

Prenatal PFAS exposure, gut microbiota dysbiosis, and neurobehavioral development in childhood

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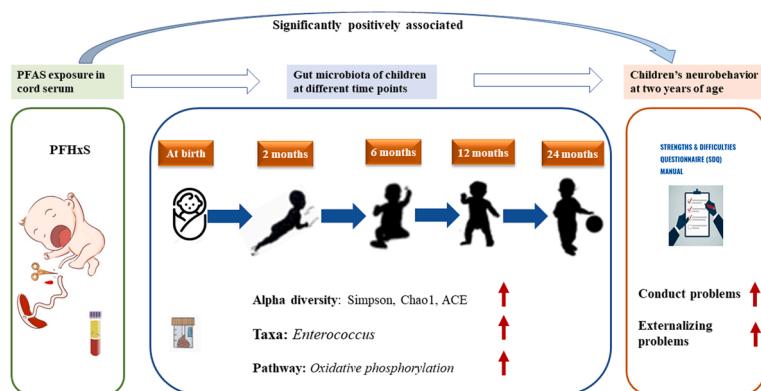
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HIGHLIGHTS

- PFHxS was associated with increased scores for conduct and externalizing problems.
- PFHxS was substantially associated with increased alpha diversity of children.
- PFHxS can affect *Enterococcus spp.* and Oxidative phosphorylation pathway abundance.
- Alpha diversity or Oxidative phosphorylation pathway was related to neurobehavior.
- Alpha diversity in neonates played a mediating role in PFAS neurotoxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

Studies on the role of the gut microbiota in the associations between per- and polyfluoroalkyl substance (PFAS) exposure and adverse neurodevelopment are limited. Umbilical cord serum and faeces samples were collected from children, and the Strengths and Difficulties Questionnaire (SDQ) was conducted. Generalized linear models, linear mixed-effects models, multivariate analysis by linear models and microbiome regression-based kernel association tests were used to evaluate the associations among PFAS exposure, the gut microbiota, and

Abbreviations: BMI, body mass index; CI, confidence interval; FDR, False Discovery Rate; LOD, limits of detection; KEGG, Kyoto Encyclopedia of Genes and Genome; OTU, operational taxonomic unit; PFAS, per- and poly-fluoroalkyl substances; PFOA, Pentadecafluorooctanoic acid; PFOS, Perfluorooctane sulphonate; PFDA, Perfluorodecanoic acid; PFUnA, Perfluoroundecanoic acid; PFDoA, Perfluorododecanoic acid; PFTra, Perfluorotridecanoic acid; PFHpA, Perfluoroheptanoic acid; PFOSA, Perfluorooctane sulfonamide; PFNA, Perfluorononanoic acid; PFHxS, Perfluorohexanesulfonic acid; KFBS, Potassium nonafluoro-1-butanesulfonate; PFBA, Perfluorobutanoic acid; PFHxA, Perfluorohexanoic acid; PFPeA, Perfluoropentanoic acid; 6:2 Cl-PFESA, 6:2 chlorinated polyfluoroctane ether sulfonate; 8:2 Cl-PFESA, 8:2 chlorinated polyfluoroctane ether sulfonate; SDQ, Strengths and Difficulties Questionnaire.

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neurobehavioural development. Perfluorohexane sulfonic acid (PFHxS) exposure was associated with increased scores for conduct problems and externalizing problems, as well as altered gut microbiota alpha and beta diversity. PFHxS concentrations were associated with higher relative abundances of *Enterococcus* spp. but lower relative abundances of several short-chain fatty acid-producing genera (e.g., *Ruminococcus gauvreauii group* spp.). PFHxS exposure was also associated with increased oxidative phosphorylation. Alpha and beta diversity were found significantly associated with conduct problems and externalizing problems. *Ruminococcus gauvreauii group* spp. abundance was positively correlated with prosocial behavior scores. Increased alpha diversity played a mediating role in the associations of PFHxS exposure with conduct problems. Our results suggest that the gut microbiota might play an important role in PFAS neurotoxicity, which may have implications for PFAS control.

1. Introduction

Due to the carbon-fluorine bond, which is the shortest and strongest single covalent bond, per- and polyfluoroalkyl substances (PFAS) are chemically and thermally stable. As a result, PFAS are extensively used in industrial manufacturing and daily life [79]. Legacy PFAS, such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), are globally regulated because of their potential health hazards [11]. In recent years, alternative compounds, such as 6:2 chlorinated poly-fluoroethersulfonic acid (6:2 Cl-PFESA) and perfluorohexanesulfonic acid (PFHxS), have replaced their predecessors; however, they are ubiquitously found in the environment as well [35,64]. As a class of persistent organic pollutants, the extreme persistence and bio-accumulative properties of different PFAS have still raised great concern regarding their prevalence [42].

Environmental exposure sources of PFAS include food, drinking water, household dust, and air [11]. The presence of PFAS in the placenta [44] and brain [83] indicates their ability to cross the placenta and blood-brain barrier. PFAS neurotoxicity is therefore becoming an increasing research topic of interest. For example, prenatal PFOA exposure was negatively associated with externalizing behavior in boys, according to the Linking Maternal Nutrition to Child Health cohort [57]. Exposure to PFOA and PFOS in early life was associated with a considerable increase in the likelihood of neurobehavioral developmental abnormalities, such as executive function abnormalities [72] and attention deficit hyperactivity disorder, in children [13]. Prenatal PFHxS exposure was associated with increased problems in both externalizing and internalizing behaviors in 5 to 8-year-old children [71], but no associations were noted for PFOA exposure [73]. The methods of measurements of exposures or outcomes and children's ages might contribute to these inconsistencies. Moreover, the majority of these studies did not examine potential mechanisms.

Recently, the impact of environmental contaminants on the gut microbiota has attracted much attention. For example, toxicological studies found that exposure to novel PFAS Cl-PFESAs could cause gut microbiota dysbiosis in zebrafish [27,78]. Both subacute and subchronic PFOA exposure caused changes in the abundances of probiotics and inflammatory and oxidative stress-related genera in mice (e.g., *Dehalobacterium* and *Bacteroides*) [77]. Additionally, exposure to legacy (PFOA and PFOS) and novel PFAS (6:2Cl-PFESA) could induce gut microbiota dysbiosis in frogs [38]. A recent epidemiological study also found a positive association of prenatal PFAS exposure with the alpha diversity of the infant gut microbiota [49]. Prenatal exposure to PFAS, especially PFOS, was associated with altered composition of the adult gut microbiota [33]. Both prenatal and postnatal factors, such as environmental factors, as well as diet and lifestyle factors, can help shape the variable gut microbiota in early life [16]. Therefore, a one-time point investigation of gut microbiota may not be able to reveal dynamic gut microbiota homeostasis [17]. Accumulating data suggest that host brain development is often influenced by microbiota development in early life, which is communicated through the microbiota-gut-brain axis [24, 65,85]. However, initial acquisition and subsequent microbiota development are rarely discussed in the effect of PFAS exposure on neurobehavioral development.

Herein, a panel study was designed to assess the effect of PFAS exposure on neurobehavioral development in childhood and to explore whether the gut microbiota could contribute to the above associations. PFAS concentrations in umbilical cord serum and neurobehavioral development in childhood were assessed. Longitudinal gut microbiota homeostasis was measured at multiple time points to capture initial acquisition and development. We hope that the population-based findings will provide more comprehensive insights into the effects of prenatal PFAS exposure on neurobehavioral development and the underlying role of the gut microbiota.

2. Methods

2.1. Study design and population

In total, 800 mother-child pairs were randomly selected from the Shanghai Maternal-Child Pairs (MCPC) study. Women aged over 20 years without serious chronic disorders such as hypertension, diabetes, and heart disease, and those who had resided in Shanghai for over a year and planned to give birth there were eligible. Among the 800 mother-child pairs, participants without umbilical cord serum samples ($n = 7$) and those with twin pregnancies ($n = 4$) were excluded. In the remaining 789 mother-child pairs, child stool samples were collected from birth to 24 months of age. The population that provided stool samples at least once was retained. Specifically, a total of 417, 156, 551, 489, and 320 faecal samples were collected at birth and at 2 months, 6 months, 12 months, and 24 months of age, respectively. A total of 462 children completed a neurobehavioral assessment at 24 months of age. Details regarding recruitment can be seen in Fig. 1.

During the enrolment process, each participant provided written informed consent. The Institutional Review Board at Fudan University approved the study (IRB[#]2016-04-0587).

2.2. Exposure assessments

Samples of umbilical cord serum were collected following centrifugation at delivery and stored at -80°C at Fudan University before assessment. An Agilent 1290–6490 triple quadrupole mass spectrometer was used to determine serum PFAS concentrations (HPLC-QQQ-MS). The methods have been previously described in detail [36] and can be found in the supplementary material (Methods of PFAS measurements and Table S1). Among the 16 PFAS monitored simultaneously, 9 were legacy long-chain PFAS, 5 were short-chain PFAS, and 2 were novel alternative PFAS. The limits of detection (LODs) for the PFAS ranged from 0.004 to 0.16 $\mu\text{g/L}$ (Table S1). PFAS concentrations below the LODs were replaced with values calculated as follows: LOD/2.

2.3. Behavioral assessments

The Strengths and Difficulties Questionnaire (SDQ) was used to assess children's behavior at 24 months of age [18,20]. Generally, the SDQ is a 25-item behavioral screening questionnaire and is frequently used in research settings. The SDQ has five scales (emotional problems, conduct problems, hyperactivity, peer relationship problems, and

prosocial behavior). Each scale has five items, with 0 indicating "not true", 1 indicating "partly true", and 2 indicating "very true". A higher prosocial score indicates preferable prosocial behavior, while higher emotional, conduct, hyperactivity, and peer relationship problem scores indicate increasing behavioral difficulties [21]. The scores of the emotional difficulty, conduct difficulty, hyperactivity difficulty, and peer relationship difficulty subscales were summed to determine the difficulty score. The internalizing difficulty score was calculated by adding the scores of the emotional and peer relationship difficulty subscales, while the externalizing difficulty score was calculated by summing the conduct and hyperactivity difficulty subscale scores [19].

2.4. Faecal microbiota sequencing

First-pass samples at birth, as well as faecal samples at 2 months, 6 months, 12 months, and 24 months of age (abbreviated as 0 d, 2 M, 6 M, 12 M, and 24 M, respectively), were obtained by the research staff. DNA extraction, as well as 16 S ribosomal RNA gene sequencing, were performed as described in our previous study [89]. In brief, 200 mg of faecal samples was used for DNA extraction with a FastDNA Spin Kit for Faeces (MP Biomedicals, California, USA). Jingnan University amplified the hypervariable region of the 16 S rRNA gene V3- V4. Extraction from a 2.0% agarose gel was followed by purification and quantification of polymerase chain reaction (PCR) products with the TIANgel Mini Purification Kit and Qubit dsDNA HS Assay Kit. An Illumina MiSeq PE300 platform was used to sequence the libraries prepared using a TruSeq DNA LT Sample Preparation Kit (Illumina, Santiago, CA, USA). Afterwards, raw sequences were quality checked, demultiplexed, read filtered, and denoised, paired reads were merged, and chimeras were checked using DADA2. After clustering the operational taxonomic units (OTUs), a taxonomy class was assigned using the SILVA databases from version 132.99 and the "assignTaxonomy" function in DADA2.

R ("vegan" package) was used to estimate alpha diversity indices at the OTU level. By calculating the Chao1 and ACE estimators, we examined microbial richness, a measure of the number of taxa in each sample. In addition, Shannon and Simpson diversity indices were used to assess evenness, a measure of the number of different taxa in each sample. We also summarized the relative abundance of each OTU from the phylum level to the genus level in R ("phyloseq" package). Phylogenetic Investigation of Communities by Reconstruction of Unobserved

States (PICRUSt) was applied for predicting Kyoto Encyclopedia of Genes and Genome (KEGG) pathways, based on OTUs abundances.

2.5. Covariates

During enrollment, trained nurses collected demographic information (e.g., maternal age, parental weight and height), sociodemographic information (e.g., educational attainment, family income), and lifestyle information (e.g., smoking, passive smoking, drinking, physical activity). On the basis of weight and height, the body mass index (BMI) was calculated (weight in kg divided by height in m²). Records of medical and reproductive history (e.g., gestational hypertension, gestational diabetes mellitus) and delivery details (e.g., infant sex, delivery mode) were obtained from the hospital. Questionnaires were also used to collect information regarding breastfeeding duration, the time of supplementary food introduction, and antibiotic usage in children.

2.6. Statistical analysis

Data are presented as the mean and standard deviation (SD) for continuous variables and as distributions (%) for categorical variables. This analysis included PFAS with detection rates exceeding 60%. The undetected PFAS concentrations were ln-transformed to ameliorate the skewed distribution.

First, a generalized linear model (GLM) was applied to evaluate the effects of exposure to different PFAS on infant neurobehavior. A linear mixed-effect model was then applied to evaluate the associations of various kinds of PFAS exposure with alpha diversity. A GLM was also used to assess the cross-sectional relationships between PFAS exposure or neurobehavior and alpha diversity at birth, 2, 6, 12, and 24 months of age. The Microbiome regression-based kernel association test (MiRKAT) was applied to assess the associations between PFAS exposure or neurobehavioral development and overall microbial composition (β diversity) at different times [87]. In brief, this method can test the association between a microbial community and a continuous/binary phenotype via a kernel function. Bray-Curtis, weighted UniFrac, and unweighted UniFrac distance matrices were used in MiRKAT. The omnibus test was conducted to simultaneously consider the three distance matrices.

Afterwards, the associations of exposure to different PFAS with the

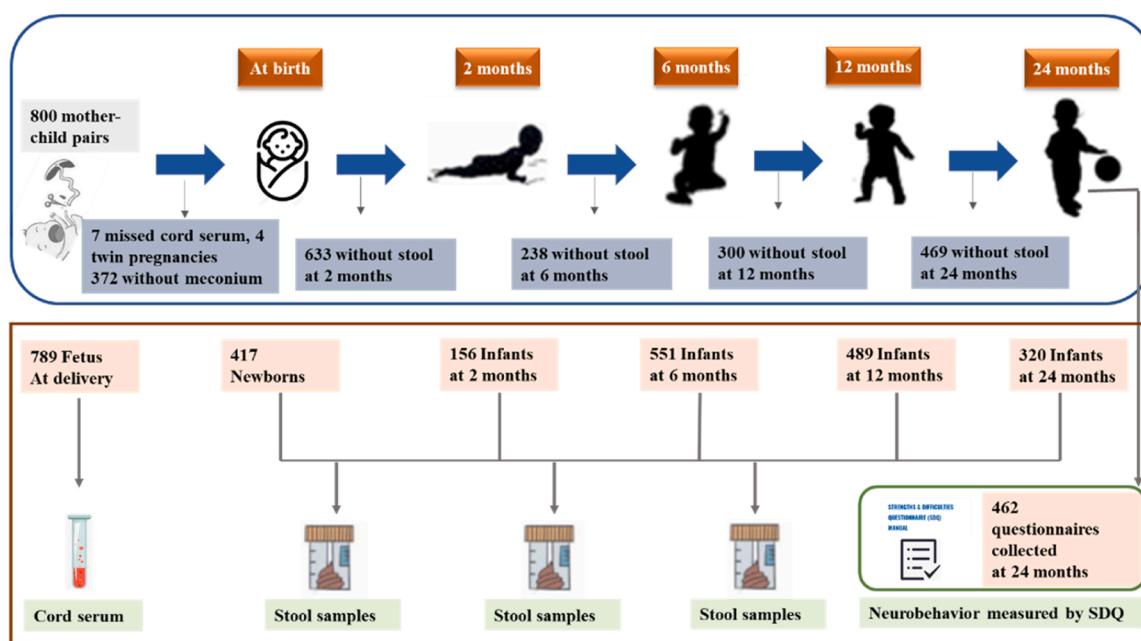


Fig. 1. Analysis design. Conceptual diagram illustrating the analysis design and collection of biospecimens questionnaires.

longitudinal relative abundances of OTUs or KEGG pathways were evaluated using multivariate analysis by linear models (R “*Maaslin2*” package). This method can maintain good statistical efficiency throughout the analysis process, with a nested GLM used to investigate the relationship between single microbial measurements and complex metadata (such as the characteristics of health outcomes, diet, and environmental factors) [46]. The main models were fitted by introducing the random-intercept for each participant as a confounder to account for within-individual correlations.

Finally, mediation analysis was performed to investigate potential associations that may underlie the relationship between the exposure variables (PFAS) and outcome variables (neurobehavior) by examining how they related to the third variable, the mediating variable (alpha diversity metrics) [70]. Based on the two linear models, the mediation was calculated as follows:

$$Mi = \beta_0 + \alpha X_i + \varepsilon_i$$

$$Y_i = \beta'_0 + \lambda Mi + \theta X_i + \eta_i$$

Here, M denotes the mediating variable (alpha diversity in the present study), X is the PFAS exposure and Y is the neurobehavior score. β_0 and β'_0 are the intercepts for M and Y. ε_i and η_i are residuals for M and Y, respectively. The proportion mediated by alpha diversity was calculated as the total effect divided by the indirect effect. The mediation analysis was conducted using R software (version: 4.1.0, “*mediation package*”).

Based on previous studies, covariates were selected [3,88]. Covariates adjusted for in the models included maternal age, prepregnancy BMI, family income, maternal educational attainment, parity, mode of delivery, infant sex, gestational age, breastfeeding status, breastfeeding duration, time of supplementary food introduction, region, and infant age (months). The statistical test was two-sided, and the significance level was 0.05. For multiple comparison correction [4], the significance level was set at 0.05 and the q-value at 0.20.

3. Results

Table 1 presents the demographic and socioeconomic characteristics of the study population. The mean age of the mothers was 29.0 (SD: 4.32) years. Before pregnancy, the average BMI of the mothers was 21.5 (SD: 2.94) kg/m². Most (75.7%) of the mothers had a junior college degree or higher, and 71.4% of the families earned over 100,000 RMB annually. A total of 46.0% of the mothers had a cesarean delivery, and 55.9% were primiparous. In this study, 437 boys and 352 girls were enrolled. Among the children, the mean birth weight was 3340 g (SD: 424) and the gestational age was 39.3 weeks (SD: 1.26). The mean breastfeeding duration was 7.23 (SD: 5.4) months and the average time for supplementary food introduction was 7.33 (SD: 1.98) months.

The distribution of PFAS concentrations is shown in **Table 2**. The detection rates of 3 legacy long-chain PFAS (perfluoroundecanoic acid (PFUnA; C11), Perfluoroheptanoic acid (PFHpA), and perfluorooctane sulfonamide (PFOSA)) and 3 short-chain PFAS (perfluorohexanoic acid (PFHxA), potassium nonafluoro-1-butanesulfonate (KFBS), and perfluoropentanoic acid (PFPeA)) were less than 60%. In over 80% of the cord serum samples, 3 legacy long-chain PFAS (PFOS, PFOA, perfluorononanoic acid (PFNA), and perfluorododecanoic acid (PFDoA)), 1 short-chain PFAS (PFHxS), and 2 novel alternatives (8:2Cl-PFESA, and 6:2Cl-PFESA) were detected. PFOA, a legacy long-chain PFAS, had the highest median concentration of 4.16 µg/L, followed by PFOS, with a median concentration of 2.66 µg/L. The novel alternative 6:2Cl-PFESA also had a relatively high concentration (median: 1.91 µg/L). The short-chain PFAS PFHxS was observed to have a median concentration of 0.92 g/L. The concentrations of most PFAS were positively correlated (**Fig. S1**). PFOS and its novel alternative 6:2Cl-PFESA, as well as PFDA and PFNA, had the highest correlation coefficient of 0.78, followed by PFDA and PFOS (0.74).

The SDQ scores are summarized in **Table S2**. Among the 789 mother-

Table 1
Characteristics of the study population (N = 789).

Variable	Mean (SD)/ n (%)	Variable	Mean (SD)/ n (%)
Maternal characteristics		Paternal characteristics	
Age (years)	29.0 (4.32)	Age (years)	30.3 (4.72)
Prepregnancy BMI (kg/m ²)	21.5 (2.94)	BMI (kg/m ²)	23.8 (3.23)
Educational attainment		Educational attainment	
High school or less	192 (24.3%)	High school or less	192 (24.3%)
Tech school/ college	297 (37.6%)	Tech school/college	290 (36.8%)
Bachelor's or higher	300 (38.1%)	Bachelor's or higher	307 (38.9%)
Family income (RMB)		Smoking	
≤ 100,000	226 (28.6%)	Yes	363 (46.0%)
100,000-300,000	510 (64.6%)	No	426 (54.0%)
> 300,000	53 (6.8%)	Alcohol consumption	
Smoking before pregnancy		Yes	420 (53.2%)
Yes	2 (0.3%)	No	369 (46.8%)
No	787 (99.7%)	Infant characteristics	
Drinking before pregnancy		Infant sex	
Yes	14 (1.8%)	Male	437 (55.4%)
No	775 (98.2%)	Female	352 (44.6%)
Passive smoking		Birth weight (gram)	3340 (424)
Yes	129 (16.3%)	Preterm birth	
No	660 (83.7%)	Yes	31 (3.9%)
Physical activity		No	758 (96.1%)
Low	314 (39.8%)	Breastfeeding duration (months)	7.23 (5.4)
Middle	436 (55.3%)	Time of supplementary food introduction (months)	7.33 (1.98)
High	39 (4.9%)	Antibiotic usage 6 months	
Pregnancy syndrome ^a			
Yes	182 (23.1%)	Yes	158 (20.0%)
No	607 (76.9%)	No	631 (80.0%)
Gestational weight gain, kg	13.8 (5.40)	12 months	
Parity		Yes	77 (9.8%)
Primiparous	441 (55.9%)	No	712 (90.2%)
Multiparous	348 (44.1%)	24 months	
Delivery mode		Yes	257 (32.6%)
Vaginal	363 (46.0%)	No	532 (67.4%)
Caesarean section	426 (54.0%)	Region	
Gestational age (weeks)	39.3 (1.26)	Songjiang	438 (55.5%)
		Pudong	351 (44.5%)

^a Pregnancy syndromes included eclampsia, gestational diabetes mellitus, and gestational hypertension.

Table 2

Distribution of PFAS concentrations in cord serum samples (N = 789).

Exposure	Detection rate (%)	Concentration				
		10 th	25 th	median	75 th	90 th
Legacy long-chain PFAS						
PFOA	99.46	2.00	2.87	4.16	6.17	8.84
PFOS	98.78	0.99	1.58	2.66	4.35	6.29
PFDA	79.84	b	0.04	0.18	0.42	0.72
PFUnA	32.34	b	b	b	0.06	0.27
PFNA	90.80	0.03	0.14	0.29	0.52	0.84
PFTrA	62.39	b	b	0.09	0.23	0.36
PFHpA	0	b	b	b	b	b
PFOSA	29.11	b	b	b	0.12	0.26
PFDoA	88.09	b	0.22	0.48	0.80	1.22
Short-chain PFAS						
PFHxS	90.26	0.11	0.32	0.92	1.59	2.16
PFBA	57.10	b	b	0.10	0.20	0.32
PFHxA	25.23	b	b	b	0.08	0.22
PPPeA	0	b	b	b	b	b
KFBS	0	b	b	b	b	b
Novel alternative PFAS						
8:2Cl-PFESA	81.06	b	0.02	0.06	0.12	0.24
6:2Cl-PFESA	99.46	0.71	1.19	1.91	3.26	5.43

b, below the LOD

child pairs, a total of 462 completed the questionnaires, including 256 pairs with male infants and 206 pairs with female infants. The mean total difficulties score was 13.6 (SD: 4.30), and the SDQ subscale scores ranged from 2.27 to 5.56. The mean scores for internalizing and externalizing problems were 5.65 and 8.00, respectively. Among the five SDQ subscales and three SDQ composites, no significant differences were found between the sexes.

The associations between PFAS exposure and behavioral development are shown in Fig. 2 and Table S3-S4. Higher concentrations of PFHxS were associated with higher scores for conduct problems ($\beta = 0.10$, 95% CI: 0.01, 0.19) and externalizing problems ($\beta = 0.17$, 95% CI: 0.002, 0.34). In addition, a negative relationship was found between PFOS exposure and prosocial behavior scores ($\beta = -0.17$, 95% CI: -0.32, -0.02). The association of PFHxS exposure with increased conduct problem scores remained significant after FDR adjustment.

The alpha diversity of the infant gut microbiota during the first two years is shown in Fig. S2 and Table S5. The evenness (Shannon and Simpson indices) and richness indices (ACE and Chao1) of the microbiota varied slightly within the first 2 years of life. Different alpha diversity indicators increased from birth to 2 months of age, declined slightly at 6 months, and then progressively increased, reaching a peak at 2 years, with an overall upward tendency.

Fig. S3A shows the relative abundances of the infant gut microbiota at the phylum and genus levels during the first two years of life. The main microbiota at the phylum level in infants were Firmicutes, Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. The Proteobacteria phylum accounting for the highest proportion of the gut microbiota in neonates, and the proportions of Actinobacteria and Firmicutes increased later. Fig. S3B shows that *Bifidobacterium*, *Escherichia*, *Enterococcus*, and *Klebsiella* were the main microbiota at the genus level. During the neonatal period, *Escherichia* accounted for the highest proportion at the genus level. The proportion of the *Bifidobacterium* genus increased later.

Fig. 3 depicts the associations of PFAS exposure with alpha diversity in infants during the first 2 years of life. According to the mixed-effect model, PFAS exposure was associated with higher alpha diversity in infancy. More specifically, each unit (ln- μ g/L) increase in the PFNA concentration was associated with an increase of 0.03 (95% CI: 0.005, 0.05) in the Shannon index, 0.02 (95% CI: 0.003, 0.05) in the Simpson index, 0.03 (95% CI: 0.004, 0.05) in the Chao1 index, and 0.03 (95% CI: 0.004, 0.05) in the ACE index. Each unit (ln- μ g/L) increase in the PFHxS concentration was associated with an increase of 0.03 (95% CI: 0, 0.06) in the Simpson index, 0.03 (95% CI: 0, 0.06) in the Chao1 index, and 0.03 (95% CI: 0.004, 0.07) in the ACE index. In addition, PFOA was positively associated with the Shannon ($\beta = 0.02$, 95% CI: 0.001, 0.05) and Chao1 ($\beta = 0.05$, 95% CI: 0.008, 0.08) indices. Broadline associations were also found between PFTrA levels and the Shannon ($\beta = 0.02$, 95% CI: 0, 0.04) and Simpson ($\beta = 0.03$, 95% CI: 0, 0.07) indices. After FDR adjustment, the above associations remained significant (Table S6).

The results of the multivariable linear regression analysis, shown in Fig. 4, were mostly consistent with the results of the longitudinal analysis. Briefly, prenatal exposure to PFAS was significantly associated with increased alpha diversity, especially in neonates. For example, PFOA concentrations were associated with elevated Shannon ($\beta = 0.09$, 95% CI: 0.01, 0.17) and Simpson ($\beta = 0.09$, 95% CI: 0.01, 0.18) indices in neonates. PFOA was positively associated with Chao1 at birth, with a borderline level of statistical significance ($P = 0.059$). PFNA concentrations were also positively associated with the Shannon ($\beta = 0.07$, 95% CI: 0.01, 0.14) and Simpson ($\beta = 0.07$, 95% CI: 0.01, 0.14) indices at birth. PFNA concentrations were positively associated with Chao1 and ACE in neonates, although not significant. In addition, PFTrA concentrations were associated with increased Shannon ($\beta = 0.08$, 95% CI: 0.01, 0.14), Chao1 ($\beta = 0.10$, 95% CI: 0.04, 0.16), and ACE ($\beta = 0.10$, 95% CI: 0.04, 0.16) indices in neonates. We also found that PFHxS concentrations were associated with increased Chao1 ($\beta = 0.08$, 95% CI: 0.02, 0.14) and ACE ($\beta = 0.11$, 95% CI: 0.04, 0.18) indices in newborns. The associations remained significant after FDR adjustment (Table S7).

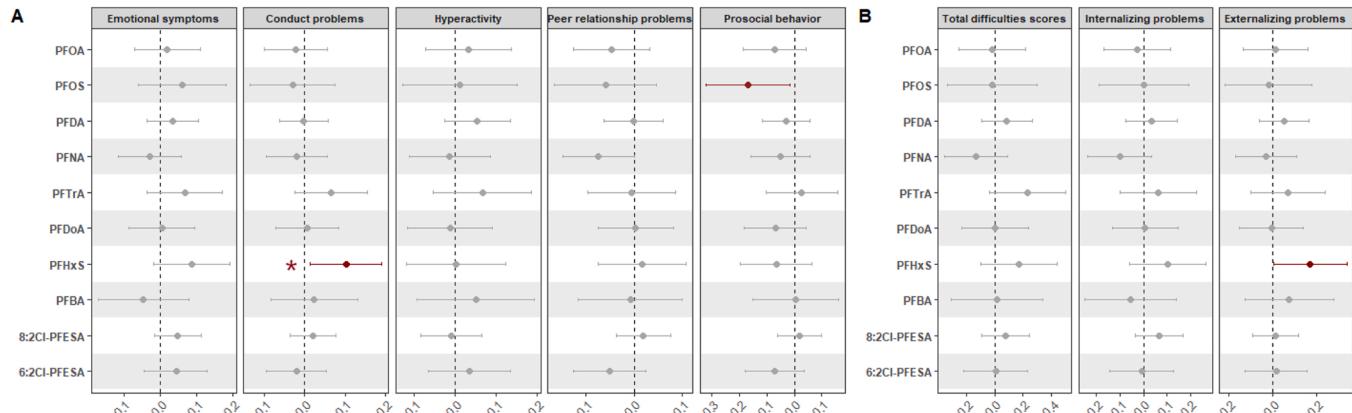


Fig. 2. Associations of PFAS exposure with behavioral development. The associations of PFAS exposure with SDQ subscale scores; B. the associations of PFAS exposure with SDQ composite scores. Red highlights denote $p < 0.05$, and * denotes a q -value < 0.25 . Models were adjusted for maternal age, prepregnancy BMI, maternal educational attainment, family income, parity, gestational age, mode of delivery, infant sex, breastfeeding duration, time of supplementary food introduction, and infant age (months).

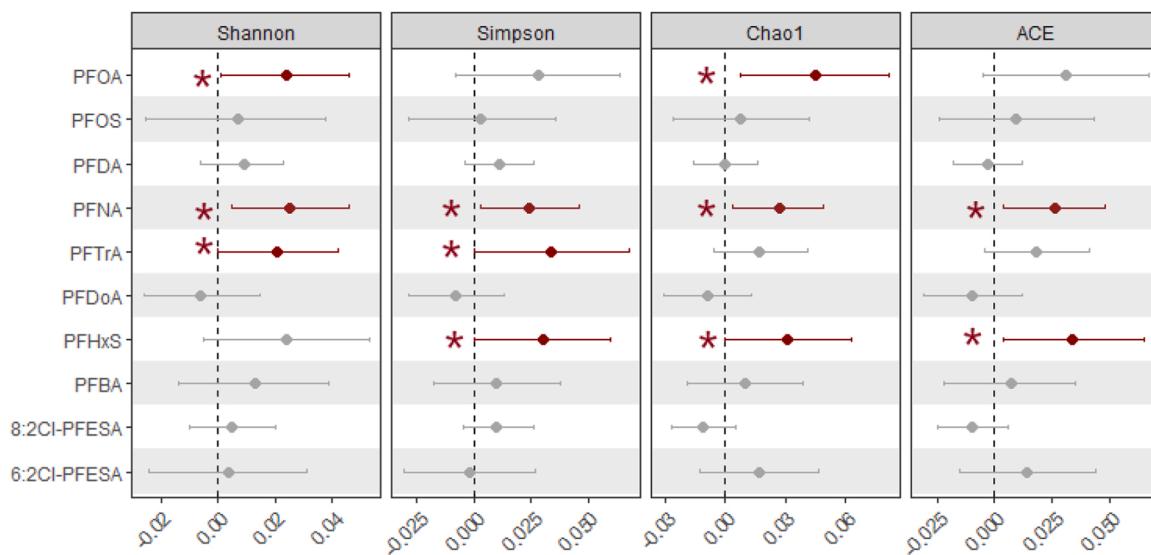


Fig. 3. Association of PFAS exposure with longitudinal alpha diversity in infant gut microbiota. Red highlights denote $p < 0.05$, and * denotes a q -value < 0.25 . Models were adjusted for maternal age, prepregnancy BMI, maternal educational attainment, family income, parity, mode of delivery, infant sex, gestational age, breastfeeding status, breastfeeding duration, time of supplementary food introduction, region, and infant age (months).

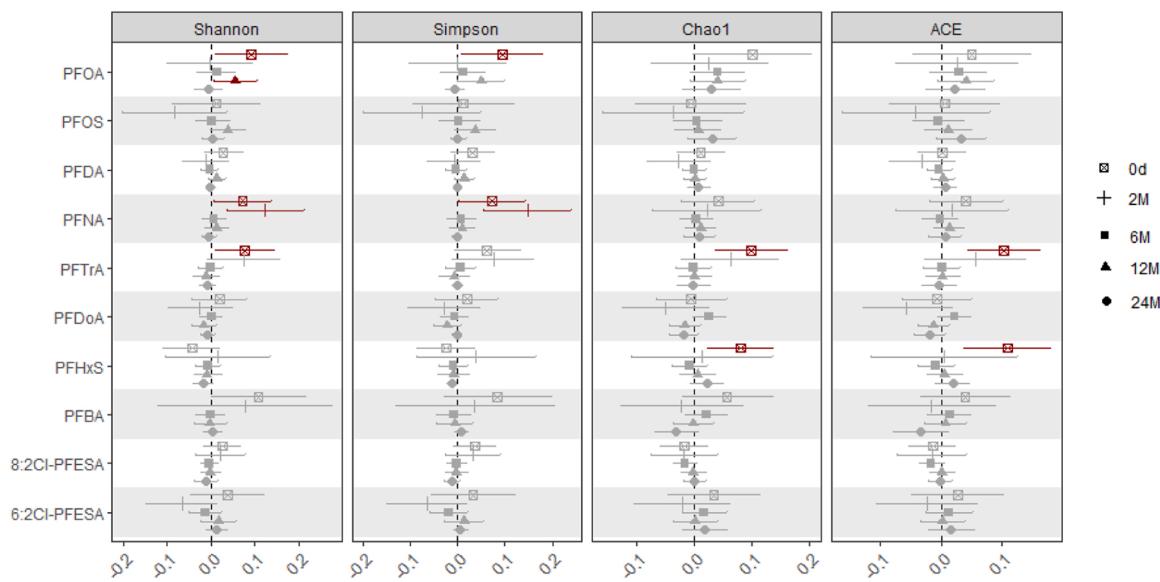


Fig. 4. Association of PFAS exposure with alpha diversity in infant gut microbiota. 0 d: 0 day (neonates); 2 M: 2 months; 6 M: 6 months; 12 M: 12 months; 24 M: 24 months. Models were adjusted for maternal age, prepregnancy BMI, maternal educational attainment, family income, parity, mode of delivery, infant sex, gestational age, breastfeeding status, breastfeeding duration, time of supplementary food introduction, region, and infant age (months).

Table 3

Association of exposure to different PFAS with the relative abundance of taxa.

Exposure	Phylum	Class	Order	Family	Genus	coefficients	P value	q-value
PFHxS	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium sensu stricto</i> 1	-0.13	< 0.001	< 0.001
PFHxS	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	<i>Ruminococcus gauvreauii</i> group	-0.11	< 0.001	< 0.001
PFHxS	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	<i>Eubacterium ventriosum</i> group	-0.07	< 0.001	0.055
PFHxS	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	<i>Dorea</i>	-0.07	< 0.001	0.055
PFHxS	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	<i>Enterococcus</i>	0.25	< 0.001	0.058
PFHxS	Actinobacteriota	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	<i>Collinsella</i>	-0.07	0.002	0.119
PFHxS	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Faecalibacterium</i>	-0.19	0.002	0.119
PFHxS	Bacteroidetes	Bacteroides	Bacteroidales	Rikenellaceae	<i>Alistipes</i>	-0.15	0.002	0.121
PFHxS	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	<i>Agathobacter</i>	-0.06	0.003	0.138
PFNA	Actinobacteriota	Coriobacteriia	Coriobacteriales	Eggerthellaceae	<i>Adlercreutzia</i>	-0.07	0.004	0.143
PFOA	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	<i>Enterococcus</i>	0.41	0.004	0.146
PFHxS	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Lachnoclostridium</i>	-0.13	0.005	0.165

The associations between PFAS exposure and overall microbial diversity are shown in the supplementary material (Tables S12-S16). According to MiRKAT, PFHxS and PFTra exposure was found to be significantly associated with microbial diversity in neonates. Significant associations were also found between PFNA exposure and β diversity in children at 24 months of age.

According to the results of MaAsLin2, PFHxS exposure was negatively associated with the relative abundances of the genera *Clostridium sensu stricto 1*, *Ruminococcus gauvreauii group*, *Eubacterium ventriosum group*, *Dorea*, *Collinsella*, *Faecalibacterium*, *Alistipes*, *Agathobacter*, and *Lachnoclostridium*. PFHxS and PFOA exposure was positively associated with the relative abundance of *Enterococcus*. In addition, PFNA exposure was negatively associated with the abundance of *Adlercreutzia*. FDR adjustment did not affect the significance of the above associations (Table 3).

In addition, prenatal PFAS exposure was found to be significantly associated with the relative abundance of several KEGG pathways. For instance, prenatal exposure to PFHxS was associated with decreased vitamin B6 metabolism, lipoic acid metabolism, and amino sugar and nucleotide sugar metabolism. In addition, PFHxS exposure was associated with increased transcription machinery, oxidative phosphorylation, secondary bile acid biosynthesis, porphyrin and chlorophyll metabolism, RNA transport, beta-lactam resistance, and bacterial chemotaxis (Table 4).

Models were adjusted for maternal age, prepregnancy BMI, family income, maternal educational attainment, parity, mode of delivery, infant sex, gestational age, breastfeeding status, breastfeeding duration, time of supplementary food introduction, region, and infant age (months).

Models were adjusted for maternal age, prepregnancy BMI, family income, maternal educational attainment, parity, mode of delivery, infant sex, gestational age, breastfeeding status, breastfeeding duration, time for supplementary food introduction, region, and infant age (months).

Fig. 5 shows the associations of alpha diversity with neurobehavior in childhood. Positive associations were found between the richness indices (Chao1 and ACE) and the scores for conduct problems ($\beta_{ACE} = 0.21$, 95% CI: 0.02, 0.39), hyperactivity ($\beta_{Chao1} = 0.32$, 95% CI: 0.07, 0.57; $\beta_{ACE} = 0.31$, 95% CI: 0.05, 0.57), total difficulties ($\beta_{Chao1} = 0.61$, 95% CI: 0.07, 1.16; $\beta_{ACE} = 0.64$, 95% CI: 0.07, 1.21) and externalizing problems ($\beta_{Chao1} = 0.50$, 95% CI: 0.15, 0.84; $\beta_{ACE} = 0.52$, 95% CI: 0.16, 0.88). After adjusting for FDR, these associations were still significant (Table S17).

The cross-sectional associations of alpha diversity with neurobehavior in childhood were shown in Fig. 6. The results further

confirmed the results of longitudinal analysis above. Positive associations were found between neonatal gut microbiota richness (Chao1 and ACE) and the scores for conduct problems ($\beta_{Chao1} = 0.38$, 95% CI: 0.17, 0.60; $\beta_{ACE} = 0.44$, 95% CI: 0.22, 0.67), hyperactivity ($\beta_{Chao1} = 0.40$, 95% CI: 0.09, 0.71; $\beta_{ACE} = 0.36$, 95% CI: 0.03, 0.68), total difficulties ($\beta_{Chao1} = 1.06$, 95% CI: 0.38, 1.74; $\beta_{ACE} = 1.13$, 95% CI: 0.41, 1.85), and externalizing problems ($\beta_{Chao1} = 0.78$, 95% CI: 0.36, 1.20; $\beta_{ACE} = 0.80$, 95% CI: 0.35, 1.24). These results did not change their significance after FDR adjustment (Table S18).

The associations of overall microbial composition with neurobehavioral development were also evaluated using MiRKAT. The results are shown in Tables S23-S27. Generally, the overall microbial composition in neonates was significantly associated with the scores for conduct problems, total difficulties and externalizing problems (Omnibus P value < 0.05).

The relative abundance of the genus *Adlercreutzia* was positively associated with the scores for peer relationship problems. The relative abundance of the genus *Ruminococcus gauvreauii group* was found to be associated with increased scores on the prosocial behavior subscale. However, the associations were not significant after FDR adjustment (Fig. 7).

Models were adjusted for maternal age, prepregnancy BMI, family income, maternal educational attainment, parity, mode of delivery, infant sex, gestational age, breastfeeding status, breastfeeding duration, time of supplementary food introduction, region, antibiotic usage, and infant age (months).

Amino sugar and nucleotide sugar metabolism was positively associated with the prosocial behavior subscale score. Oxidative phosphorylation was associated with increased scores on the conduct problems, hyperactivity subscale, and externalizing problem subscales. Secondary bile acid biosynthesis was negatively associated with the prosocial behavior subscale score but positively associated with the externalizing problem composite score. In addition, vitamin B6 metabolism was positively associated with the prosocial behavior subscale score but negatively associated with the hyperactivity and externalizing problem subscale scores. Most associations remained significant after FDR adjustment. For example, the associations of oxidative phosphorylation, secondary bile acid biosynthesis and vitamin B6 metabolism with the externalizing problem subscale scores were significant after FDR adjustment (Fig. 8).

Models were adjusted for maternal age, prepregnancy BMI, family income, maternal educational attainment, parity, mode of delivery, infant sex, gestational age, breastfeeding status, breastfeeding duration, time of supplementary food introduction, region, antibiotic usage, and infant age (months).

Table 4
Association of PFAS exposure with KEGG pathways.

Exposure	Level 1	Level 2	Pathway name	Coefficients	P value	q-value
PFHxS	Brite Hierarchies	Protein families: genetic information processing	Transcription machinery	0.054	< 0.001	< 0.001
PFHxS	Metabolism	Energy metabolism	Oxidative phosphorylation	0.033	< 0.001	0.003
PFHxS	Metabolism	Lipid metabolism	Secondary bile acid biosynthesis	0.154	< 0.001	0.006
PFHxS	Metabolism	Metabolism of cofactors and vitamins	Vitamin B6 metabolism	-0.045	0.001	0.013
PFHxS	Metabolism	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism	0.08	0.001	0.034
PFNA	Brite Hierarchies	Protein families: genetic information processing	Ubiquitin system	0.44	0.002	0.041
PFDoA	Metabolism	Biosynthesis of other secondary metabolites	Penicillin and cephalosporin biosynthesis	-0.225	0.004	0.084
PFHxS	Metabolism	Metabolism of cofactors and vitamins	Lipoic acid metabolism	-0.077	0.004	0.087
PFHxS	Genetic Information Processing	Translation	RNA transport	0.103	0.004	0.095
PFHxS	Metabolism	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism	-0.031	0.005	0.098
PFHxS	Human Diseases	Drug resistance: antimicrobial	beta Lactam resistance	0.179	0.007	0.138
PFDA	Genetic Information Processing	Replication and repair	Base excision repair	-0.02	0.009	0.175
PFHxS	Cellular Processes	Cell motility	Bacterial chemotaxis	0.213	0.011	0.204
PFOA	Organismal Systems	Immune system	Haematopoietic cell lineage	0.381	0.011	0.21

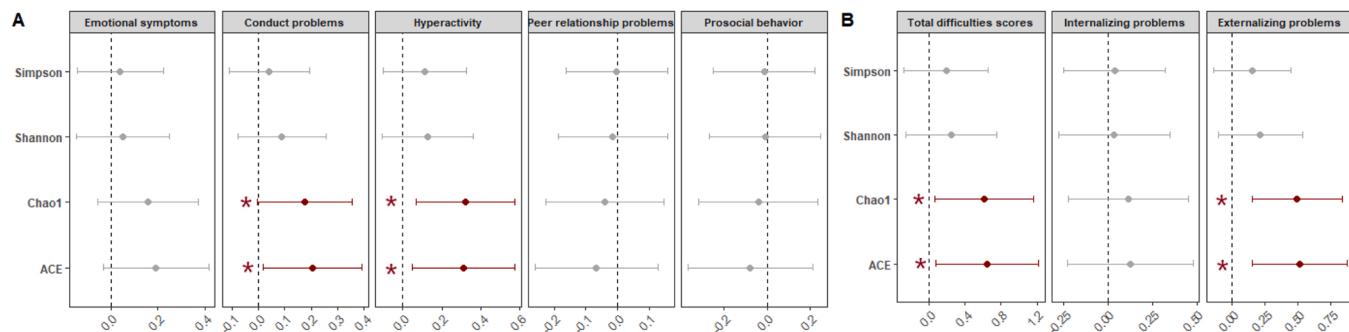


Fig. 5. Association of the alpha diversity of gut microbiota with neurobehavior in childhood. A. The association of alpha diversity with the SDQ subscale scores; B. the association of alpha diversity with the SDQ composite scores. Red highlights denote $p < 0.05$ and * denotes a q -value < 0.25 . Models were adjusted for maternal age, pre-pregnancy BMI, maternal educational attainment, family income, parity, mode of delivery, infant sex, gestational age, breastfeeding status, breastfeeding duration, time of supplementary food introduction, region, antibiotic usage, and infant age (months).

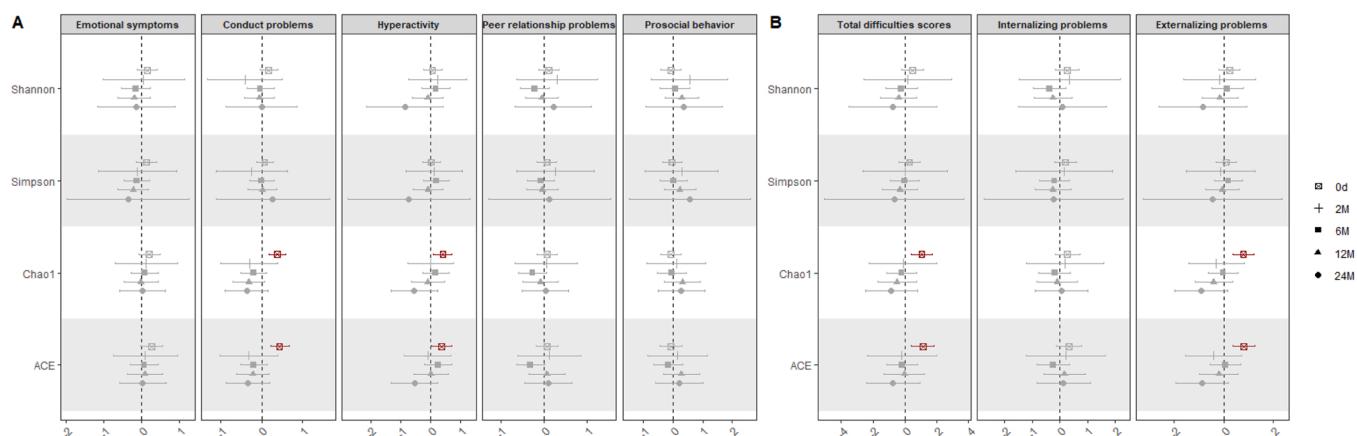


Fig. 6. Association of the alpha diversity of gut microbiota with neurobehavior in childhood. 0 d: 0 day (neonates); 2 M: 2 months; 6 M: 6 months; 12 M: 12 months; 24 M: 24 months. A. the association of alpha diversity with the SDQ subscale scores; B. the association of alpha diversity with the SDQ composite scores. Models were adjusted for maternal age, prepregnancy BMI, maternal educational attainment, family income, parity, mode of delivery, infant sex, gestational age, breastfeeding status, breastfeeding duration, time of supplementary food introduction, region, antibiotic usage, and infant age (months).

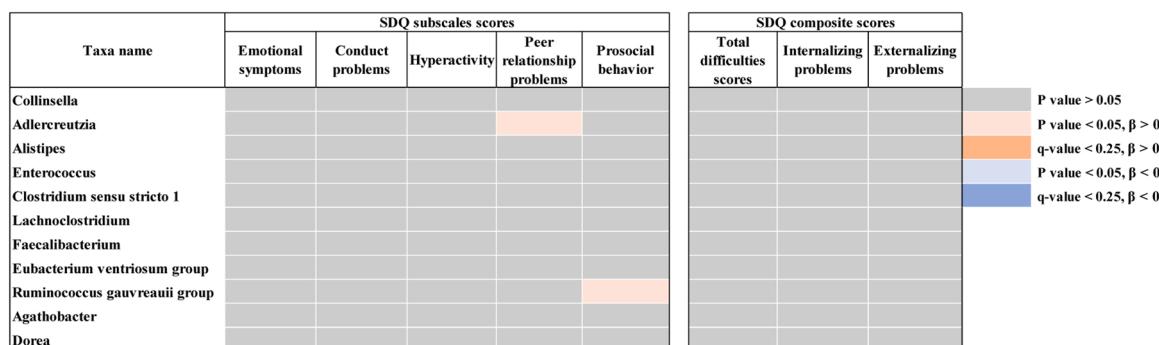


Fig. 7. Associations of taxa with neurobehavior in childhood.

The above analysis confirmed the screening for mediating variables. First, prenatal PFHxS exposure was associated with increased scores on the conduct problem subscale. Second, prenatal PFOA, PFNA, PFTra, and PFHxS exposure was found to be positively associated with the alpha diversity of the gut microbiota in children, especially in neonates. Third, we observed positive associations between alpha diversity (Chao1, ACE) and the conduct problem, hyperactivity, total difficulty, and externalizing problem subscale scores. Ultimately, the Chao1 and ACE indices were selected as the mediators underlying the association between prenatal exposure to PFHxS and scores of conduct problem score.

As shown in Fig. 9, the mediation analysis suggested that neonatal Chao1 and ACE accounted for 29.5% and 46.9% of PFHxS exposure-associated changes in the conduct problem score, respectively.

The models were adjusted for maternal age, prepregnancy BMI, family income, maternal educational attainment, parity, mode of delivery, infant sex, gestational age, breastfeeding status, breastfeeding duration, time of supplementary food introduction, region, antibiotic usage, and infant age (months).

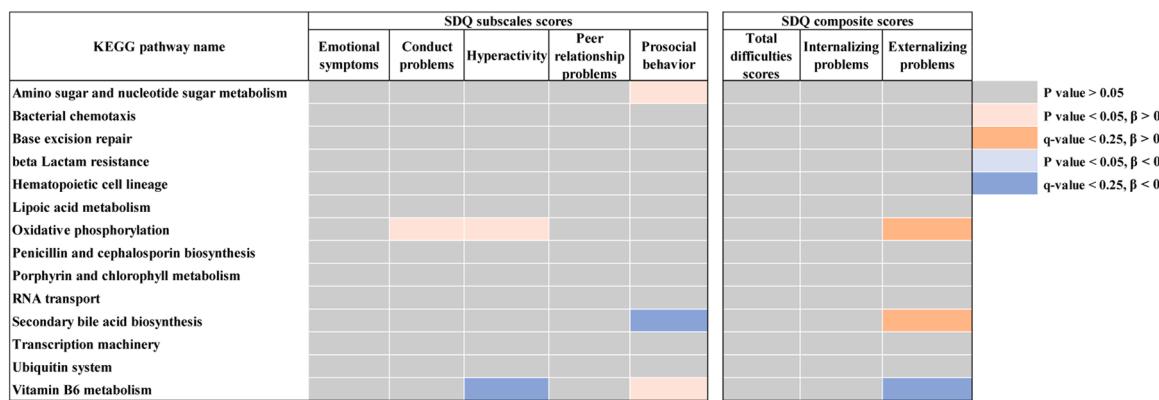


Fig. 8. Associations of KEGG pathways with neurobehavior in childhood.

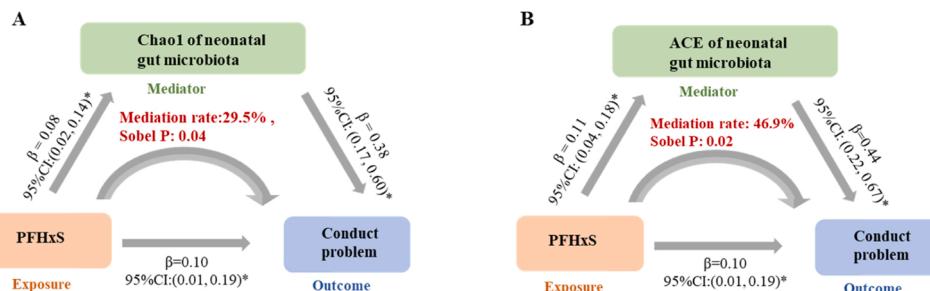


Fig. 9. Mediation analysis of richness indices on the interaction between PFAS exposure and neurobehavior.

4. Discussion

This study investigated the associations among prenatal PFAS exposure, infant gut microbiota in the first 2 years of life, and neurobehavioral development based on the Shanghai MCPC. PFHxS exposure was found to be positively associated with the conduct problem subscale score and the externalizing problem composite score. PFHxS exposure was mainly associated with increased alpha diversity during the first 2 years of life and decreased relative abundances of several taxa associated with short-chain fatty acid (SCFA) production, such as *Dorea spp.*, *Collinsella spp.*, *Alistipes spp.*, *Faecalibacterium spp.*, *Lachnoclostridium spp.*, *Ruminococcus gauvreauii group sp.*, *Eubacterium ventriosum group sp.*, and *Agathobacter spp.*. The microbial composition was also altered after PFHxS exposure. Higher concentrations of PFHxS in umbilical cord serum were also negatively associated with the abundance of *Enterococcus spp.* during the first 2 years of life. PFHxS was mainly found to be positively associated with several metabolic pathways (e.g., oxidative phosphorylation, secondary bile acid biosynthesis, and porphyrin and chlorophyll metabolism) but negatively associated with vitamin B6 metabolism, lipoic acid metabolism, and amino sugar and nucleotide sugar metabolism. Moreover, the alpha diversity (e.g., Chao1 and ACE) and microbial composition of infant gut microbiota were found to be associated with the conduct problem and hyperactivity subscale scores, as well as the externalizing problem composite score. The abundances of *Adlercreutzia spp.* and *Ruminococcus gauvreauii group sp.* were positively associated with the peer relationship problem and prosocial behavior scores, respectively. Amino sugar and nucleotide sugar metabolism and vitamin B6 metabolism were associated with increased scores on the prosocial behavior subscale, while oxidative phosphorylation was positively associated with the externalizing problem subscale score, especially the conduct problem and hyperactivity scores. Childhood alpha diversity, especially the richness indices (Chao1 and ACE) in neonates, may play a mediating role in the association of PFAS exposure with conduct problems.

The concentrations of PFAS in this study were comparable to those in other studies. PFOA, a representative legacy long-chain PFAS, had greater concentrations (4.16 µg/L) than previously reported, such as those reported by the Wuhan birth cohort study (1.65 µg/L) [40], Jiangsu Sheyang cohort study (2.21 µg/L) [22], and Maoming Birth Cohort study (1.04 µg/L) [37]. Levels of PFOA and PFOS (2.66 µg/L) were lower than those of the Health Outcomes and Measure of the Environment (HOME) study (14.3 µg/L) conducted in the USA [34]. The level of the short-chain PFAS, PFHxS (0.92 µg/L), reported in this study was also found to be lower than that reported in the HOME study [34] but higher than that reported in the Maoming Birth Cohort study (0.16 µg/L) [37]. In addition, 6:2Cl-PFESA (1.91 µg/L), an alternative for PFOS, had a higher concentration in this study than in other studies in China (0.90 µg/L [53] and 0.63 µg/L [37]).

The current study found that PFHxS exposure was related to higher conduct problem subscale scores. This finding was consistent with the results that antenatal PFHxS exposure was associated with higher SDQ, behavioral issues, and executive problem scores in children in these studies [25,26]. The HOME cohort also discovered a substantial relationship between prenatal PFHxS exposure and externalizing behavioral issues in 8-year-olds [73]. However, there is some inconsistency between the effects of PFAS and neurobehavioral development. In the Danish birth cohort, for instance, it was discovered that PFAS exposure during pregnancy, particularly PFNA exposure, was strongly linked to externalizing behavioral issues in children between the ages of 7 and 11 years old, although PFHxS exposure had no effect [45]. The Fine Cohort found no evidence of a significant connection between third-trimester PFAS exposure (PFOA, PFOS, PFHxS, PFNA, and PFDA) and neurobehavioral development in 7-year-old children, but childhood PFOA, PFNA, and PFDA exposure (5 and 7 years old) was linked to higher behavioral problem scores in 7-year-olds [52].

Microbiota colonization in early life is a continual process. Aerobic or facultative anaerobic bacteria (e.g., *Enterobacteriaceae spp.*, *Enterococcus spp.*, *Staphylococcus spp.*, and *Streptococcus spp.*) colonize firstly,

followed by strictly anaerobic bacteria such as *Bifidobacterium spp.*, *Bacteroidetes spp.*, and *Clostridium spp.* Milani et al., (\$year\$) [47,48]. The alpha diversity of the gut microbiota increased throughout early life, which was consistent with prior observations [2,59]. The intestinal flora colonize in vast quantities after birth and is influenced by dietary structure and environmental factors [54,7,9]. In this study, PFAS exposure was found to be significantly associated with altered alpha and beta diversity, especially in neonates. The effect of pollutant exposure on the alpha diversity of gut microbiota can be influenced by exposure dose, exposure time, and developmental status [1]. Animal experiments found that low-dose PFAS exposure increased the Simpson index in the gut of mice, while high-dose exposure decreased the Simpson index [75]. A recent cohort study found that levels of PFAS in the umbilical cord serum were associated with increased alpha diversity in neonatal faeces [49], which corresponded with our findings.

Furthermore, this study investigated the correlation between foetal PFAS exposure and the relative abundance of infant gut microbiota taxa. In this study, PFAS exposure was related to a decreased abundance of taxa associated with probiotics, such as *Ruminococcus gauvreauii group spp.*, *Dorea spp.*, *Collinsella spp.*, *Ruminococcaceae spp.*, and *Alistipes spp.* in the infant gut. Animal studies found that PFAS exposure was associated with reductions in the relative abundances of *Ruminococcaceae spp.* Shi et al., (\$year\$) [62,63,75,76], which corresponded with our findings. Probiotics regulate intestinal ecology, reduce oxidative stress, and limit inflammation [51], primarily through the synthesis of SCFAs, which activate G protein-coupled receptors on host cells, affecting intestinal epithelial cells. The integrity of the intestinal barrier has a regulatory influence, regulating the body's metabolism and inflammatory response [12], and being involved in cognition and memory, all of which contribute to body health. The most common probiotic, *Bifidobacterium spp.*, alongside *Collinsella spp.*, and *Blautia spp.* can produce SCFAs, such as acetic acid, maintaining intestinal homeostasis, and reducing inflammation [41]. The structure of PFAS is comparable to that of fatty acids, and it has a similar affinity for serum proteins. As a result, PFAS can affect the transport and metabolism of fatty acids in the body by competitively binding to the binding site of fatty acids [8]. In addition, animal experiments found that PFAS [62,75] exposure significantly reduced the levels of SCFAs in the intestinal tract of mice, which further supported the findings of this study.

PFHxS exposure was also found to be significantly associated with the increased relative abundance of *Enterococcus spp.* PFAS exposure has been reported to modify innate lymphocyte responses, increase inflammatory cytokines in the gut, and reduce mucin formation, all of which are detrimental to the clearance of bacterial infections in the gut [66]. The hypothalamus-pituitary-adrenal axis was affected by PFAS in human studies [40]. Elevated cortisol levels can cause alterations in gut microbiota and lead to an increase in the relative abundance of opportunistic taxa [55]. Growing evidence indicates that PFAS may alter intestinal barrier function [10]. For instance, PFOS [75] and PFOA [63] exposure in mice can cause gut microbiota dysbiosis, intestinal barrier damage and increased intestinal permeability, which ultimately lead to peripheral inflammation and oxidative stress. PFHxS has been shown to diminish the amount of cadherin [56], implying that PFHxS can increase intestinal barrier permeability.

Prenatal PFAS exposure, especially PFHxS exposure, was associated with altered metabolism pathways. For example, vitamin B6 metabolism, lipoic acid metabolism, and amino sugar and nucleotide sugar metabolism were found to decrease after PFHxS exposure, which was consistent with previous studies showing that PFAS exposure is associated with amino acid and lipid metabolism [23]. PFAS can interact with peroxide-proliferation-activated receptors to regulate metabolic functions [67], including downregulation of lipid metabolism and amino acid metabolism [23]. The increase in secondary bile acid biosynthesis corresponded with previous studies [60], which indicated that PFAS exposure might disrupt enterohepatic circulation.

The gut microbiota is critical for metabolic and immunological

maturity of the body, from a low-diversity microbiome in early life to a rapidly maturing microbial ecosystem [82], while disturbance of normal microbial succession may alter long-term development and intergenerational abnormalities [58,68]. For example, microplastic exposure altered the microbiota profile of *Eriocheir sinensis* and provoked immune inhibition [43]. Increased microbiota diversity and decreased inflammatory markers were found in humans with a high-fermented-food diet [80]. Even though the relationships between the composition of the gut microbiota at various stages of neurobehavioral development were examined, significant relationships were discovered only between neonatal gut microbiota and SDQ scores. Previously, a higher alpha diversity indicated a more mature community, and low alpha diversity was always associated with negative health outcomes [61]. However, in terms of neurobehavior, this hypothesis is not entirely accurate. Higher alpha diversity was found in adults with major depressive disorders and schizophrenia [29,32]. A recent study found that alpha diversity in neonatal faeces was negatively associated with children's prosocial behavior [81]. In a study with 39 infants, the alpha diversity of gut microbiota at 1 year of age was negatively correlated with connectivity among the amygdala, right anterior insula, and anterior cingulate, suggesting that higher levels of alpha diversity may be associated with ineffective emotion processing mechanisms [14].

Taxa abundances of *Adlercreutzia spp.* and *Ruminococcus gauvreauii group sp.* were found to be significantly associated with increased peer relationship problems and prosocial behavior scores, respectively. *Adlercreutzia spp.* was found to be negatively correlated with the expression of brain-derived neurotrophic factor (BDNF) in the brain in mice, which further induced anxiety/depression-like behaviors [84]. *Ruminococcus gauvreauii group sp.* is usually considered a probiotic and can produce SCFAs, which can be beneficial for neurodevelopment [30].

Amino sugar and nucleotide sugar metabolism and vitamin B6 metabolism were found to be positively associated with scores on the prosocial behavior subscale. Previous evidence supported the beneficial role of vitamin B6 metabolism in the process of neurotransmitter synthesis [69] and amino sugar and nucleotide sugar metabolism in neuron metabolic regulation [31]. Positive associations were also found between oxidative phosphorylation and the scores for externalizing problems, especially the conduct problem and hyperactivity subscale scores. Oxidative phosphorylation is crucial for neuronal differentiation. Altered oxidative phosphorylation under environmental pollutants can affect dopaminergic neurogenesis and promote neuron degeneration [28]. Bile acids and their metabolic products have been implicated in the communication between the gut microbiota and the brain [74]. Associations have been found between secondary bile acid biosynthesis and neuroinflammation [50], which can help explain our findings of significant associations between secondary bile acid biosynthesis and prosocial behavior and externalizing problems scores.

Additionally, this study found a significant mediating effect of neonatal richness indices on the associations of PFHxS exposure with neurobehavior. An important role of the neonatal microbiota in early life neurodevelopment has been suggested by a recent study indicating that the composition of the neonatal microbiota predicted cognitive development in children at 36 months of age [24]. Through the establishment of structural and functional synapses, neural development begins during foetal life and develops into a fully developed central nervous system [39]. Because of the plasticity of the developing brain, even minute alterations in its structure and function during foetal life may eventually be amplified and have a lasting impact on the brain's functional connections [5,6]. As a result, PFAS exposure in early life might lead to predisposed microbiota colonization, such as pathogenic or opportunistic bacteria [15,86], increased inflammatory cytokines in the gut [66], and dysfunctional intestinal barrier [56,75], and then finally influence neurodevelopment via the gut-brain axis [66].

Several advantages can be drawn from our study. First, the current study provided an appropriate opportunity to examine the effects of

prenatal PFAS exposure owing to its prospective design. The scientific rigor was enhanced in this research due to the consideration of a list of potential confounders, including breastfeeding duration. Second, the longitudinal gut microbiota data during early life made the investigation into the relationship between PFAS exposure and gut microbiota colonization more comprehensive. Third, concentrations of PFAS in cord serum provided a more direct indicator of foetal exposure during early life.

However, several limitations of this study cannot be ignored. Although several kinds of PFAS were detected, the effects of PFAS mixtures were not considered in this study. The combined effects of PFAS exposure should be the focus of future studies. Furthermore, 16 S rRNA gene sequencing was incapable of identifying specific taxa at the species level. Additionally, functional profiling could not be performed with this method. As a result, metagenomic sequencing should be applied to uncover the underlying pathways, rather than the predicted KEGG pathways, in future studies.

5. Conclusions

In conclusion, we found that higher concentrations of PFAS, especially PFHxS, were associated with increased scores on the conduct problem subscale in children at 2 years of age. Prenatal exposure to PFHxS was associated with altered alpha and beta diversity and decreased taxa (e.g., *Ruminococcus gauvraeuii group sp.*) and pathway abundances (e.g., vitamin B6 metabolism, amino sugar and nucleotide sugar metabolism). Altered alpha diversity, taxa, and pathways were found significantly associated with children's neurobehavior. The richness indices (Chao1 and ACE) of neonates played a mediating role in the association of PFHxS exposure with conduct problems.

Environmental implications

PFAS are widely used in daily life. Abundant evidence has indicated that PFAS exposure in early life can affect individual neurodevelopment. Therefore, PFAS can be considered "hazardous materials". Evidence has shown that early-life PFAS exposure might induce neurobehavioral problems, but few studies have focused on the effect of PFAS substitutes, as well as the potential role of gut microbiota. Understanding the associations between exposure to different PFAS and neurodevelopment and exploring the role of gut microbiota can simply and effectively reduce the burden of neurological disease.

CRediT authorship contribution statement

Yunhui Zhang: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Wenwei Lu:** Methodology. **Huijing Shi:** Project administration. **Hang Wang:** Investigation. **Pengpeng Wang:** Supervision. **Qiang Li:** Funding acquisition, Investigation, Methodology. **Liyi Zhang:** Investigation, Methodology. **Yuhan Zhou:** Formal analysis, Funding acquisition, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing.

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 authors do not have permission to share data.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.133920.

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