad level, these pathways were involved in coenzyme A and vitamin biosynthesis, cell wall production, nucleotide and GABA degradation, and amino acid and secondary metabolite biosynthesis (Fig. 4). Observed differences in vitamin biosynthesis potential were related to the production of B vitamins, including thiamine (B₁), phosphopantothenate (B₅), pyridoxal 5'-phosphate (B₆), and folate (B₉), as well as menaquinol (Vitamin K₂). Amino acid biosynthesis differed specifically in relation to L-ornithine (an amino acid previously shown to play a role in gut barrier function [59]), and L-lysine.

To ascertain that the increase in *Bacteroides* spp. within VB infant microbiomes was responsible for the functional alterations observed, differential pathway abundances were stratified by species

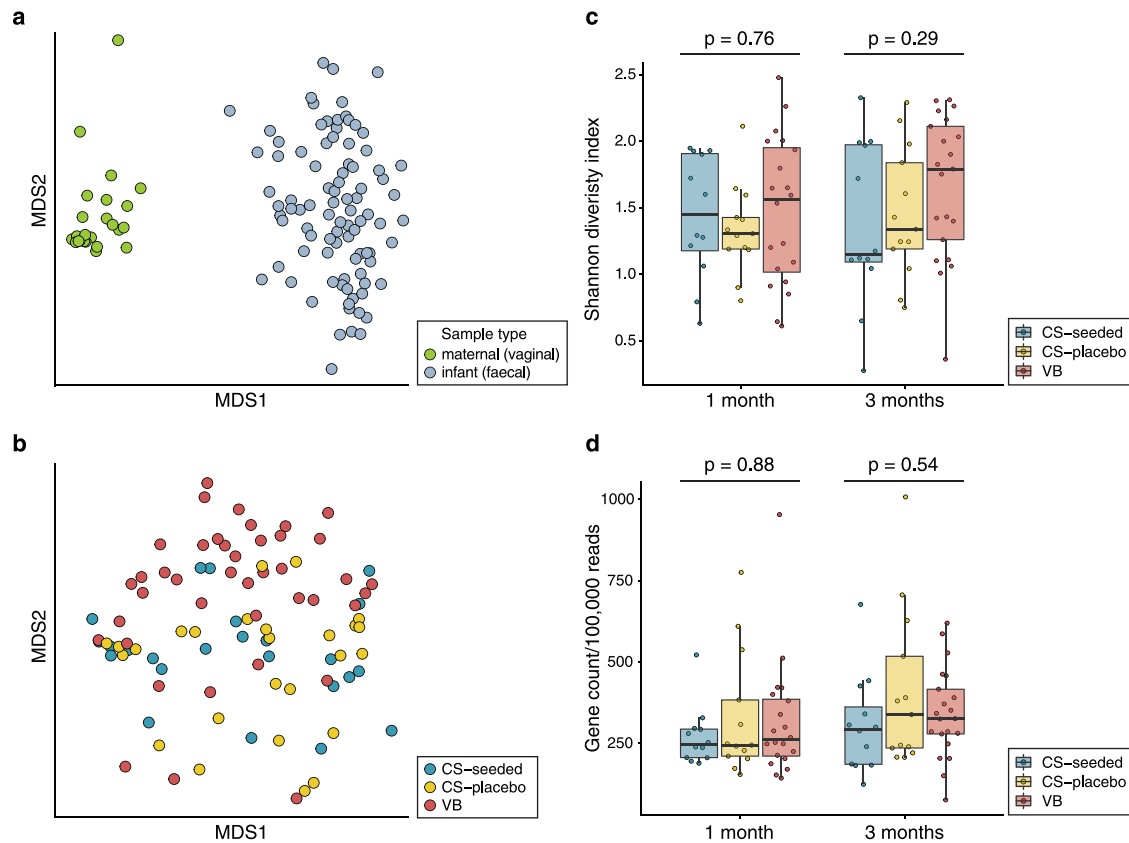


Fig. 2. Comparisons of microbiome composition and diversity in infants born by caesarean section who received vaginal seeding (CS-seeded) or placebo (CS-placebo), and infants born vaginally (VB). Non-metric multi-dimensional scaling plots based on genus-level Bray Curtis dissimilarities showing the variation in microbiome composition in: (a) maternal vaginal and infant faecal samples at 1 month and 3 months of age; and (b) faecal samples from infants in the three study groups at 1 month and 3 months of age. Significant differences in infant microbiome composition based on birth mode, intervention group, feeding mode, and sex was assessed by PERMANOVA (see Supplementary Table 3). (c) Shannon diversity index and (d) gene richness, normalised by sequencing depth, for infant faecal microbiomes at 1 month and 3 months of age; each box represents the median and inter-quartile range (IQR), and whiskers the range of the data (expanding up to 1.5 x IQR). Group differences assessed by Kruskal–Wallis test.

contribution; these were then compared between VB and CS samples to identify the species driving the increase in overall pathway abundance. For the majority of differentially abundant pathways (14/20), *Bacteroides* spp. were responsible for the increase in pathway potential (Wilcoxon rank sum test, FDR adjusted $q < 0.2$). Specifically, pathway abundances were found to be higher in VB infants for *B. uniformis*, *B. vulgatus*, *B. dorei*, *B. fragilis*, *B. faecis*, and *Parabacteroides distasonis* (Supplementary Fig. 2).

3.5. Limited colonisation of the infant gut by maternal vaginal microbes

To assess engraftment of maternal vaginal strains within the infant gut in relation to vaginal seeding, we performed strain-level profiling for maternal vaginal samples and infant faecal samples collected from the CS intervention groups (CS-seeded and CS-placebo). No strain profiling was performed for the VB group due to the absence of maternal vaginal samples from VB participants. Within the CS group, only four species were present in both the maternal vaginal and infant faecal profiles at a sufficient sequencing depth for strain-level identification. These included strains of *Bifidobacterium breve*, *Bifidobacterium longum*, *Lactobacillus gasseri*, and *Lactobacillus casei paracasei*. Infants were more likely to share strains from these species with their own mother (6/7, 85.7% of intra-pair comparisons) than with unrelated mothers ($n = 2/79$, 2.5% of inter-pair comparisons). The two examples of strain matches between infants and unrelated mothers involved *Lactobacillus gasseri*.

At 1 month of age, there were five probable maternal strain transmission events, four within CS-seeded infants and one within a CS-placebo infant (Fig. 5a). By 3 months of age, only one of these maternally-

derived strains (*Bifidobacterium breve*) was still present in the gut of a CS-seeded infant (Fig. 5b). In another case, the maternal strain of *Bifidobacterium longum* that was present in the infant gut at 1 month of age had been replaced by a genetically distinct strain at 3 months. In the remaining three cases of maternal transmission, the species was either absent from the infant gut or present but below the detection threshold for strain identification at 3 months of age. There were no other examples of maternal strain transmission at 3 months of age.

For species that were present within the infant gut at both 1 month and 3 months of age, we compared the genetic similarity of the dominant strain. Across all infants and including all species, we identified a mean dominant strain replacement rate of $46 \pm 25\%$, indicating that strain fluctuation in the infant microbiome was highly dynamic. Dominant strain replacement rates did not vary between infant groups (ANOVA, $p = 0.18$). The most common strains to be replaced between 1 month and 3 months of age belonged to *Veillonella parvula* (17/18, 94%) and *Haemophilus parainfluenzae* (12/15, 80%). Both of these commensal species are regarded as opportunistic pathogens that are typically found across multiple sites, including the oral cavity, gut, and vagina [60–62]. *Veillonella parvula* in particular, is known to bloom in infancy, and has been shown to be important in immune development [63]. The high rates of strain turnover we observed for these two species could reflect a lack of stable colonisation within the infant gut.

3.6. Growth outcomes

As a result of the New Zealand-wide lockdown imposed by the government due to the COVID-19 pandemic, a subset of clinical

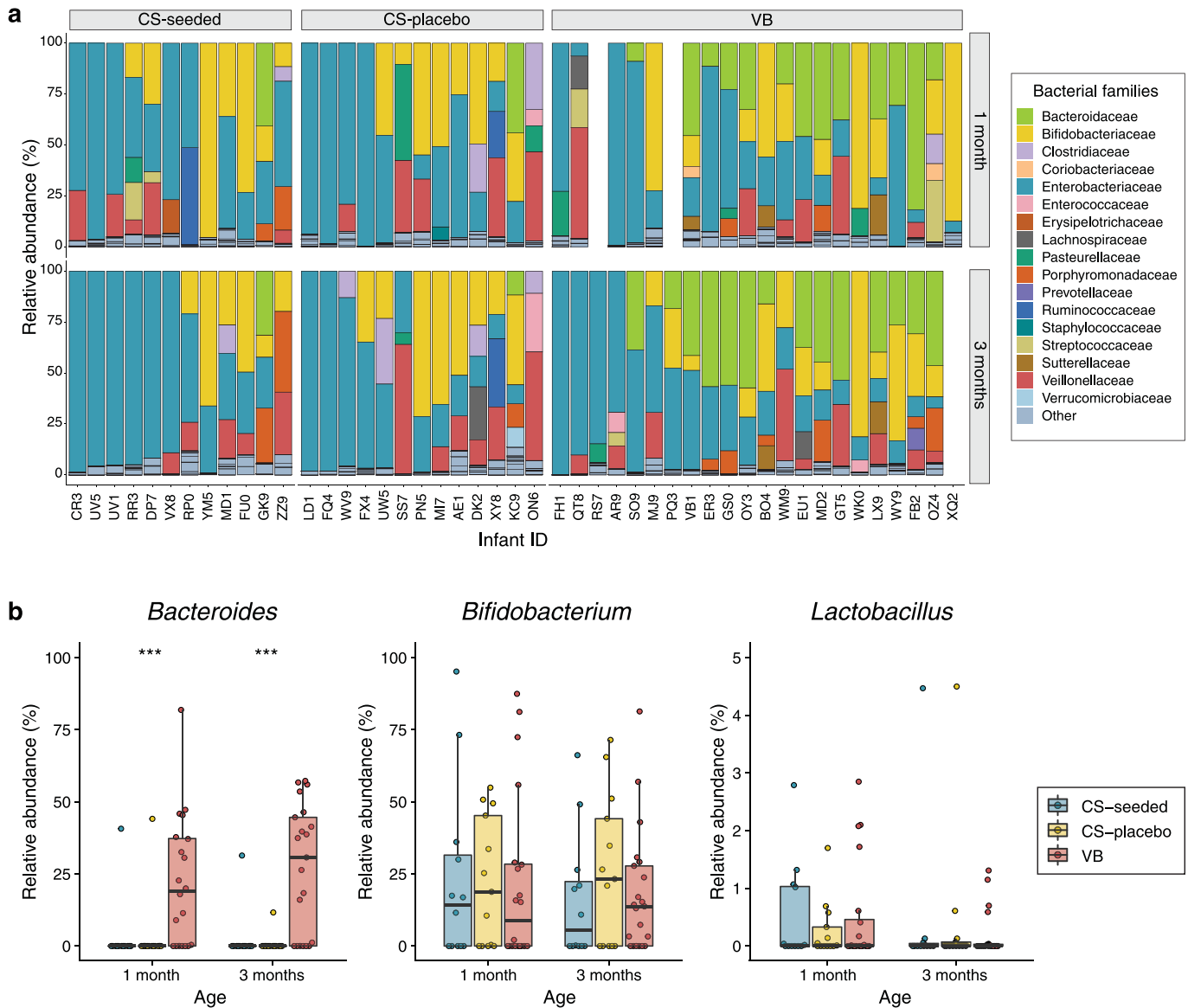


Fig. 3. Taxonomic differences in infant faecal microbiomes at 1 and 3 months of age in infants born by caesarean section who received vaginal seeding (CS-seeded) or placebo (CS-placebo), and infants born vaginally (VB). (a) Relative abundance of bacterial families in infant faecal microbiomes. Bacterial families whose relative abundances were <1% are categorised as "Other". (b) Relative abundances of *Bacteroides*, *Bifidobacterium*, and *Lactobacillus* genera in infant faecal microbiomes. Each box represents the median and inter-quartile range (IQR), and whiskers the range of the data (expanding up to 1.5 x IQR). *** $p < 0.001$ for an overall difference in relative abundances among the three study groups, assessed by a Kruskal–Wallis test.

assessments that should have occurred at 1 month ($n = 5$) and 3 months ($n = 11$) of age took place over the phone. Consequently, there were fewer anthropometric measurements and body composition scans obtained than originally aimed for (Supplementary Fig. 3), and some anthropometric data were either recorded by the attending healthcare professional or taken by the parents themselves. From the data we did obtain, there were no observed differences in anthropometry or body composition between the three infant groups at 1 month or 3 months of age, including total body fat (a key secondary outcome; Table 2). Similarly, there were no differences in growth outcomes when comparing both CS groups combined to VB infants (Supplementary Table 6)

3.7. Adverse events

There were no serious adverse events recorded in the study. There were no cases of early onset postpartum infection or high fever after birth. Neonatal hypoglycaemia ($n = 5$; 2 CS-seeded, 3 CS-placebo) and

neonatal care unit/hospital admission ($n = 5$; 4 CS-seeded, 1 CS-placebo) were the most frequently recorded adverse events (Supplementary Table 6). There was one case of colic (as defined by the modified Wessel criteria [64]) in a CS-placebo infant and two cases of reflux (1 CS-seeded, 1 CS-placebo). However, these were considered to be unrelated to the treatment by the study's data monitoring committee and the research team.

4. Discussion

Vaginal seeding is a largely untested procedure designed to restore gut microbiome development in infants born by CS, with the intention of reducing their risk of developing metabolic and immune disorders later in life. Here, we show that oral administration of maternal vaginal microbiota suspended in water did not alter the structure or function of the gut microbiome in infants born by planned unlaboured CS at 1 month or 3 months of age. With the inclusion of a reference group of infants born vaginally, we

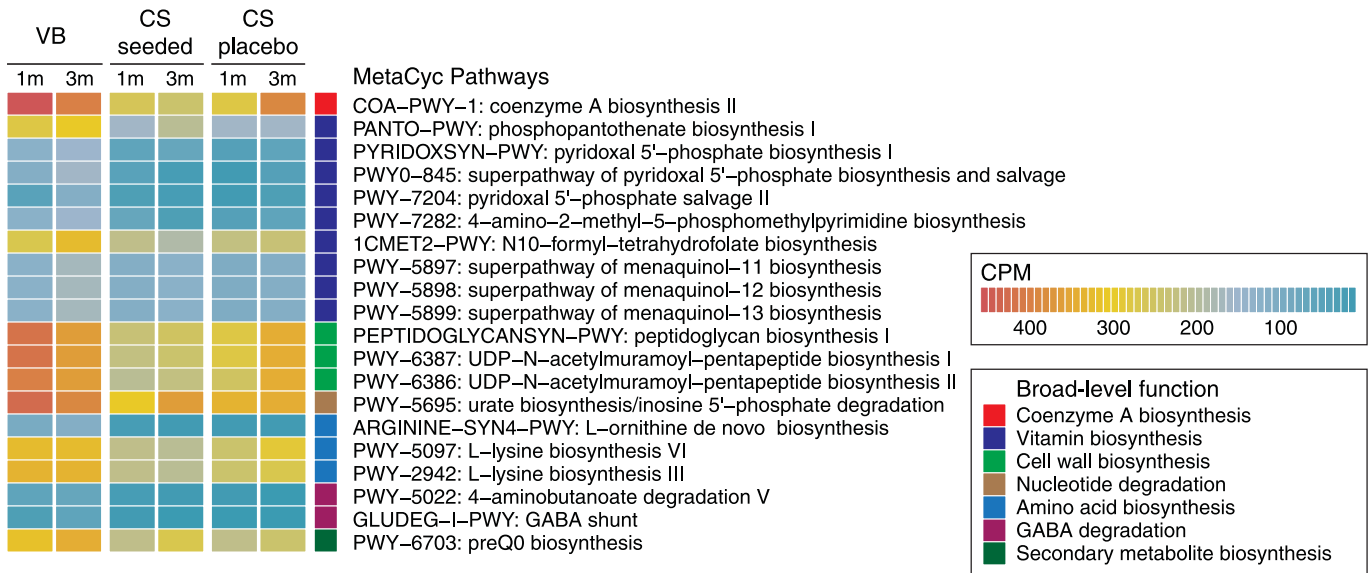


Fig. 4. Microbial metabolic pathways that were more abundant in the faecal microbiomes of infants born vaginally (VB) in comparison to infants born by caesarean section who received vaginal seeding (CS-seeded) or placebo (CS-placebo). Differences in MetaCyc pathway abundances were assessed using general linearised models, as implemented in MaAsLin2, and were adjusted for feeding mode and sex. Cells represent the mean pathway abundance expressed in copies per million (CPM) for each infant group at 1 month (1m) and 3 months (3m) of age. Broad-level functions were categorised based on MetaCyc “superclasses”.

corroborated what many others have shown before [7–14,65], i.e., that CS-born infants have lower relative abundances of *Bacteroides* spp.. Our method of vaginal seeding was unable to restore *Bacteroides* levels in these infants. This characteristic signature of low *Bacteroides* abundance led to functional differences in the metabolic potential of the microbiome of CS infants with a number of biosynthesis pathways underrepresented. While we acknowledge that our reduced participant numbers might have impaired our ability to detect subtle differences in microbiota composition and function between CS-seeded and CS-placebo infants, our findings suggest that vaginal seeding alone does not have an appreciable impact on gut microbiome development. Furthermore, vaginal seeding had no detectable effect on infant anthropometry and body composition within the first 3 months of life.

Our results extend those of a previously published pilot study by Dominguez-Bello and colleagues who used an alternative method of vaginal seeding, in which the face and body of four CS neonates were wiped with maternal vaginal fluids [41]. The authors reported partial restoration of the neonatal microbiome across multiple body sites, most notably the oral and skin microbiomes. Using anal swabs as a proxy for the gut microbiome, *Bacteroides* restoration by vaginal seeding was observed in only one of the four infants from the second week of age. Although we were limited to studying infant gut microbiota at just two time points (i.e., 1 month and 3 months of age), these preliminary findings suggest that vaginal seeding, irrespective of administration technique, is unlikely to sufficiently restore the gut microbiome of infants born by CS, particularly with respect to *Bacteroides* colonisation and abundance.

This result is not particularly surprising given that *Bacteroides* spp. are not typical residents of the vaginal microbiome [66]. In our study, *Bacteroides* spp. were detected in only 8/25 (32%) of CS mothers, at a mean relative abundance of $0.069 \pm 0.13\%$. While we did not collect maternal stool samples in our study, *Bacteroides* spp. are known to be abundant members of the adult gut microbiome [67]. Thus, exposure to the maternal faecal, rather than vaginal, microbiota may be a more effective method for restoring *Bacteroides* levels in infants born by CS. In support of this, a group in Finland recently demonstrated that oral administration of maternally-derived faecal microbiota (FMT) in a small volume of breastmilk led to greater restoration of the early gut microbiome in seven CS neonates compared to vaginal seeding

[68]. In particular, *Bacteroides* spp. abundances in the FMT group during the first four weeks of life were comparable to VB neonates, although their abundance had declined by 12 weeks of age [68]. Recent studies assessing maternal strain transmission have also revealed that maternal enteric strains, rather than those from the vagina, are more likely to reside within the infant gut with higher proportions of maternal strain sharing in VB infants compared to CS infants [9,10,18,65,69]. These findings are consistent with the very low levels of maternal vaginal strain engraftment we observed within CS infants in our study.

Faecal microbiomes of CS infants at 3 months of age were enriched in *Haemophilus* spp. and *Streptococcus* group *mitis/oralis/pneumonia*. These taxa have previously been found to negatively correlate with *Bacteroides* spp. abundances suggesting a potential antagonistic relationship [8,65,70]. Given the consistency of this observation, research should be directed towards understanding the specific interactions that occur between these species. It may be possible that the low *Bacteroides* abundance in CS infants is not strictly related to a lack of maternal exposure, but rather, an outcompetition by other species present with the infant gut microbiome. This notion is supported by the recent observation that *Bacteroides* abundance is similar between CS and VB neonates during the first week of life, but subsequently diminishes within CS neonates one week later [65]. Further research should be focused into understanding the cause of this low *Bacteroides* profile, particularly given up to 49% of VB microbiomes also have low *Bacteroides* [9]. Moreover, the possibility that CS-associated microbiomes are pre-set *in utero* due to prenatal microbial exposures warrants further investigation [6].

The impact of any seeding approach, be it faecal or vaginal, is likely to be compromised by the routine intrapartum antibiotic prophylaxis (IAP) given to women to reduce their risk of surgical infection during CS. Under WHO guidelines [71], IAP are typically administered preoperatively while the neonate is still *in utero*. As a consequence, not only do neonates born by CS have reduced exposure to maternal microbes at birth, they're also exposed to broad spectrum antibiotics during one of the most critical periods of gut microbiome acquisition. Interestingly, a previous study found that VB neonates whose mothers received IAP developed a perturbed microbiome during the first month of life that was also characterised by low *Bacteroides* [9]. While the microbiome effects of IAP are difficult

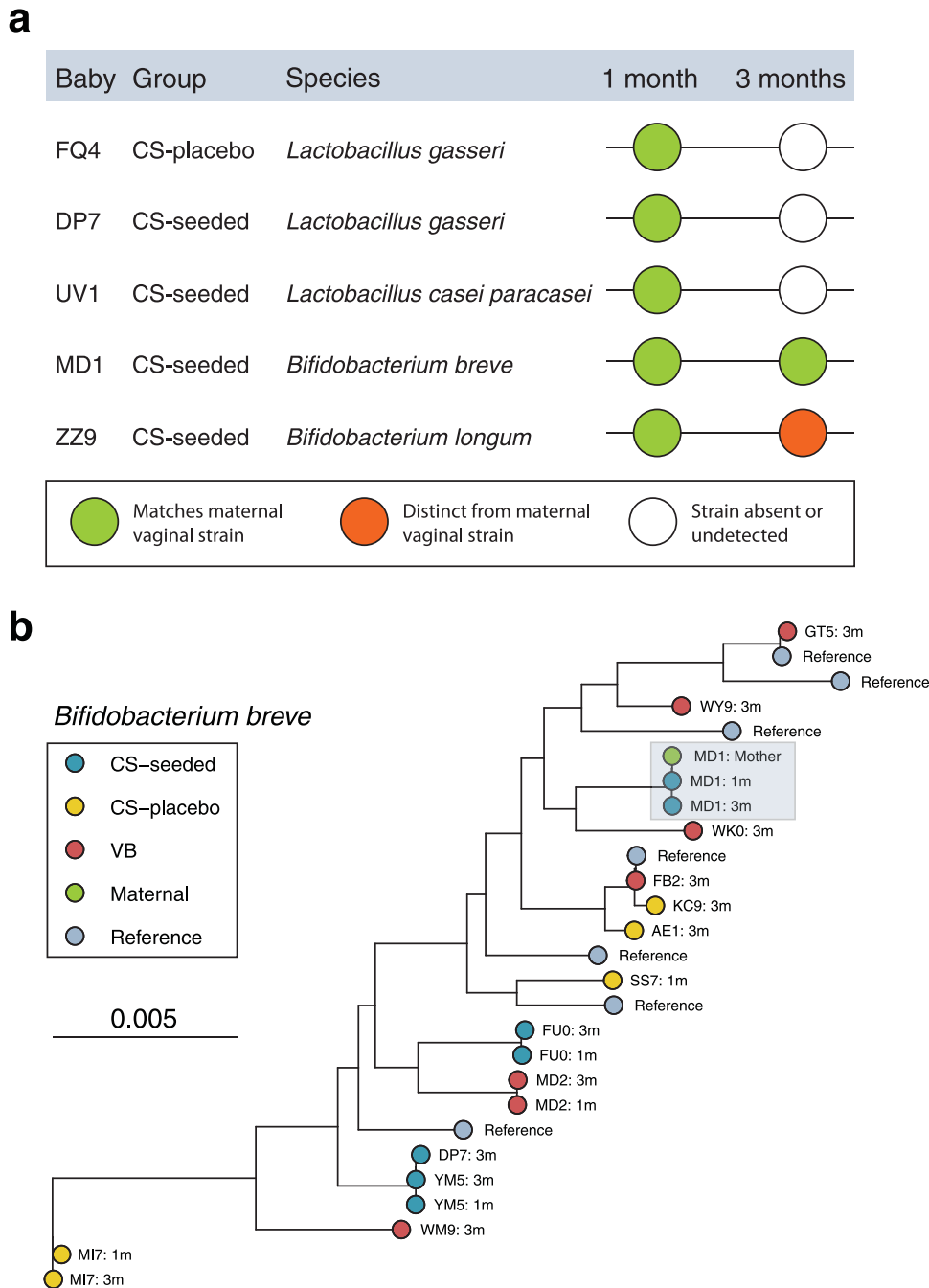


Fig. 5. Maternal vaginal strains detected at 1 month (1m) and 3 months (3m) of age, in faecal samples of infants born by caesarean section who received vaginal seeding (CS-seeded) or placebo (CS-placebo). (a) The five maternal vaginal strains that were detected in the faecal microbiomes of CS infants. (b) Phylogenetic tree of different *Bifidobacterium breve* strains from infant faecal samples and vaginal samples from CS mothers. Scale bar signifies difference in sequence similarity between strains as determined by single nucleotide polymorphism (SNP)-based haplotyping. Strains from reference genomes and infants born vaginally (VB) are included for context. An example of a probable maternal strain transmission event is highlighted in the grey box.

to disentangle from the effects of CS, it is likely that neonatal antibiotic exposure in our study could have detrimentally affect the viability of 'seeded' microbes, thereby reducing their likelihood of successful colonisation. Delaying IAP administration until after cord clamping may help mitigate these effects and improve gut microbiome development which is particularly pertinent given a recent study of 55,901 women found no differences in surgical site infections depending on whether IAP were administered before or after cord clamping [72].

In our study, vaginal seeding was performed without incident and no serious adverse events were reported. However, the short- and

long-term safety of vaginal seeding is still yet to be determined. Pathogen screening is paramount to minimising infection risk, particularly given the recent case report of a neonate contracting herpes following vaginal seeding from an unscreened herpes-positive mother [73]. In our trial, 20% of enrolled women were excluded after testing positive for potentially pathogenic organisms, which was slightly lower than the 35% exclusion rate reported by Korpela et al. who performed faecal seeding [68]. Therefore, if "seeding" practices were to become routine in the future, maternal pathogen screening would limit the number of neonates who could potentially benefit from these therapies. Moreover, due to their personalised nature,

Table 2

Anthropometry and body composition in babies born by caesarean section who received vaginal seeding (CS-seeded) or placebo (CS-placebo) and in babies born vaginally (VB).

	CS-seeded	CS-placebo	VB
Anthropometry (1 month of age)			
<i>n</i>	12	13	19
Weight z-score	0.23 (-0.26, 0.71)	0.25 (-0.21, 0.72)	0.35 (-0.02, 0.72)
Length z-score	1.08 (0.56, 1.61)	1.22 (0.72, 1.71)	0.99 (0.58, 1.41)
BMI z-score	0.03 (-0.56, 0.63)	0.07 (-0.49, 0.63)	0.40 (-0.07, 0.87)
Ponderal index (g/cm ³)	2.59 (2.44, 2.75)	2.60 (2.45, 2.75)	2.67 (2.55, 2.80)
Anthropometry (3 months of age)			
<i>n</i>	9	12	20
Weight z-score	0.13 (-0.36, 0.63)	0.30 (-0.18, 0.78)	0.62 (0.24, 0.99)
Δ weight z-score/month	-0.14 (-0.36, 0.09)	-0.08 (-0.28, 0.12)	0.02 (-0.14, 0.17)
Length z-score	0.82 (0.24, 1.39)	1.26 (0.74, 1.77)	0.96 (0.56, 1.36)
Δ length z-score/month	-0.10 (-0.32, 0.13)	-0.03 (-0.23, 0.17)	-0.08 (-0.24, 0.08)
BMI z-score	-0.14 (-0.82, 0.54)	-0.18 (-0.77, 0.40)	0.34 (-0.12, 0.80)
Δ BMI z-score/month	0.005 (-0.28, 0.29)	-0.06 (-0.30, 0.19)	0.07 (-0.12, 0.26)
Ponderal index (g/cm ³)	2.69 (2.51, 2.86)	2.64 (2.49, 2.80)	2.77 (2.65, 2.89)
DXA (3 months of age)			
<i>n</i>	8	10	17
Sex (females)	5 (63%)	6 (60%)	7 (42%)
Total body fat (%)	38.2 (34.5, 41.8)	36.9 (33.8, 40.0)	40.2 (37.8, 42.6)
Trunk fat (%)	10.8 (8.9, 12.7)	9.5 (7.8, 11.3)	10.5 (9.2, 11.7)
Fat-free mass (%)	68.8 (65.8, 71.9)	71.0 (68.2, 73.8)	69.6 (67.6, 71.7)

Δ, delta (change) expressed as the average change in z-score per month between birth and the 3-month assessment; BMI, body mass index; DXA, whole body dual-energy x-ray absorptiometry.

Anthropometric data are the adjusted mean and the respective 95% confidence interval (CI) from a repeated measures analysis, whose model included trial group allocation, assessment (1- and 3-month visits), and their interaction term, as well as sex and the baseline value of the outcome; Δ data are the mean and respective 95% CI, adjusted for sex and the baseline value of the outcome; and DXA data are mean and 95% CI, adjusted for sex and BMI z-score at birth. There were no statistically significant pairwise differences between groups (at $p < 0.05$) for any of the reported study outcomes.

maternally-derived therapies would be difficult to scale to a population level.

Administration of standardised probiotic formulations may be a safer, more inclusive, and practical therapy for microbiota restoration, as was recently demonstrated in a moderately sized ($n = 422$ infants) multi-strain probiotic randomised controlled trial [74]. Future research should also focus on investigating other factors outside of microbial interventions that might help restore gut microbiome development. For example, presence of an older sibling has been shown to help normalise the gut microbiome of CS-born infants during their first year of life, subsequently removing their heightened risk of developing childhood asthma [75]. Greater understanding of how the microbiome adapts and develops during infancy may provide alternative avenues for microbiota restoration. Future research is also still required to determine whether microbiome restoration can meaningfully reduce the risk of developing other CS-associated disorders, such as obesity.

In conclusion, our pilot trial found that oral administration of maternal vaginal microbiota had no detectable effect on the structure and function of the early gut microbiome of infants born by CS. Although infection risk was minimised with maternal pathogen screening, the risks involved with transplanting microbiota, particularly in neonates with underdeveloped immune systems, warrants continual caution. Given that this procedure could not revert gut microbiome development, the argument for its utility in reducing disease risk in CS-infants may now be moot.

Contributors

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 Microbiome investigation: BCW, TV
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 Supervision: JGBD, TV, JMO, WSC
 Writing draft manuscript: BCW
 Manuscript revision: BCW, ÉMB, CPG, JGBD, VC, NW, ST, CC, AJR, TV, JMO, WSC

Data sharing statement

Post-processed metagenomic sequencing files and accompanying metadata are deposited in NCBI's SRA database (BioProject PRJNA701480). The BioBakery workflow script is available at <https://github.com/brookewilson/ecobabe>. The BioBakery output files are available at FigShare (doi: <https://doi.org/10.17608/k6.auckland.14390939.v1>). Flow cytometry data is available at FigShare (doi: <https://doi.org/10.17608/k6.auckland.13542992.v1>).

Declaration of Competing Interest

The authors have nothing to disclose.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103443.

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