Gut microbiota composition during infancy and subsequent behavioural outcomes

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ABSTRACT

Background: Despite intense interest in the relationship between gut microbiota and brain development, longitudinal data from human studies are lacking. This study aimed to investigate the relationship between the composition of gut microbiota during infancy and subsequent behavioural outcomes.

Methods: A subcohort of 201 children with behavioural outcome measures was identified within a longitudinal, Australian birth-cohort study. The faecal microbiota were analysed at 1, 6, and 12 months of age. Behavioural outcomes were measured at 2 years of age.

Findings: In an unselected birth cohort, we found a clear association between decreased normalised abundance of Prevotella in faecal samples collected at 12 months of age and increased behavioural problems at 2 years, in particular Internalizing Problem scores. Recent exposure to antibiotics was the best predictor of decreased Prevotella.

Interpretation: Our findings demonstrate a strong association between the composition of the gut microbiota in infancy and subsequent behavioural outcomes; and support the importance of responsible use of antibiotics during early life.

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1. Introduction

The gut microbiome appears to play an important role in stimulating neurodevelopment via neuronal, hormonal, and immunological signalling [1]. Disruptions to gut microbiota cause aberrant hypothalamic-pituitary-adrenal axis stress-response, decreased expression of brain-derived neurotrophic factor, and impaired social behaviour in germ-free mice [2]. Both gut microbiota and the central nervous system mature rapidly during early life, and in mice, this appears to be a key period of gut-brain interaction [3].

Clinical studies show altered gut microbiota in people with established conditions such as autism and schizophrenia [4]; however, gut-brain associations in normal human neurodevelopment remain understudied. One study examining cross-sectional relationships between behaviour and gut microbiota in 77 human infants at age 18–27 months reported an association between phylogenetic diversity and temperamental problems, particularly in boys [5]. The only
Informed consent before participating.

Health (reference 10/24) and mothers of all infants gave written consent before participating.

Sample and study design

The Barwon Infant Study (BIS) is a longitudinal, Australian birth cohort study (n = 1074 infants) [8]. Sociodemographic, family history, and maternal health data were collected during pregnancy, along with faecal samples from infants at 1, 6, and 12 months of age. 16S rRNA gene sequencing was performed in a random subsample (n = 324), of whom 201 had sufficient data available and were included in the present study. Parents completed the Child behaviour Checklist [9] (CBCL) when children were two years old.

Ethics

The study was approved by the ethics committee at Barwon Health (reference 10/24) and mothers of all infants gave written informed consent before participating.

2. Materials & methods

2.1. Sample and study design

The Barwon Infant Study (BIS) is a longitudinal, Australian birth cohort study (n = 1074 infants) [8]. Sociodemographic, family history, and maternal health data were collected during pregnancy, along with faecal samples from infants at 1, 6, and 12 months of age. 16S rRNA gene sequencing was performed in a random subsample (n = 324), of whom 201 had sufficient data available and were included in the present study. Parents completed the Child behaviour Checklist [9] (CBCL) when children were two years old.

2.2. Microbiota

Stool samples were stored at −80°C. In a random subsample of n = 324, microbial DNA was extracted and 16S rRNA sequencing conducted. DNA was extracted using the Qiagen PowerSoil® DNA Isolation Kit, Cat# 12888–100. The lysis of the bacteria was optimised by performing two bead shearing steps (5 min each), in conjunction with the treatment with lysis buffer, and two heat incubation steps (85 °C × 2). The DNA was transported to the J. Craig Venter Institute, Rockville, MD, USA. The V4 hypervariable region of the 16S rRNA gene was sequenced on the Illumina MiSeq platform. USEARCH software was used to merge corresponding paired-end reads, filter, cluster into OTUs at 97% identity, identify OTU representative sequences, and remove chimeras. Mothur software was used to assign representative sequences to taxa described in the SILVA v123 Nr99 taxonomic database. The final descriptions of OTUs present in each sample were composed in USEARCH. Samples with fewer than 2500 read pairs were excluded. Additional aliquots of faecal samples were transported on dry ice to the Commonwealth Science and Industrial Research Organisation laboratories, Adelaide, Australia, where short-chain fatty acids (SCFAs) were quantified by capillary gas chromatography (GC; 5890 series II Hewlett Packard, Australia). For SCFA determination, samples (0.5–1 g) were diluted 3-fold with an internal standard (1.68 mmol/L heptanoic acid, Sigma, NSW), centrifuged (3000 × g for 15 min at 5 °C) and the pH of the resultant supernatant measured by inserting an appropriate glass probe. An aliquot (150 μL) of supernatant were then acidified with 30 μL of 0.16 mol/L orthophosphoric acid and distilled under vacuum. Individual SCFA in the distillates were separated and quantified by capillary gas chromatography (GC; 5890 series II Hewlett Packard, NSW, Australia). The GC was equipped with a flame ionisation detector, split-less injector and a Zebon ZB-FFAP 30 m × 0.53 μm capillary column with 0.1 μm film thickness (Phenomenex, NSW, Australia). Injector and detector temperatures were both 210 °C, and the column temperature program was 120 °C held for 0.5 min and then raised at 30 °C/min to reach a final column temperature of 190 °C. Helium was used as the carrier gas (head pressure 50 kPa) and an injection volume of 0.2 μL was used. Faecal SCFA concentrations were calculated as (mmol/L) × wet faecal weight x faecial moisture content (g/100 g) × 10.

2.3. Statistical analysis

Statistical analysis was conducted in the statistical software environment R, using the phyloseq package for microbiome data management [12], and the vegan package for beta-diversity [13]. Alpha diversity within samples was computed as the Shannon, Simpson, Chao1, and Observed species indices. Beta diversity between samples was computed as the weighted UniFrac distance [14]. The association between beta diversity and the binary behavioural outcome was examined via the PERMANOVA test; PERMDISP2 was used to examine whether the findings of PERMANOVA could

3.1. Microbiota

Evidence before this study

In rodents, gut microbiota composition has been shown to influence neurodevelopment and behaviour. In humans, cross-sectional associations have been observed between the composition of gut microbiota and neuropsychiatric outcomes including temperament, attention and impulsivity, autism, schizophrenia, bipolar disorder, and depression; with recent evidence of an association between gut microbiota composition at 2.5 months and temperament at 6 months. However there is a lack of longitudinal data regarding gut microbiota and longer term behavioural outcomes.

Added value of this study

We present findings from an Australian birth cohort study demonstrating a prospective association between a decrease in a genus of bacteria in the faecal microbiota, Prevotella, at 1 year of age, and adverse behavioural outcomes at 2 years of age. Strengths of the study include the unselected sampling strategy, large sample size, and comprehensive assessment of potential confounding factors. The study adds novel evidence from a human birth cohort study regarding a longitudinal association between gut microbiota and behaviour.

Implications of all the available evidence

Accumulating evidence supports the role of the infant gut microbiota for neurodevelopment and mental health in later life. The findings of this study suggest a developmental window in late infancy in which increased Prevotella in the gut microbiota may predict a reduced risk of subsequent anxiety.

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have arisen from differing dispersion between groups. Both tests were based on 999 permutations.

The voom method from the limma package [15] was used for differential normalised abundance testing. Voom outperforms competing methods (e.g. DESeq2) for highly variable library sizes, which are typical in 16S sequencing studies [16]. The log-fold change reported by voom is based on abundances normalised using relative log expression with pseudo-counts added to avoid taking the log of 0 and including voom’s precision weighting scheme (see Supplement 1). This metric is described as normalised abundance. We adjusted p-values for multiple testing using the Benjamini-Hochberg method and refer to corresponding false-discovery rates as q-values. As the SILVA taxonomy divides accepted genera into multiple clades (e.g. Tyzzerella_x, where x can be 3 or 4), we generated an intermediate taxonomic level between the SILVA family and genus levels by truncating the genus name before its first underscore. This has a secondary effect of consolidating genus level groupings of unnamed clades within families, e.g. Ruminococcaceae_UCG-xxx where xxx can be 013, 014, 010 etc. Logistic and linear regression and chi square tests were used to assess associations between categorical and linear outcomes as appropriate.

Analyses of differential normalised abundance were adjusted for the methodological variables:

(i) any storage of faecal samples in the home freezer (typically −18C) prior to delivery to the laboratory, and
(ii) duration of storage at −80C.

To evaluate adjustment for potential covariates, twenty six candidate domains of relevance to infant microbiota and/or early childhood behaviour were identified and used to construct a directed acyclic graph (DAG; Supplement 2a) using DAGitty v3.0. For each of these domains, a number of candidate variables available in the BIS dataset were specified. Correlation matrices were constructed to inspect linear relationships between candidate variables, candidate microbial exposures, and behavioural outcomes of interest. For each construct, the one with the strongest associative relationships with exposures and/or outcomes was selected for inclusion in the directed acyclic graph (Supplement 2b). Minimal sufficient adjustment sets for estimating the total effect of the microbial exposure on the behavioural outcome, were then generated from DAGitty. We investigated potential confounding using the minimum adjustment set with the most complete data, which comprised: gestational age, mode of birth, antibiotic use during labour, breastfeeding at four weeks, number of siblings, and household pet ownership. Sex and child’s age in months at time of questionnaire completion were additionally adjusted for in separate analyses given sex differences in neurodevelopment [17] and reduce potential outcome measurement variability respectively.

3.4. Data statement

Regarding access of data, the microbiome sequencing data is available from the Sequence Read Archive under accession number PRJNA576314. Anonymised data relating to the additional variables used in the analysis, results and conclusions reported in this paper will be made available upon reasonable requests for purposes of reproducing or extending the analysis.

4. Results

4.1. Descriptive statistics of study sample

The baseline characteristics of the inception birth cohort (n = 1074) and randomly selected subgroup with adequate 12 month faecal microbiota and behavioural outcome data (n = 201) were similar (Table 1).
Of the 201 participants in the study sample, 22 were classified as cases on the basis of ‘elevated’ behavioural problems ($T \geq 60$; $n = 14$ Externalizing subscale, $n = 9$ Internalizing subscale, $n = 10$ Total Problems subscale; see Supplement 3 for density plot of $T$ score distributions and scores in case and non-case groups). 182 infants had one-month and 190 had six-month faecal microbiota samples available.

4.2. Sequencing summary

Faecal samples from the three time-points were sequenced across two runs. Summary statistics of the sequencing are presented in Supplement 4a. A small number of biological and technical duplicates were sequenced. amongst the 12-month samples, all replicates of the same sample clustered together according to weighted UniFrac distance (Supplement 4b).

4.3. Child microbiota and behavioural outcomes

4.3.1. One- and six-month child microbiota

There was no evidence of associations between one- or six-month microbiota alpha diversity (as measured by Shannon Index), or beta diversity (weighted and unweighted UniFrac distances) and the behavioural outcome at age 2 (see Supplements 5, 6). Nor was there evidence of differential normalised abundance in the one-month faecal microbiota of behavioural case infants versus non-case infants ($n = 182$). In the six-month faecal samples ($n = 190$), the normalised abundance of the genus Sutterella appeared to be lower in the case group ($\log FC = -0.37, p = 0.0002, q = 0.02$) but this association was attenuated following adjustment for any storage in a domestic freezer and duration of storage at $-80^\circ C$ ($p = 0.0016, q = 0.18$).

4.4. 12-month child microbiota

4.4.1. Alpha and beta diversity

There was weak evidence that a higher Shannon index alpha diversity at 12 months was associated with increased risk of elevated behaviour problems at two years (OR: $2.42 [0.92–6.97], p = 0.087$). A similar pattern was observed using alternative measures of alpha diversity (see Fig. 1). None of the processing or potentially confounding variables caused a change of $>10\%$ to the estimate of the odds ratio.

PERMANOVA applied to unweighted UniFrac distances suggested differences in microbiota community structure between behavioural groups ($R^2 = 0.0092, p = 0.018$). PERMDISP2 indicated that this may reflect differential multivariate dispersions ($F = 9.33(1, 199)$).

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**Fig. 1.** Number of observed OTUs, Chao1, Shannon and Simpson indices of alpha diversity of 12-month faecal microbiota of infants with elevated behaviour problems (case) and normative behaviour (non-case) at two years of age.
4.4.2. Differential normalised abundance

The normalised abundance of two bacterial groups was substantially different in the 12-month faecal microbiota of behavioural case infants versus non-case infants: Prevotella (logFC = −1.46, \( p < 0.0001 \), \( q < 0.0001 \)) and the unspecified genera of the Lachnospiraceae family (logFC = 2.09, \( p = 0.0009 \), \( q = 0.054 \); see Fig. 2). Prevotella was detected in only 4% (1/22) of case infants compared with 44% (79/179) of non-case infants (\( q = 0.0001 \)). In contrast, the association between increased normalised abundance of Lachnospiraceae at one year and the behaviour case group persisted follow-up to two years (\( q = 0.499 \); see Fig. 2).

4.4.4. Investigation of reverse causation

To assess reverse causation we considered associations between early temperament and both candidate bacteria at subsequent timepoints. There were however no associations evident between child temperament at one, six, and 12 months and presence or abundance of subsequent \( \text{Prevotella} \) or Lachnospiraceae at 12 months of age. Nor was the association between the normalised abundance of \( \text{Prevotella} \) and behaviour at two years attenuated by adjusting for infant temperament at 1-, 6-, or 12 months of age (\( q < 0.001 \) in all cases). For Lachnospiraceae, \( q \) values were 0.15, 0.01, and 0.01 respectively (for...
1-, 6-, and 12-month infant temperament). Thus, the prospective association between *Prevotella* and subsequent behaviour persisted after accounting for early life infant temperament.

4.4.5. Child behaviour checklist subscales

The association between decreased *Prevotella* at 12 months and two-year behaviour outcomes was primarily related to the Internalizing subscale (Supplement 9). There was an analogous trend for Lachnospiraceae.

4.5. OTU members of the *Prevotella* genus and Lachnospiraceae family

Amongst 12-month samples from the random subcohort, OTU41 comprised 95% of all OTUs identified as belonging to the genus *Prevotella*. The sequence of 253 base pairs characterising OTU41 was 100% identical to base pairs 529–781 of the *P. copri* strain JCM 13,464 16S rRNA gene (Accession No: AB649279). The next most common *Prevotella* OTU was OTU697 at 1.7%. The sequence characterising OTU697 differed from that of OTU41 by only eight base pairs (96.8% identity). OTU697 was only evident in samples in which OTU41 was identified, suggesting that OTU697 may have arisen from sequencing errors in reads otherwise destined to be classified as OTU41.

The group of Lachnospiraceae OTUs was mostly composed of OTU35 (56%) and OTU70 (22%), both classified to the Lachnospiraceae NK4A136 group. A BLAST search for the representative sequences of these two OTUs was inconclusive. The NK4A136 group accounted for only 5% of all family Lachnospiraceae OTUs counted in 12-month samples.

4.6. Predictors of 12-month *Prevotella* and Lachnospiraceae carriage

There was no evidence of a relationship between infant feeding practices, sibling number, or pet ownership and carriage of *Prevotella* at 12 months (all p > 0.05). Having a pet in the household during the first postnatal year was positively associated with Lachnospiraceae carriage (OR: 2.03 [1.05–3.89], p = 0.033). Antibiotic use from 9 to 12 months (i.e. the three months prior to faecal sample collection) was associated with reduced presence of *Prevotella* (OR 0.37 [0.17–0.75], p = 0.007), but not Lachnospiraceae (OR 0.67 [0.34–1.33], p = 0.24; Supplement 11). However, antibiotic use did not relate to 2 year behaviour (χ² = 0.047, df = 1, p = 0.83).

4.7. Faecal short-chain fatty acids

There was no evidence of associations between the concentration of faecal SCFAs and child behaviour, presence of *Prevotella* genus in 12-month faecal microbiota or antibiotic use from 9–12 months (butyrate, propionate, acetate, caproate, valerate, isobutyrate, isovalerate; and a sum of butyrate, acetate, propionate, and valerate; Supplement 10).

5. Discussion

In a study of 201 infants from an Australian birth cohort assembled using an unselected sampling frame, we report a clear association between reduced normalised abundance of the genus *Prevotella* in infancy and increased risk of being in a behaviour case group at 2 years of age. This association primarily related to the Internalizing subscale, and was independent of a range of potential confounding factors. Recent exposure to antibiotics was the best predictor of *Prevotella* absence. Although *Prevotella* abundance has been associated with both autism [18] and Parkinson’s disease [19] in cross-sectional studies, this is the first study to show an association between a low abundance of *Prevotella* in early life and subsequent adverse behavioural outcomes.

The mechanisms by which *Prevotella* may influence brain development and behaviour are poorly characterised but may include stimulation of the vagus nerve, release of cytokines or enzymes, tryptophan metabolism, interaction with the peripheral immune system, effects on brain-derived neurotrophic factor and the production of SCFAs [20,21]. We found no evidence of associations between short-chain fatty acid abundance in stool and *Prevotella*, recent use of antibiotics (a predictor of *Prevotella* carriage in this sample) or behaviour problems. Of relevance, one study of women demonstrated positive associations between *Prevotella* abundance, complexity of frontal cortex and insula connections as measured by functional magnetic resonance imaging, and reduced emotional responses to distressing stimuli [22]. It is of course possible that *Prevotella* is simply a biomarker for broader aspects of the microbiome, host genotype, or metabolism. Further studies are required to assess causality and underlying mechanisms.

*Prevotella* is a gram-negative bacterial genus that is more abundant amongst populations living in non-westernised environments [23]. The low rate and abundance of *Prevotella* carriage amongst westernised populations is thought to relate to low dietary intake of plant polysaccharides, which provide important cellulose and xylans substrates [24]. High rates of antibiotic exposure, as reported in our cohort [25], may also be relevant; consistent with evidence from mouse studies demonstrating that antibiotic exposure in early life influences gut microbiota, and alters brain cytokines and behaviour [26]. Estimates regarding the duration of disruption to the gut microbiota following exposure to antibiotics vary widely, and are likely to depend on the nature of the antibiotic agent [27]. However here, antibiotic exposure between ages 9 and 12 months, but not earlier, predicted lower carriage of *Prevotella* at 12 months, suggesting that *Prevotella* carriage may recover over longer intervals. Host, diet, and environmental factors influencing the likelihood and rate of reconstitution are yet to be determined.

We found weak evidence of an association between higher alpha diversity and subsequent behavioural problems. Although decreased microbial diversity is typically associated with westernised populations and adverse health outcomes in adults, we note that associations between diversity and health status is mixed in children [28]. Our finding is consistent with recent evidence of an association between increased microbiota diversity at 12 months of age and subsequent adverse cognitive outcomes [8]. We acknowledge that microbial diversity provides a summary of a highly dimensional dataset, and it is widely recognised that more biologically meaningful descriptors are required.

The association we observed between increased normalised abundance of Lachnospiraceae and adverse behavioural outcomes is noteworthy. Lachnospiraceae is a family of anaerobic bacteria from the order Clostridiales. Some members of this family are ‘fibre fermenters’ and produce butyrate, a SCFA shown to have positive neurological effects on tight junction proteins of the blood brain barrier and to reduce neuroinflammation [29]. Decreased carriage of Lachnospiraceae has been associated with depression [30] and Parkinson’s disease [31]. Our findings however highlight the likely complexity of gut-brain relationships and the importance of considering the adverse outcomes of strategies to promote carriage of specific taxa. The single finding to emerge from the six-month microbiota of reduced normalised abundance of *Sutterella* in the case group requires replication in a larger sample as the effect did not persist beyond adjustment for processing variables.

The only previous study to relate gut microbiota and behaviour beyond infancy in humans was limited by cross-sectional design [5]. We found no evidence of a cross-sectional association between gut microbiota and child temperament at 12 months of age, suggesting the longitudinal association between decreased *Prevotella* and adverse behaviour is unlikely to reflect reverse causation. Further, temperament measured during the first postnatal year did not predict *Prevotella* carriage.

In contrast to recent evidence of an association between microbiota composition at 2.5 months and temperament at 6 months [7], we found little evidence linking faecal microbiota composition at one
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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.2020.102640.

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