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Research paper

Maternal prenatal gut microbiota composition predicts child behaviour

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ABSTRACT

Background: Murine studies demonstrate that maternal prenatal gut microbiota influences brain development and behaviour of offspring. No human study has related maternal gut microbiota to behavioural outcomes during early life. This study aimed to evaluate relationships between the prenatal faecal microbiota, prenatal diet and childhood behaviour.

Methods: A sub-cohort of 213 mothers and 215 children were selected from a longitudinal pre-birth cohort. Maternal prenatal exposure measures collected during the third trimester included the faecal microbiota (generated using 16S rRNA amplicon sequencing), and dietary intake. The behavioural outcome used the Childhood Behaviour Checklist at age two. Models were adjusted for prenatal diet, smoking, perceived stress, maternal age and sample batch.

Findings: We found evidence that the alpha diversity of the maternal faecal microbiota during the third trimester of pregnancy predicts child internalising behaviour at two years of age (−2·74, (−4·71, −0·78), p = 0·01 (Wald test), R^2=0·07). Taxa from butyrate-producing families, *Lachnospiraceae* and *Ruminococcaceae,* were more abundant in mothers of children with normative behaviour. A healthy prenatal diet indirectly related to decreased child internalising behaviours via higher alpha diversity of maternal faecal microbiota.

Interpretation: These findings support animal studies showing that the composition of maternal prenatal gut microbiota is related to offspring brain development and behaviour. Our findings highlight the need to evaluate potential impacts of the prenatal gut microbiota on early life brain development.

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1. Introduction

Murine studies have shown that alteration of maternal gut microbiota composition during pregnancy influences brain structure, function, and behaviour in the offspring [1-3]. Proposed pathways include: modulating maternal serum and foetal brain metabolites

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Research in Context

Evidence before the study

Animal studies demonstrate that maternal gut microbiota during pregnancy influence brain development and behaviour in offspring. To date, no human studies have reported the relationship between maternal gut microbiota during pregnancy and behavioural outcomes in children.

Added value of the study

We present the first evidence of a longitudinal association between the composition of maternal faecal microbiota during pregnancy and internalising behaviour in the offspring, which is strongly associated with subsequent anxiety disorders. We found evidence that increased alpha diversity of prenatal gut microbiota predicts reduced internalising symptoms amongst children at age 2 years, with no evidence of mediation via the offspring's gut microbiota during infancy. Taxa from the butyrate-producing families, *Lachnospiraceae* and *Ruminococcaceae,* were more abundant in mothers of children with normative behaviour. In addition, we present moderate evidence that a healthy prenatal maternal diet may reduce internalising behaviours in children via higher alpha diversity of maternal faecal microbiota. The strengths of the study include an unselected sampling frame, longitudinal design, collection and analysis of maternal and infant faecal samples and detailed consideration of potential confounding factors within a causal framework.

Implications of all the available evidence

Our findings support the evidence from animal studies and suggest that there may be a developmental window during pregnancy where aspects of the maternal prenatal gut microbiota influence foetal brain development, and in turn, behaviour in offspring. Further human studies are required to replicate these findings and delineate the underlying mechanisms.

that promote foetal thalamocortical axonogenesis [3], induction of systemic Th17-mediated inflammation during pregnancy [2], and maternal to offspring transfer of the gut microbiota [1], which we and others have related to subsequent behavioural outcomes [4,5]. To date, however, there are no human studies relating prenatal gut microbiota to offspring behaviour.

Prenatal maternal diets low in fruit, vegetables and fibre, and high in fats and sugars have been linked to child behavioural dysregulation in three large birth cohorts [6-8]. The mechanism of these associations is unknown, but the composition and metabolic products of the gut microbiota may be involved. Diets high in fruit and vegetables are associated with higher alpha diversity in adult [9] and pregnancy cohorts [10] and increased production of short chain fatty acids (SCFAs), which can cross the placenta and have potent anti-inflammatory properties [11,12].

Our hypotheses were that: (a) aspects of the maternal gut microbiota (diversity, composition and short chain fatty acid metabolites) predict child behaviour, and (b) the prenatal gut microbiota mediates the relationship between the maternal prenatal diet and child behaviour. In a human pre-birth cohort, we show evidence that increased alpha diversity in the maternal prenatal gut microbiota, but not short chain fatty acids, was associated with reduced behaviour problems in children after adjustment for confounding factors, with no evidence of mediation via the offspring's gut microbiota during infancy. Mothers of children with normative behaviour had a higher abundance of *Lachnospiraceae* and *Ruminococcaceae* during pregnancy. Further, we report moderate evidence that a higher quality prenatal diet may have a beneficial influence on offspring behaviour indirectly via increased alpha diversity of the maternal prenatal gut microbiota.

2. Methods

2.1. Study design and sample

The Barwon Infant Study (BIS) is an Australian birth cohort study consisting of 1064 mothers and their 1074 children [13]. Between June 2010 and June 2013 pregnant women were recruited and enroled. Women were included if they were: Australian residents based in the Barwon region; less than 32 completed weeks pregnant at enrolment; and planning to give birth at the University Hospital Geelong or St John of God Hospital. Women that were unable to give consent, complete the questionnaires, or had moved out of the region, were excluded. Women completed baseline data collection immediately following enrolment. The baseline questionnaires covered a range of domains [13] including: sociodemographic, household composition, medical history, dietary intake (Dietary Questionnaire for Epidemiological Studies (DQES), Version 2 [14]), stress (Perceived Stress Scale (PSS) [15]) and depression symptoms (Edinburgh Depression Scale (EDS) [16]), along with lifestyle and smoking. 16S rRNA gene amplicon sequencing was performed on maternal faecal samples collected in week 36 of pregnancy in a random subsample of 324 mother-infant dyads. Parents completed the standardised Child Behavior Checklist (CBCL) [17] to measure behaviour and emotional development for children at two years of age (n = 675/1074). The analysis dataset includes mothers (n = 213) and children (n = 215, including two sets of twins), for whom maternal 16S rRNA sequencing and data were complete for an *a priori* selected set of potential confounders.

2.2. Ethics

The BIS was approved by the ethics committee at Barwon Health (reference 10/24) and mothers provided written informed consent prior to participating.

2.3. Exposure measures

Our primary exposure variables were maternal microbiota and their metabolites (short chain fatty acids). Maternal microbiota was measured using 16S rRNA gene amplicon sequencing and diversity profiling. At 36 weeks' gestation, maternal faecal samples were collected at home into a sterile specimen jar in accordance with detailed participant instructions. Specimens were transported on ice to the University Hospital, where they were aliquoted and stored at −80 °C. DNA extraction used the Qiagen PowerSoil® DNA Isolation Kit, Cat#12888-100. Samples of DNA (100 ng) were sent to J. Craig Venter Institute, Rockville, MD, USA for 16S rRNA gene amplification using the V4 variable region (primers, forward: 5′-GTGCCAGCMGCCGCGGTAA-3′, reverse: 5′-GGACTACHVGGGTWTCTAAT-3′). Amplicons were generated and sequenced using Illumina MiSeq in accordance with the manufacturer's specifications [18]. Corresponding paired-end reads were merged, filtered and clustered into 97% identity OTUs using USEARCH software [19]. Sequences were assigned to taxa described in the SILVA v123Nr99 taxonomic database using the mothur software suite [20] and final OTU descriptions were composed in USEARCH. Samples with fewer than 2500 read pairs were excluded from analysis. The primary exposure measure was the Shannon index of alpha diversity. Secondary diversity measures included: inverse Simpson index, Faith's phylogenetic diversity, Fisher's alpha diversity index and Chao1, and beta diversity using Bray-Curtis dissimilarity, weighted UniFrac distance, unweighted UniFrac distance and Jensen-Shannon divergence.

Aliquots of maternal faecal samples and maternal serum samples (collected in week 28 of pregnancy) were transported on dry ice to the CSIRO laboratories, Adelaide, Australia, for quantification of SCFA concentrations using capillary gas chromatography (GC; 5890 series II Hewlett Packard, Australia). The SCFA exposure measures used in the data analysis included logarithmically transformed molarity of faecal acetate, butyrate, propionate, and serum acetate, butyrate, propionate and measures of their relative concentrations.

2.4. Outcome measure

The outcome was measured using the Child Behaviour Checklist; this was selected because it is a widely-used validated, parent-reported screening tool for emotional, behavioural and social problems [17]. The CBCL yields T-scores for internalising, externalising and total problem behaviour symptoms. This study investigates three CBCL outcomes: a binary outcome, and internalising and externalising subscale T-score measures (continuous measures). The case group classifies cases as children with elevated behavioural problems, namely T-scores of 60 or above on any of the internalising, externalising or total problem behaviours subscales of the CBCL questionnaire [4]. Elsewhere, this case group definition predicted psychiatric disorders diagnosed later in childhood with high specificity (88−96%), but low sensitivity (25−34%) [21]. Hence, the case group definition has high accuracy at predicting that a child will not develop a disorder when T-scores are under 60, but lower accuracy at predicting a disorder when T-scores are at or above 60 [21].

2.5. Derived covariate measures

2.5.1. Maternal diet

Principal component analysis (PCA) was used to derive prenatal dietary patterns from the DQES [14]. PC1 was identified as a modern healthy dietary pattern with high loadings on fish, nuts, eggs, green vegetables, wholegrains, and low loadings on white bread, sugar, full-cream milk and hamburgers (Supplementary Figure 1). PC2 was identified as a Western dietary pattern with high loadings on pasta, chips, meat and take-away foods, sweet biscuits and confectionery. The PCA used varimax rotation over the daily food items (grams) for the full cohort (n = 1064) [22]. Each participant had a centred and scaled z-score for the two dietary patterns, where negative z-scores indicated intakes lower than the mean and positive z-scores indicated intakes above the mean for each pattern.

2.5.2. Maternal stress

The Perceived Stress Scale [15] was selected as an *a priori* potential confounder. Mothers completed a prenatal PSS (n = 163/213, 76.5%) at enrolment (prior to week 32), and at one (n = 184/213, 86.4%) and six months (n = 204/213, 95.3%) postnatally. The prenatal PSS was introduced into the BIS protocol approximately eight months after recruitment began. As such, prenatal PSS data were missing in 50/213 (23.5%) of the analysis subcohort. In this context we generated a 'combined PSS' score, which comprised the mean PSS score from the available pre and postnatal PSS measures. Amongst those with prenatal PSS scores available (n = 163), the prenatal PSS and combined PSS scores were highly correlated (r = 0.87, p<0.001).

2.6. Statistics

2.6.1. Design framework

We prespecified the hypothesised relationships between the maternal prenatal gut microbiota, maternal diet (and other covariates) and CBCL outcomes using directed acyclic graphs (DAGs) [23,24] (Supplementary Figures 2 − 4). The DAGs were informed by published associations and theoretical considerations and were used to identify the minimum adjustment set of covariates to control for confounding bias on the main effect (e.g., alpha diversity of maternal gut microbiota on child CBCL outcomes, Supplementary Figure 2).

2.6.2. Microbiota

The R statistical software environment (https://www.r-project.org) was used for all analyses. The phyloseq package [25] was used to manage community composition data. We selected the Shannon index *a priori* as the primary measure of alpha diversity as it accounts for richness and evenness. We included four other measures of alpha diversity and four measures of beta diversity to evaluate whether the results were robust across the different estimators- that is, were not biased by rare taxa or dominant taxa, phylogeny or undersampling. Phyloseq [25] was used to calculate Shannon, inverse Simpson, Chao1 and Fisher's alpha diversity indices, and picante [26] was used to calculate Faith's phylogenetic diversity. Vegan [27] and phyloseq [25] were used to calculate the beta diversity matrices for Bray-Curtis dissimilarity, weighted UniFrac distance, unweighted UniFrac distance and Jensen-Shannon divergence. For the binary outcome, odds ratios for behaviour problems predicted by the microbial and SCFA exposures were estimated using binomial regression with a logit link function. We used linear regression to estimate the relationship between each exposure and the internalising and externalising T-score outcomes. Models for alpha diversity and SCFA exposures were adjusted using multivariable regression on an *a priori* selected set of potential confounders (method detailed below): PCA-derived healthy dietary pattern, smoking during pregnancy, combined perceived stress score, maternal age and sequencing batch number. The linear model assumptions were evaluated and where the homoscedascity assumption was violated by heteroscedasticity-robust standard errors were calculated and reported [28,29]. Semi-parametric, Permutational Multivariate Analysis of Variance (PERMANOVA) analyses were performed with adonis2 [27] to estimate the fraction of the overall variance in the beta diversity that could be explained by the three CBCL outcomes. Exploratory differential abundance analysis was performed on the binary outcome to estimate the log-fold change in taxa between mothers of cases and controls using the voom function of the limma package [30], this model was adjusted for the *a priori* selected set of potential confounders. The statistical tests relating to the *a priori* defined hypotheses were not adjusted for multiple testing. Our exploratory differential abundance testing tested 373 taxa as response variables and used a Benjamini-Hochberg false discovery rate adjustment.

2.6.3. Adjustment for potential confounders

The prespecified potential confounders identified in the DAG (Supplementary Figure 2) comprised: the PCA-derived modern healthy and Western dietary patterns, alcohol, smoking during pregnancy, maternal age, country of birth, household income, number of siblings at home, and combined perceived stress score. Processing factors included were: the duration of faecal storage, whether the sample was fresh (collected in the research unit and immediately processed) or frozen (collected at home, frozen and then transferred to the research unit), and the sequencing batch number. We prespecified that only those covariates that changed the main effect estimate by more than 10% would be retained in the adjusted models. Therefore, the potential confounders were: healthy dietary pattern, smoking during pregnancy, combined perceived stress score, maternal age and batch number.

Given the paucity of literature regarding factors associated with the composition of maternal microbiota during pregnancy, we then conducted exploratory analyses using data-driven approaches to further investigate relationships between parental, child and household characteristics and CBCL outcomes (Supplementary Table 1 outlines the full list of covariates that were considered). First, covariates were evaluated as potential antecedents (but not causes of the outcome), mediators or potential confounders for each exposure-outcome

relationship via stratification [31]. Second, univariate relationships were tested using individual univariate linear or logistic regression models and potential confounders that changed the main estimate by more than 10% in a two-term model were considered.

2.6.4. Dietary predictors of alpha diversity

Linear regression models were used to evaluate whether the dietary patterns predicted the Shannon alpha diversity index when adjusted for maternal age and household income (based on DAG in Supplementary Figure 3).

2.6.5. Mediation between maternal diet and child CBCL by alpha diversity of maternal faeces

Mediation analyses were performed using a counterfactual framework (medflex R version 0·6-6) [32] to investigate dietary pattern z-scores, alpha diversity and CBCL outcomes (including the binary outcome, and continuous T-scores for internalising and externalising; Supplementary Figure 5). Nested counterfactuals were imputed using linear regression to decompose the total effect into natural direct and natural indirect effects via the mediator. The total effect was the difference in CBCL outcome when z-scores for the healthy dietary pattern were changed from a reference level of 1 standard deviation (SD) below the mean (representing a poorer overall diet quality due to lower intakes of healthy foods), to 1 SD above the mean (representing high diet quality due to higher intakes of healthy foods). Standard errors were estimated using 10,000 bootstrap samples, and Wald-type 95% confidence intervals were calculated for each effect. A new DAG, which modelled diet as the exposure was used to identify potential confounders (Supplementary Figure 4) and models were adjusted for maternal age, household income and country of birth.

2.7. Sensitivity analysis

We then conducted a sensitivity analysis restricted to those with prenatal PSS data available to evaluate whether estimates obtained in analyses adjusted for the prenatal PSS were materially different to the estimates obtained in analyses adjusted for the combined PSS.

2.8. Role of funding source

The funders did not have any role in the design, implementation or reporting of this study.

3. Results

3.1. Descriptive characteristics

The analysis subcohort consisted of 215 mother-infant dyads from the Barwon Infant Study that had both maternal gut microbiota profiling during the 3rd trimester of pregnancy and standardised Child Behaviour Checklist (CBCL) [17] measures in children at 2 years of age. Participant characteristics were similar between the total inception cohort and the analysis subcohort, except for smoking during pregnancy (Table 1). Twenty (9·3%) of the 215 children were classified as belonging to an elevated behavioural problem group. The remaining children were classified as belonging to a normative behaviour group.

Table 1
Descriptive characteristics of the inception birth cohort and analysis cohort for this study.

	Inception birth cohort	Analysis subcohort
Children (n)	1074	215
Maternal age (mean (SD))	31·3 (4·8)	32·5 (4·1)
Maternal country of birth (%)		
Australia	967 (90·0%)	196 (91·2%)
Other non-English speaking country	48 (4·5%)	4 (1·9%)
Other predominantly English-speaking country: New Zealand, UK/Ireland, USA/Canada	57 (5·3%)	15 (7·0%)
Missing	2 (0·2%)	0 (0%)
Household size (mean (SD))	2·9 (1·0)	2·9 (0·8)
Socio-Economic Index for Area (%)		
Low SEIFA (most disadvantaged)	272 (25·3%)	51 (23·7%)
Medium SEIFA	206 (19·2%)	49 (22·8%)
High SEIFA (least disadvantaged)	593 (55·2%)	115 (53·5%)
Missing	3 (0·3%)	0 (0%)
Maternal education (%)		
Less than year 10	12 (1·1%)	1 (0·5%)
Year 10 or equivalent	80 (7·4%)	13 (6·0%)
Year 12 or equivalent	162 (15·1%)	27 (12·6%)
Trade/Cert/Dip	266 (24·8%)	42 (19·5%)
Bachelor's degree	354 (33·0%)	77 (35·8%)
Postgraduate degree	194 (18·1%)	54 (25·1%)
Missing	6 (0·6%)	1 (0·5%)
Lone parent status (%)		
No	1028 (95·7%)	210 (97·7%)
Yes	43 (4·0%)	5 (2·3%)
Missing	3 (0·3%)	0 (0%)
Maternal combined Perceived Stress Score (mean (SD))	18·8 (6·5)	17·8 (6·5)
Maternal smoking during pregnancy (%)		
No smoking	892 (83·1%)	199 (92·6%)
Any smoking	169 (15·7%)	16 (7·4%)
Missing	13 (1·2%)	0 (0%)
Passive smoking during pregnancy (%)		
No environmental tobacco smoke exposure	957 (89·1%)	201 (93·5%)
Less than 1 hour a day	44 (4·1%)	7 (3·3%)
One or more hours a day	70 (6·5%)	7 (3·3%)
Missing	3 (0·3%)	0 (0%)
Maternal prenatal antibiotics (%)		
No	891 (83·0%)	180 (83·7%)
Yes	180 (16·8%)	35 (16·3%)
Missing	3 (0·3%)	0 (0%)
Maternal pre-pregnancy BMI (kg/m^2) (%)		
Underweight (BMI < 18·5)	18 (1·7%)	4 (1·9%)
Normal weight (18·5 < BMI < 25)	366 (34·1%)	69 (32·1%)
Overweight (25 < BMI < 30)	192 (17·9%)	45 (20·9%)
Obese (BMI > 30)	135 (12·6%)	20 (9·3%)
Missing	363 (33·8%)	77 (35·8%)
Prenatal dietary fibre (g/day) (mean (SD))	21·3 (7·4)	21·5 (6·7)
Prenatal dietary fat (g/day) (mean (SD))	74·3 (26·7)	74·6 (24·8)
Prenatal dietary omega-3 PUFA (g/day) (mean (SD))	1·4 (0·6)	1·4 (0·6)
Child age (months) at behavioural assessment (mean (SD))	29·5 (1·8)	29·3 (1·8)
Child sex (%)		
Female	519 (48·3%)	90 (41·9%)
Male	555 (51·7%)	125 (58·1%)
Binary outcome (%)		
Normative behaviour (non-case)	619 (57·6%)	195 (90·7%)
Elevated behaviour problem (case)	56 (5·2%)	20 (9·3%)
Unmeasured	399 (37·2%)	0 (0%)
Child internalising T-score (mean (SD))	41·8 (9·2)	41·6 (9·3)
Child externalising T-score (mean (SD))	44·9 (9·3)	45·5 (9·9)

BMI: Body mass index, PUFA: Polyunsaturated fatty acids, SEIFA: Socio-economic indexes for areas.

3.2. Association between maternal prenatal alpha diversity of the gut microbiota and child behaviour

Analysis of the V4 region of the 16S rRNA gene in microbial DNA from maternal prenatal faecal samples demonstrated that, on average, the odds ratio of case status per unit increase in Shannon index of alpha diversity was 0·42 (95% CI: (0·22, 0·82), p = 0·01 (Wald test)) (Fig. 1). This finding was essentially unchanged following adjustment for prenatal healthy dietary pattern, smoking during pregnancy, combined perceived stress score, maternal age, sequence batch number (Table 2). The Shannon index was inversely associated with internalising T-scores after adjustment for potential confounding factors (Table 2). This finding was consistent for the Inverse Simpson index, indicating that the results were not influenced by taxa rarity or

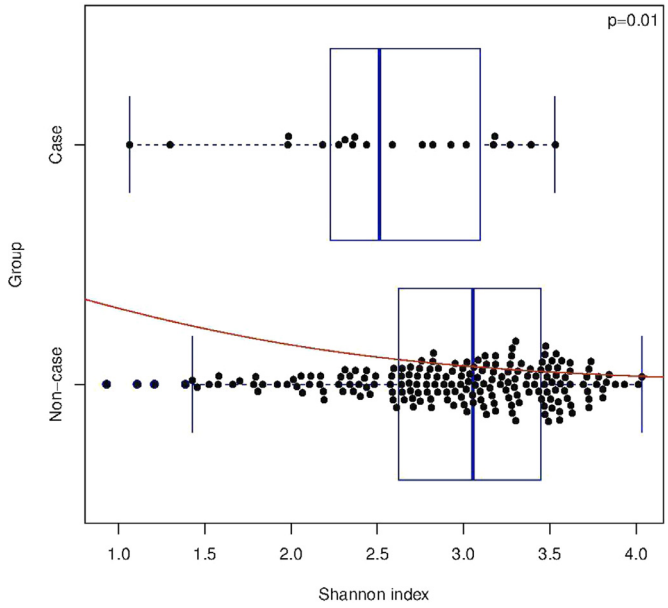

Fig. 1. Shannon index of maternal prenatal gut microbiota plotted against elevated behavioural problem case status in two year old offspring. Box and whisker plots show median (bar), first and third quartiles (box), with whiskers at the most extreme data points less than 1•5 times the interquartile range from the quartiles. Small deviations in the vertical direction separate points within the two bands. The red line shows a predicted probability of being a case given the Shannon index, obtained from a univariable logistic model (*p*-value indicated corresponds to the odds ratio for this fit). *N* = 213 mothers and 215 children.

dominance (Table 2). The three estimators of richness (Faith's phylogenetic diversity, Fisher's alpha diversity index and Chao1), were also inversely associated with internalising T-scores after adjustment for confounders (Table 2), indicating that these results were robust to the different alpha diversity estimators. In each of these models, alpha diversity of maternal gut microbiota explained between 5 and 7% of the variance in the offspring's internalising T-scores. There was, however, no evidence of an association between prenatal alpha diversity and externalising behaviours. A sensitivity analysis restricted to those with prenatal PSS data available, demonstrated that the point estimates obtained in analyses adjusted for the prenatal PSS were similar to the estimates obtained in analyses adjusted for the combined PSS (Supplementary Table 2).

3.3. Maternal prenatal beta diversity of the gut microbiota in children with problem vs normative behaviour

Beta diversity analysis revealed differences in the structure of the maternal prenatal gut microbiota communities between the mothers of children in the elevated behavioural problem group in comparison to mothers of infants in the normative behaviour group. This was primarily driven by the association between the beta diversity of the maternal microbiota and internalising symptoms in the offspring. Adjustment for potential confounding factors made no material difference to the findings (Table 3). Evidence of an association between beta diversity of maternal gut microbiota and offspring behaviour was apparent across the four beta diversity distance measures, where a small proportion (between 0·7 and 1·1%) of the variance in maternal prenatal beta diversity related to the offspring's CBCL binary outcome status. However, there was no visual separation in beta diversity of gut microbiota between the mothers of offspring in the elevated behavioural problem versus normative groups (Supplementary Figure 6). There was a small change in internalising T-scores driven through the second principal coordinate of the Bray-Curtis dissimilarity (Supplementary Figure 7).

3.4. Differential normalised abundance of maternal prenatal gut microbiota by child behaviour group

Amongst mothers of children in the normative behaviour group, there was higher differential normalised abundance of eight genus-

Table 2
Relationship between alpha diversity of the maternal prenatal gut microbiota and Child Behaviour Checklist outcomes.

	Shannon Index	Inverse Simpson Index	Faith's Phylogenetic Diversity	Fisher's alpha diversity index	Chao1
Elevated behavioural problems (odds increase by a factor of OR per unit increase in diversity (OR, 95%CI)					
Unadjusted	0·42	0·89	0·97	0·96	1
	(0·22, 0·82)	(0·8, 0·98)	(0·79, 1·16)	(0·91, 1·02)	(0·99, 1·01)
	p = 0·01	*p* = 0·02	*p* = 0·77	*p* = 0·18	*p* = 0·89
	pseudo R^2=0·05	pseudo R^2=0·05	pseudo R^2=0	pseudo R^2=0·01	pseudo R^2=0
Adjusted*	0·37	0·87	0·87	0·94	1
	(0·18, 0·76)	(0·77, 0·97)	(0·66, 1·11)	(0·88, 1·00)	(0·99, 1·01)
	p = 0·01	*p* = 0·02	*p* = 0·28	*p* = 0·08	*p* = 0·44
	pseudo R^2=0·11	pseudo R^2=0·11	pseudo R^2=0·07	pseudo R^2=0·08	pseudo R^2=0·06
Internalising T-score (change in T-score per unit increase in diversity, 95%CI)					
Unadjusted	−2·63	−0·25	−0·24	−0·15	−0·02
	(−4·56, −0·71)	(−0·44, −0·05)	(−0·74, 0·26)	(−0·29, −0·01)	(−0·04, 0·01)
	p = 0·01	*p* = 0·01	*p* = 0·35	*p* = 0·03	*p* = 0·17
	R^2=0·03	R^2=0·02	R^2=0	R^2=0·02	R^2=0
Adjusted*	−2·74	−0·23	−0·9	−0·23	−0·04
	(−4·71, −0·78)	(−0·43, −0·03)	(−1·57, −0·22)	(−0·39, −0·08)	(−0·07, −0·01)
	p = 0·01	*p* = 0·03	*p* = 0·01	*p* = 0·003	*p* = 0·01
	R^2=0·07	R^2=0·05	R^2=0·06	R^2=0·07	R^2=0·07
Externalising T-score (change in T-score per unit increase in diversity, 95%CI)					
Unadjusted	−1·54	−0·17	−0·23	−0·10	−0·01
	(−3·62, 0·54)	(−0·38, 0·05)	(−0·76, 0·31)	(−0·25, 0·05)	(−0·03, 0·01)
	p = 0·15	*p* = 0·13	*p* = 0·41	*p* = 0·2	*p* = 0·44
	R^2=0·01	R^2=0·01	R^2=0	R^2=0	R^2=0
Adjusted*	−1·24	−0·11	−0·72	−0·13	−0·02
	(−3·37, 0·89)†	(−0·3, 0·08)†	(−1·47, 0·03)†	(−0·3, 0·04)†	(−0·05, 0·01)†
	p = 0·25	*p* = 0·25	*p* = 0·06	*p* = 0·12	*p* = 0·13
	R^2=0·05	R^2=0·05	R^2=0·06	R^2=0·06	R^2=0·06

OR, Odds ratio. All measures rounded to 2 decimal places. A pseudo R^2 is quoted for the elevated behavioural problem based on the logistic regression model deviance. Internalising and externalising linear regression models adjusted R^2 value is quoted. Unadjusted and adjusted models report on *n* = 213 mothers, *n* = 215 infants.

* Adjusted for prespecified potential confounders: prenatal healthy dietary pattern, smoking during pregnancy, combined perceived stress score, maternal age, batch.

† Uses heteroscedasticity-robust standard errors for reporting confidence intervals..

Table 3
Descriptive analysis of relationships between maternal faecal beta diversity and Child Behaviour Checklist outcomes.

	Bray-Curtis dissimilarity	Weighted UniFrac distance	Unweighted UniFrac distance	Jensen-Shannon divergence
Elevated behavioural problems	1·0%, p = 0·01	1·0%, p = 0·03	0·7%, p = 0·02	1·1%, p = 0·01
Internalising T-score	0·9%, p = 0·02	1·1%, p = 0·03	0·5%, p = 0·16	1·0%, p = 0·04
Externalising T-score	0·6%, p = 0·16	0·8%, p = 0·09	0·5%, p = 0·29	0·6%, p = 0·18

Percent of variance in maternal microbiota communities explained by CBCL outcomes for unadjusted PERMANOVA models, p-value.

Table 4
Differential normalised abundance of maternal gut microbiota taxa between children in the elevated behavioural problem case group relative to normative behaviour group adjusted for the prespecified potential confounders.

Predominant OTU in taxon	Genus	Log_2 fold change*	p-value	q-value†
OTU 230	*Lachnospiraceae FCS020 group*	−0·99	<0·001	0·001
OTU 75	*Ruminococcus 1*	−1·09	<0·001	0·001
OTU 93	*Lachnospira*	−0·77	<0·001	0·004
OTU 112	*[Eubacterium] ventriosum group*	−0·80	0·001	0·029
OTU 117	*Ruminococcaceae NK4A214 group*	−0·92	0·001	0·037
OTU 198	*Lachnospiraceae UCG-004*	−0·37	0·001	0·037
OTU 13	*Fecalibacterium*	−1·75	0·001	0·037
OTU 24	*[Eubacterium] coprostanoligenes group*	−1·68	0·002	0·045

The differential OTUs belonged to the *Clostridia* class.
*The negative log fold change indicates that the abundance was greater in the normative behaviour group compared to the elevated behavioural problem group (voom model).
† q-value: the p-value after Benjamini-Hochberg adjustment for multiple hypothesis testing.

level taxa from the *Lachnospiraceae* and *Ruminococcaceae* families of the *Clostridia* class (Table 4) compared to the elevated behavioural problem group. The strongest evidence related to taxa dominated by OTU 230 *(Lachnospiraceae FCS020 group),* OTU 75 (*Ruminococcus 1)* and OTU 93 *(Lachnospira)* (Fig. 2a). The taxon *Lachnospiraceae FCS020* was only present in 40% (8/20) of the case group compared to 69% (134/195) of the normative behaviour group (Fig. 2b). *Ruminococcus 1* was present in 30% of the case group compared to 56% (109/195) of the normative behaviour group (Fig. 2c). *Lachnospira* was only present in 15% of the case group compared to 45% (89/195) of the normative behaviour group (Fig. 2d). However, the relative abundance of each of these OTUs was low.

3.5. Association between short chain fatty acid concentrations in maternal blood serum and faeces and child behaviour

There was no evidence of associations between the concentrations of the SCFAs acetate, butyrate and propionate in maternal serum or faeces and any of the CBCL outcomes in either unadjusted or adjusted models (Supplementary Table 3).

3.6. Dietary predictors of the maternal prenatal Shannon index of the gut microbiota

Dietary predictors included two PCA-derived prenatal dietary patterns, identified as a healthy dietary pattern (high intakes of fish, nuts, eggs, green vegetables, whole-grains, and low intakes of white bread, sugar, full-cream milk and hamburgers) and a Western dietary pattern (high intakes of pasta, chips, meat and take-away foods, sweet biscuits and confectionery) (Supplementary Figure 1). Higher scores on the healthy dietary pattern were associated with higher prenatal Shannon indices. After adjustment for age and income, each standard deviation increase in healthy dietary pattern score was related to a 0.12 unit increase in Shannon index (95% CI: (0·02, 0·21), p = 0·02 (Wald test), adjusted R^2=0·02). There was no evidence of a relationship between the maternal Western dietary pattern and the Shannon index (coefficient 0·001, 95%CI: (−0·09, 0·1), p = 0·87 (Wald test), adjusted R^2=−0·01).

3.7. Mediation of the effect of maternal diet on child behaviour by the Shannon index of the maternal prenatal gut microbiota

On the basis of associations between the healthy dietary pattern and Shannon index, and Shannon index and child behaviour, we evaluated mediation of the prenatal diet on behaviour via changes in the prenatal Shannon index (Fig. 3). We found moderate evidence of an indirect pathway between the healthy maternal dietary pattern and lower child internalising behaviours that was mediated through increased alpha diversity (Shannon index) (Fig. 3b). There was however no evidence of an overall association between healthy dietary pattern scores and child behavioural dysregulation.

Higher scores on the Western dietary pattern (indicating high intakes of discretionary foods, and a poor-quality diet) were related to higher internalising scores (coefficient 2·07, (95% CI: 0·77, 3·37), p = 0·002 (Wald test), adjusted R^2=0·07). Mediation was not evaluated using the Western dietary pattern as an exposure, as we found no evidence that the Western dietary pattern was related to the maternal prenatal Shannon index. There was no evidence of an association between the Western dietary pattern and externalising behaviour (0·83, 95% CI: (−0·59, 2·25), p = 0·25 (Wald test), adjusted R^2=0·2), nor the elevated behavioural problem case group (OR 1·35, 95% CI: (0·82, 2·19), p = 0·22 (Wald test), pseudo R^2=0·12).

3.8. Mediation between maternal microbiota and child behaviour by infant microbiota

We found no evidence that maternal alpha diversity (measured by Shannon index) predicted child alpha diversity at one or six months, nor was there evidence that child alpha diversity or microbiota composition at one or six months predicted child behaviour at two years; hence, no mediation analyses were performed. We recently reported that infant carriage of *Prevotella* at one year was associated with reduced behavioural problems at two years [4]. However, in the current study we found no evidence of a relationship between maternal Shannon index and child carriage of *Prevotella* at one year (OR 1·2, (95% CI: 0·75, 1·94) p = 0·46 (Wald test), adjusted R^2= −0·006).

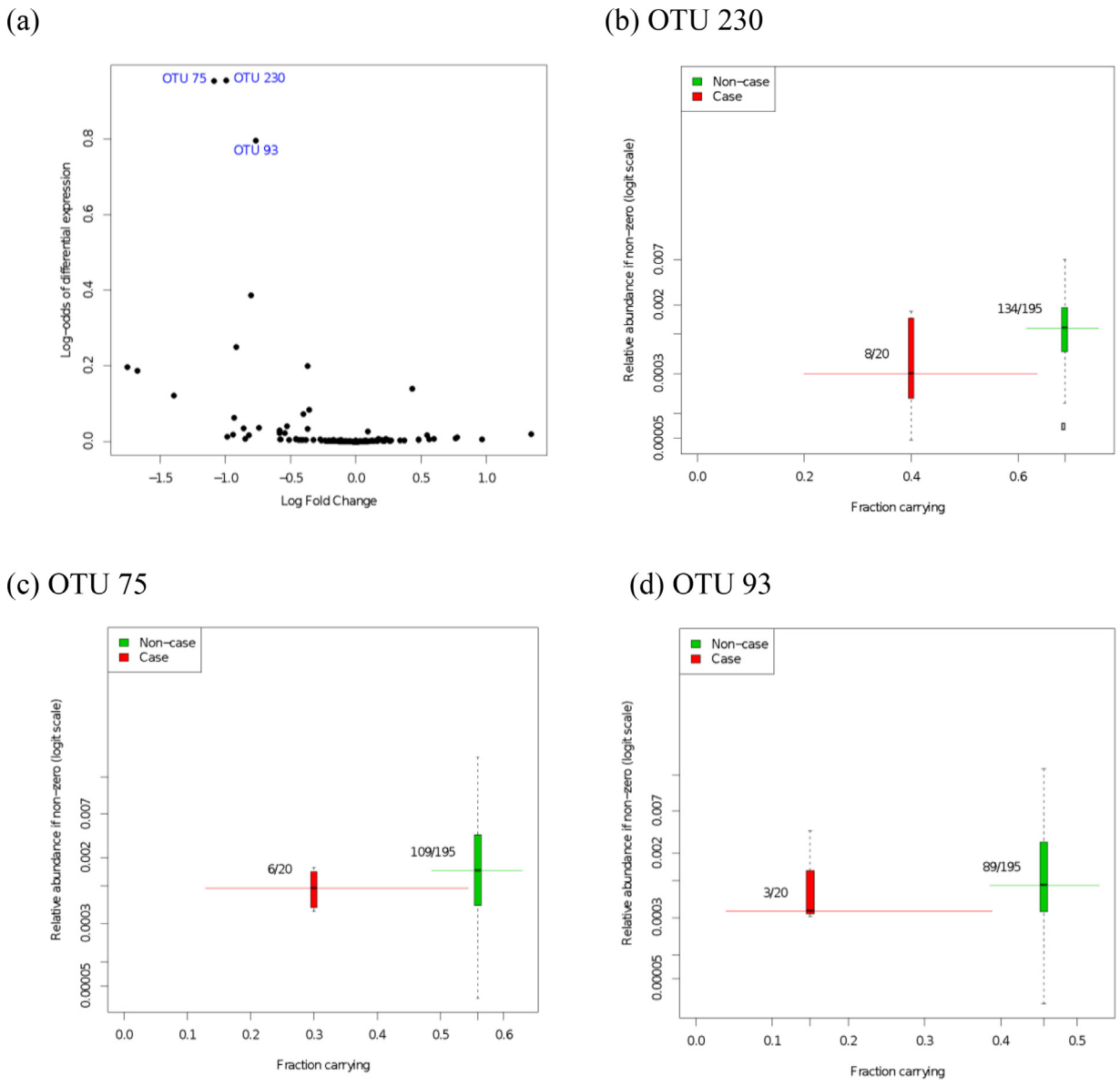

Fig. 2. Differential abundance of microbiota at the genus level. a. Volcano plot showing taxon log fold change against log odds of differential abundance. A log odds approaching 1.0 implies very strong evidence of differential abundance. Evidence of differential abundance was strongest for OTU 230 (*Lachnospiraceae FCS020 group*), OTU 75 (*Ruminococcus 1*) and OTU 93 (*Lachnospira*). **b.** The fractional carriage and relative abundance of OTU 230, **c.** OTU 75, and **d.** OTU 93 amongst mothers of children in the elevated behavioural problem group (red) and normative behaviour group (green). The X-axis shows the fraction of mothers carrying the OTU, and the Y-axis shows the relative abundance-when-present. Figures **b.-d.**: Horizontal solid lines: 95% confidence intervals. Box and whisker plots show median (bar), first and third quartiles (box), with whiskers at the most extreme data points less than 1•5 times the interquartile range from the quartiles, and all data outside the whiskers shown explicitly; parentheses indicate the 95% CI of the median. *N* = 213 mothers and 215 children.

4. Discussion

This is the first human study to investigate the relationship between maternal faecal microbiota during pregnancy and behavioural outcomes in children. Higher maternal alpha diversity was associated with better behavioural outcomes at two years of age, primarily due to lower internalising symptoms. At the genus level, taxa from the *Lachnospiraceae* and *Ruminococcaceae* families of the Clostridia class were more abundant in mothers of the children in the normative behavioural group. In addition, there was evidence of an indirect pathway between the healthy prenatal diet, increased alpha diversity of maternal microbiota, and lower internalising behaviours amongst children.

In mice, a high fat diet during pregnancy reduces the diversity of maternal faecal microbiota and promotes adverse behavioural outcomes in the offspring [1]. Consistent with this, in our study, foods comprising the healthy maternal prenatal dietary pattern were generally low in saturated fat, and this pattern was positively associated with faecal alpha diversity. We found evidence of a mediation pathway linking higher intakes of the healthy dietary pattern during pregnancy to increased maternal alpha diversity and reduced child internalising behaviours. However, the absence of an overall association between the healthy dietary pattern and child behaviour limits the evidence of causality that can be inferred from the reported indirect pathway between maternal diet, maternal microbiota and offspring behaviour. As previous studies of maternal diet and child behaviour had large sample sizes and modest magnitudes of association [6-8], it is likely that this component of our analysis is substantially underpowered. Further studies are needed to replicate the association between maternal diet, alpha diversity of maternal

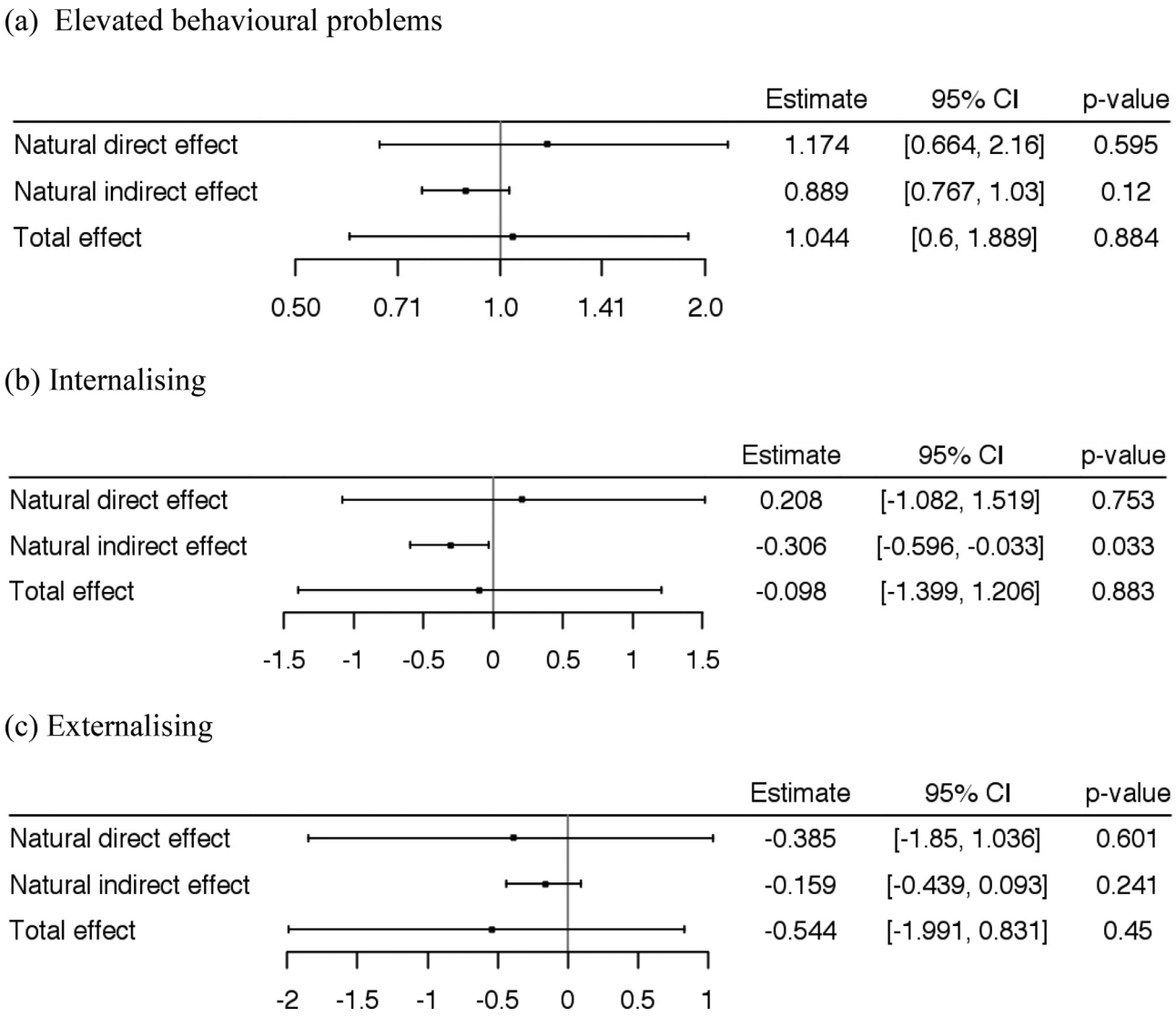

Fig. 3. Effect decomposition plots modelling the prenatal maternal Shannon index as a mediator between maternal healthy dietary pattern (PC1) and child behaviour. Outcomes are **a**. Elevated behavioural problems (case group) **b.** internalising T-scores (continuous), **c.** externalising T-scores (continuous). Plots show the effects should propensity to follow the healthy dietary pattern be increased from lower diet quality (1 SD below the mean) to higher diet quality (1 SD above the mean). The natural direct effect represents the difference in CBCL outcome should propensity to follow the healthy diet pattern be changed from lower to higher adherence, while fixing the Shannon index at natural levels occurring with low adherence. The natural indirect effect represents the difference in CBCL outcome should the healthy dietary pattern be fixed at low adherence but the Shannon index be changed to levels that would occur normally in participants with high propensity to follow to the healthy dietary pattern. Estimates and confidence intervals correspond to **a.** odds ratio for the specified change in diet; **b., c.** change in T-score for the specified change in diet. *N* = 213 mothers and 215 children.

microbiota, and child behaviour in larger cohorts and, importantly, these studies should explore underlying mechanisms.

We found that mothers of children in the normative behaviour group had a higher differential normalised abundance of taxa from the *Clostridia* class (Table 4). A recent murine study demonstrated that colonising microbiota-depleted dams prior to conception prevented neurodevelopmental abnormalities [3]. In particular, colonisation with taxa from the *Clostridia* class prevented sensory behavioural disturbances in offspring and elevated metabolites involved in axonogenisis in the maternal sera and foetal brain [3]. Although the *Clostridia* taxa identified in the current study differed from those identified the murine study, it is possible that similar mechanistic pathways are relevant.

In mice, butyrate inhibits Th17 cell development and promotes production of the anti-inflammatory cytokine IL10 [2, 33]. The association between higher maternal carriage of the butyrate producers *Lachnospiraceae* and *Ruminococcaceae* (to which most key butyrate-producing species belong [34]), and children with improved behaviour is consistent with the mounting evidence that butyrate is neuroprotective [35]. Although not all OTUs belonging to these families are able to generate SCFA, some may assist in their production. For example, *Ruminococcus bromii* does not produce butyrate [34], but is thought to play a significant role in butyrate production in humans by its ability to break down complex polysaccharides such as resistant starch and then provide accessible substrates to butyrate producers. However, it should be noted that in our study the relative abundances of these butyrate-producing OTUs were relatively low.

Microbiota-derived SCFAs, including butyrate, have well established anti-inflammatory properties [36] and in mice have been shown to promote the integrity of the blood brain barrier [37]. However, we were unable to show a relationship between SCFA concentrations in maternal serum or faeces and child behaviour. SCFAs are rapidly consumed by large bowel microbiota and colonocytes, and accordingly, their concentrations drop substantially between the caecum and the anus [38]. It is therefore possible that faecal measures are a poor proxy for the SCFA exposure of the foetus, as supported by

a recent study in adults wherein a marked increase in butyrate-producing bacteria and genes associated with carbohydrate fermentation, as a result of a Mediterranean diet intervention, was not reflected in faecal SCFA levels [39].

It has been over 15 years since murine studies first demonstrated that the infant gut microbiota influences rodent brain development and behaviour [40]. More recently, human studies have found associations between infant gut microbiota composition and temperament [5], cognitive development [41], and neurodevelopment [42]. We recently reported that, in the BIS cohort, the genus *Prevotella* in 12-month infant gut microbiota predicts improved scores on the same 2-year CBCL measure used in the present study [4]. Vertical transmission of microbiota from mother to infant has been clearly demonstrated, with transmitted strains apparent in the infant gut until up to four months of age [43,44]. We therefore considered whether the association between maternal microbiota and child behaviour may be mediated by the infant's microbiota, but found no evidence to support this hypothesis: neither the alpha diversity nor composition of the infant gut microbiota at one and six months related to either maternal alpha diversity or child behaviour at two years. The lack of association may be because the infant gut microbiota is still developing and, until three years of age, is far less diverse [45]. It is possible that metagenomic analyses will reveal patterns of transmission and associations that are not apparent in 16S rRNA gene amplicon sequencing data. Nevertheless, our current evidence is more compatible with an *in utero* effect rather than a postnatal effect. The bases of the associations we have reported in this study remain to be determined.

4.1. Caveats and limitations

The strengths of our study include the longitudinal design, collection of both prenatal maternal and postnatal infant faeces, and detailed measurement of a suite of relevant covariates. Our analyses investigated multiple covariates as either antecedents, mediators or confounders. In addition to adjustment for prenatal factors, where relevant we evaluated other factors such as mode of birth, preterm delivery, siblings and breastfeeding, as well as accounting for processing variations. There are however several important limitations. Our cohort was largely comprised of Australian born, non-smoking women from medium to high socioeconomic areas who were not parenting alone. It is therefore uncertain whether our findings are generalisable to the broader community. Our study relies on one sample collected in the third trimester of pregnancy. It is unclear how representative these microbiota data would be of the whole pregnancy because the alpha and beta diversity of the gut microbiota fluctuates between trimesters one and three [46,47]. Towards the end of pregnancy as progesterone increases, the alpha diversity of the gut microbiota decreases [47]. Further studies are required to investigate the impact of maternal gut microbiota across the course of pregnancy on behavioural and neurodevelopmental outcomes in their children. As the prenatal PSS was introduced after the study commenced, this measure was missing in 23.5% of the participants included in the study. We therefore derived a mean PSS from all available and pre- and postnatal PSS measures. In those with available data, the prenatal PSS and the combined PSS were highly correlated. In a sensitivity analysis restricted to those with prenatal PSS data available, the direction and magnitude of associations between maternal alpha diversity measures and CBCL outcomes were similar to the estimates obtained in the main analysis although the precision was somewhat reduced (as expected with a smaller sample size). Hence the use of the combined PSS score rather than only the prenatal PSS is unlikely to have influenced our results. Sixty-eight (23.6%) of 228 mother-child dyads in the random subsample with adequate sequencing read counts had failed to complete the CBCL at two years. It is possible that this introduced a retention bias. Our analyses did not include measures of child diet, which could be a proxy for unmeasured antecedent confounding factors such as family dynamics and eating behaviours [6,48]. It is likely that our maternal dietary analyses are substantially underpowered and larger studies are needed. Data from 16S rRNA gene amplicon sequencing provides minimal information from which to infer a potential mechanistic basis for the observed associations. Metagenomic and metabolomic analyses may inform mechanistic insights. Finally, our behavioural measures were parent-reported and the children were relatively young. Objective measures amongst older children would improve the precision of case definition. Nonetheless, our findings are novel and provide a compelling basis for future work regarding the potential role of the maternal microbiome in the early life origins of adverse neurodevelopment and mental illness.

5. Conclusion

This study supports the importance of the prenatal diet and composition of the maternal gut microbiota during pregnancy in determining behavioural outcomes in children. Independent of confounding factors, higher prenatal microbial alpha diversity was related to lower internalising scores in the children, and mothers of children with normative behaviour showed a greater carriage of specific butyrate-producing organisms. Larger studies incorporating metagenomic sequencing–based investigation of mechanistic pathways are now needed to replicate and extend these findings.

Data sharing statement

Microbiota sequencing reads can be accessed at the Sequence Read Archive under accession number PRJNA576314 (ncbi.nlm.nih.gov/sra/?term=PRJNA576314). The cohort data used in this paper can be requested through the BIS Steering Committee by contacting the corresponding author. Project information, including descriptions of the cohort data, and access procedure, is available at the project's website barwoninfantstudy.org.au.

Contributors

PV, ALP, MLKT, SR, and RS conceived of the study and designed the protocol. FC was responsible for sample processing and storage and DNA isolation. SLD and MOH verified the underlying data. SLD, MOH, CS, PV, ALP and MMB planned the analyses, and SLD and MOH conducted the analyses. SLD and PV wrote the original draft of the manuscript. SLD, MOH, FNJ, ALP, CS, AL, FC, MMB, PS, DB, MLKT, RS, SR, MAC, LCH, SB, KK, PV contributed to the interpretation of study findings, critically revised successive drafts of the paper, and approved its final version.

Declaration of Competing Interest

Dr. O'Hely reports grants from National Health & Medical Research Council (Australia), during the conduct of the study; other from Prevatex Pty Ltd, outside the submitted work. Dr. Symeonides reports grants from NHMRC (Australian Government), grants from Shepherd Foundation (philanthropic foundation), during the conduct of the study. Dr. Loughman has a patent 'Behavioural Treatment' PCT/AU2019/050878 issued. Dr. Burgner reports grants from National Health and Medical Research Council (NHMRC) Australia, during the conduct of the study. Dr Jacka 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. Dr. Tang reports personal fees from Prota Therapeutics, Abbott Nutrition Nestle health Science, Nestle Nutrition Institute, Nutricia and Bayer Pharmaceuticals, outside the submitted work; In addition, Dr. Tang has a patent 'Methods and compositions for

determining and for minimizing the likelihood of development of allergy in infants' WO2018112553 pending to MCRI, and a patent 'Behavioural Treatment' WO2020037364 licensed to MCRI. All other authors have nothing to disclose.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103400.

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