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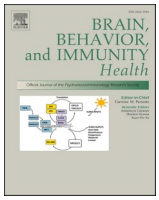

Absence of association between maternal adverse events and long-term gut microbiome outcomes in the Australian autism biobank

Svetlina Vasileva [a], Chloe X. Yap [b,c,d], Andrew J.O. Whitehouse [e], Jacob Gratten [b,d], Darryl Eyles [a,f,*]

[a] Queensland Brain Institute, University of Queensland, Brisbane, Australia
[b] Mater Research Institute, The University of Queensland, Translational Research Institute, Brisbane, Australia
[c] Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia
[d] Cooperative Research Centre for Living with Autism (Autism CRC), Long Pocket, Brisbane, Australia
[e] Telethon Kids Institute, University of Western Australia, Australia
[f] Queensland Centre for Mental Health Research, Wacol, Australia

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ABSTRACT

Introduction: Maternal immune activation (MIA) and prenatal maternal stress (MatS) are well-studied risk factors for psychiatric conditions such as autism and schizophrenia. Animal studies have proposed the gut microbiome as a mechanism underlying this association and have found that risk factor-related gut microbiome alterations persist in the adult offspring. In this cross-sectional study, we assessed whether maternal immune activation and prenatal maternal stress were associated with long-term gut microbiome alterations in children using shotgun metagenomics.

Methods: This cross-sectional study included children diagnosed with autism (N = 92), siblings without a diagnosis (N = 42), and unrelated children (N = 40) without a diagnosis who were recruited into the Australian Autism Biobank and provided a faecal sample. MIA exposure was inferred from self-reported data and included asthma/allergies, complications during pregnancy triggering an immune response, auto-immune conditions, and acute inflammation. Maternal stress included any of up to 9 stressful life events during pregnancy, such as divorce, job loss, and money problems. Data were analysed for a total of 174 children, of whom 63 (36%) were born to mothers with MIA and 84 (48%) were born to mothers who experienced maternal stress during pregnancy (where 33 [19%] experienced both). Gut microbiome data was assessed using shotgun metagenomic sequencing of the children's faecal samples.

Results: In our cohort, MIA, but not MatS, was associated with ASD. Variance component analysis revealed no associations between any of the gut microbiome datasets and neither MIA nor MatS. After adjusting for age, sex, diet and autism diagnosis, there was no significant difference between groups for bacterial richness, α-diversity or β-diversity. We found no significant differences in species abundance in the main analyses. However, when stratifying the cohort by age, we found that *Faecalibacterium prausnitzii E* was significantly decreased in MIA children aged 11–17.

Discussion: Consistent with previous findings, we found that children who were born to mothers with MIA were more likely to be diagnosed with autism. Unlike within animal studies, we found negligible microbiome differences associated with MIA and maternal stress. Given the current interest in the microbiome-gut-brain axis, researchers should exercise caution in translating microbiome findings from animal models to human contexts and the clinical setting.

1. Introduction

The concept that "the womb may be more important than home", initially posited by David Barker in the 1990s, has influenced our understanding of infants' physical and psychological health trajectories (Barker, 1990). Psychiatric and neurodevelopmental conditions such as

* Corresponding author. Queensland Brain Institute, University of Queensland, Brisbane, Australia.
E-mail address: eyles@uq.edu.au (D. Eyles).

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schizophrenia and autism spectrum disorders (ASD) have long been linked to prenatal factors. Notably, two prenatal risk factors have garnered attention: maternal immune activation (MIA) – the activation of an expectant mother's immune system triggered by infectious stimuli (Brown and Meyer, 2018; Patterson, 2009) – and maternal exposure to stress during pregnancy (MatS) (Lipner et al., 2019; Walder et al., 2014). The increased risk for neuropsychiatric conditions associated with these risk factors has been supported by both epidemiological studies, including those on influenza epidemics and famine (Barr et al., 1990; Malaspina et al., 2008), and case-control studies using clinical diagnoses, self-report, and biological biomarkers, such as inflammatory cytokines and cortisol (Lipner et al., 2019).

Animal studies have proposed the gut microbiome as a potential mediator explaining these associations. Inducing MIA or MatS in pregnant animals, mainly via the viral mimic polyinosinic:polycytidylic acid (poly I:C) or environmental stressors, respectively, leads to social and cognitive behaviours in offspring of potential relevance to autism and schizophrenia (Abbott et al., 2018; Lammert and Lukens, 2019; Meyer and Feldon, 2012). In their study using a poly:IC model in mice, Hsiao et al. (2013) found that some intestinal permeability disruptions, MIA-related behavioral phenotypes, and changes in gut microbiome composition were corrected by oral treatment with *Bacteroides fragilis* in the offspring during weaning. Another study in mice by Kim et al. (2017) found that a specific maternal microbiome profile is required to induce hyperinflammation and its related behavioural phenotypes. They further proposed that changing the maternal gut microbiome (e.g., through antibiotics) may be protective against MIA-related phenotypes in the offspring. Notably, both studies suggest that MIA-related changes in the gut microbiome are long-lasting and can be observed in the animal offspring during adolescence and adulthood.

Animal studies have also provided evidence for a mediating role of the gut microbiome concerning MatS and behavioural changes relevant to psychiatric conditions. Studies in mice and rats have found that MatS causes alterations in the gastrointestinal system, hyper-responsiveness to stress, and changes in gut microbiome composition in infancy (Jašarević et al., 2017, 2018) and early adulthood (4 months; Golubeva et al., 2015; Gur et al., 2017). Furthermore, through a series of well-designed cross-fostering studies, Jasarevic and colleagues (2018) found that the maternal gut microbiome partially mediates the behavioural effects of early prenatal stress, consistent with Kim et al.'s (2017) findings that changes in the maternal microbiome can mediate MIA-related behaviours in the offspring.

The evidence from animal studies suggesting that the gut microbiome may mediate the long-term effects of adverse gestational events on psychiatric outcomes in offspring indicates that the microbiome may be a promising therapeutic avenue in psychiatry across various life stages. However, human studies remain limited and inconclusive. A recent study found differences in bacterial relative abundance between HIV-exposed uninfected and HIV-unexposed infants at 6, 28, and 62 weeks post-partum (Jackson et al., 2022), indicating some evidence for microbiome-and-MIA associations in human populations. Some human studies have also replicated the short-term associations between MatS and gut microbiome alterations in neonates (Weiss and Hamidi, 2023) and infants (Aatsinki et al., 2020; Galley et al., 2023; Zijlmans et al., 2015), but specific taxa and the directions of findings vary widely. In addition, a recent large case-control study using data from the Australian Autism Biobank which incorporated lifestyle and behavioural measures found limited evidence for an association between ASD and the gut microbiome (Yap et al., 2021).

Within the human studies investigating the relationship between the gut microbiome and maternal adverse events, none have explored this in the context of psychiatric outcomes or in children beyond infancy. Furthermore, the long-lasting changes observed in pre-clinical studies are often contingent on strictly controlled environmental factors such as diet, housing conditions and the microbiome of the dam (Kim et al., 2017). Considering the inter-species differences in developmental stage duration and overall lifespan (two years for mice, three years for rats, and 80 years for humans on average) (Dutta and Sengupta, 2016; Sengupta, 2013), and the lack of defined psychiatric syndromes in rodents, there is a need to investigate whether long-lasting microbiome associations in animal studies are also observed in humans beyond infancy.

To address this, we analysed shotgun metagenomics data from 174 stool samples from children and adolescents aged 2–17 recruited into the Australian Autism Biobank, an initiative of the Cooperative Research Centre for Living with Autism (Autism CRC) (Alvares et al., 2018). We used data from psychological questionnaires, lifestyle surveys, and family history to determine MIA and MatS retrospectively. We performed variance component, bacterial diversity, and relative abundance analyses to test for an association of MIA and MatS with gut microbiome composition while covarying for age, sex, diet.

2. Methods

This microbiome study was reported according to the Strengthening The Organization and Reporting of Microbiome Studies (STORMS) checklist (Mirzayi et al., 2021) (Table S1).

2.1. Participants

This case-control study included 199 participant mother-child dyads who came from the Australian Autism Biobank dataset (Alvares et al., 2018). Data were collected between 2013 and June 2018. Participants were children diagnosed with ASD (n = 99, recruited from autism clinics and research centres across Australia's largest cities: Sydney, Melbourne, Brisbane, and Perth, no exclusion criteria), their siblings (SIB) without a diagnosis (n = 51), and unrelated children (UNR) without a diagnosis (n = 49, recruited from the community, exclusion criteria: no ASD diagnosis). 25 participants were excluded (see Fig. S1) due to a genetic condition (n = 1), missing dietary data (n = 1), and incomplete data across the family questionnaires relevant to MIA and MatS (n = 23). See Fig. 1 for an overview of the study design and Table 1 for descriptive and summary statistics of the participants' demographics. ASD was not investigated as a main phenotype as this participant cohort was part of a larger study which found limited evidence for associations between the gut microbiome and ASD (Yap et al., 2021).

2.2. Defining maternal phenotypes

2.2.1. Maternal immune activation

Maternal Immune Activation (MIA) was inferred by reviewing family history questionnaires completed by a parent for each individual child at the time of data collection (see Alvares et al., 2018 for questionnaire details). MIA was compiled from a combination of self-reported questions regarding maternal medication history, reported complications during pregnancy, and chronic illness (and age of onset) and was based on previous methodology (Patel et al. (2018, 2020). Four main types of MIA events were recorded: acute inflammation (N = 23 pregnancies), chronic immune-related conditions (allergy/asthma; N = 20), complications during pregnancy (pre-eclampsia, urinary tract infection (UTI), gestational diabetes, diabetes type II; N = 24), and autoimmune disease (diabetes type I, thyroid problems, other; N = 11). MIA was established if a mother experienced at least one event (n = 63) noting that some mothers experienced more than one type of MIA event (N = 11). Acute inflammation was determined if mothers specified that they experienced an infection (e.g. parvovirus, swine flu, bronchitis, pneumonia, shingles, listeria, flu, thrush) and/or indicated the intake of antibiotics, anti-inflammatory medication, or corticosteroids during the pregnancy.

2.2.2. Maternal stress

Maternal stress was indicated by the presence of at least one stressful event experienced by the mother *during* pregnancy. Mothers were asked whether or not they experienced any of 9 major life stress events selected

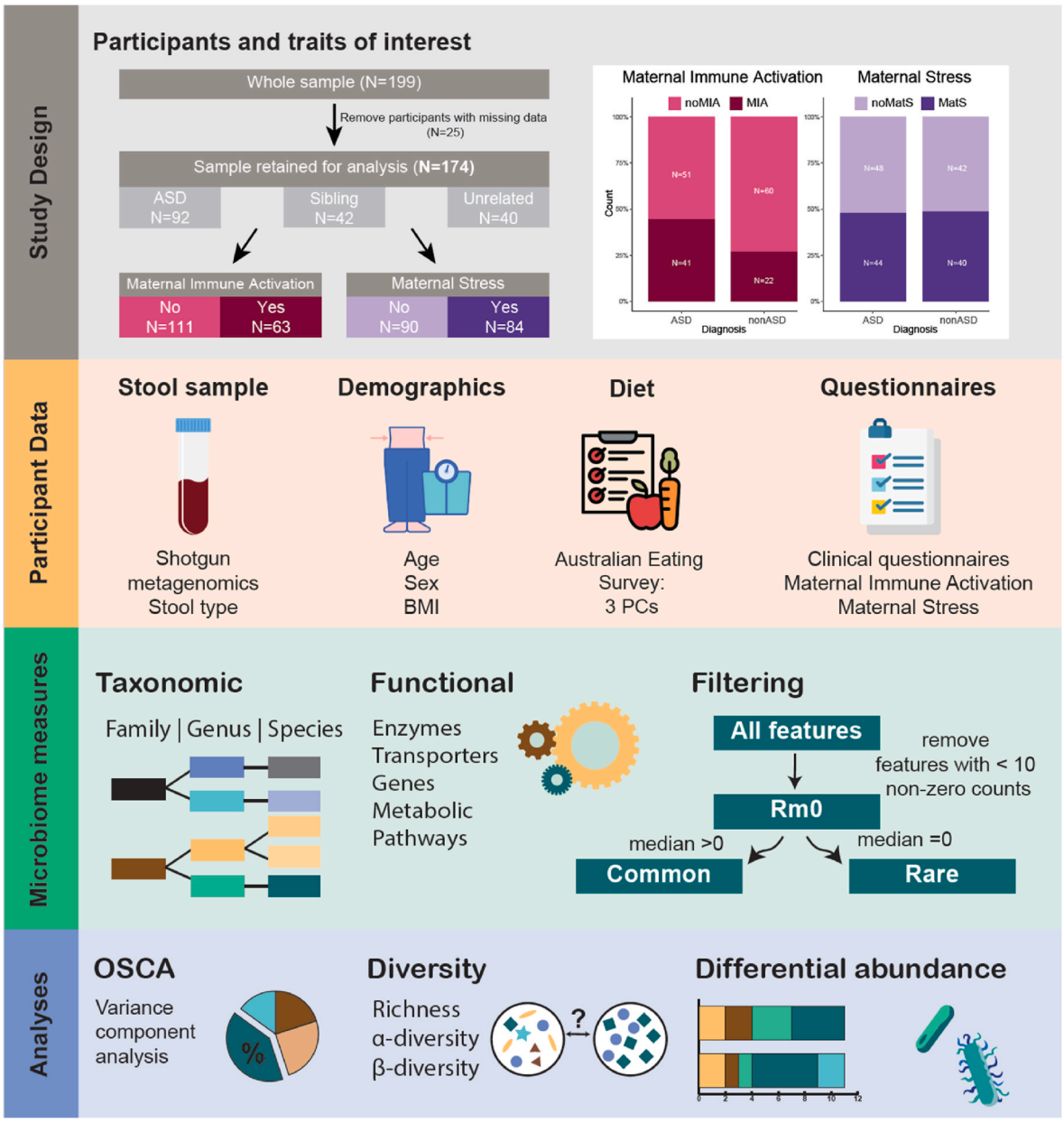

Fig. 1. Study design, measures, and analyses.

from a stress inventory (Tennant and Andrews, 1976). Events included death of a close friend or relative, separation or divorce, marital problems, problems with children, job loss (involuntary), partner's job loss (involuntary), money problems, residential move, or "other stressful event". "Other stressful" events included work/study stress, stressful living situation, and serious illness in the family, among others (see Table S2 for a complete list of stressful events and their occurrence). MatS was established if at least one of these events occurred (n = 84). Importantly, these events did not include maternal infection and were independent of MIA.

The figure shows 1) study design and groups, 2) clinical and demographic measures, 3) microbiome datasets obtained through shotgun-metagenomic sequencing and how features from each dataset were filtered, and 4) analyses performed on the microbiome datasets.

Abbreviations: ASD = autism spectrum disorder; MIA = maternal immune activation; MatS = maternal stress; BMI=Body Mass Index, PC = principal components.

2.2.3. *Maternal adverse event*

We also established a maternal adverse event (ME) category where the presence of ME was established if a mother experienced at least one MIA *or* MatS event (noME = 60, ME = 114). ME was used in secondary variance component (OREML) and diversity analyses.

2.3. *Secondary measures*

2.3.1. *Diet*

Dietary data were collected through the Australian Eating Survey (AES, toddler's and children's versions), a food frequency questionnaire validated in the Australian population, which was predominantly completed by a parent. The AES records frequency for the consumption of 123 different food items from which 4 main types of variables are derived: percentage energy (pe) from 13 core foods (vegetables, fruit, meat, alternative proteins, grains, dairy, sweet drinks, packed snacks, confectionary, baked products, takeaway, condiments, fatty meats), micronutrients (multiple vitamins and minerals), macronutrients

Table 1
Demographics table.

		ASD (N = 92)	SIB (N = 42)	UNR (N = 40)	Total (N = 174)	p value
Age	Mean (SD)	8.7 (3.8)	8.2 (4.5)	6.0 (3.0)	8.0 (3.9)	0.001
	Range	2.1–17.6	2.2–16.9	2.1–13.8	2.1–17.6	
Sex	F	24 (26.1%)	19 (45.2%)	20 (50.0%)	63 (36.2%)	0.012
	M	68 (73.9%)	23 (54.8%)	20 (50.0%)	111 (63.8%)	
BMI	Mean (SD)	18.5 (4.3)	18.2 (4.0)	17.0 (3.0)	18.1 (4.0)	0.153
	Range	10.4–32.5	13.8–33.1	8.1–26.5	8.1–33.1	
MIA	noMIA	51 (55.4%)	34 (81.0%)	26 (65.0%)	111 (63.8%)	0.017
	MIA	41 (44.6%)	8 (19.0%)	14 (35.0%)	63 (36.2%)	
MatS	noMatS	48 (52.2%)	24 (57.1%)	18 (45.0%)	90 (51.7%)	0.542
	MatS	44 (47.8%)	18 (42.9%)	22 (55.0%)	84 (48.3%)	
Bristol	N-Miss	8	0	1	9	0.350
Stool	Mean (SD)	3.5 (1.3)	3.7 (1.2)	3.3 (1.0)	3.5 (1.2)	
Type	Range	1–7	1–7	1–5	1–7	
Diet	Mean (SD)	−0.3 (1.5)	0.1 (1.3)	0.1 (1.0)	−0.1 (1.3)	0.120
PC1	Range	−4.7–3.6	−3.4–2.7	−1.9–2.4	−4.7–3.6	
Diet	Mean (SD)	−0.1 (1.5)	−0.1 (1.4)	0.4 (0.9)	−0.0 (1.4)	0.134
PC2	Range	−5.4–1.9	−3.4–2.1	−1.8–2.1	−5.4–2.1	
Diet	Mean (SD)	−0.2 (1.6)	0.3 (0.7)	0.0 (0.6)	0.0 (1.2)	0.088
PC3	Range	−7.1–2.4	−1.8–2.1	−2.1–1.6	−7.1–2.4	

(multiple types of carbohydrates, fats and proteins) and the Australian Recommended Food Score (ARFS) which measures diet quality. We performed Principal Component Analysis (PCA) on the centre log-ratio (clr) transformed percentage energy intake data (details of how this was obtained are provided in Yap et al., 2021). We then used the top 3 principal components (PCs, explaining 43% of the total variance) as measures of diet, where each PC could be explained as:

- **PC1:** high in vegetables, fruit, and alternative proteins and low in sweet drinks, snacks, and confectionary.
- **PC2**: high in dairy, low in grains and takeaway
- **PC3**: high in meat and low in grains and dairy

2.3.2. Bristol Stool Chart

Stool type was recorded by the children or their parents at the time of defecation using the Bristol Stool Chart (Lewis and Heaton, 1997) (BSC). The BSC is a measure of stool consistency from 1 to 7, where lower values are indicative of dryer stool (1, constipation), middle values are indicative of healthy stool (3–4), and higher values are indicative of softer stool (7, diarrhea). To normalise the distribution of the data, we collapsed types 4 and 5 into one variable for some analyses (1–6; for variance component analyses and diversity measures).

2.3.3. Medication data

We extracted data for medications (antibiotics, antipsychotics, gastrointestinal [GI] medications) and complementary therapies (probiotics) taken by the children at the time of the completion of the family history questionnaire. In the questionnaire, families were asked about the medication history of the child and indicated whether the medication was currently taken by selecting "Yes", "No", or "Only when needed". We used this data for sensitivity analyses in the variance component analyses, where we excluded participants who took these medications at the time of data collection (N = 10 for antibiotics, N = 41 for probiotics, N = 5 for antipsychotics, N = 18 for GI medication) Therefore, the sample sizes for these analyses varied as follows: Full sample, (N = 174), No SIB (no sibling, N = 132), No AB (no antibiotics, N = 164), No PB (no probiotic, N = 133), No AAP (no antipsychotics; N = 169), No GI meds (no gastrointestinal medications, N = 156).

2.3.4. Age

For sensitivity analyses invesitgating whether there was an age-associated effect on gut-microbiome-maternal-exposure associations, we stratified the cohort into three age groups – Early (2–5 years; N = 64), Intermediate (6–10 years; N = 68), Older (11–17 years; N = 42). These groups correspond roughly to early childhood, late childhood/early adolescence, and adolescence, respectively, and allowed for relatively equally sized strata.

2.4. Sample preparation

2.4.1. Fecal sample collection

As described in Alvares et al. (2018) and Yap et al. (2021), teaspoon-sized stool samples were collected by parents at home either by scraping diapers or a toilet bowl liner and placing the sample in 4 mL RNAlater. Samples were subsequently sent to the Institute for Molecular Bioscience at the University of Queensland, where they were processed immediately (time from shipping to processing was between 12 and 72 h). Each sample underwent vigorous homogenization prior to being aliquoted to 3 x 1 mL tubes, which were then stored long-term in a −80 °C freezer. Samples went through only one freeze-thaw cycle (during sequencing). Stool samples were processed by the University of Queensland Human Studies Unit and were transported to the Microba Life Sciences laboratory for randomization, DNA isolation and preparation of sequencing libraries.

2.4.2. DNA extraction

DNA was extracted using the QIAamp 96 PowerFecal QIAcube HT Kit in 2 ml deep well plate format as per manufacturer's instructions with a modified mechanical lysis step on the QIAcube HT DNA extraction system (Qiagen 9001793). Prior to processing, the samples were transferred to a 96-well plate and were washed twice with ice cold PBS in accordance with the manufacturer's instructions.

2.4.3. Library preparation

Libraries were prepared as per the manufacturer's protocol using the Nextera XT Library Preparation Kit (Illumina #FC-131-1096), with a reduction of volume to allow processing in a 384-plate format. Resulting libraries were indexed with NexteraXT v2 384 Index A-D (Illumina FC-131-2001-4), quantified and assessed for appropriate quality control (QC) including fluorometric quantification and gel analysis.

2.4.4. Library pooling, QC, loading and sequencing

Illumina libraries were pooled at equimolar amounts to create a sequencing pool. The collective pool was quantified, and quality control was again performed via gel analysis and fluorometric quantification. The pooled library was prepared for sequencing on the Illumina NovaSeq6000 platform, as per the manufacturer's instructions. Sequencing was carried out using 2 x 150bp paired-end chemistry in the Microba Life Sciences laboratory to a target depth of 3 Gb per sample, minimum 2 GB, which was equivalent to approximately 7–16 million paired-end reads.

2.5. Bioinformatics and pre-processing

2.5.1. Quality control (QC) and quantification of taxa, gene, and pathway abundances

QC of metagenomic sequencing data, bioinformatic processing and quantification of microbial taxonomic and functional abundances were performed by Microba Life Sciences Limited as described in Yap et al. (2021).

Briefly, quantification of species in the metagenomic samples was performed using the Microba Community Profiler (MCP) v2.0.2 with the Microba Genome Database (MGDB) v2.0.0 as a reference (Parks et al., 2021). Reads were mapped to the database, and relative cellular abundance of species clusters with sufficient evidence to be considered reliable was estimated and reported. After the exclusion of participants with missing data (N = 25), we identified a total of 1,751 species, including 1,731 bacteria, 12 archaea, and 8 eukaryota. Quantification of genes and metabolic pathways was performed using the Microba Gene and Pathway Profiler (MGPP) 2.0.0 against the Microba Genes (MGENES) database v2.0.0, in two stages. In stage one, all open reading frames (ORFs) from all genomes in MGDB were clustered against UniRef90 (Suzek et al., 2015) release 2019/09 using 90% identity over 80% of read length with MMSeqs2 Release 10-6d92c (Steinegger and Söding, 2018). Next, gene clusters were annotated using the UniRef90 identifiers and linked to the Enzyme Commission (EC) and Transporter Classification Database (Saier et al., 2016) annotations via the UniProt ID mapping service (www.uniprot.org/uploadlists/). Enzyme Commission annotations were used to establish the encoding of MetaCyc pathways (Caspi et al., 2020) in each genome with enrichM (https://github.com/geronimp/enrichM) and pathways that were >80% complete were classified as encoded and retained for further analysis. In stage two, all DNA sequencing read pairs that aligned with one or more base to the gene sequence from any protein within a MGENES protein cluster were summed. Pathway abundances were estimated by averaging the gene counts of each pathway present within all genomes of all species reported by MCP.

2.5.2. Pre-processing and filtering

Due to the compositional (proportional) nature of metagenomics data, we normalised count tables. Count tables were first transposed (such that participant IDs were rows, metagenomic features were columns) and normalised by centre log ratio (clr) transformation (0-offset = 0.001) to be later used as the input data for the variance components analysis using the Omics Restricted Maximum Likelihood (OREML) method and the beta diversity analyses (see Fig. S1 for a flowchart of filtering and transformations). For the differential abundance analysis, additive log ratio transformation (default method) was applied on the counts within the ANCOM-BC package (0-offset = 1). For alpha diversity measures we used the non-transformed counts from the 1731 bacterial species present. For the variance component and beta diversity analyses, we removed low prevalence features (taxa with <10% non-zero counts, "rm0 filter"). For the variance analysis we split these further into common (present in >50%) and rare (present in 10–50% of the sample) features as detailed in Yap et al. (2021). For OREML, we used both taxonomic (species, family, genus) and functional (enzyme, metabolic pathway, gene) data to capture different aspects of gut microbiome composition. At the taxonomic level, we identified 91 "common" and 418 "rare" species out of 1,751 species in total, 30 common families (out of 131), and 65 common genera (out of 605). At the functional level, we used the common features classified with Enzyme Commission at level 4 (1,610 out of 2,311), at level 3 (87 out of 99), MetaCyc pathways (547 out of 2,870), TCDB transporter (212 out of 1,272), and Microba genes (234,142 out of 4,547,555). For ANCOM-BC we used the default filter setting (prv_cut = 0.1) which filters out species with prevalence of <0.1, leaving 357 bacterial species.

2.6. Statistical analyses

Except for the variance component analysis, all analyses were performed in the R statistical software (version 4.2.1).

2.6.1. Variance component analysis

We performed variance components analyses using the open-source software tool OSCA (OmicS-data-based Complex trait Analysis), as previously described in Yap et al. (Yap et al., 2021; Zhang et al., 2019). First, we constructed omics-data-based relationship matrices (ORM) between all pairs of study participants (N = 174) based on different microbiome features [common species (N = 91), rare species (N = 420), common families (N = 30), common genera (N = 75), common enzymes level 3 (N = 87), common enzymes level 4 (N = 1,610), common metabolic pathways (N = 547), common transporters (N = 212), common genes (N = 234,142)]. Next, we applied the OREML method to estimate the proportion of phenotypic variance for the primary traits (e.g., MIA and MatS) and secondary traits (e.g., age) that was associated with the focal ORM (e.g., common species) while adjusting for covariates including age, sex and dietary PCs 1–3. OREML efficiently estimates the effects of predictors on a response variable while considering the correlation structure of the predictors and potential covariates. We summarise the experimental design in Fig. 1. We used OREML to estimate the proportion of phenotypic variance associated with taxonomic and functional gut microbiome features for a total of 10 traits (MIA, MatS, ASD, age, sex, BMI, stool type, and dietary PCs 1–3). We performed an FDR-adjustment (adjusting for N = 10 traits) on the data shown in Fig. 2, and those values are reported throughout the paper.

2.6.2. Diversity measures

Alpha diversity was measured by species richness (number of species observed), Shannon Index, and Simpson Index, with and without covariates (age, sex, diet PCs 1–3) using all non-transformed bacterial species counts. After establishing that the normality assumptions for the measures were not met using Shapiro-Wilk and Levene's tests (Table S3), all alpha diversity measures were normalised using rank-based inverse normal transformation (INT). We adjusted the normalised values for covariates (age, sex, diet Pcs 1–3) using ordinary least squares regression and then performed a series of independent samples t-tests on the residuals to investigate differences between groups.

To calculate beta diversity, we generated a weighted Unifrac distance matrix (clr-transformed, with rm0 filter), which incorporates information about species relative abundance and their phylogenetic relationships. We used non-metric multidimensional scaling (NMDS) to visualize beta diversity. NMDS is a non-linear technique which preserves the relative dissimilarities between samples in a low-dimensional space and can handle the non-linear relationships between microbial community data. We used permutational multivariate ANOVA (PERMANOVA) to test for group differences between the centroids of the groups while including covariates (Anderson, 2017). We used PERMDISP2 (Anderson et al., 2006) to test for a difference in the variance within a group compared to another group, which does not allow for covariates.

2.6.3. Differential abundance

We used Analysis of Composition of Microbiome with Bias Correction (ANCOM-BC) to test for differential abundance between groups of bacterial taxa at the species, genus, class and phylum level using the default parameters (Lin and Peddada, 2020). The ANCOM-BC method models taxonomic absolute abundance using a linear regression framework while correcting for bias created by different sampling fractions across samples and controlling for multiple comparisons using false discovery rate (FDR) correction. We filtered out features present in <10% of the whole sample (rm0 filter, 357 species, 12 phyla, 64 classes, 204 genera) and adjusted for age, sex and dietary PCs 1–3 in all analyses.

3. Results

3.1. Cohort characteristics

Data were analysed for a total of 174 (111 male) participants aged between 2 and 17. Of those children, 92 were diagnosed with ASD, 42 were siblings without a diagnosis (SIB) and 40 were unrelated (UNR; see Tables 1 and S4). Within the sample, 111 children were born to mothers who did not experience MIA (noMIA) and 63 to mothers who did (MIA). Regarding maternal stress, there were 90 noMatS children and 84 MatS

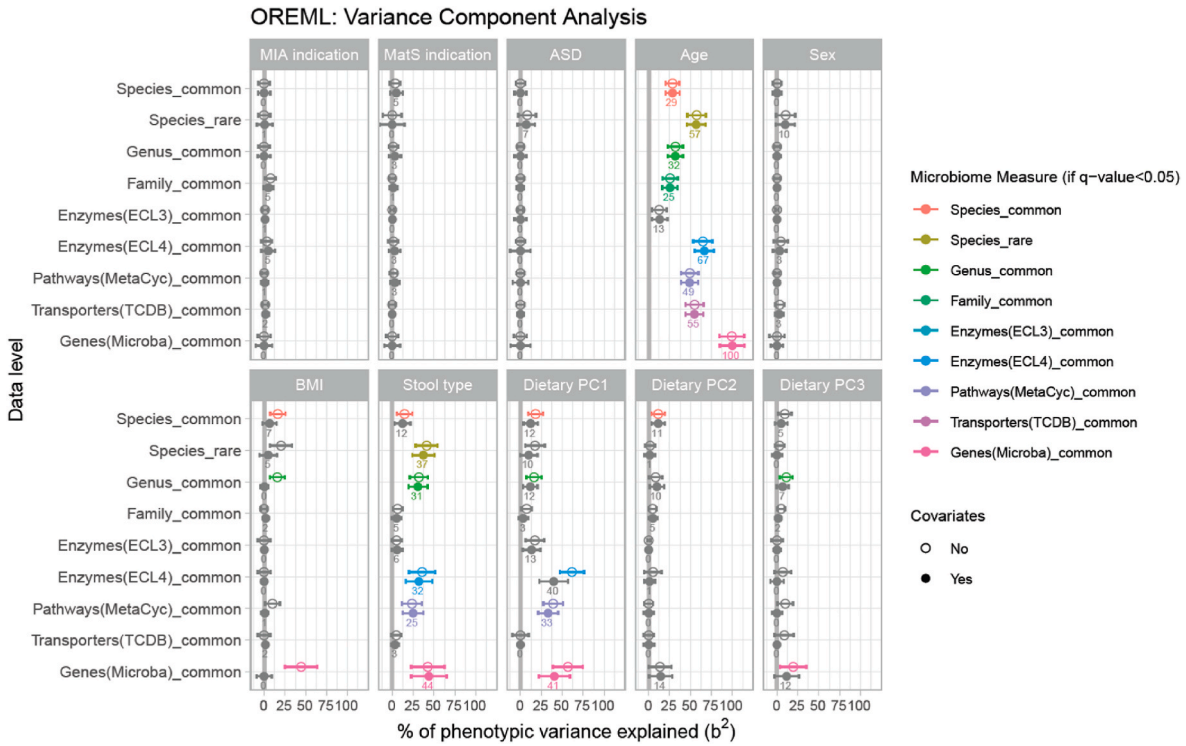

Fig. 2. Percentage of phenotypic variance associated with microbiome composition.

children. Considering both phenotypes, there were 33 children with mothers who experienced both MIA and MatS, 30 with MIA only, 51 with MatS only and 60 with mothers who experienced neither (see Table S5 for MIA and MatS participant numbers in relation to ASD diagnosis). After confirming that MIA and MatS were not associated with each other ($\chi^2(1, N = 174) = 0.7$, $p = 0.4$), we performed a logistic regression to investigate whether MIA, MatS, age and sex were associated with ASD in our sample. This analysis revealed a statistically significant relationship between ASD diagnosis and MIA (OR = 2.21, 95% CI: 1.16 to 4.18, p = 0.015), age (OR = 1.11, 95% CI: 1.02 to 1.2, p = 0.019) and sex (OR = 2.46, 95% CI: 1.27 to 4.77, p = 0.008), but not MatS (OR = 1.07, 95% CI: 0.56 to 2.029, p = 0.84).

3.2. Variance components analyses

We used OREML to estimate the proportion of phenotypic variance associated with the gut microbiome for a total of 10 traits. We generated omics-data-based relationship matrices (ORMs) based on taxonomic (species, genus, family) and functional (enzyme, metabolic pathway, genes) microbiome features, and performed analyses with and without covariates, including age, sex and dietary PCs 1–3 (see Methods). For MIA and MatS there was no evidence for association with any of the microbiome features, including in analyses adjusted for covariates (b^2 = 0%–5%, SE = 2%–15%, adjusted p > 0.05; Fig. 2, Table S6.1). We also observed consistent results in sensitivity analyses in which ASD and stool type were included as additional covariates (b^2 = 0%–9%, SE = 2%–16%, p > 0.05; Table S6.2), as expected given that a larger study encompassing this cohort found no association between gut microbiome measures and ASD (Yap et al., 2021). Moreover, after multiple testing correction, we observed no microbiome associations with either phenotype (adjusted p-values>0.05) in sensitivity analyses where we excluded participants: i) who were siblings (N = 42), ii) taking antibiotics at the time of data collection (N = 10), iii) taking probiotics (N = 41), iv) taking antipsychotics (N = 5), and v) taking gastrointestinal medications (N = 18; Fig. S2, Table S6.2). This was also the case for both MIA and MatS when we stratified the cohort into early, intermediate and older age groups (FDR-adjusted p > 0.05; see Fig. S2, Table S6.2). We also restricted the sample to include individuals who experienced no exposure ("true negative control", N = 60) and participants with exposure to either only MIA (to investigate MIA, N = 30) or MatS (to investigate MatS, N = 51). The results of these analyses were consistent with the original MIA and MatS analyses whereby there was no association with either phenotype. Finally, considering that most MIA models in rodents utilise acute inflammation, we investigated the MIA phenotype when restricting the sample only to individuals who experienced acute MIA (N = 23) and the true negative control (N = 60), which also provided null results (FDR-adjusted p > 0.05; Fig. S2; Table S6.2).

We also observed no microbiome associations in a secondary analysis using maternal adverse event (ME) as a phenotype (p > 0.05; Table S6.2). In secondary analyses where we investigated some of the covariates as the phenotypes of interest, we found strong and significant microbiome associations with age (most features, except EC level 3; b^2 = 25%–100%, SE = 8%–15%, FDR-adjusted p < 0.001; covariates: sex), stool type (multiple features including common genera, EC level 4, MetaCyc pathway, genes, and rare species; b^2 = 25%–44%, SE = 11%–22%, FDR-adjusted p-values<0.05; covariates: age, sex, dietary PCs) and dietary PC1 (metaCyc pathways, b^2 = 33%, SE = 12%, FDR-adjusted p = 0.023; and common genes, b^2 = 41%, SE = 19%, FDR-adjusted p = 0.015; covariates: age, sex). In contrast, after covariate and multiple testing adjustment, we found no significant associations for ASD, sex, BMI and dietary PCs 2 and 3 (b^2 = 0%–14%, SE = 0%–15%, FDR-adjusted p-values>0.05; Fig. 2).

On the x-axis is the percentage of phenotypic variance (b^2) associated with the relevant microbiome measure where error bars are standard errors (SE). On the y-axis are the microbiome datasets/features (all represented by a different colour) used to create the omics-data-based relationship matrices (ORMs). Grey circles represent analyses for which the adjusted p-value was >0.05, whereas coloured circles represent analyses for which the p-value was ≤0.05 after false discovery rate (FDR) adjustment (N = 10). Each panel represents a phenotypic trait. "Common" denotes microbiome features with median count>0, whereas "rare" denotes features with a median = 0. Unfilled points represent

analyses without covariates, filled points represent analyses with covariates. Covariates included were as follows: for MIA, MatS, ASD, BMI and Stool type (covariates: age, sex, and dietary PCs 1–3); for Sex (covariate: age), for Age (covariate: sex), for Dietary PCs 1–3 (covariates: age and sex). The number denotes the b^2 estimate for the focal trait in the analysis with covariates. Abbreviations: MIA = maternal immune activation; MatS = maternal stress; ASD = autism spectrum disorder; BMI=Body Mass Index; PC = principal component; EClevel3 = Enzyme Commission Level 3; EClevel4 = Enzyme Commission Level 4; MetaCycpathway = MetaCyc Metabolic pathway; TCDB = Transporter Classification DataBase.

3.3. Diversity analysis

We quantified alpha diversity using species richness, Shannon index and Simpson index and found no evidence for significant differences in the MIA or MatS groups (independent samples *t*-test, $p > 0.05$), irrespective of covariate adjustment (sex, age and dietary PCs 1–3; Fig. 3; Table S7). We also observed consistent results in sensitivity analyses in which ASD was included as an additional covariate (Table S7). In contrast to MIA and MatS, we found significant associations between age and all measures of alpha diversity (bacterial species richness p-value = 1.07e-6; Shannon index p-value = 9.44e-5; Simpson index, p-value = 0.03), and for dietary PC2 with richness and Shannon index (p-values = 6.00e-3). There were no significant associations for sex, dietary PCs 1 and 3 ($p > 0.05$).

Weighted Unifrac was used to assess beta diversity and was analysed with PERMANOVA and PERMIDSP2. There were no significant associations for MIA using PERMANOVA (pseudo-F = 0.66, p = 0.7, covariates: age, sex and dietary PCs) or PERMDISP (pseudo-F = 0.03, p = 0.9, no covariates). We also found no associations for MatS (PERMANOVA: pseudo-F = 0.73, p = 0.6; PERMDISP: pseudo-F = 0.3, p = 0.6; see Table S8). We also performed sensitivity analyses on maternal adverse event [ME] (no, N = 60; yes, N = 114), and found no differences between groups in alpha or beta diversity (see Fig. S3 and Table S7).

Panels A) and C) show the residuals from linear regression adjusting for covariates (age, sex, dietary PCs 1–3) for bacterial species richness, Shannon index and Simpson index (y axis) for MIA and MatS (x axis), respectively. Alpha diversity analyses used independent samples t-tests to compare groups after regressing out covariates. Panels B) and D) show beta diversity analyses (Weighted Unifrac) plotted using nonmetric multidimensional scaling (NMDS). The PERMANOVA analysis included age, sex and dietary PCs 1–3 as covariates, PERMDISP2 did not include covariates. Large dots represent the centroid for each group.

3.4. Differential abundance analysis

ANCOM-BC was used to test for differential abundance of 357 bacterial species in the MIA and MatS study groups. We found no significant associations with MIA or MatS after FDR correction (p-adjusted >0.05; Fig. 4; Tables S9.1 and S9.2). This was the case in analyses with and without covariates (age, sex and dietary PCs). Noting that previous studies have reported differences at the level of phyla, family and genera, we also investigated these taxonomic levels and found no significant differences between groups (Tables S10.1-10.3). From the covariates, age was significantly associated with 15 species (Tables S9.1 and S9.2).

Volcano plots for analyses without and with covariates (age, sex, dietary PCs) which show the log-fold change (LFC, x-axis) and the -log10-transformed p-value (y axis) for every bacterial species (N = 357). Dashed line represents significance threshold (p-value<0.05). The text names the bacteria with the smallest p-values. Circle colour signifies whether taxa were significantly downregulated (red), significantly upregulated (blue), or not significant (black) after FDR-adjustment. No species were significant after FDR-adjustment.

We found no significant differences in sensitivity analyses where we compared the individuals with no exposure ("true negative controls") to participants with exposure to 1) MIA only, 2) MatS only, or 3) acute MIA

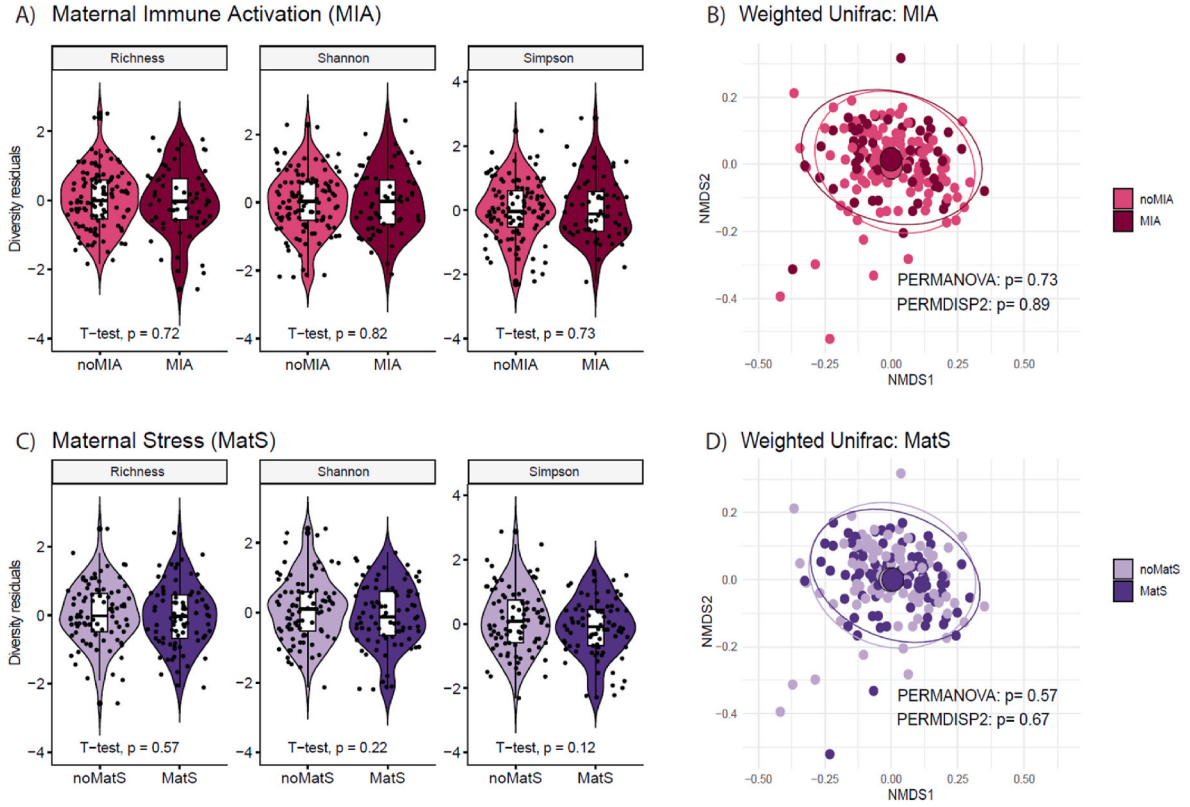

Fig. 3. Diversity analyses with covariate adjustment.

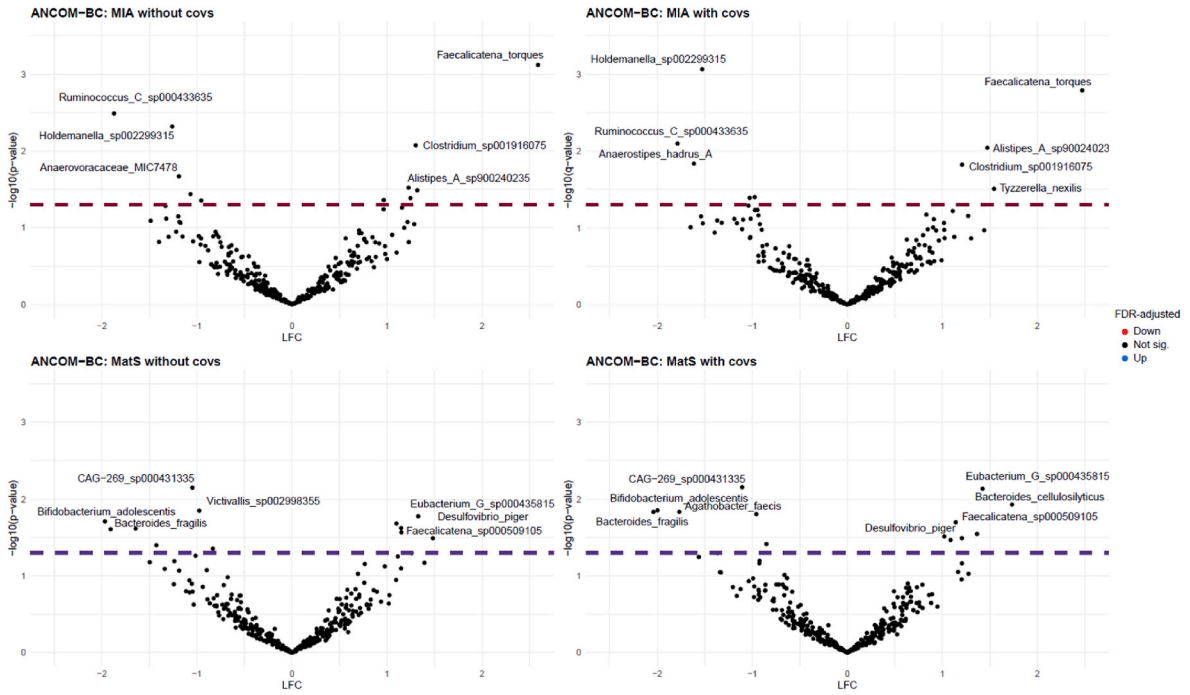

Fig. 4. Differential Abundance (ANCOM-BC) for bacterial species.

only (Tables S11.1-11.5). We also performed a series of sensitivity analyses, where we stratified the cohort in three age groups. Results from these analyses were largely consistent with the main analyses for MIA and MatS, finding no associations between MIA/MatS with the child's gut microbiome (Tables S12.1-12.5). However, in analyses investigating MIA in the older group (11–17) where we adjusted for sex, age, and diet, we found that two bacterial species, *Faecalibacterium prausnitzii E* (LFC = −4.53, q-value = 0.019) and *UBA11452 sp003526375* (LFC = −3.35, q-value = 0.014), were significantly decreased in the MIA group (Table S12.6).

4. Discussion

In this retrospective, cross-sectional shotgun gut metagenomics study, we investigated if two common prenatal maternal adverse events, maternal immune activation (MIA) and maternal stress (MatS), were associated with long-term changes in offspring gut microbiome composition. In line with large epidemiological studies (Brown et al., 2014; Jiang et al., 2016), we found that children born to mothers with MIA were over two-times more likely to be diagnosed with ASD. However, we found no association between ASD and MatS, and importantly, no associations between MIA or MatS and the child's gut microbiome through variance components, diversity, and differential abundance analyses. This was the case for both the taxonomic and functional microbiome measures studied. In the absence of a direct association between the child's microbiome and MIA or MatS, it is highly unlikely that the microbiome mediates the relationship between MIA or MatS and ASD diagnosis.

Our results are partially consistent with previous animal and human literature. The lack of alpha and beta diversity differences between groups is consistent with most human studies investigating infant microbiome diversity associated with MIA (Jackson et al., 2022) and MatS (Aatsinki et al., 2020; Zijlmans et al., 2015). Two previous studies investigating MatS found differences in alpha diversity. However, their findings were conflicting as one found it to decrease with stress (Galley et al., 2023), and another found it to increase (Weiss and Hamidi, 2023). We also found no evidence for differential abundance at the phyla, family, genus, or species level. Whereas former animal (Golubeva et al., 2015; Gur et al., 2017; Hsiao et al., 2013) and human studies (Aatsinki et al., 2020; Galley et al., 2023; Jackson et al., 2022; Weiss and Hamidi, 2023; Zijlmans et al., 2015) have reported differences, the identified taxa are highly inconsistent. Even in the laboratory context, where environmental conditions such as diet, day-night cycles, and social interactions are highly controlled, differences have been inconsistent in terms of both specific taxa and direction. For example, whereas Gur et al. (2017) found MatS-related differences for the families Muribaculaceae, Rikenellaceae, and Bifidobacteraceae in mice, Golubeva et al. (2015) found a MatS-associated decrease in the Streptococcaceae family in rats, and Jašarević et al. (2017) reported an increase in mice. This highlights pervasive replication issues in this field and raises a question about the clinical implications of these findings.

When performing sensitivity analyses for differential abundance, we found that two species – *UBA11452sp003526375* and the butyrate-producing *Faecalibacterium prausnitzii E* – were decreased in the older groups of MIA-exposed children (aged 11–17). *F. prausnitzii* is known for its anti-inflammatory properties (Qiu et al., 2013) and is consistently decreased in psychiatric populations (Nikolova et al., 2021) such as those with schizophrenia (Vasileva et al., 2024), and immune-mediated inflammatory bowel disease including Chron's disease (Pascal et al., 2017) and ulcerative colitis (Varela et al., 2013). *F. prausnitzii* is a late gut coloniser whose abundance peaks between 8 and 17 years (Miquel et al., 2014) which may explain why this difference was observed only in the late age group. Due to the limited sample size in this cohort, we were not powered to explore whether this decrease was mediated by other factors such as ASD or stool type, so future larger studies will need to explore this further.

It is also possible that our measures for adverse maternal events were not sufficiently sensitive. Epidemiological studies investigating the associations of prenatal MatS with psychiatric and neurodevelopmental conditions suggest an effect of adverse event type, stage of pregnancy, and sex of the offspring. Specifically, *chronic* exposure to stress and prenatal stress during the *first* or *third* trimester, but not the second, are associated with an increased incidence of psychiatric and neurodevelopmental conditions (Lipner et al., 2019). Therefore, it is possible that the absence of associations for MatS with both ASD and the gut microbiome we observed is due to the low sensitivity of our measure.

In contrast to the weak associations with maternal adverse events, we found significant microbiome associations for age, stool type and dietary PCs which is consistent with findings in a larger study including this cohort (Yap et al., 2021). Considering the rapid development of the child's gut microbiome in the first years of life, it is possible that these, and other, environmental factors may dominate, thereby overwhelming any effects of the prenatal environment on the gut microbiome. For instance, a few studies have now provided evidence that the method of birth delivery affects the infant's gut microbiome in the early stages of life, however these effects are limited beyond infancy (Bäckhed et al., 2015; Shao et al., 2019). Furthermore, one study which investigated the effect of human immunodeficiency virus (HIV) exposure on the infant's gut microbiome over the first 62 weeks of life (Jackson et al., 2022), found that whereas there were 12 genera, 5 families, and 1 phylum differentially abundant at week 6, there were only 2 genera and 2 families by week 62, suggesting that gut microbiome changes in infants exposed to infection during pregnancy are restored over time. A longitudinal study in mice also found multiple differentially abundant families associated with MIA exposure at postnatal day 30, but no differences in taxa, or microbiome diversity, at postnatal day 180 (Juckel et al., 2021). Therefore, it is possible that lifestyle factors which have well-known associations with the gut microbiome such as diet (David et al., 2014), physical exercise (Quiroga et al., 2020) and sleep (Smith et al., 2019) might overshadow microbiome alterations which otherwise are present earlier in life. This is in line with two other studies coming from our group, suggesting that diet and medication are mediators of gut microbiome changes in autism (Yap et al., 2021) and schizophrenia respectively (Vasileva et al., under review).

Notwithstanding our findings that neither MIA nor MatS were associated with long-term gut microbiome changes in children, several limitations exist in the present study. Firstly, this was a retrospective study which collected data from self-report questionnaires distributed at the time of collection of the child's faecal sample. As such, we relied on the memory of the parents about adverse events that occurred during pregnancy and thus our MIA and MatS measures might be imprecise; for example, the relationship between reported MIA and ASD diagnosis may be explained by recall bias. Second, as this dataset came from the Australian Autism Biobank, this sample had a high proportion of children diagnosed with ASD, potentially limiting the generalizability of these findings to the wider population. Third, the absence of an association between MatS and ASD raises questions regarding the construct validity of our MatS measure. Fourth, due to sample size restrictions and missing data, we were not able to perform analyses on stage of pregnancy or the specific types of MIA/MatS event. Finally, we did not have access to the maternal or neonatal microbiome of the child which would have allowed us to assess the change of the child's microbiome over time. To address these limitations, prospective studies which follow the mother and child pre- and post-birth are needed. These studies should collect biological samples and data on inflammatory markers and hormones, along with questionnaires about stress and inflammation, which would enable more rigorous measurement of MIA and MatS phenotypes. Additionally, the collection of maternal prenatal microbiome and child microbiome samples across infancy and childhood would enable investigation of how the infant microbiome changes in response to postnatal environmental factors and its relationship to the maternal microbiome.

Despite pre-clinical and clinical studies suggesting that the gut microbiome acts as a mediator between psychiatric symptoms and risk factors such as MIA and MatS, the results reported in the literature vary widely. Researchers and clinicians should be cautious about translating findings from animal research to human populations, due to the large contrast between the highly controlled environments (in terms of diet, sleep cycles and social environment) in animal studies, the variability encountered in human lifestyles, and the distinction between behavioural measures in animals and psychiatric phenomena in humans.

Data and code availability

The microbiome and demographic data analysed in this article is available by application to the Autism CRC: https://www.autismcrc.com.au/biobank. The code used to process and analyse the data will be made available upon publication through UQ eSpace.

CRediT authorship contribution statement

Svetlina Vasileva: Data curation, Formal analysis, Writing – original draft. **Chloe X. Yap:** Data curation, Formal analysis. **Andrew J.O. Whitehouse:** Writing – review & editing. **Jacob Gratten:** Data curation, Formal analysis, Methodology, Supervision, Writing – original draft. **Darryl Eyles:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data is available upon request to the Autism CRC. The codes will be uploaded on UQ eSpace/GitHub upon publication.

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Appendix A. Supplementary data

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