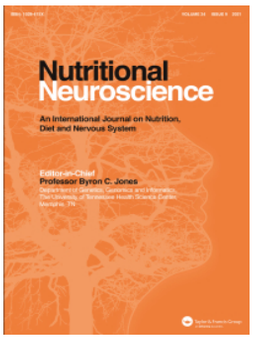

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Nutritional Neuroscience

An International Journal on Nutrition, Diet and Nervous System

ISSN: 1028-415X (Print) 1476-8305 (Online) Journal homepage: www.tandfonline.com/journals/ynns20

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To cite this article: Samantha L. Dawson, Gerard Clarke, Anne-Louise Ponsonby, Amy Loughman, Mohammadreza Mohebbi, Tiril Cecilie Borge, Adrienne O'Neil, Peter Vuillermin, Mimi L.K. Tang, Jeffrey M. Craig & Felice N. Jacka (2025) A gut-focused perinatal dietary intervention is associated with lower alpha diversity of the infant gut microbiota: results from a randomised controlled trial, Nutritional Neuroscience, 28:6, 694-708, DOI: 10.1080/1028415X.2024.2413233

To link to this article: https://doi.org/10.1080/1028415X.2024.2413233

NUTRITIONAL NEUROSCIENCE
2025, VOL. 28, NO. 6, 694–708
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A gut-focused perinatal dietary intervention is associated with lower alpha diversity of the infant gut microbiota: results from a randomised controlled trial

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ABSTRACT

Objectives: In experimental models, the prenatal diet influences gut microbiota composition in mothers and offspring; however, it is unclear whether this occurs in humans. We investigated the effects of a gut-focused perinatal dietary intervention on maternal and infant gut microbiota composition four weeks after birth.

Methods: This randomised controlled trial randomised pregnant women to receive dietary advice as part of standard care, or additionally receive a dietary intervention focused on the Australian Dietary Guidelines and increasing prebiotic and probiotic/fermented food intakes (ACTRN12616000936426). Study assessments occurred from gestation week 26 (baseline) to four weeks postpartum (follow-up). Faecal samples, collected at baseline for mothers, and follow-up for mothers and infants, underwent 16SrRNA sequencing. The primary outcome was a between-group mean difference in infant faecal Shannon index. Secondary outcomes included between-group differences in other microbiota measures, including maternal change from baseline CLR-transformed *Prevotella* abundance.

Results: Forty-four women and 45 infants completed the study. The mean Shannon index of infants in the intervention group was −0.35 (95% CI: −0.64, −0.06, SD: 0.52) units lower than control group infants, corresponding to a medium effect size (Cohen's D: −0.74, 95% CI: −1.34, −0.13). The findings were similar using other metrics of α-diversity. There were no between-group differences in β-diversity, nor any differentially abundant taxa in infants. The intervention increased abundances of the genus *Prevotella* in mothers compared to controls.

Discussion: This gut-focused perinatal dietary intervention was associated with differences in the maternal and infant gut microbiota composition. Larger studies are required to replicate and extend these findings.

KEYWORDS
Dietary intervention; pregnancy; infant; gut microbiota; randomised controlled trial

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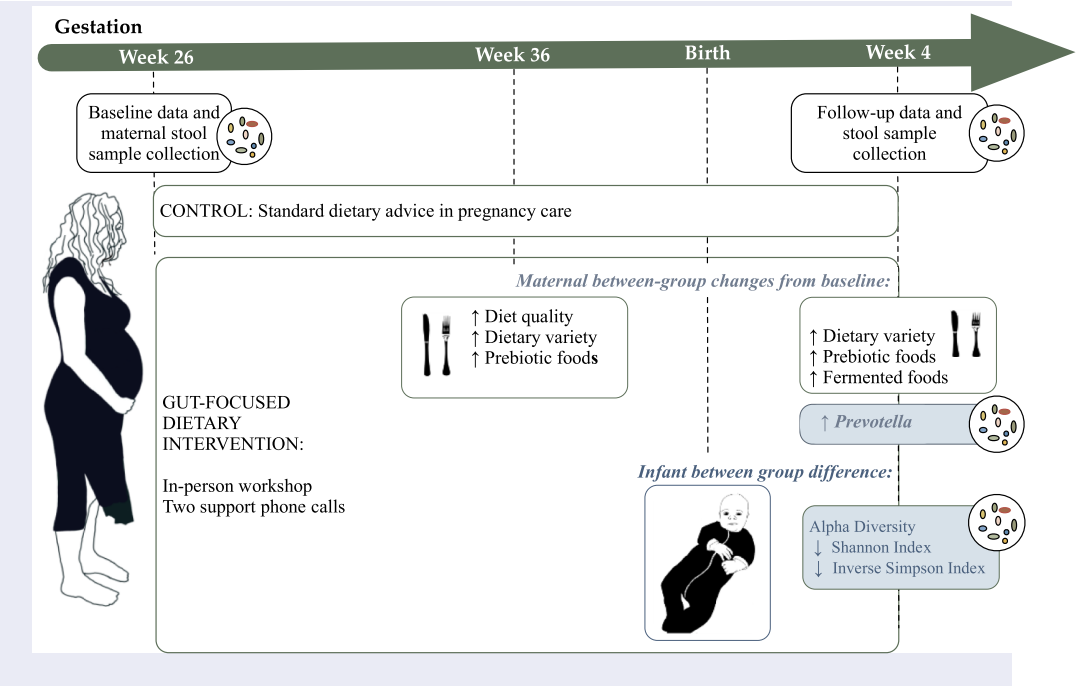
Supplemental data for this article can be accessed online at https://doi.org/10.1080/1028415X.2024.2413233.



This study presents the gut microbiota outcomes from a perinatal randomised controlled trial of an educational dietary intervention throughout the third trimester of pregnancy and first month postpartum. The intervention targeted '*eating for the gut microbiota*' as a behaviour change mechanism. We have reported previously that the intervention increased mothers' diet quality, variety and intakes of prebiotic foods before birth [1], and that increases in dietary variety and prebiotic foods were sustained after birth. Intakes of fermented foods were increased from baseline in the intervention group [1]. The results of the present study are denoted in blue, showing the primary outcome that infants in the intervention group had lower α-diversity (Shannon and inverse Simpson indices) at 4-weeks of age compared to the control group. In mothers, the between-group differential change from baseline to 4 weeks after birth showed a significant increase in the CLR-transformed abundance of the genus *Prevotella*. These results provide evidence that maternal diet may influence the maternal and infant gut microbiota.

Introduction

Preclinical studies demonstrate that high-fat or low-fibre maternal prenatal diets alter the maternal gut microbiota and offspring gut microbiota assembly [2,3], however it is still unclear whether this occurs in humans. Observational studies in humans show relationships between the prenatal diet and infant gut microbial composition [4–6]. For example, infants born to mothers consuming a high-fat prenatal diet (compared to lower fat) had gut microbiota differences at birth which remained detectable after six weeks [4]. The initial mechanism of transmission includes inoculation of the maternal microbiota upon delivery [7,8]. The maternal gut microbiota accounts for a high proportion of the variation in the gut microbiota of vaginally born infants [9], however taxa from the maternal gut microbiota are also present in caesarean-born infants [10]. Randomised controlled trials that investigate whether the prenatal diet shapes the maternal gut microbiota and infant microbial colonisation are now the next important step.

There is intense interest in the potential for the prenatal diet to modulate the early life gut microbiota, as this may influence early life programming. For example, in mice a high-fibre maternal diet shapes the offspring gut microbiota in a manner that reduces its susceptibility to lower respiratory tract infections by promoting a healthy pattern of early life immune development [11]. More broadly, preclinical studies demonstrate causal relationships between the diversity and composition of the maternal gut microbiota and offspring immunity

[12], brain development [13] and behaviour [14]. Our recent work explored the relevance of this in mother–child dyads in the Barwon Infant Study, a prospective birth cohort in Victoria, Australia [15,16]. We reported that the presence of *Prevotella copri* (a fibre-fermenting species) during the third trimester of pregnancy was associated with protection against food allergy in their children [15]. Within this same cohort, a healthy prenatal dietary pattern was associated with a higher diversity of the maternal prenatal gut microbiota, which in turn was associated with fewer internalising behaviour problems in two-year old children [16]. Prior to this study, we had reported that a higher abundance of *P. copri* in infants at 12 months was associated with fewer internalising behaviour problems at 2 years [17]. Concordantly, infant gut microbiota composition during the first year of life has been related to subsequent temperament [18], behaviour [17], and cognitive [19] outcomes. Importantly, as it pertains to the current study, α-diversity in infancy has been associated with better behaviour outcomes, specifically temperament and fear behaviour outcomes [18,20]. These studies provide a key rationale for targeting the prenatal and infant gut microbiota during early life to promote better offspring health. Yet intervention studies in humans are needed to understand the potential for the maternal diet to shape infant microbial colonisation.

The Healthy Parents, Healthy Kids (HPHK) study was a pre-registered, randomised controlled trial (RCT) that aimed to evaluate the efficacy of a perinatal educational dietary intervention from the third trimester of pregnancy, for altering the gut microbiota in mothers and their infants measured four weeks after birth [21]. We have reported that compared to standard care, the intervention increased total diet quality (adherence to the Australian Dietary Guidelines (ADG)) [22] throughout the third trimester of pregnancy with a large effect size Cohen's D: 0.82 (SE: 0.33), however this change was not sustained at follow-up four weeks after birth [1]. Compared to the control group, the intervention sustained increases in dietary variety and intakes of prebiotic and probiotic/fermented foods throughout the third trimester through to four weeks after birth with large effect sizes [1]. We hypothesised that intervening in the maternal diet from the third trimester (as it is most proximal to birth) would alter the maternal and infant gut microbiota. This study reports the intervention effects on maternal and infant gut microbiota four weeks after birth.

Methods

Study design

The HPHPK study was registered prospectively in the Australia and New Zealand Clinical Trials Registry on 14th July 2016 (ACTRN12616000936426). The study protocol [21] has been published and intervention effects on maternal diet [1] have been reported. In accordance with the CONSORT 2017 guidelines for intervention studies [23], this study reports the study's primary outcome, which was a between-group mean difference in the Shannon index of α-diversity in infant faecal samples 4 weeks after birth. We also report secondary outcomes for infant faecal microbiota composition at follow-up included between-group differences in other α-diversity measures (mean: inverse Simpson index, observed species count, phylogenetic diversity), community dissimilarity and differential ASV abundance, including *Prevotella* abundance. For mothers, between-group changes from baseline at four weeks after birth were investigated for the above outcomes. To provide 80% power to detect a difference of at least 0.25 Shannon index units in infants at four weeks of age, assuming a standard deviation of 0.4 and a two-tailed type 1 error of 0.05, 80 women would be required [21]. Therefore, the recruitment target was 90 allowing a loss to follow-up of 10 women.

We hypothesised that, compared to the control group, the faecal microbiota of mothers and infants in the intervention group would have: (a) higher α-diversity (primary outcome); (b) dissimilarity in community structure (β-diversity) and differentially abundant taxa; (c) and that mothers in the intervention group would have higher relative abundance of the fibre-degrading genus *Prevotella* [21].

Recruitment

Healthy pregnant women were recruited online and within the Melbourne community to participate from gestation week 26. Informed consent was obtained prior to enrolment. Participants were eligible if they were able to attend a dietary workshop and were not: under 18 years of age; living with a BMI of 30 or higher; diagnosed with a bowel condition, mental illness or diabetes mellitus; lacking dietary autonomy or on an exclusion diet; using illicit drugs; taking antibiotics, prebiotic or probiotic supplements in the previous month [21]. Participants were randomised according to a concealed 1:1 allocation ratio that used permuted block sizes. An external statistician prepared the randomised schedule and loaded it into a module in the *Research*

Electronic Data Capture (REDCap) system [24]. REDCap was used to securely administer the study and collect participant data. After baseline data collection, participants were randomised and the study administrators were informed of group allocation to enable study execution.

Procedures

The control condition was standard pregnancy care, which in the Australian public health system comprises regular appointments with midwives and obstetricians who provide ADG-based advice. In addition to standard care, the intervention condition received a three-hour workshop between weeks 26–29 of pregnancy and two support phone calls in weeks 31 and 36 [21]. The intervention targeted the behaviour '*eating for the gut microbiota*' and the education and dietary advice was based on the ADG and increasing intakes of prebiotic and probiotic/fermented foods. The intervention design incorporated behaviour change techniques [25] to support sustained dietary behaviour change, including: setting three dietary goals, goal monitoring and support via two phone calls, and dietary monitoring via a self-reported FFQ [21]. Intervention adherence was high, as evaluated by participation and engagement rates across the intervention activities: all 22 participants in the intervention group attended the workshop, set their dietary goals, and completed their week 31 support call [1]. The week 36 support call was completed by 21 of 22 (95%) women [1]. Self-reported dietary intakes were completed by 20/22 (90%) mothers at week 31, and 21/22 (95%) of mothers at week 36 [1].

Data and faecal sample collection

Data collection included individual and household demographics, maternal and infant physical health, wellbeing, diet and lifestyle [21]. Data were collected at baseline, gestational week 26 (prior to randomisation), and at follow-up four weeks after birth. Mothers provided faecal samples at baseline (during gestation week 26) and at follow-up four weeks after birth. The infant faecal sample collection occurred four weeks after birth, this proceeded the hepatitis B vaccination administered at birth and preceded subsequent vaccinations occurring at 2-months of age [26].

Faecal sample processing

Mothers brought their baseline samples on ice to the Royal Children's Hospital, where they were stored at −80°C. Follow-up samples were collected during a home visit and were transported on dry ice for storage at −80°C. DNA extraction and 16S rRNA sequencing was performed by the Australian Genomic Research Facility (AGRF). PCR amplicons using the V3-V4 hypervariable region of the 16S rRNA gene were generated with the forward primer, 341F, 5'- CCTAYGGGRBGCASCAG-3' and reverse primer, 806R, 5'-GGACTACNNGGGTATCTAAT-3'. Purified PCR amplicons were sequenced on the Illumina MiSeq platform (San Diego, CA, U.S.A.) with a V3, 600 cycle kit (2 × 300 base pairs paired-end) in accordance with the manufacturer specification and AGRF protocols. Cutadapt [27] was used to trim primers from the demultiplexed raw reads. The DADA2 pipeline [28] was used to generate the ASV table. Specifically, the *dada2* package in R was used to inspect read quality, filter, merge paired reads, remove chimeras, generate an amplicon sequence variants (ASV) table, and assign taxonomy against release 138.1 [29]. of the SILVA [30] database.

Statistical methods

Hypothesis testing followed an *a priori* protocol using a modified intention-to-treat principle, where participants with least one valid post-baseline follow-up were included [21]. Statistical significance was considered at the $p < 0.05$ level [31] for the *a priori* hypotheses and for differential abundance testing a q-value for of <0.05. Our microbiota data analysis methods deviated from the protocol [21] through the use of updated methods: compositional data analysis approach (CoDA) to microbiome analysis, as is currently recommended best practice [32], and the use of ASVs (rather than OTUs).

Alpha diversity

Four α-diversity outcome measures were calculated for maternal and infant faecal samples using the R packages *phyloseq* [33] (Shannon Diversity index, inverse Simpson, observed species count) and *vegan* [34] (Faith's phylogenetic diversity). Normality of distributions were assessed with visual inspection of quantile-quantile plots. To test for between-group mean differences in infant α-diversity measures, independent student's *t* tests were conducted where data were normally distributed, otherwise Wilcoxon–Mann–Whitney *U* tests were used. Where linear regression model assumptions of homoscedasticity were violated [35], then heteroscedasticity-robust standard errors approach were implemented [36]. The sample storage duration (in days) was evaluated as a technical artefact that may have influenced the precision of the results. To increase the precision on

the main effect, if the sample storage duration related to any of the α-diversity measures then secondary analysis was performed using linear regression models adjusted for this duration.

For maternal α-diversity outcome measures, linear mixed-effect (LME) models were used to estimate the between-group change from baseline α-diversity. The random effect term was the participant ID, and the interaction between group and timepoint was used to predict each α-diversity outcome. For maternal α-diversity outcomes, change from baseline α-diversity measures were created and these were used to report descriptive summary data and effect size. Cohen's D effect was calculated to estimate the intervention effect size for the maternal change from baseline α-diversity measures, and the infant follow-up α-diversity measures, with D of 0.2, 0.5, 0.8, 1.2 corresponding to small, medium, large and very large effects [37].

β-diversity

After evaluating alpha diversity we performed unsupervised prevalence filtering using a threshold of 5% as described by per Callahan et al. [38]. CoDA matrices representing ASV abundances were created by agglomerating taxa at the genus-level and applying a centre log-ratio (CLR) transform to the count data using the *Tjazi* package [39]. For maternal data, we created a matrix representing the change from baseline CLR-transformed ASV abundances. Aitchison distances were calculated for maternal and infant data to evaluate differences in β-diversity. Group-based separation was inspected using Principal Components Analysis (PCA) plots. Permutational multivariate analysis of variance (PERMANOVA) [40] using the *adonis2* R package [34] with 999 permutations was used to evaluate the statistical significance of any group-based separation in the β-diversity matrices.

Maternal Prevotella *abundance*

To evaluate our *a priori* hypothesis that compared to the group, mothers in the intervention group would have higher relative abundance of *Prevotella,* we tested for a between-group differential change from baseline four weeks after birth in maternal *Prevotella* abundance. A CLR transformation was applied to the maternal count data at the genus-level, and a linear-mixed effects model with the participant ID as a random effect was used. The linear mixed model contained nominal fixed effects of time, intervention group and the two-way interaction between time and group allocation. In this setting the two-way interaction estimated the intervention effect on CLR-transformed *Prevotella* abundance. Due to our recent results regarding maternal *P. copri* carriage and offspring food allergy [15], we performed an exploratory analysis to test for a between-group change from baseline four weeks after birth for maternal *P. copri* abundance using the same methods described for *Prevotella* but at the species-level.

Differential CLR-transformed ASV abundance testing

Maaslin2 was used for differential abundance testing at the genus level. A Benjamini-Hochberg *p*-value adjustment was applied with significance considered at a false discovery rate (FDR) of <0.05. If our linear-mixed effects model reported a between-group change from baseline in maternal *Prevotella* abundance, then *Prevotella* would also be expected to appear as a discovery in the differential abundance analysis. In this case, the FDR threshold would not be interpreted for *Prevotella* given that this was independently evaluated as an *a priori* hypothesis.

Additional multivariable analysis

We considered the relationships between the potential determinants of α-diversity, and α and β-diversity (Supplementary Figure 1). The potential determinants included antibiotic exposure (mothers' and infants'), birth mode, gestational age, and mode of feeding. All participants with follow-up data for these determinants were included in the analysis. Where relationships existed between these determinants and α diversity, then potential effect modification of the intervention was evaluated using linear regression with an interaction term between the group and α-diversity determinant. Similarly, where relationships existed between a determinant and β-diversity, then possible effect modification of the intervention was evaluated statistically using PERMANOVA models with an interaction term between group and the determinant.

Sensitivity analyses

To evaluate the influence of infant antibiotic use on the infant α-diversity results, sensitivity analysis was performed by excluding the infants that had used antibiotics. A second sensitivity analysis investigated the influence of potential outliers in sequencing depth on the α-diversity outcomes by excluding samples with a sequencing depth 1.5 interquartile ranges (IQR) below the first IQR or above the third IQR.

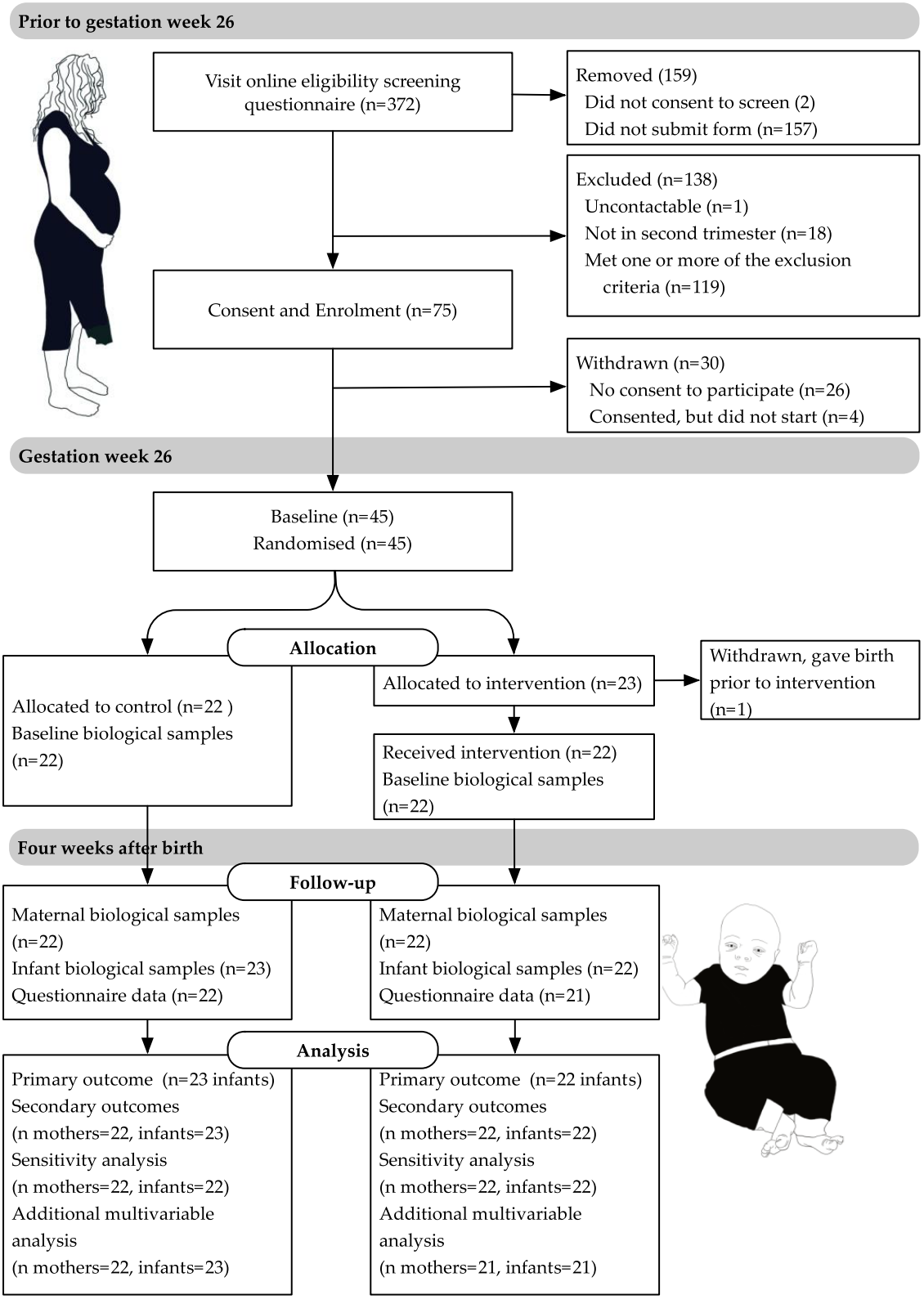


Figure 1. CONSORT flow chart showing participant flow through the study and numbers used for each analysis. Adapted from [1].

Ethics

This study was approved by the Royal Children's Hospital Human Ethics Committee on 17th December, 2015 (HREC 35200), and Deakin University Human Ethics Committee on 16th February, 2016 (DUHREC 2016-036).

Table 1. Participant characteristics.

	Control	Intervention
Mothers		
n	22	22
Antibiotic use (between 26 weeks gestation and four weeks postpartum) %		
No	15 (68.2)	14 (63.6)
Yes	7 (31.8)	7 (31.8)
Unknown	0 (0.0)	1 (4.5)
Antacid or acid suppressing medication use[a] (between 26 weeks gestation and four weeks postpartum) %		
No	6 (27.3)	8 (36.4)
Yes	15 (68.2)	11 (50.0)
Unknown	1 (4.5)	3 (13.6)
Baseline sample storage duration at −80°C (mean weeks (SD))	44.6 (12.5)	44.3 (12.5)
Follow-up sample storage duration at −80°C (mean weeks (SD))	29.2 (12.5)	28.1 (12.8)
Baseline α-diversity		
Shannon index (mean (SD))	4.06, (0.46)	4.1, (0.45)
Inverse Simpson (mean (SD))	33.13, (19.22)	34.19, (16.62)
Phylogenetic diversity (mean (SD))	204.27, (51.5)	204, (68.39)
Observed richness (mean (SD))	4.06, (0.46)	4.1, (0.45)
Infants		
n	23	22
Sex (%)		
Female	11 (47.8)	10 (45.5)
Male	12 (52.2)	11 (50.0)
Unknown	0 (0.0)	1 (4.5)
Birth, gestation weeks (%)		
Less than 32 weeks	2 (8.7)	0 (0.0)
32–37 weeks	1 (4.3)	0 (0.0)
37–42 weeks	19 (82.6)	21 (95.5)
42 weeks or more	1 (4.3)	0 (0.0)
Unknown	0 (0.0)	1 (4.5)
Birth mode (%)		
Vaginal	20 (87.0)	17 (77.3)
Caesarean	3 (13.0)	4 (18.2)
Not reported	0 (0.0)	1 (4.5)
Mode of feeding (%)		
Receiving breast milk only at four weeks	18 (78.3)	15 (68.2)
Not exclusively receiving breast milk	5 (21.7)	6 (27.3)
Unknown	0 (0.0)	1 (4.5)
Infant antibiotic use during first four weeks of life (%)		
No	22 (95.7)	17 (77.3)
Yes	1 (4.3)	4 (18.2)
Unknown	0 (0.0)	1 (4.5)
Birth weight (mean g (SD))	3230.6 (671.3)	3385.9 (484.0)
Sample storage duration at −80°C (mean days (SD))	29.9 (12.3)	28.3 (12.7)

[a]Antacids such as Zantac, Losec, Nexium, Motilium, Mylanta, Quickeze, Gaviscon, Rennie.

Results

Participant characteristics

Of 213 participants who completed eligibility screening, 75 were eligible. Of these, 45 were randomised starting from July 2016 (Figure 1 for reasons for non-participation) [1]. One participant from the intervention group withdrew due to premature delivery prior to receiving the intervention workshop. Forty-four women and 45 infants (including one set of twins) completed the study. The final follow-up was in October 2017. All completers provided faecal samples and all but one provided follow-up questionnaire data. Following the modified intention-to-treat protocol, this mother/infant dyad were included in all of the main outcome analyses, and were only excluded from the additional multivariable analysis describing the predictors of alpha and beta diversity (e.g. birth mode, antibiotic use etc.) presented in supplementary material.

The maternal baseline demographic characteristics including diet and lifestyle factors are presented elsewhere [1]. Briefly, there were no between-group differences for education, income, employment, marital status, mean BMI, type of pregnancy care, and baseline dietary intake. Participants were generally Australian born, first time parents, in full time work with higher education, and none met all of the ADG recommendations [1]. There were no differences between groups in maternal antibiotic use, faecal sample storage duration or baseline α-diversity (Table 1). There were no between-group differences in infant characteristics including factors known to influence infant gut microbiota such as modes of birth and feeding, however the intervention group had slightly higher infant antibiotic use during the first four weeks of life (Table 1). The median sequencing depth of all maternal samples was 14716 reads per sample (IQR: 8606) with one outlier identified using the IQR method (sequencing depth 39184, Supplementary Figure 2 (a)). The median sequencing depth of the 45 infant samples was 20066 (IQR: 13986), with two potential high outlying samples identified (sequencing depths 54884, 55142) (Supplementary Figure 2(a)).

Between-group differences in α-diversity

The primary outcome, infant faecal Shannon index was 0.35 units lower in infants of mothers in the intervention group compared to the control group (Figure 2, Table 2). Likewise, the inverse Simpson index was lower in infants of the intervention group compared to control group. These differences represented a medium magnitude of effect. The concordance between Shannon and inverse Simpson indices suggests that rare or dominant taxa did not influence the results. There was no evidence of between-group differences for Faith's phylogenetic diversity, nor observed species in infants. However, the duration that the sample was stored at −80°C was negatively associated with the infant observed species count (Supplementary Table 1). After adjustment for storage duration, the between-group difference estimates for the infant α-diversity models increased slightly, with all being lower in the intervention group ($p < 0.05$) (Supplementary Table 2). For mothers, there was no evidence

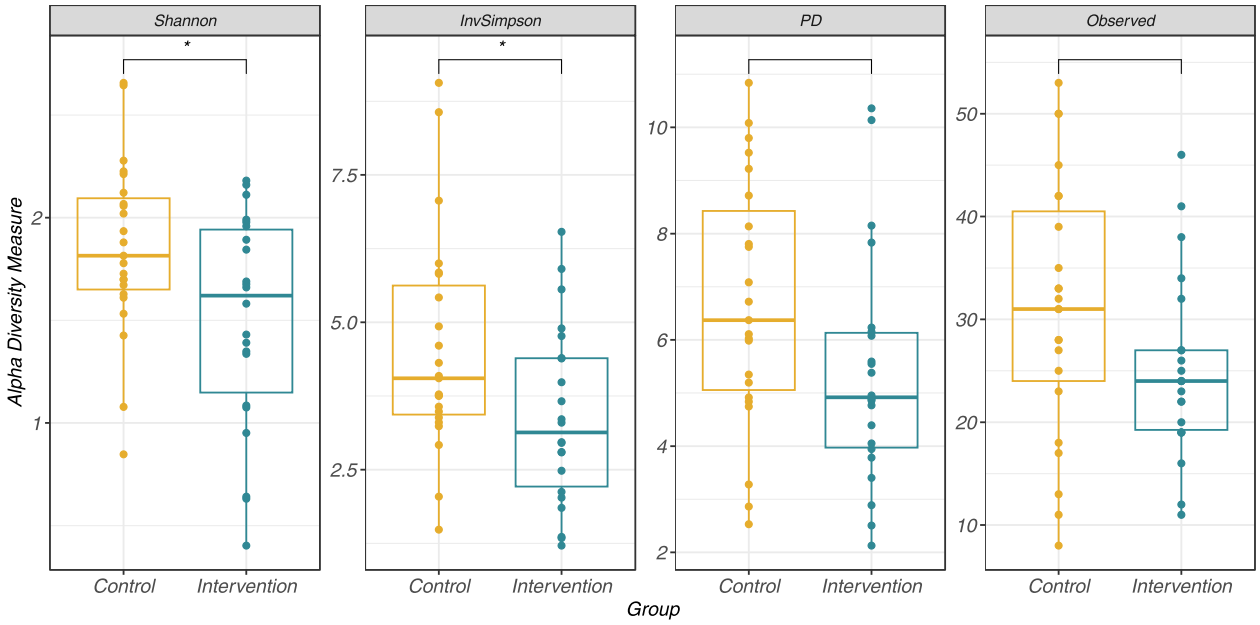


Figure 2. Box and whisker plot showing between group differences in infant gut microbiota α-diversity indices.

of any between-group differences across the α-diversity measures (Table 2), nor was the storage duration associated with these measures (Supplementary Table 2).

There were no clear patterns of association between the potential predictors of α-diversity (antibiotics, infant gestational age, birth mode, mode of feeding) and the α-diversity measures in mothers and infants (Supplementary Table 1). Therefore, except for sensitivity analysis involving antibiotic usage in infants, additional analyses were not performed for these factors. Antibiotic usage was slightly higher in infants in the intervention group; however, this did not explain the lower Shannon index in the intervention group, as sensitivity analysis with antibiotic users removed did not attenuate the results, all α-diversity indices were lower in the intervention group ($p < 0.05$) and the mean difference in infant Shannon index was −0.38, (95% CI: −0.72, −0.04), $p = 0.03$ (Supplementary Table 3). Similarly, sensitivity analysis indicated that infant α-diversity results were not influenced greatly by the two infant samples with high read counts (Shannon index mean difference: −0.37, (95% CI: −0.67, −0.07), $p = 0.02$, Supplementary Table 3).

Between-group differences in community structure (β-diversity)

There was no evidence of any between-group differences in for the maternal pre-to-post intervention change β-diversity measure, nor for the infant β-diversity measure. Maternal dietary group allocation explained 2.26% ($p = 0.48$) of the variance in Aitchison distance for maternal samples, and 2.14% ($p = 0.49$) for infant samples (Figure 3). Of the potential predictors of infant β-diversity, birth mode explained 6.72% of the variation in infant Aitchison distance ($p = 0.001$) and feeding mode explained 4.55% ($p = 0.02$) (Supplementary Figure 3); however, there was no evidence that either modified the intervention effect (Supplementary Table 4).

Maternal Prevotella *abundance*

There were no between-group differences in CLR-transformed *Prevotella* abundance at baseline (Figure 4, top). In mothers, the between-group differential change from baseline to 4 weeks after birth showed a significant increase of 0.66 in the CLR-transformed *Prevotella* abundance (95% CI: 0.21, 1.1, $p = 0.005$) (Figure 4, bottom).

Maternal P. copri *abundance*

There were no between-group differences in CLR-transformed *P. copri* abundances at baseline (Supplementary Figure 4). After the study, there was no evidence of any between group differences in the change in *P. copri* abundances (0.34, 95%CI: −0.11, 0.79, $p = 0.14$).

Differential CLR-transformed ASV abundance

In mothers, there were between group differences in the pre-to-post intervention change in abundances

Table 2. Between-group differences in infant and maternal measures of α-diversity measured at 4-weeks postpartum.

Measure	Descriptive statistics		Test statistics[a]				Effect size[b]	
Infant samples	Control $n = 23$	Intervention $n = 22$						
	Mean, (SD)	Mean, (SD)	Mean difference	95% CI	*p*-value	r^2	Cohen's D	95% CI
Shannon index	1.85, (0.43)	1.5, (0.52)	−0.35	(−0.64, −0.07)	0.02	0.1	−0.74	(−1.36, −0.12)
Inverse Simpson	4.54, (1.87)	3.39, (1.52)	−1.15	(−2.17, −0.12)	0.03	0.09	−0.67	(−1.29, −0.06)
Faiths PD	6.69, (2.34)	5.36, (2.19)	−1.33	(−2.69, 0.04)	0.06	0.06	−0.58	(−1.2, 0.03)
Observed Species	31.04, (12.59)	25.05, (8.75)	−6	(−12.51, 0.55)	0.07	0.05	−0.55	(−1.16, 0.06)
Maternal samples	$n = 22$	$n = 22$						
	Differential change from baseline, (SD)	Differential change from baseline, (SD)	Between-group differential change from baseline 4-weeks after birth	95% CI	*p*-value		Cohen's D	95% CI
Shannon index	−0.08, (0.44)	−0.06, (0.3)	0.02	(−0.2, 0.25)	0.82		0.07	(−0.54, 0.67)
Inverse Simpson	−4.38, (18.79)	−1.34, (14.72)	3.03	(−6.99, 13.05)	0.55		0.18	(−0.43, 0.79)
Faiths PD	2.09, (6.65)	−0.18, (6.79)	−2.28	(−6.27, 1.71)	0.26		−0.34	(−0.95, 0.27)
Observed Species	20.77, (54.18)	3.95, (62.76)	−16.82	(−51.62, 17.99)	0.34		−0.29	(−0.9, 0.32)

SD, Standard Deviation; PD, phylogenetic diversity.
[a]For infant samples, the test statistics represent mean differences using independent *t*-tests. For maternal samples, the test statistics represent the two-way interaction between time and group allocation from a linear mixed-effect models with participant as a random effect term.
[b]For maternal samples, Cohen's D effect sizes are based on mean change from baseline measures for each group.

for eight genera, including increased Prevotella_9 ($p < 0.05$) however the evidence for all eight attenuated after adjustment for multiple testing (Table 3). Likewise, in infants, there was a between group difference in abundances of two genera, however after adjustment the differences were attenuated (Table 3).

Discussion

Summary of main findings

To our knowledge, this is the first RCT to evaluate whether a gut-focused prenatal dietary intervention can alter the maternal and infant gut microbiota. This study failed to meet the recruitment target and therefore was underpowered. The dietary intervention was associated with a moderate reduction in infant α-diversity (Shannon and Simpson indices), and no change to maternal α-diversity measured four weeks after birth. In mothers, the intervention was associated with higher *Prevotella* abundances at follow-up. This study adds to our previous finding that it is feasible to improve women's dietary intake during the latter stages of pregnancy [1], and shows that this modulation was associated with alterations in the maternal and infant gut microbiota at four weeks of age.

Infants in the intervention group had a lower average α-diversity than those in the control group that was not explained by the slightly higher rate of antibiotic use in the intervention group. This result does not support our *a priori* hypothesis that prenatal dietary improvement would increase infant α-diversity. Our study design was based on a now outdated premise that higher α-diversity was associated with better infant health (as was the case in adults) [41,42]. It is understood that the diversity of the infant gut microbiota is initially low, particularly in breast fed infants [43], and it increases with dietary complexity to adult levels by around three years of age [43,44]. Recent systematic reviews report inconsistent associations for infant α-diversity and outcomes, such as allergy [45], and cognition [46]. In this context, α-diversity is also now considered to be an overly simplistic summary metric with limited biological meaning or clinical relevance. Due to differences in microbiota measurement and reporting we were unable to meaningfully compare our α-diversity difference measures with those that informed our original power calculation [21], or those reported in recent cognitive outcome studies [18,20].

We expected that higher diet quality and/or dietary variety would be associated with higher microbial diversity in the mothers, as reported elsewhere [16,47]. The

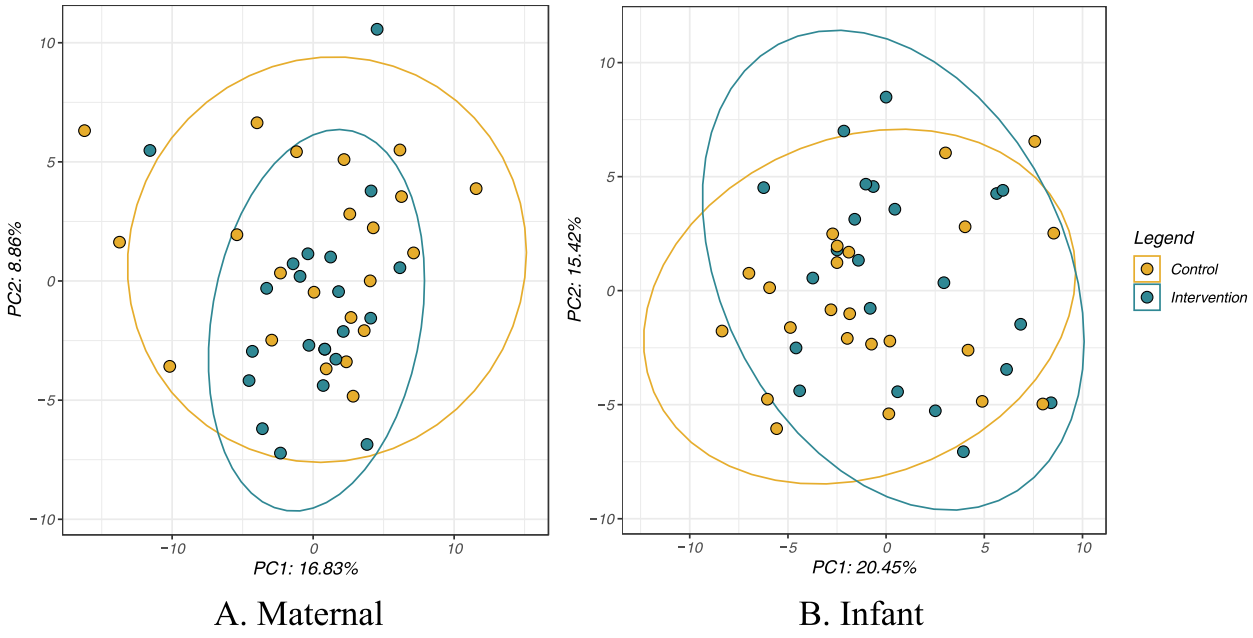


Figure 3. Group-wise β-diversity PCA plots of the first two principal components. A. Maternal Aitchison distance pre-to-post intervention change, B. Infant Aitchison distances.

apparent lack of effect may be due to timing of sample collection, or the dose, duration, or magnitude of dietary change being insufficient for eliciting longer-lasting changes in maternal α- or β-diversity. Alternatively, it may be a true effect, as an observational dietary study compared the gut microbiota between adults consuming an omnivorous diet, plant-based vegetarian, or vegan diets ($n = 153$) and found no between-group differences in α or β-diversity [48], however the fibrous plant-based diets were associated with greater abundances of the fibre-degrading genus *Prevotella* [48]. We report that mothers in the intervention group had an increased relative abundance of *Prevotella* compared to controls, this is in support of our original hypothesis. This increase in *Prevotella* may have been driven the higher intakes of fibrous and prebiotic foods (containing inulin, fructooligosaccharides and/or oligosaccharides) achieved in the intervention group [1], as other dietary intervention studies trialling fibrous foods report increases in *Prevotella* abundance [49,50]. The potential for a gut-focused dietary intervention to increase *Prevotella* is of interest, as we previously reported that maternal prenatal carriage of *Prevotella* was associated with a subsequent lower risk of food allergy in children [15].

We hypothesised that changes in the infant microbiota would occur via changes to the maternal gut microbiota, but as maternal sample faecal samples were not collected just prior to birth we are unable to evaluate transmission. The magnitudes of maternal dietary change were higher at the 36-week dietary follow-up than 4-weeks after birth when the faecal samples were collected [1], therefore there may have been an unmeasured difference in the maternal gut microbiota around birth, when vertical transmission is thought to occur [7]. It is possible that the between-group difference in infant α-diversity was driven unmeasured diet-related differences in the maternal breastmilk composition. The maternal pre- and postnatal diet modulates the nutritional and microbial composition of breastmilk [51]. In animals, a higher fibre maternal diet impacts the offspring gut microbiome via changes in the composition of breast milk [11], however further studies are needed to determine how this relates to humans [52].

Strengths and limitations

The strengths of this study includes its randomised controlled trial design, the intervention's success in altering maternal dietary intakes, and the *a priori* analysis plan [21]. The groups were well balanced and retention rates were high [1]. However, this study was underpowered and lacks a clinically meaningful outcome measure. Due to the smaller than anticipated sample size, the between-group differences reported for our primary outcome of infant alpha diversity may be alternatively explained by random variation in the context of limited statistical power. Our results may also be influenced by other factors. For example, we could not investigate other specific potential predictors of maternal or infant gut microbiota composition such as the use of proton pump

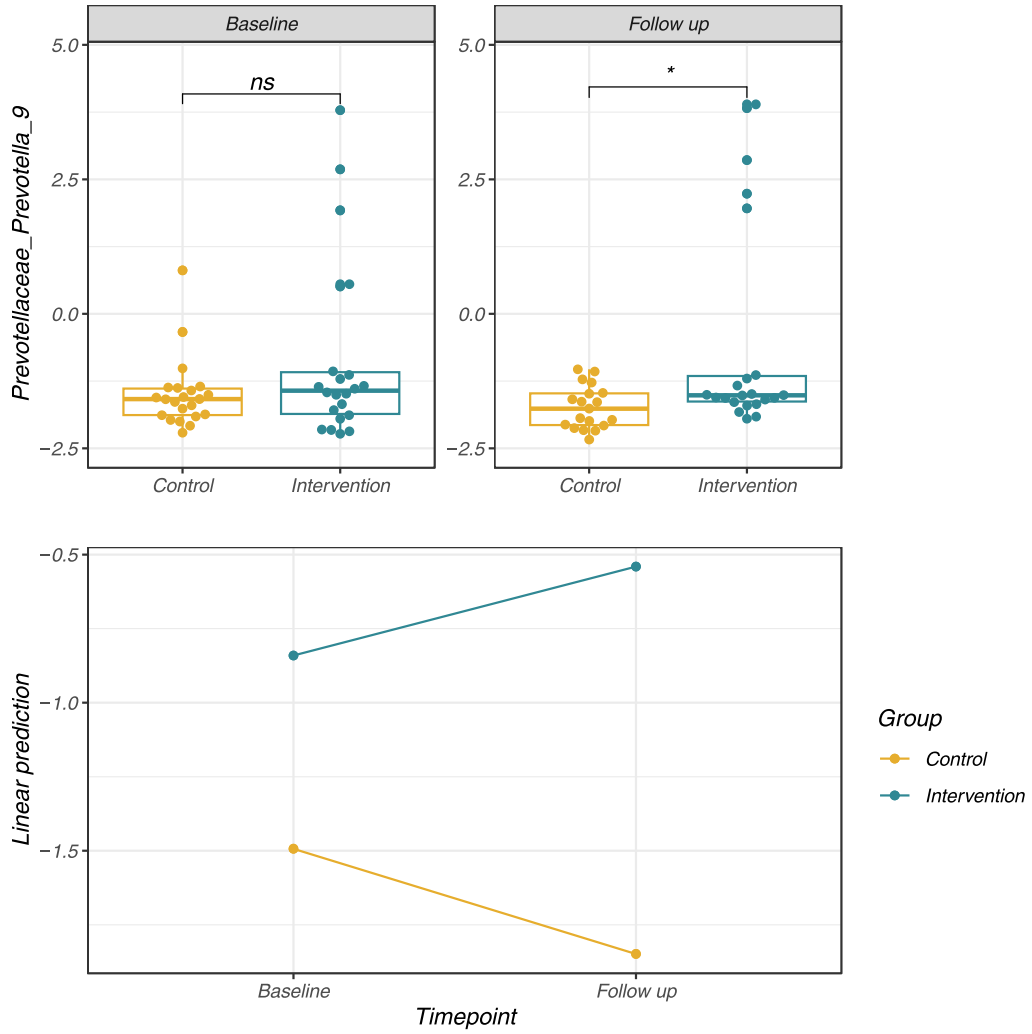


Figure 4. Maternal CLR-transformed *Prevotella* abundances for each group. Top panel shows baseline and follow-up boxplots and points for *Prevotella* abundances, with between-group *t*-test comparison results (ns indicates $p \geq 0.05$). The bottom panel shows the direction of change in *Prevotella* abundance for both groups using an interaction-style plot of the estimated marginal means based on the linear mixed effect model.

inhibitors [53,54] as our questionnaire did not distinguish between antacids or acid suppressing medication, and we did not record steroid treatments [55], maternal and infant vitamin D levels [56], or fucosyltransferase2 (FUT2) secretory statuses [57]. We anticipate that any potential bias of these factors would be evenly distributed between groups due to randomisation.

As there was no evidence that mode of birth or feeding were related to the α-diversity measures we were unable to explore whether the intervention effect on infant α-diversity differed by these factors. Our estimates of whether the intervention effect on infant β-diversity differed by these factors were likely underpowered given the low rates of caesarean-births and infants not exclusively receiving breastmilk.

A further limitation of this study is that there was only one follow-up timepoint for infant faecal samples, therefore it is unclear how robust or enduring these proof-of-principle results are. Further, we likely missed important changes to the maternal gut microbiota as the maternal faecal sample was collected four weeks after birth instead of prior to birth when the magnitude of dietary change was highest. These results may be influenced by the microbial remodelling that occurs throughout pregnancy, with reduced α-diversity in the third trimester, and an overall shift in microbial community structure between trimesters one to three [58]. The effect of microbial remodelling would likely balance out due to randomisation; however, it is still unclear how personalised remodelling is. Finally, the generalisability of our findings are limited to healthy, generally well-educated Australian pregnant women with a BMI of less than 30.

Table 3. Differential CLR-transformed ASV abundance between mothers and infants in the intervention group relative to the control group.

Feature (Family Genus)	Coef	Stderr	*P*-value	*Q*-value
Maternal change from baseline				
Prevotellaceae Prevotella_9	0.66	0.23	0.01	0.42
Oscillospiraceae Colidextribacter	−0.82	0.32	0.01	0.42
Lachnospiraceae Anaerostipes	0.97	0.39	0.02	0.42
Barnesiellaceae Barnesiella	−0.68	0.28	0.02	0.42
Lachnospiraceae Dorea	0.88	0.36	0.02	0.42
Ruminococcaceae Incertae.Sedis	−0.87	0.40	0.03	0.60
Erysipelotrichaceae Turicibacter	0.88	0.41	0.04	0.60
Butyricicoccaceae UCG.009	0.37	0.18	0.05	0.62
Christensenellaceae Christensenellaceae.R.7.group	−0.65	0.33	0.05	0.62
Atopobiaceae Olsenella	0.48	0.25	0.07	0.70
Infant				
Carnobacteriaceae Dolosigranulum	0.30	0.14	0.04	0.70
Atopobiaceae Atopobium	0.44	0.21	0.04	0.70
Bifidobacteriaceae Scardovia	0.40	0.21	0.06	0.70
Rikenellaceae Alistipes	−0.53	0.32	0.11	0.70
Bacteroidaceae Bacteroides	−1.50	0.91	0.11	0.70
Coriobacteriaceae Collinsella	−0.95	0.62	0.14	0.70
Micrococcaceae Rothia	0.37	0.25	0.15	0.70
Lachnospiraceae Ruminococcus..gnavus.group	0.93	0.70	0.19	0.70
Lachnospiraceae Ruminococcus..torques.group	−0.49	0.38	0.20	0.70
Erysipelatoclostridiaceae Erysipelatoclostridium	−0.67	0.53	0.21	0.70

Mothers $n = 44$; Infant $n = 45$. *Q*-value uses the Benjamini-Hochberg method, significance considered at $Q < 0.05$. Reporting shows the top 10 Maaslin2 results. Positive coefficients (Coef) indicate that the CLR-transformed ASV abundance at the genera level was higher in the intervention group compared to the control group. Maternal dataset reflects the CLR change from baseline.

Implications for future research or clinical practice

In relation to infant health, new theories now consider the developmental trajectory of the infant gut microbiota [44,59]. Laursen et al. [44] consider the timing of the progression to a more diverse microbiota to be more important when evaluating the health-relevance of α-diversity than measurements at a single time point. New data from the field of allergy show that differences in the compositional trajectory of the infant gut microbiota in late infancy relate to allergy risk [59,60]. For cognitive outcomes, Carlson et al. [19] posited that the better cognitive results seen in one-year old infants with lower α-diversity may have been due to delayed maturation of the gut microbiota, which perhaps influenced a longer period of cortical plasticity. It is likely that α-diversity is too simplistic of a summary statistic to subsequently link to health because it is not specific to particular taxa, communities, or microbial functions. Coupling diversity with other measures of microbial composition and function may be more informative. This suggests the need to consider the developmental trajectory of the infant gut microbiota and function across multiple time points in relation to later clinically relevant outcomes.

Further research is needed to inform dietary interventions aiming to alter the infant gut microbiota via the pre- and/or postnatal maternal diet and infant feeding practices. To adequately power such studies, further research is needed to determine what a meaningful change in the infant gut microbiota would be. Future studies should further interrogate dietary strategies for impacting the infant microbiome, for example, by initiating dietary improvement earlier in pregnancy, continuing for a longer duration, and investigating different magnitudes of dietary change. Additionally, further research is needed to investigate whether the maternal diet exerts effects on the infant gut microbiome via diet-related changes to the maternal milk microbiome [52], as this could have implications for continuing dietary strategies throughout the lactation period.

Our findings need to be replicated in larger RCTs that are adequately powered to evaluate the impact of maternal diet on the infant gut microbiota, while also considering the microbial remodelling that occurs during pregnancy, and the influence of postnatal factors such as infant birth and feeding modes. It will be important that future trials investigate neurodevelopmental, immune and metabolic impacts of these changes, and that they are sufficiently large to evaluate impacts on clinically relevant outcomes. These trials should include a more diverse population starting in the preconception phase, and collect maternal vaginal, faecal and breastmilk samples at birth, and infant faecal samples across multiple timepoints.

Conclusion

Although underpowered/exploratory, this study provides evidence of an association between the perinatal diet and infant gut microbiota composition. Compared to a control condition consisting of standard dietary advice during pregnancy, a gut-focused perinatal dietary intervention was associated with differences in aspects of the maternal and infant gut microbiota measured 4 weeks after birth. In mothers, the abundance of the genus *Prevotella* was higher in the intervention group compared to controls. Infants born to mothers in the intervention group had a lower alpha diversity compared to infants in the control group. Larger trials are now needed to replicate and extend our exploratory findings. Ideally these trials would start prior to conception and continue throughout infancy to track the contribution of the maternal diet on the developmental trajectory of the infant gut microbiome over time. Future research is needed to provide insights

into the possible clinical implications of maternal dietary impacts on the infant gut microbiota.

Acknowledgements

The authors thank the HPHK study team for assisting with the study conduct; Dr Yuk Jing Loke and Dr Pamela Leong for biological sample collection training and support; Mrs Jann Parkes for the mother and baby artwork. We acknowledge support from the Victorian Department of Education and Training.

Author Contributions

All authors met the criteria for authorship, and all approved this final manuscript. FNJ conceived of the study and SLD and FNJ designed the study in consultation with JMC and GC. ALP advised on recruitment and retention. SLD implemented the study and delivered the intervention. Senior authors FNJ and JMC supervised the conduct of the study. TCB performed home visits, data collection, and sample collection and preparation. SLD performed the statistical analyses and drafted this manuscript. AL, ALP, GC, MM advised and reviewed the statistical methods and results. AL, ALP, AO, FNJ, GC, JMC, MM, TCB, MT, PV provided feedback and revisions on this manuscript.

Disclosure statement

FNJ reports industry support for research from Meat and Livestock Australia, Woolworths Limited, the A2 Milk Company, and Be Fit Foods, and two relevant books for commercial publication. AL, ALP, MLKT and PV are named inventors on a patent 'Behavioural Treatment' PCT/AU2019/ 050878 regarding the role of Prevotella in childhood health. GC. has received honoraria from Janssen, Probi and Apsen as an invited speaker, is in receipt of research funding from Pharmavite, Fonterra, Tate and Lyle, Nestle and Reckitt, and has received payments as a consultant for Yakult and Zentiva; this support neither influenced nor constrained the content of this article. AO is supported by a National Health & Medical Research Council Emerging Leader 2 Fellowship (2009295). All other authors report no disclosures.

Funding

This study was funded by the Food & Mood Centre, Deakin University and received in-kind support from the Murdoch Children's Research Centre and the Royal Children's Hospital.

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Data availability statement

The participants of this study did not give written consent for their data to be shared publicly, so due to the sensitive nature of the research supporting data is not available.

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