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Original article

Maternal prebiotic supplementation during pregnancy and lactation modifies the microbiome and short chain fatty acid profile of both mother and infant

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SUMMARY

Background & aims: Improving maternal gut health in pregnancy and lactation is a potential strategy to improve immune and metabolic health in offspring and curtail the rising rates of inflammatory diseases linked to alterations in gut microbiota. Here, we investigate the effects of a maternal prebiotic supplement (galacto-oligosaccharides and fructo-oligosaccharides), ingested daily from <21 weeks' gestation to six months' post-partum, in a double-blinded, randomised placebo-controlled trial.

Methods: Stool samples were collected at multiple timepoints from 74 mother–infant pairs as part of a larger, double-blinded, randomised controlled allergy intervention trial. The participants were randomised to one of two groups; with one group receiving 14.2 g per day of prebiotic powder (galacto-oligosaccharides GOS and fructo-oligosaccharides FOS in ratio 9:1), and the other receiving a placebo powder consisting of 8.7 g per day of maltodextrin. The faecal microbiota of both mother and infants were assessed based on the analysis of bacterial 16S rRNA gene (V4 region) sequences, and short chain fatty acid (SCFA) concentrations in stool.

Results: Significant differences in the maternal microbiota profiles between baseline and either 28-weeks' or 36-weeks' gestation were found in the prebiotic supplemented women. Infant microbial beta-diversity also significantly differed between prebiotic and placebo groups at 12-months of age. Supplementation was associated with increased abundance of commensal *Bifidobacteria* in the maternal microbiota, and a reduction in the abundance of Negativicutes in both maternal and infant microbiota. There were also changes in SCFA concentrations with maternal prebiotics supplementation, including significant differences in acetic acid concentration between intervention and control groups from 20 to 28-weeks' gestation.

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Conclusion: Maternal prebiotic supplementation of 14.2 g per day GOS/FOS was found to favourably modify both the maternal and the developing infant gut microbiome. These results build on our understanding of the importance of maternal diet during pregnancy, and indicate that it is possible to intervene and modify the development of the infant microbiome by dietary modulation of the maternal gut microbiome.

1. Introduction

The "first 1000 days" from conception play a crucial role in establishing immune, metabolic, and neurological development [1]. Microbial exposure and colonization patterns play an important role in shaping these developing pathways. Loss of key microbial species, particularly in the gut, is implicated in the global increase of noncommunicable diseases (NCDs) by disrupting immune and metabolic regulation throughout life [2,3]. While multifactorial, diet imposes the strongest force on gut microbiota community composition [4–6]. This includes dietary effects on the maternal microbiome in pregnancy, which have implications for both fetal physiology and postnatal colonization [7]. Therefore, modulation of the maternal diet to promote a favorable community of maternal gut microbiota for early-life infant exposure, is, an attractive strategy for promoting development of a beneficial early-life gut microbiome, which can lead to long-term immune-metabolic health.

One of the most well described methods for positively modulating the microbiome is with prebiotic fiber supplements, which is typically utilised by the gut microbiota as the main source of carbon [8]. Prebiotic fibre-based dietary supplements, in particular, fructo-oligosaccharide (FOS) and galacto-oligosaccharide (GOS) are commonly used as discussed in several reviews [8–10]. FOS and GOS have also demonstrated beneficial effects when added to prebiotic infant formula [11]. Each of these soluble fibres are well known for the ability to maintain a healthy gut ecosystem by favouring the growth of commensal bacteria (most notably *Bifidobacterium* spp.), regulating levels of short chain fatty acids (SCFA), and reducing gut pH [12]. SCFA are involved in several important pathways in the gut including dampening inflammation in colon epithelial cells and inhibiting the inflammatory response by monocytes [13]. The quantify and production of SCFA in the gut is almost exclusively determined by the resident gut microbiota, which is the major reason why prebiotic fibres can improve gut health.

The maternal microbiome also has recognised antenatal effects on multiple aspects of fetal development, including the development of the infant gut microbiome, that may influence future childhood health outcomes [2,14]. Microbial products, including genetic material and metabolites absorbed into the maternal blood, have the potential to cross the placental barrier and act as important external exposures which modulate fetal immune programming [2,15].

After birth, the establishment of the infant gut microbiome is further influenced by factors including breast milk composition, solid food diet, environmental exposures and antibiotic administration. Breastmilk also acts as both a pre- and pro-biotic, containing both human milk oligosaccharides (HMOs) and microbes which support the growth of mutualistic microorganisms in the infant gut [16]. As the composition of breastmilk changes over time, and with maternal dietary habits [17], prebiotic fibre supplementation during lactation may also have the potential to influence infant gut health through this additional pathway. While there is much interest in exploring the impact of maternal diet on enhancing maternal and infant gut health [18], we are unaware of any research investigating the effectiveness of maternal prebiotic supplementation in the development of the infant gut microbiome.

In this study, we examined the effect of maternal prebiotic (GOS and FOS) supplementation on the gut microbiome and SCFA concentrations of mother–infant pairs. The mothers were enrolled in a double-blinded, randomised controlled, allergy prevention trial investigating the effects of maternal prebiotic supplementation during pregnancy and lactation [19].

2. Methods

2.1. Study design and faecal sample collection

Stool samples were collected as part of a larger, two-arm (1:1 allocation), parallel-design, double-blinded, superiority randomised controlled allergy intervention trial [19], known as the SYMBA Study, which randomised a total of 652 pregnant women. The SYMBA Study was nested within The ORIGINS Project [20], a unique long-term cohort study, recruiting 10,000 families from the communities of Western Australia (WA). Full methodological details of the SYMBA Study, including exclusionary criteria are in the protocol paper [19]. Briefly, the participants of the SYMBA Study were randomised to one of two groups (1:1 allocation); one group allocated to consume 14.2 g per day of prebiotic powder (GOS and FOS in ratio 9:1 as seen in a previous RCT [21]), and the other receiving a placebo powder consisting of 8.7 g per day of maltodextrin. The energy content of the powder supplements (143 KJ per day) was matched between groups. The trial intervention period was from <21 weeks' gestation until 6 months' postnatal infant age. A subset of these mother–infant pairs (selected based on time series completeness) was included in this present microbiome analysis (Table 1). Using G*Power we determined that a total sample size of 52 mothers was required to determine (with a power of 80% and two-tailed alpha = 0.05) statistically significant differences in stool acetate concentrations (Faul et al., 2007); the effect size (r = 0.81) was based on publicly available data [22].

In this current study, complete stool sampling at all timepoints was obtained from 65 mother–infant pairs. At 20-weeks' gestation a baseline was established by collecting a single stool sample from

Table 1
Stool sample collection timepoints during pregnancy (weeks) and after birth (months). The number of samples received and analysed in this study is shown at each timepoint.

	20-w	28-w	36-w	2-m	4-m	6-m	12-m
Maternal samples recieved							
Placebo group	32	32	32	32	32	32	
Prebiotic group	39	39	39	39	39	38	
Infant samples received							
Placebo group				32	32	32	32
Prebiotic group				39	39	39	37

each mother prior to consumption of the trial prebiotic supplement or placebo. Stool samples were then collected from each mother at 5 timepoints during pregnancy and in the post-natal period, and infant stool samples were collected at 4 timepoints during the first year of life (Table 1). At each sampling point, a single stool was collected into three identical specimen jars, and frozen in the household freezer within 15 min of collection. The frozen samples were then taken to Joondalup Health Campus (Joondalup, WA) in a supplied Styrofoam pack by each participant. From Joondalup Health Campus, the stool samples were transferred on dry ice to the Western Diagnostic pathology laboratories in Myaree, WA, and biobanked at −80 °C, at the Telethon Kids Institute, located in the Perth Children's Hospital, Nedlands WA until sample processing.

2.2. Library preparation, sequencing, and SCFA quantification

Stool sample processing, DNA extraction, and sequencing are described in full in the supplementary methods. In brief, stool samples were categorised using the Bristol Stool Form Scale (BSFS) [23] prior to sample aliquoting for downstream analyses. Samples for SCFA analysis were immediately frozen at −80 °C, and then transferred to the Science Analytical Facility at Edith Cowan University, WA, for SCFA quantification using GC–MS as previously described (Jones et al., 2021). Microbial DNA was extracted from stools using QIAamp PowerFecal Pro DNA kit (QIAGEN, Hilden, Germany), and the bacterial 16S rRNA gene (V4 region) was amplified. Amplicon libraries were prepared in-house, and sequencing was performed at Curtin University, WA, using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Contamination was accounted for using NTCs during the DNA extraction and PCR steps. To provide a positive reference control, the microbial DNA from eight randomly selected individuals were added to each sequencing run. Mock communities (https://www.atcc.org/products/msa-1006) were also added to evaluate recovery across sequencing runs.

Quality filtering, trimming, and merging of sequences was performed prior to inferring amplicon sequence variants (ASVs) using DADA2 [24]. Chimeric errors were removed using the default method, and sequences variants were classified using the Genome Taxonomy reference database (Version 202) formatted for use with DADA2 (https://zenodo.org/record/4735821#.YN18Om4RWis). The species assignments for the top 200 ASVs were also confirmed by BLAST using the same databases, at 100% identity. ASVs with up to three matches were annotated to include all three potential species assignments, whereas any ASV with more than three identical matches was annotated with the genus name followed by “spp”. Where taxa were not fully resolved, the lowest available rank name and sp., gen., or fam. have been annotated for each lower taxonomic level. Potential contamination was removed using the package microDecon [25], and final data filtering removed ASVs with unassigned phylum, plus those with a prevalence of less than 1 read in 50 (2%) samples.

2.3. Statistical analysis

A detailed description of the analyses is supplied in the supplementary methods. Briefly, enterotypes were assigned to the maternal microbiota (https://enterotype.embl.de), and a McNemar's test was used to compare the counts of Firmicutes-dominated enterotypes over time. DistLM was used to identify factors with a significant contribution to the composition of the maternal and infant gut microbiota. Differential abundance testing between intervention and placebo groups was performed at the genus, family, and phylum taxonomic levels, using MaAsLin [26]. Lastly, a Wilcoxon signed rank test was used to test both groups for an increase in *Bifidobacterium* and *Lactobacillus* abundance. P values were corrected using Benjamini-Hochburg adjustment (BH) (Benjamini & Hochberg, 1995), with significance was set at $p = 0.05$. Beta-diversity was visualized using Principal Coordinates Analysis (PCoA) and was calculated using Euclidian distances of centre-log-ratio transformed counts of ASVs. Differences in beta-diversity were determined with PERMANOVA, PRIMER-e v7 [27].

Alpha-diversity estimates were calculated on decontaminated reads and those alpha-diversity indices that were significantly correlated using Pearson correlation (PD) to library size were calculated on rarefied reads. Individual SCFA concentrations were analysed as mM/g of faeces, with the concentration variance stabilized by a log10 transformation prior to analysis. Untransformed concentrations shown in plots. Differences in alpha-diversity and SCFA concentrations over time within each randomised group were determined using linear mixed-effects models, performed using lme4 in RStudio. Pairwise tests were restricted to 5 timepoint comparisons for maternal samples, and 3 timepoint comparisons for infant samples, and the p value of these post-hoc comparisons were accepted only if $p < 0.010$ or $p < 0.017$, respectively, according to the Bonferroni correction method. A one-way ANOVA was used to compare the relative change in both SCFA concentrations and alpha-diversity from baseline to 28-weeks' and 36-weeks' gestation between the intervention and placebo groups. Extreme outliers (see supplementary) were removed prior to analysis. Multiple comparisons were corrected and accepted only if $p < 0.025$, according to the Bonferroni correction method. Amplicon sequencing and statistical analyses were completed while blinded from the treatment group allocation.

3. Results

3.1. Data quality and participant characteristics

Across 6 sequencing runs, and after all filtering (Supp. table 1), a total of 40,153,233 sequences ranging from 20,820 to 105,769 per sample were present. ASVs totalling 1,478 ranged from 20 to 242 and averaged 78.5 ± 48.7 per infant sample, and from 92 to 449 and averaged 285.6 ± 69.6 ASVs per maternal sample. Faith's phylogenetic diversity (PD) was correlated to read count ($R = 0.99$, $p < 0.001$) and therefore rarefied counts were used. Mock communities (Supp. Figure 1), replicate samples (Supp. Figure 2), and negative controls, were also sequenced alongside sample data to assess the quality of the data and depth of species assignments across all runs. A full description of the data quality is provided as well as PCO plots showing samples by sequencing run (Supp. Figure 3).

To describe the participant cohort within this microbiome analysis sub-set, each of the randomised groups were characterised at baseline and post-randomisation (Table 2). Some participants did not provide information for every variable. Mothers and infants who received any antibiotics over the course of the trial were not excluded from this analysis. While antibiotic use is a potential microbiome modifier, there was a high incidence of antibiotic use during the intervention, making it impractical to exclude a large proportion of women and infants due to antibiotic use. Furthermore, given the variable timepoints of maternal antibiotic use over the 11-month long intervention period controlling for antibiotic use was not feasible. Significantly more mothers in the placebo group had previously used prebiotics prior to the start of the trial (Chi-squared, $p = 0.03$), and infants in the prebiotic group weighed significantly more at 6-months of age (t-test, $p = 0.02$) compared to the placebo group. The duration of breastfeeding and age introduced to infant formula have also been summarised as proportions for each intervention group (Supp. Table 2). No other significant

Table 2
Baseline and post-randomisation characteristics for mother–infant pairs. Data are presented as mean and standard deviation. Percentages are used to show the proportion of individuals for which the characteristic is true. P values are for t-test or Chi-Squared tests; significant differences are highlighted in bold.

Baseline characteristic	Placebo (n = 33)	Prebiotic (n = 41)	p
Maternal age in years	32.97 ± 4.05	33.09 ± 3.79	0.89
Ethnicity Caucasian: Other	1(n = 28) 2(n = 5)	1 (n = 40), 2(n = 1)	0.12
Previous use of probiotic supplements	21 (63.6%)	23 (56.1%)	0.67
Previous use of prebiotic supplements	15 (45%)	8 (19%)	**0.03**
Pre-pregnancy BMI (kg/m2)	24.8 ± 4.87	24.9 ± 4.79	0.93
Weight at randomization (kg)	71.64 ± 13.57	74.42 ± 13.61	0.38
BMI at randomization (kg/m2)	26.61 ± 4.78	26.28 ± 4.72	0.95
Maternal history of allergic disease	27 (81.2%)	33 (80.5%)	0.88
Infant sex ratio female: male	18:15	20:21	0.80
Post-randomisation characteristic			
Maternal weight at 36 weeks' gestation in kg	79.98 ± 13.5	83.20 ± 14.10	0.33
Maternal BMI at 36 weeks' gestation	29.72 ± 4.75	29.88 ± 4.77	0.89
Maternal consumption of probiotics during the intervention	4 (12.12%)	10 (24.4%)	0.30
Maternal antibiotic use prior to 36 weeks' gestation	5 (15.2%)	7 (17.1%)	0.92
Maternal antibiotic use during the intervention	20 (60.6%)	29 (70.7%)	0.50
Infant birth weight in kg	3.36 ± 0.37	3.44 ± 0.46	0.39
Infant gestational age at birth in weeks	38.63 ± 1.16	38.5 ± 1.24	0.66
Preterm birth <37 weeks	0	3 (7.3%)	NA
Infant birth vaginal	15 (45.5%)	17 (41%)	0.73
Infant birth caesarean section	18 (54.5%)	24 (58.5%)	0.52
Ever breastfed	33 (100%)	40 (97.6%)	NA
Infant breastfed until 6 months of age	27 (81%)	28 (68%)	0.29
Average breastfeeding duration in months	9.20 ± 3.67	8.03 ± 4.07	0.20
Infant given any infant formula during intervention	28 (84.8%)	34 (82.9%)	0.92
Age introduced to any formula (months) in infants given formula	2.14 ± 2.86	2.28 ± 3.27	0.87
Infant consumption of prebiotics (in infant formula) during the intervention	15 (45.5%)	18 (43.9%)	0.92
Infant consumption of probiotics during the intervention	18 (54.5%)	17 (41.4%)	0.38
Infant antibiotic use during the intervention period[a]	12 (36.4%)	24 (58.5%)	0.09
Age at introduction to solid foods in months	5.05 ± 0.65	4.91 ± 0.69	0.36
Infant weight at 3 months of age in kg	6.04 ± 0.77	6.38 ± 0.75	0.06
Infant weight at 6 months of age in kg	7.56 ± 0.89	8.07 ± 0.91	**0.02**

[a] Dose not include antibiotics given prophylactically during labour.

participant characteristic differences were observed between the groups, and gastrointestinal symptoms were balanced between the intervention and control groups. All maternal and infant serious adverse events (SAE) were deemed not related to the study powders nor study protocol by an Independent SAE Committee.

3.2. Impact of the intervention on the maternal microbiome community structure

Beta-diversity of the maternal microbiota changed significantly from baseline in the prebiotic group to 28-weeks ($p = 0.023$, pseudo-$F = 1.4$), and to 36-weeks ($p = 0.004$, pseudo-$F = 1.6$). There was no significant change in the placebo group over the same time periods ($p > 0.16$, pseudo-$F < 1.1$). The relative abundance of microbiota was plotted using PCoA over the pregnancy period, showing the effects of the intervention over time in each group (Fig. 1A). The prebiotic supplementation did not significantly impact microbial alpha-diversity during pregnancy (Fig. 1B). However, in the prebiotic group, a decreased relative change in phylogenetic diversity (PD), Shannon, Fisher's alpha, and species richness occurred in 55% to 60% of individuals from baseline to 28-weeks. In comparison, only 39% to 54%, of individuals in the placebo group had a decreased relative change in the same alpha diversity measures over the same time. This difference was maintained until 36 weeks' gestation (16 weeks of supplementation), where 48% to 58% of participants in the placebo group and 68% to 72% of participants in the prebiotic group had a negative relative change across all alpha diversity estimates. The difference in the proportion of negative relative change values between the prebiotic and placebo groups was approaching significance when comparing Fisher's alpha ($p = 0.065$) and species richness ($p = 0.086$) at 28-weeks, and Fisher's alpha ($p = 0.04$), and species richness ($p = 0.051$) at 36-weeks. This indicates prebiotic supplementation may impact alpha diversity during pregnancy.

Enterotypes were assigned to maternal samples to observe shifts in these important taxonomic groups over time and between randomised groups. Twenty-eight post-natal maternal samples and 15 ante-natal maternal samples were not compositionally similar to the MEtaHIT reference samples and therefore confidence in those assignments is lower. However, the gut microbiome of pregnant mothers is expected to look characteristically different to that of a non-pregnant female [28], which might explain some of the low similarity. Of the 417 assignments, 190 were Firmicutes enriched (F-enterotype), 143 were *Bacteroides* enriched (B-enterotype), and 84 Prevotella enriched (P-enterotype). At the 20-week baseline, 65% and 63% of participants had an F-enterotype in the placebo and prebiotic groups, respectively. At the 36-week timepoint, the proportion of the F-enterotype decreased to 52% in the placebo group, and 45% in the prebiotic group. Over the pregnancy period, the shift from a F-enterotype to either a B-enterotype or P-enterotype was significant in the prebiotic group (X^2, $p = 0.048$) but not the placebo group (X^2, $p = 0.45$).

Lactobacillus, and *Bifidobacterium* were expected to increase in abundance in response to the prebiotic supplement. Therefore, the abundance of these genera at baseline (20-weeks) was compared to the abundance at 28 and 36-weeks within each intervention group. *Lactobacillus* did not change significantly in abundance in either group, and was not further investigated. Bifidobacteriaceae gen (a group which was unresolved past the family level). and *Bifidobacterium* both significantly increased from 20 to 28-weeks (Wilcoxon, $p < 0.02$) and from 20 to 36-weeks (Wilcoxon, $p < 0.01$) in the prebiotic group only. The baseline abundance of these bacterial

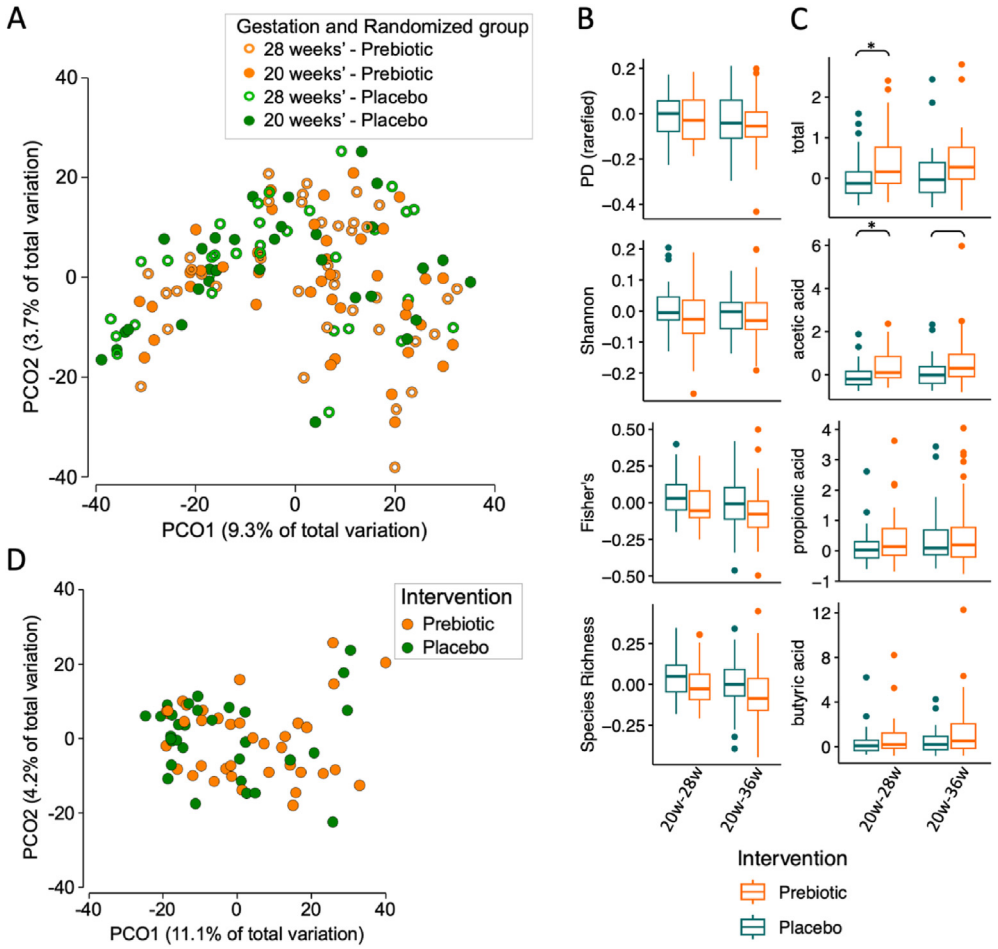

Fig. 1. Impact of the prebiotic supplement on the maternal and infant microbiome and SCFA concentrations. Distribution of the maternal microbiome composition according to Euclidian distances at 20 and 28-weeks's gestation (A). The infant microbiome composition according to Euclidian distances at 6-months of age (B). The relative change in alpha-diversity, including phylogenetic diversity (PD) and SCFA concentrations, from baseline to 28 and 36-weeks' gestation, within both the prebiotic and placebo groups (B). Box plot whiskers extend 1.5 times the interquartile range. Significant differences are indicated by ($p < 0.05$)*, and differences approaching significance ($p < 0.06$) are indicated by an empty single bracket. The infant microbiome composition according to Euclidian distances at 12-months of age (D).

groups was then also compared to the 2, 4, and 6-month timepoints. In the prebiotic group Bifidobacteriaceae gen remained significantly higher than baseline at the 2, 4 and 6-month post-natal timepoints, (Wilcoxon, $p < 0.01$); *Bifidobacterium* remained significantly higher than baseline at the 4 and 6-month post-natal timepoints, (Wilcoxon, $p < 0.02$) but not at the 2-month timepoint, where there was no significant difference to baseline (Fig. 2A). When considered Bifidobacterium abundance relative to Enterotype, the F-enterotype compared to the B and P-enterotype was found to have a higher proportion of Bifidobacterium species, regardless of randomised treatment group. Furthermore, the proportion of *Bifidobacterium infantis* and *Bifidobacterium* spp. increased predominantly in the F-enterotype group during pregnancy in the prebiotic group. Although these species do increase in all enterotype groups at 28-weeks, this may be partially confounded by the individuals who switch from F-enterotype to either P or B-enterotype during pregnancy (Fig. 2B).

The relative change in maternal SCFA concentration from baseline throughout the pregnancy period was also evaluated. From baseline to 28-weeks, 60% vs 29% of the participants in the prebiotic and placebo groups respectively had increased concentrations of acetic acid. Similarly, from baseline to 36-weeks, 68% vs 48% of the participants in the prebiotic vs placebo group had increased acetic acid. The proportion of women with a positive relative change in acetic acid was significantly higher in the prebiotic than the placebo group from baseline to 28-weeks ($p = 0.004$), but not from baseline to 36-weeks ($p = 0.053$), showing more women in the prebiotic group had an increase in gut acetate concentrations during pregnancy. Including all acids quantified (total SCFA), the magnitude of change was significantly different between randomised groups from 20 to 28-weeks ($F(69) = 7.34$, $p = 0.018$). Also, the relative change in acetic acid was significantly difference between the randomised groups from baseline to 28-weeks ($F(69) = 9.17$, $p = 0.006$) but not 36-weeks ($F(68) = 5.03$, $p = 0.056$). In both cases (total SCFA concentration and acetic acid concentration), the magnitude of change was higher in the prebiotic group, indicating a more rapid increase in acetate in the prebiotic group. There was no significant difference in butyric acid and propionic acid between the groups (Fig. 1C).

Changes to relative community abundance, SCFA concentrations and alpha-diversity (except Shannon index which did not shift significantly over time) were observed both during and after the pregnancy period within each randomised group (Fig. 3A). During pregnancy from 20 to 36 weeks, Fisher's index ($p = 0.008$), and Phylogenetic alpha-diversity (PD) ($p = 0.004$) decreased significantly, while butyric acid increased significantly ($p = 0.004$) in the prebiotic group only. During this time Actinomycetia expanded (2.4%) and receded (0.5%) in the prebiotic and placebo groups,

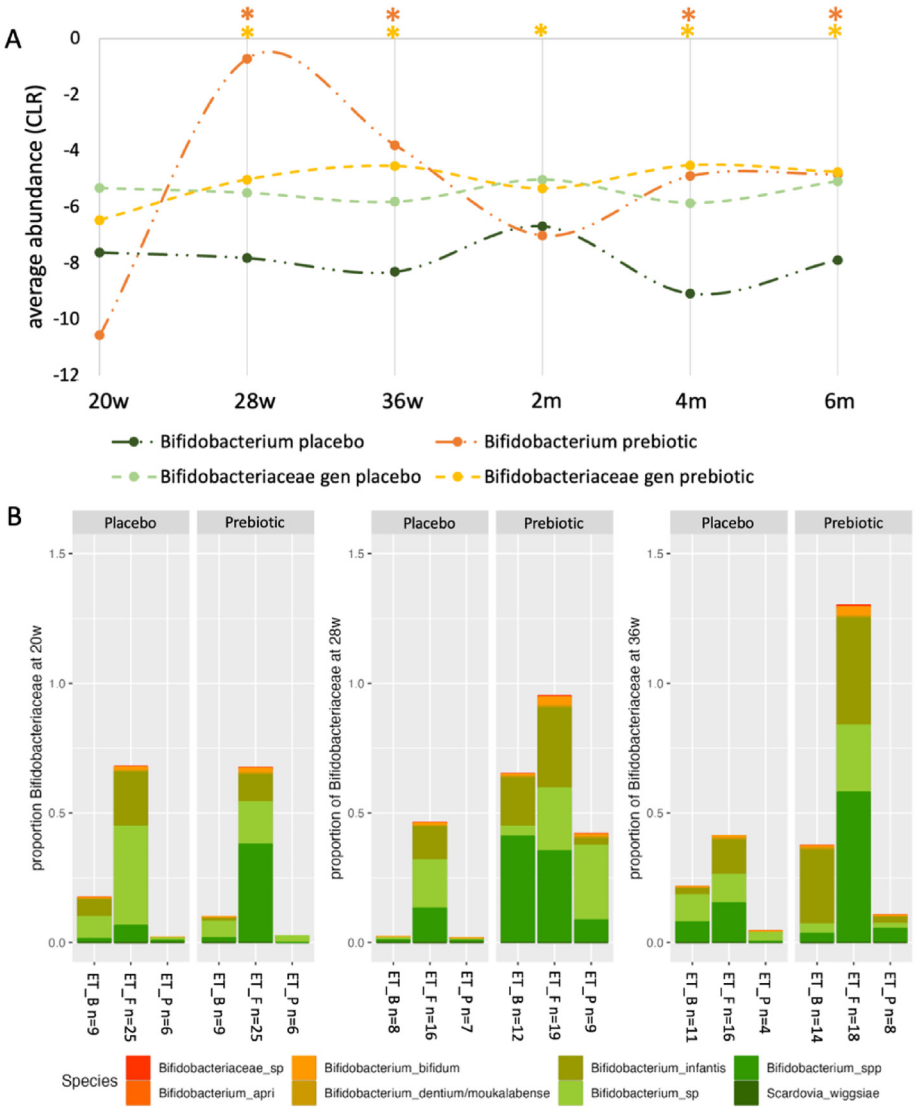

Fig. 2. Abundance of Bifidobacterium in the maternal microbiome overtime. (A) Shifts in the CLR transformed abundance of Bifidobacterium over six maternal timepoints (20, 28, 36-weeks' gestation, and 2, 4, and 6-months post birth). (A) Negative abundance does not indicate a deficit in abundance, but rather an abundance less than the mean centre abundance. Timepoints with significantly higher abundance of Bifidobacterium compared to baseline are indicated with a star in the colour of that genus on the plot, and colours compare between prebiotic (orange) and placebo (green) groups. (B) Proportional abundance of species within Bifidobacteriaceae according to maternal enterotype (Firmicutes enriched (ET_F) Bacteroides enriched (ET_B), and Prevotella enriched (ET_P)) and compared between prebiotic and placebo groups. Timepoints shown are baseline (20-weeks) and 28- and 36-weeks' gestation.

respectively. From 36-weeks' gestation to 2-months post-pregnancy, propionic acid levels dropped significantly in both randomised groups ($p < 0.009$), and over the same time-period acetic acid ($p < 0.001$) and butyric acid ($p < 0.001$) concentrations dropped significantly, but only in the prebiotic group. Across both intervention groups, Fisher's index, PD, and species richness were also significantly higher at 20-weeks' gestation than at 6-months post-birth ($p < 0.008$); indeed, the average of all alpha diversity measures was lowest at 6-months in this study (Fig. 3B).

3.3. Impact of the intervention on the infant microbiome community structure

The infant gut microbiota was found to be significantly different between groups at 12-months of age ($p = 0.034$, pseudo-$F = 1.4$); this difference in beta-diversity was visualised using PCoA at 12-months (Fig. 1D). The between group differences in beta-diversity at this time were hypothesised to be partially driven by differences in the abundance of *Bifidobacterium* species, which have a high prevalence in the infant microbiome. Over the first year of life, infants hosted three different *Bifidobacterium* species with an average abundance greater than 0.04%. *B. infantis* was most abundant (average 23%), followed by *Bifidobacterium* spp. (3.7%) and *Bifidobacterium bifidum* was least abundant (average 1.6%). A comparison of these species at 2-, 4-, 6- and 12-months of age showed no significant difference in abundance between the randomised groups ($p > 0.29$). Breastfeeding is also known to promote the growth of *Bifidobacterium* species; however, at 2-, and 4-months there were only 7 and 13 infants who had not been breastfed up until the respective time-point, rendering any further comparisons highly speculative.

The infant microbiome also changed over the study period, most noticeably from 4 to 12-months (Fig. 3B), where both alpha-diversity and total SCFA concentrations increased, with some

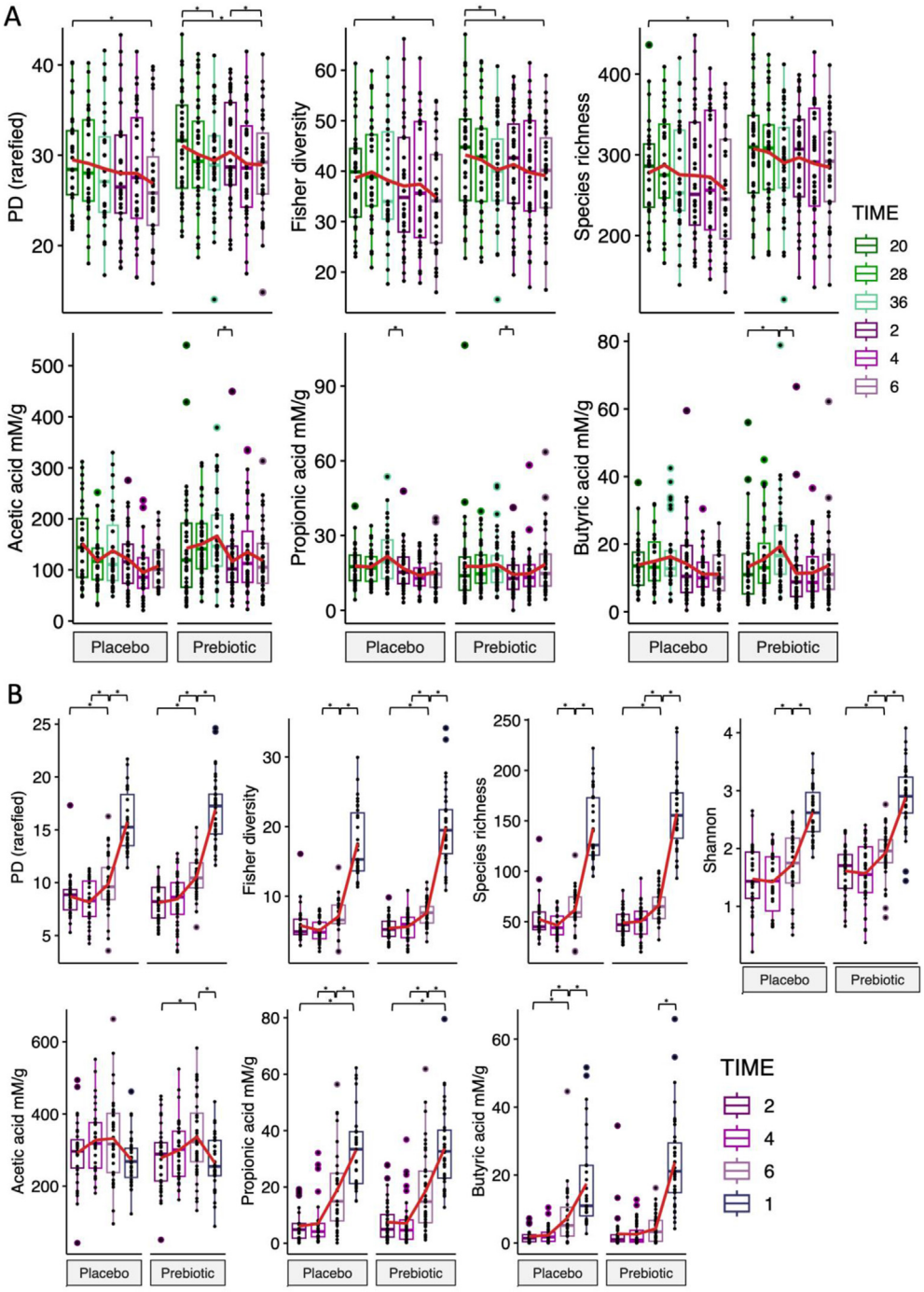

Fig. 3. Average maternal and infant alpha-diversity, and SCFA concentrations per randomised group. Box plots of the alpha-diversity including phylogenetic diversity (PD) and SCFA concentrations are also shown over the same period and split by intervention group for mothers (A), and infants (B). A red line connects the average at each timepoint per group, and the whiskers extend 1.5 times the interquartile range.

differences identified between the prebiotic and placebo groups. Acetic acid concentrations were relatively high compared to all other acids quantified at 2-months of age, increasing significantly from 2 to 6-months of age in the prebiotic group only ($p = 0.002$), while butyric acid increased significantly ($p = 0.02$) in the placebo group only over the same period. Also, from 2 to 6-months, all alpha-diversity measures increased significantly in the prebiotic group ($p < 0.003$), while only PD increased significantly in the placebo group ($p = 0.02$). All diversity measures significantly increased from 4 to 6-months of age across both groups. Over the same time-period, all alpha diversity measures, propionic acid, and butyric acid concentrations continued to increase significantly ($p < 0.001$) in both groups, while acetic acid levels dropped significantly ($p = 0.004$) in the prebiotic group only.

3.4. Differential abundance of taxa between intervention groups

The prebiotic and placebo groups were assessed for differentially abundant bacteria during and after pregnancy in the maternal microbiome, and over the entire study period for the infant

microbiome. There were no significant differences in bacterial abundance in the maternal gut microbiota after correcting for multiple testing (Table 3). However, prior to correction, 3 taxa including Bifidobacteriaceae gen., were enriched in the prebiotic group and 8 taxa including the Negativicutes class were enriched in the placebo group during pregnancy. After birth, 8 taxa including Akkermansiaceae were enriched in the prebiotic group, and 16 taxa including 6 families from Lachnospiraceae were enriched in the placebo group. Concerning the infant microbiota, Peptostreptococcaceae, *Acetatifactor*, *Clostridium*, and *Romboutsia* were found with significantly enriched abundance in the prebiotic group (Table 4). In the placebo group Negativicutes, Megasphaeraceae and 4 other families, and 9 genera were enriched. The Negativicutes, (including at the family level Selenomodaceae) were enriched in both the infant and (prior to FDR correction) maternal microbiome of the placebo group. Bacteria associated with antibiotic use prior to 36 weeks' gestation was also investigated, showing a significant reduction in Verrucomicrobiota, and *Akkermansia*, and an increase in Actinomycetaceae, *Corynebacterium,* Enterococcaceae, and *Enterococcus* (adjusted $p < 0.03$).

To determine bacterial taxa that were commonly shared among mothers and infants in both prebiotic and placebo groups, core bacterial ASVs were identified for maternal (36-weeks), infants (2-months), prebiotic, and placebo groups (Fig. 4). Core members had a minimum per sample abundance of 0.1% and were shared by at least 60% of group members to ensure a minimum of 1 core ASV shared between all groups. No core taxa were shared among all members of any group. At 36-weeks (which was the closest time-point to childbirth), there were a total of 1,399 ASVs present, with 40 ASVs making up the maternal core microbiota irrespective of intervention group; thirty-eight and 41 ASVs made up the core microbiota within the prebiotic and placebo groups, respectively. At 2-months, there were 541 ASVs present, with three ASVs making up the infant core microbiota. These were *B. infantis* (ASV 1), *Streptococcus* spp. (ASV22), and *Staphylococcus* (ASV150). The same three ASVs were shared as core members among mothers and infants in the prebiotic group, while two additional ASVs (ASV24, *Enterobacter* D; ASV242, *Streptococcus* spp) were also shared between mothers and infants in the placebo group.

4. Discussion

The breakdown of dietary fibre in the large intestine is one of the most important drivers of gut microbiota composition, with prebiotic supplements showing positive effects on the gut microbiome of both infants and adults [29,30]. One of the most predictable responses to a prebiotic supplement with FOS and GOS is an increase in the abundance of Bifidobacteria [31]. Indeed, despite the considerable inter-individual variations observed, Bifidobacteria were significantly more abundant in the prebiotic group, both during and after pregnancy, demonstrating the ability of the prebiotic supplementation to elevate the abundance of this important genus in both the antenatal and post-natal periods. *B. infantis,* along with *Streptococcus* and *Staphylococcus,* were identified as core members of the infant gut microbiota (with >60% prevalence in infants), with no difference in abundance according to the randomised groups. This is consistent with two other randomised placebo-controlled trials that assessed the

Table 3
Phyla, families, and genera identified with differential abundance over both the pregnancy and post birth period between the prebiotic and placebo groups. Bolded taxa indicate the taxonomic level at which changes were assessed. All results shown are significant prior to BH correction ($p < 0.05$), and corrected (qval) p values are also shown.

Prebiotic group enriched			Pregnancy			After birth		
Phylum/Class	Family	Genus	coef	pval	qval	coef	pval	qval
Actinomycetia	Bifidobacteriaceae	**Bifidobacteriaceae gen**	0.67	0.02	0.27			
Bacilli	UBA660	**UBA6985**				0.79	<0.01	0.11
Clostridia	CAG-272	**CAG-272**	0.81	0.01	0.24	0.74	0.01	0.18
Clostridia	**CAG-272**		0.93	0	0.12	0.78	0.01	0.22
Clostridia	Lachnospiraceae	**UBA7160**				0.78	0.03	0.28
Clostridia	Ruminococcaceae	**UBA1394**				0.77	0.03	0.29
Verrucomicrobiae	Akkermansiaceae	**Akkermansia**				1.22	0.01	0.22
Verrucomicrobiae	**Akkermansiaceae**					1.22	0.01	0.24
Verrucomicrobiota						1.12	0.01	0.21
Placebo group enriched								
Alphaproteobacteria	CAG-239	**CAG-495**				−0.72	0.03	0.28
Bacilli	Bacilli	**Bacilli**				−0.24	0.02	0.28
Bacilli	**Bacilli**					−0.24	0.04	0.27
Bacilli	Erysipelatoclostridiaceae	**Erysipelatoclostridium**				−0.71	0.03	0.28
Bacilli	Erysipelatoclostridiaceae	**Longibaculum**				−0.43	0.02	0.28
Bacilli	Lactobacillaceae	**Lacticaseibacillus**				−0.24	0.03	0.29
Bacilli	Lactobacillales	**Lactobacillales**				−0.61	0.01	0.18
Bacilli	**Lactobacillales**					−0.62	0.01	0.24
Clostridia	Butyricicoccaceae	**Intestinibacillus**	−0.66	0.02	0.27			
Clostridia	BX7	**BX7**				−0.25	0.02	0.28
Clostridia	**BX7**					−0.25	0.03	0.27
Clostridia	Lachnospiraceae	**Blautia**	−1.45	0.01	0.22	−1.64	<0.01	0.11
Clostridia	Lachnospiraceae	**Enterocloster**				−0.67	0.02	0.28
Clostridia	Lachnospiraceae	**Lachnospiraceae**				−0.33	0.02	0.28
Clostridia	Lachnospiraceae	**Mediterraneibacter**				−0.92	0.01	0.2
Clostridia	Lachnospiraceae	**Ruminococcus**	−1.19	0.02	0.27			
Clostridia	Lachnospiraceae	**Schaedlerella**	−1.19	0	0.11			
Clostridia	Lachnospirales	**Lachnospirales**	−1.03	0.02	0.27			
Clostridia	**Lachnospirales**		−1.04	0.02	0.18			
Firmicutes C/Negativicutes			−0.99	0.01	0.1			
Gammaproteobacteria	Enterobacteriaceae	**Enterobacter**				−0.53	0.03	0.28
Gammaproteobacteria	**Enterobacteriaceae**					−0.52	0.04	0.27
Negativicutes	**Selenomodaceae**		−0.63	0.03	0.29			

Table 4
Phyla, families, and genera identified with differential abundance between the 2 randomised groups over all study period timepoints for infants. All results shown are significant prior to BH correction ($p < 0.05$), and corrected (qval) p values are also shown, with significant results in bold. Bolded taxa indicate the taxonomic level tested.

Prebiotic group enriched					
Phylum/Class	family	genus	coef	p val	q val
Clostridia	Lachnospiraceae	**Acetatifactor**	0.32	0.02	0.06
Clostridia	Lachnospiraceae	**Clostridium**	0.21	0.03	0.07
Clostridia	Peptostreptococcaceae	**Romboutsia**	0.45	0.02	0.06
Clostridia	**Peptostreptococcaceae**		0.77	0.02	**0.04**
Placebo group enriched					
Actinomycetia	Mycobacteriaceae	**Corynebacterium**	−0.31	0.03	0.07
Bacilli	Erysipelotrichaceae	**Holdemanella**	−0.27	<0.01	**0.01**
Bacilli	Gemellaceae	**Gemella**	−0.47	0.01	**0.03**
Bacilli	**Gemellaceae**		−0.44	0.02	0.04
Campylobacteria	Campylobacteraceae	**Campylobacter**	−0.27	0.02	0.04
Campylobacteria	**Campylobacteraceae**		−0.24	0.04	0.09
Firmicutes C/Negativicutes		−0.95	<0.01	**<0.01**	
Gammaproteobacteria	Pasteurellaceae	**Pasteurellaceae**	−0.42	0.02	**0.04**
Negativicutes	Dialisteraceae	**Allisonella**	−0.08	0.04	0.09
Negativicutes	Megasphaeraceae	**Aeroglobus**	−0.48	<0.01	**0.01**
Negativicutes	**Megasphaeraceae**		−0.44	0.01	**0.02**
Negativicutes	Negativicutes	**Negativicutes**	−0.49	0.01	**0.03**
Negativicutes	**Negativicutes**		−0.46	0.02	**0.04**
Negativicutes	Selenomodaceae	**Selenomodaceae**	−0.23	0.04	0.08
Negativicutes	**Veillonellaceae**		−0.89	<0.01	**0.01**

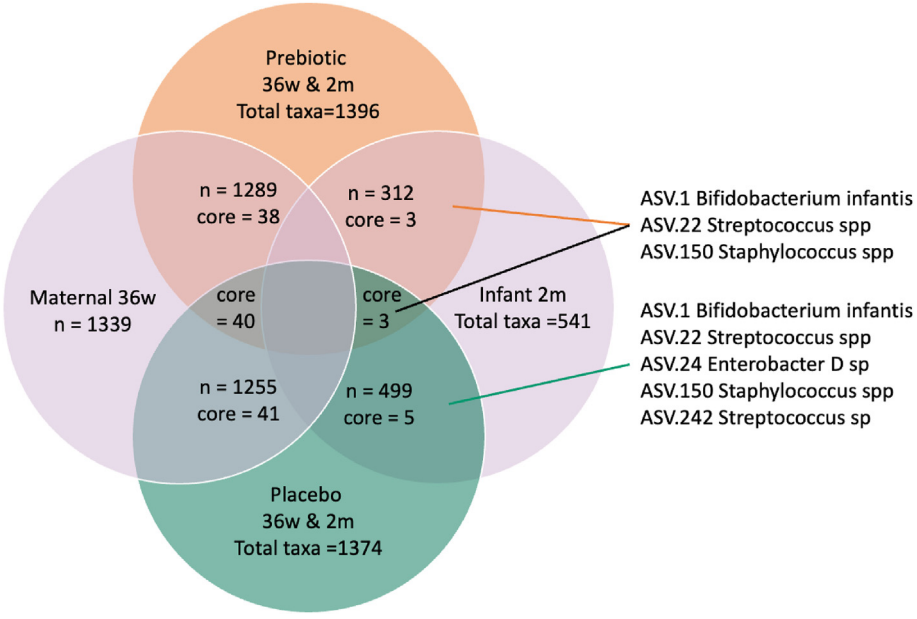

Fig. 4. Core bacterial ASVs of mother infant pairs. The number of core ASVs present within each of the maternal and infant groups, as well as in the placebo and prebiotic groups (mothers and infants) are shown in the outer-most circles. Core bacterial members (abundance of 0.1%) that are shared by at least 60% of group members are shown in the inner circles. Weeks (w) and months (m) have been abbreviated.

impact of maternal supplementation with FOS/GOS using fluorescent in situ hybridization [32] or qPCR [21]. Both studies reported a significant increase in the abundance of *Bifidobacterium* spp during the third trimester of pregnancy after FOS or GOS supplementation, whereas neither study found an increase in the number of *Bifidobacterium* spp in infant samples.

At 2-months postpartum *Bifidobacterium* contracted in the prebiotic group, and increased in the placebo group, resulting in a comparable abundance in these two groups at this timepoint. In a previous observational study, the microbiome of 47 healthy women were compared across pregnancy and the postpartum period; three of their time-points (two during pregnancy and one postpartum) match closely with three time-points in this study. They observed an increase in the relative abundance of Actinobacteria and *Bifidobacterium* beginning at 33- to 38-week's gestation that peaked at 6-weeks postpartum [33]. In line with this study, the similar abundance of *Bifidobacterium* at 2-months postpartum in both groups may indicate a characteristic fluctuation in the microbiome that occurs after pregnancy, possibly due to the energy demanding breastfeeding period. Additionally, infants delivered via caesarean section (over 50% in this study), expose the mother to antibiotics and pain relief medications that impact the gut microbiota community. Therefore, the prebiotic supplement was likely not the main driver of declining Bifidobacteria abundance at 2-months postpartum in the prebiotic group.

Unlike the maternal microbiome, factors that influence the assembly of the developing microbiome are predicted to have more long-lasting affects compared to those that occur after community establishment. In the present study, evidence that the infant microbiome may have been impacted by the maternal intervention included a significant difference in beta diversity at 12-months of age between the prebiotic and placebo groups, and enrichment of Clostridia from Lachnospiraceae and Peptostreptococcaceae in the microbiome of infant from the prebiotic group. Factors that drive increased abundance of gut Peptostreptococcaceae within the first year of life are conflicting within the literature, but such changes have been linked with household pet exposure [34], consumption of formula containing partially hydrolysed cow milk protein [35], lack of breastfeeding compared to exclusive breastfeeding [36], and exclusively breastfeeding compared to combined feeding [37]. In this study there was no difference in the proportion of infants breastfed until 6-months of age between the randomised groups. Therefore, it is likely that the differences in maternal prebiotic intake are responsible for the changes in Peptostreptococcaceae abundance. Furthermore, in the placebo group, Firmicutes C (Negativicutes) including Selenomodaceae were enriched in both the infant and maternal microbiome. In previous work, the Negativicutes class were found significantly enriched in caesarean section-born, mixed fed (breastmilk and formula) infants compared to vaginally born breastfed infants, [38]. While the role of this bacterial family in the human gut is not entirely clear, our study indicates that Negativicutes, including Selenomodaceae, are potentially less competitive in a prebiotic-rich nutritional environment.

During the pregnancy period, we also found that the prebiotic supplement increased the maternal stool concentrations of acetate. Unlike other abundant gut residents such as *Bacteroides* and Clostridia, *Bifidobacterium* ferment primarily carbohydrates, producing lactate and acetate [31]. Therefore, increased acetate in the prebiotic group during pregnancy is likely due in part to the increase in Bifidobacteria. An increase both *Bifidobacterium* abundance and acetate concentrations has been reported in response to a GOS prebiotic mixture in pregnant mice [39], and in an anaerobic fermenter [40]. On the other hand, in a clinical study the abundance of *Bifidobacterium* increased in healthy adults after supplementation with either FOS or GOS, although stool acetate concentrations did not increase [22]. The effect of prebiotic supplementation is likely related to the health and life stage of an individual, with the additional demands of pregnancy (nutrient acquisition, immune system remodelling, and hormonal changes) expected to impact gut health. In this study, levels of acetate, propionate, and butyrate were higher in all women during the antenatal period compared to the postnatal period, which may be related to the fundamental metabolic changes necessary to support the developing foetus [41]. However, both acetate and alpha diversity showed directional shifts over the pregnancy period, that were different according to the randomised group. In the prebiotic group, acetate concentrations increased consistently towards later pregnancy, while alpha diversity decreased; this was not observed in the placebo group. As the most prevalent organic acid in the gut acetate contributes substantially to lowering the gut pH. This may impose a selective pressure on the microbial community that can be detected as decreased alpha diversity [42]. Indeed, gut microbial diversity has been shown to decline during pregnancy, with a loss of butyrate producing bacteria and increase in lactic acid-producing members [14].

Maternal gut butyrate concentrations also increased significantly during pregnancy in the prebiotic group, but remained relatively similar to baseline concentrations in the placebo group. However, the difference in the gestational age-dependent changes between the two groups was not statistically significant. In the infant gut, there was also no significant difference in the average butyrate concentrations between the intervention groups at any time. However, in the placebo group only, butyrate concentration increased significantly from 4-months to 6-months of age, which corresponded to average age of the infants commencing on solid foods. Butyrate is mainly produced from the breakdown of carbohydrates or lactate [6] by butyrogenic bacteria including members of Clostridia, as well as *Streptococcus* spp. [43], both of which were identified as core members of the infant gut microbiome in this study, regardless of intervention group. Butyrate is rapidly transported across the epithelium [44], and is utilised preferentially by colonocytes [45]. Therefore, increased butyrate could be due to either a decreased transit time, which impairs the efficiency of butyrate uptake, or increased butyrate production. FOS has been shown to increase the production of butyrate through cross-feeding of lactate produced by *Bifidobacterium* [46]. Consequently, prebiotic supplementation in this trial may have contributed to increased butyrate production in mothers, and supported gut health during pregnancy, but there may be less impact on butyrate production in infants.

This continual expansion and diversification of the gut community is a necessary progression that will ideally lead to a relatively stable adult microbiome. Indeed, once maturation of the microbiome has begun (~4–6 months of age), reduced diversity in the infant microbiome is associated with higher incidence of allergy [47], whereas increased diversity is more often associated with healthy outcomes [48–50]. However, it may be that the low diversity structure of initial colonisers (*Bacteroides* and *Bifidobacteria*) who perform evolutionarily evolved processes, may need to predominate the gut for a certain period to establish a host benefit for immune system development. While exposure to microbes in the environment is protective and necessary, potentially a rapid premature colonisation of more adult associated microbes may disrupt this critical process. The maternal prebiotic supplement may therefore assist in the migration of commensal microbes during pregnancy and breastfeeding by supporting the health of the maternal gut during this time.

Both the metabolic environment and gut microbiome of the mother play a major part in the initial establishment of the gut microbiome, however in this microbiome sub-set analysis, differences in infant seeding patterns as a potential benefit of the maternal prebiotic supplementation was not fully investigated. Future analysis of the meconium collected as part of this trial will focus on the relationship between the maternal and neonatal gut microbiome. Also, it was not possible to collect complete maternal and infant dietary intakes over the 11-month trial intervention period; thus, we are unable to describe changes to the microbiome caused by this major driver of microbiome composition. Further, we were unable to determine if a more pronounced impact of prebiotic supplements occurred in individuals who consumed a lower fibre diet. The impact of antibiotic exposure during pregnancy and the first year of life, was also a potential confounder of this study. However, the rate of antibiotic use was not different between the intervention and control groups. Furthermore, given the variable timepoints of maternal antibiotic use over the 11-month long intervention period and only 74 participants, additional sub-group antibiotic use statistical analyses would have lacked power for any useful interpretation. Lastly, commensal gut microbes have the capacity to process many substrates and alter the gut metabolome without necessarily changing in absolute abundance. Therefore, assessing changes to the total metabolomic environment either directly or via metagenomic analysis may be another way to better capture these effects, to deepen our understanding of changes induced by maternal prebiotics supplementation.

Over the trial intervention period, which involved both antenatal and postnatal interventions, the administration of maternal prebiotics had a significant impact on both the gut microbiome

composition and SCFA concentrations. The abundance of *Bifidobacterium,* and the concentration of acetate significantly increased in the prebiotic group during pregnancy. For the first time in humans, this study also showed that maternal prebiotic supplementation impacts the composition of the infant microbiome, including a significant reduction in the abundance of Negativicutes, compared to the abundance in the placebo group. These results enhance our understanding of the impact of maternal diet on infant gut health, indicating that it is possible to intervene and modify the development of the infant microbiome *in utero* and during breastfeeding by dietary modulation of the maternal gut microbiome. The long-term follow-up of these infants will elucidate the impact of this intervention on the prevalence of childhood allergic diseases, and help clarify how early-life microbiome structure and function may influence childhood disease development.

Data availability

Once the primary outcome of the trial is published, the SYMBA Study data will be available for data sharing. Data sharing requests will need approval by the SYMBA Study Investigator Team. Please send requests to Associate Professor Debbie Palmer (debbie.palmer@telethonkids.org.au). The Australian National Health and Medical Research Council (NHMRC) supports the sharing of outputs from NHMRC funded research including publications and data. All recipients of NHMRC grants must therefore comply with all elements of the NHMRC Open Access Policy (15 January 2018).

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Author contributions

Conceptualization, D.J.P. and S.L.P.; Data curation, J.M.J., C.T.C., D.J*.P. and* S.L.P.; Funding acquisition, D.J.P., J.K., and S.L.P.; Methodology, J.M.J., S.N.R., M.M-D., C.T.C., D.J.P., J.K., J.G., M.C.J., R.S., D.S. and S.L.P.; Project administration, C.T.C., S.N.R., D.J.P. and S.L.P.; Resources, C.T.C., S.N.R., S.L.P., D.J.P., J.G. and D.S.; Supervision, S.N.R., C.T.C., and D.J.P.; Writing—original draft, J.M.J.; Writing—review & editing, J.M.J., S.N.R., M.M-D., C.T.C., D.J.P., J.K., J.G., K.S., M.C.J., R.S., D.S. and S.L.P.

Institutional review board statement

The trial is being conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Joondalup Health Campus (Human Research Ethics Committee Approval number 1451r on 2 February 2016); the University of Western Australia also granted reciprocal ethical approval for the trial (Approval number RA/4/1/8137 on 7 March 2016).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Conflict of interest

J.G. is a part-time employee of Danone-Nutricia research and receives research funding/grants from EU, Dutch Government, Bill Gates Foundation, public and private research funding from the Netherlands and some companies such as, Danone-Nutricia research, Nutricia research foundation, Friesland Campina, DSM. M.C.J. has received funding for a clinical trial and honoraria for lectures from BioGaia AB, as well as consultant fees and travel support from Danone-Nutricia and Abigo Medical. S.L.P. has received speaker's fees from Danone-Nutricia. The authors D.J.P., J.K., R.S., J.M.J, C.T.C, S.N.R, M.M-D, and D.S. declare no conflict of interest. The funders have no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2024.02.030.

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