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Associations between pre- and post-natal exposure to phthalate and DINCH metabolites and gut microbiota in one-year old children[☆]

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ABSTRACT

The gut microbiota is a collection of symbiotic microorganisms in the gastrointestinal tract. Its sensitivity to chemicals with widespread exposure, such as phthalates, is little known. We aimed to investigate the impact of perinatal exposure to phthalates on the infant gut microbiota at 12 months of age. Within SEPAGES cohort (Suivi de l'Exposition à la Pollution Atmosphérique durant la Grossesse et Effet sur la Santé), we assessed 13 phthalate metabolites and 2 di(isononyl) cyclohexane-1,2-dicarboxylate (DINCH) metabolites in repeated urine samples collected in pregnant women and their offspring. We obtained stool samples from 356 children at 12 months of age and sequenced the V3-V4 region of the 16S rRNA gene, allowing gut bacterial profiling. We used single-chemical (linear regressions) and mixture (BKMR, Bayesian Kernel Machine Regression) models to examine associations of phthalates and DINCH metabolites, with gut microbiota indices of α -diversity (specific richness and Shannon diversity) and the relative abundances of the most abundant microbiota phyla and genera. After correction for multiple testing, di(2-ethylhexyl) phthalate (Σ DEHP), diethyl phthalate (DEP) and bis(2-propylheptyl) phthalate (DHP) metabolites 12-month urinary concentrations were associated with higher Shannon α -diversity of the child gut microbiota in single-chemical models. The multiple-chemical model (BKMR) suggested higher α -diversity with exposure to the phthalate mixture at 12 months, driven by the same phthalates. There were no associations between phthalate and DINCH exposure biomarkers at other time points and α -diversity after correction for multiple testing. Σ DEHP metabolites concentration at 12 months was associated with higher *Coprococcus* genus. Finally, Σ DEHP exposure at 12 months tended to be associated with higher phylum Firmicutes, an association not maintained after correction for multiple testing. Infancy exposure to phthalate might disrupt children's gut microbiota. The observed associations were cross-sectional, so that reverse causality cannot be excluded.

1. Introduction

The microorganisms that inhabit the human digestive tract, including bacteria, archaea, parasites, fungi, and viruses, are known as the human gut microbiota. The gut microbiota communicates with other

organs through various pathways and plays a role in digestive, metabolic, trophic, immune, and neurological functions (Fujimura et al., 2010; Linares et al., 2016; Schneider et al., 2005; Turrioni et al., 2008). An imbalance or "dysbiosis" in the gut microbiota has been linked to various inflammatory and autoimmune conditions (Campeotto et al.,

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2007; Tanaka and Nakayama, 2017), as well as neurological (Cryan and Dinan, 2012; Dobbs et al., 2000; Keshavarzian et al., 2015; Pistollato et al., 2016; Sherwin et al., 2016) and neurodevelopmental disorders such as autism spectrum disorders (Hsiao et al., 2013; Krajmalnik-Brown et al., 2015; Navarro et al., 2016). The gut microbiota evolves qualitatively and quantitatively during the first few years of life and stabilizes at about three years (Rodríguez et al., 2015). Given the impact of the gut microbiota on human health, it is crucial to identify controllable determinants of the gut microbiota in early life. Accumulating evidence suggests that the diversity and composition of the gut microbiota are influenced by environmental factors such as diet and antibiotics (Bäckhed et al., 2015; Gibson et al., 2016; Guaraldi and Salvatori, 2012; Wu et al., 2011). The extent to which exposure to phthalates may influence the gut microbiota has little been studied.

Phthalates are diesters of orthophthalic acid, some of which have been produced since the 1970s, with a variety of uses, ranging from consumer to industrial products (Fillol et al., 2021). The wide and varied uses of phthalates lead to ubiquitous contamination of the environment. Exposure to phthalates can occur through food, but also through air or direct contact with equipment and products in which they are present (Wittassek et al., 2011). Some phthalates metabolites have been detected in the placental tissue, demonstrating that they can cross the placental barrier (Mose et al., 2007b, 2007a). Di(isononyl) cyclohexane-1,2-dicarboxylate (DINCH) is an alternative plasticizer and replaces phthalates such as di(2-ethylhexyl) phthalate (DEHP) and di(isononyl) phthalate (DiNP) in medical devices, toys, and food packaging materials (Schütze et al., 2012). Cohort and biomonitoring studies document widespread exposure to phthalates and DINCH in adults, pregnant women, and children (Fillol et al., 2021; Philippat et al., 2021; Vogel et al., 2023).

Both pre- and post-natal exposure to phthalates has been associated with diverse biological or pathophysiological consequences, including lipid metabolism, reproductive and hepatic functions and neurodevelopment in both toxicological (Batool et al., 2022; Gray, 2023, 2023; Jia et al., 2016; Xu et al., 2020; Zhang et al., 2021) and epidemiological studies (Axelsson et al., 2015; Guilbert et al., 2021; Lee et al., 2023; Mustieles et al., 2023; Rolland et al., 2023; Swan et al., 2015; Xia et al., 2018). Exposure to diethyl phthalate (DEP) (Deng et al., 2020; Hu et al., 2016), DEHP (Deng et al., 2020; Fan et al., 2020; Fu et al., 2021; Su et al., 2022; Yu et al., 2021; Zhao et al., 2020) and di-n-butyl phthalate (DnBP) (Xiong et al., 2020; Zhang et al., 2022) has been associated with disturbances of the gut microbiota in rodent models, but the underlying biological mechanisms that could explain these associations remain unknown.

In humans, the literature on this topic is very limited. Yang et al. conducted a small prospective study of newborns who were given intravenous infusions starting from postnatal day 1 to postnatal day 5, and were thus considered as a DEHP-exposed group ($n = 15$); they were compared to newborns who did not receive intravenous infusions (control group, $n = 10$). The urinary DEHP metabolites level was significantly higher in the exposed group than in the control group. Lower abundances of *Rothia* species and *Bifidobacterium longum* in the first 10 days of life were identified in the group exposed to DEHP (Yang et al., 2019). In a cross sectional study, mono-isobutyl phthalate (MiBP) was associated with variations in the composition of gut microbiota at the genus and phylum levels in 57 adults with active Crohn's disease (Xiong et al., 2023). To our knowledge, no other epidemiological study investigating the effects of phthalates on gut microbiota diversity and composition has been published. Given the results of these previous (mostly toxicological) studies, perturbations of gut microbiota diversity and composition in early life may be expected with exposure to DEHP, DnBP, and DEP. The objective of our study was to investigate the sensitivity of the gut microbiota of 12-month-old children to phthalate and DINCH exposure during pregnancy and infancy.

2. Material and methods

Most of the methods have been previously described elsewhere (Davias et al., 2024; Philippat et al., 2021).

2.1. Design of SEPAGES couple-child cohort and data collection

2.1.1. Study population

We relied on a subsample of the French couple-child cohort SEPAGES (Suivi de l'Exposition à la Pollution Atmosphérique durant la Grossesse et Effet sur la Santé), that recruited pregnant women between July 2014 and July 2017 in 8 obstetrical ultrasonography practices located in the Grenoble area (Lyon-Caen et al., 2019). From the 484 families included in the SEPAGES cohort, our study was restricted to 360 families with full outcome data. All required ethical and data privacy agreements (including from Comité de Protection des Personnes (CPP) and from the French data privacy institution (Commission Nationale de l'Informatique et des Libertés, CNIL)) were obtained; all parents gave informed prior consent for themselves and their child. Participants were given the possibility to accept or refuse some parts of the study.

2.1.2. Biospecimens collection

Parents collected urine samples from three micturitions per day during follow-up weeks that took place at median 18 (25th-75th percentiles, 16-19) gestational weeks and 34 (32-35) gestational weeks in mothers, and in their child in median at 53 (52-55) weeks of age (Table S1). Samples were collected in plastic vials whose composition did not include phthalates. At the end of each follow-up week, urine samples were pooled within-subject and within-period (pools of equal volume aliquots of each of the samples collected over a follow-up week). This within-subject pooling method, which does not account for the urinary dilution of each individual sample, has been validated as an accurate reflection of the urine concentrations that would have been obtained by pooling the total volume of all micturitions (Philippat and Calafat, 2021). It was demonstrated that when equal volumes from each sample are pooled, as was done in the SEPAGES cohort, normalization using specific gravity or creatinine is unnecessary (O'Brien et al., 2017) and may even bias estimated concentrations for certain compounds (Philippat and Calafat, 2021). Parents were asked to collect faecal samples from their offspring in a sealed container at any time during the 1-year urine collection week that took place in median at 54 (25th-75th centiles, 52-55) weeks. Biospecimen collection is described in more detail in Davias et al. (2024).

2.2. Assessment of exposure biomarkers

In the three weekly urine sample pools of SEPAGES mothers and children, 13 metabolites of phthalates (5 metabolites of DEHP: mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-2-carboxymethyl hexyl phthalate (MMCHP); 1 metabolite of DnBP: mono-n-butyl phthalate (MnBP); 3 metabolites of DiNP: mono-4-methyl-7-hydroxyoctyl phthalate (ohMiNP), mono-4-methyl-7-oxooctyl phthalate (oxoMiNP) and mono-4-methyl-7-carboxyoctyl phthalate (cxMiNP); 1 metabolite of di-isobutyl phthalate (DiBP): mono-isobutyl phthalate (MiBP); 1 metabolite of benzylbutyl phthalate (BBzP): monobenzyl phthalate (MBzP); 1 metabolite of DEP: monoethyl phthalate (MEP); 1 metabolite of bis(2-propylheptyl) phthalate (DPHP): 6-hydroxy monopropylheptyl phthalate (ohMPHP) and 2 metabolites of DINCH: (2-(((hydroxy-4-methyl-7-oxooctyl)oxy)carbonyl) cyclohexanecarboxylic acid (ohMINCH) and 2-(((4-methyl-7-oxooctyl)oxy)carbonyl) cyclohexanecarboxylic acid (oxoMINCH)) were assessed (Table S1). Phthalate metabolites were assessed using isotope dilution high performance liquid chromatography and electrospray ionization tandem mass spectrometry (Sabaredzovic et al., 2015). Procedural blanks, quality assurance

samples (in-house quality controls and standard reference material (SRM 3673) from National Institute of Standards and Technology) were analyzed along with the samples. The accuracy was from 70% to 125%, and the precision given as relative standard deviation was below 25% for all the metabolites. Measurements below the limit of detection (LOD) and the limit of quantification (LOQ) were singly imputed using the compound's probability distribution (Helsel, 1990). We applied a natural logarithm transformation to the biomarker concentrations (ng/mL) to limit the impact of extreme values on the regression results. The molar sums of the phthalate metabolites were calculated for the phthalates for which we measured more than one metabolite (formulas for the calculation of molar sums ($\mu\text{M/L}$) are: $\Sigma\text{DEHP} = \text{MEOHP}/292 + \text{MECPP}/308 + \text{MEHP}/278 + \text{MEHHP}/294 + \text{MMCHP}/308$; $\Sigma\text{DiNP} = \text{ohMiNP}/308.2 + \text{oxoMiNP}/306.2 + \text{cxMiNP}/322.2$; $\Sigma\text{DiNCH} = \text{ohMINCH}/314.42 + \text{oxoMINCH}/312.4$). All chemical analyses were performed at the Norwegian Institute of Public Health in Oslo (NIPH).

2.3. Assessment of the gut microbiota

2.3.1. Sequencing and data processing

Faecal samples were aliquoted from 360 children at 12 months (~150 mg). Total DNA was extracted from all samples, the V3-V4 hypervariable region of the 16S rRNA gene was amplified and amplicon sequence variant (ASV) taxonomical classification was performed using a classifier from the RDPTools suit (Cole et al., 2014). We removed 4 samples that had a low DNA or sequencing quality (samples with a number of reads below 2500). To control for variation in sequencing depth for diversity analyses, the ASV matrix was rarefied to 5000 sequences, which led to the removal of 6 additional samples ($n = 350$). Taxa counts were converted to relative abundances, which enabled us to maintain the sample size of 356 for the taxonomy analyses. More details about 16S rRNA gene sequencing and data processing can be found elsewhere (Davias et al., 2024).

2.3.2. Gut microbiota composition: α -diversity, β -diversity and taxonomy analyses

α -diversity (i.e. diversity within a sample) was estimated with 2 indices: the specific richness and the Shannon diversity (Shannon and Weaver, 1964), that were computed from the rarefied ASVs table (5000 sequences) with R version 4.2.2 (R core team, 2022), package Phyloseq version 1.27.6 (McMurdie and Holmes, 2013). β -diversity between each given pair of gut microbiota samples was calculated using the Bray-Curtis dissimilarity metric (Bray and Curtis, 1957), defined on the rarefied ASVs table (5000 sequences). Taxonomic affiliation was described at all taxa levels. Taxonomy analyses were conducted for the 4 most abundant phyla and the 46 most abundant genera (detected in at least 30% of the children). 16S rRNA gene sequencing cannot distinguish between *Shigella* species and *Escherichia coli* due to the high nucleotide similarity. Consequently, in our study, *Escherichia* and *Shigella* genera were collectively analyzed as a single taxon (Devanga Ragupathi et al., 2017).

2.4. Statistical analyses

The gut microbiota parameters considered in relation to biomarkers of exposure to phthalates were the 3 above-mentioned indices of diversity, as well as the 4 most abundant phyla and the 46 most abundant genera (detected in at least 30% of the children). We imputed missing values of adjustment covariates using simple imputation by predictive mean matching (Little, 1988; Rubin, 1986). Correlations between biomarkers of exposure to phthalates, between biomarkers of exposure to phthalates and covariates, and between gut microbiota parameters were assessed using Pearson and Spearman's rank correlation coefficient.

Based *a priori* on the literature, we selected covariates for regression models based on directed acyclic graphs (DAG) we developed (Fig. S1). The single-chemical analyses relied on multiple linear regression models

(one model per exposure – outcome pair) adjusted for: MiSeq 16S rRNA gene sequencing run (run 1 vs. run 2), child age at feces collection (continuous coding), delivery mode (cesarian section vs. vaginal delivery), interpregnancy interval (under 2 years; 2 years and more; primiparous), child sex (female vs. male), period of introduction of solid food (between 0 and 6 months of age; between 6 and 12 months; not introduced at 12 months), breast-feeding status up to 48 weeks (not breastfed; breastfed up to <24 weeks; breastfed during 24–47 weeks; still breastfed at 48 weeks), antibiotics use during the first year of life (no vs. yes), presence of pets at home (no vs. one or more), passive smoking during the first year of life (no vs. yes), and maternal characteristics (parity (none vs. one child or more), age (continuous coding) and body mass index (BMI) before pregnancy (<19 kg/m²; 19–23.9 kg/m²; ≥ 24 kg/m²), education (2 years or less after graduation; 3–4 years after graduation; ≥ 5 years after graduation), and smoking status during pregnancy (no vs. yes). In addition, models involving postnatal exposures were also adjusted for gestational duration (continuous coding) and child weight (<3 kg; 3–3.4 kg; ≥ 3.5 kg) and length (<50 cm; 50–51 cm; ≥ 52 cm) at birth. To limit associations due to multiple comparisons, a family-wise error rate (FWER) correction to the p -value threshold was applied. The correction uses a Bonferroni procedure extended to handle correlated tests: the actual number of associations being tested (T) is replaced by a smaller value called the effective number of independent tests (Te). Te value is estimated by the effective number of independent biomarkers of exposure to phthalates $\sum_{i=1}^M [I_{(\lambda_i > 1)} x (\lambda_i - 1)]$ multiplied by the effective number of independent outcomes $\sum_{i=1}^N [I_{(\lambda_i > 1)} x (\lambda_i - 1)]$, where $I(x)$ is an indicator function and λ_i are the eigenvalues of the matrix of correlations between the M biomarkers of exposure to phthalates and the eigenvalues of the matrix of correlations between the N outcomes. The p -value threshold to control FWER is then α/Te (adapted from (Li et al., 2012)).

Tier-1 (microbiota diversity) analyses: For α -diversity analyses, the 24-time window-specific biomarkers of exposure to phthalates (8 biomarkers of exposure to phthalates across 3 time windows) corresponded to 14 independent predictors and the 3 α -diversity and β -diversity indices altogether corresponded to 3 independent outcomes. The corrected significance level was $0.05/(14 \times 3) = 0.0012$.

To identify any mixture effect of multiple chemical exposures assessed at each time window on α -diversity indices, we used Bayesian kernel machine regression (BKMR), a statistical approach for analyzing multi-chemical mixtures (Bobb et al., 2015), using the R package bkmr version 0.2.2. All metabolites of the same parent compound were first summed and then included in the mixture. The model was adjusted for the same covariates as the single-chemical regression models. The combined effects of the mixtures were assessed by estimating the expected change in indices of α -diversity associated with concurrent changes in all of the components of the mixture from their 10th percentile concentrations. The posterior inclusion probabilities (PIP) were also investigated to identify the most important contributors to the mixture. The estimated effects of an increase from the 25th to 75th percentile in a single exposure biomarker on α -diversity indices when all other exposure biomarkers are fixed at either the 25th, 50th, or 75th percentiles were also investigated.

We investigated group differences in Bray-Curtis β -diversity for low (1st tertile), medium (2nd tertile), and high (3rd tertile) exposure biomarker levels considered separately at each time window (one model per exposure biomarker) by testing significance of pairwise groups of exposure biomarker levels with Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA), using R program version 4.2.2, package Vegan version 2.6–4, Adonis2 function.

Tier-2 (microbiota taxonomy) analyses: We used independent linear regression models with as outcome the 4 most abundant phyla (Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria) and the 46 most abundant genera, adjusted for the same covariates as the diversity analysis (*tier-1 analyses*), and each exposure biomarker as explanatory

variable. Null values in genera variables were replaced by the value 1/5000 before the variables were log-transformed. We applied a FWER multiple comparison correction for all associations between taxa and exposure biomarkers. The 24 time window-specific biomarkers of exposure to phthalates (8 biomarkers of exposure to phthalates across 3 time windows) corresponded to 14 independent predictors and the 50 gut microbiota taxonomy variables (4 phyla and 46 genera) to 33 independent outcomes. The corrected significance level (FWER correction) was thus $0.05/(14 \times 33) = 0.00011$. For exposure biomarkers associated with one of the diversity indices, we estimated the association between the phylum with which they were most strongly associated and the diversity index with which they were associated, to see if variations in this phylum were likely to explain variations in diversity.

Finally, a BKMR model was applied for phylum outcomes, using the same methodology as for the α -diversity analyses, in order to identify any mixture effect of multiple chemical exposures for each exposure window on the relative abundances of the 4 most abundant phyla.

Sensitivity analyses: As sensitivity analyses, we repeated the α -diversity analyses using a different threshold of rarefaction (10,000 instead of 5000 in our main analysis). We originally decided not to correct our analyses for a marker of urine dilution (Philippat and Calafat, 2021). However, we performed sensitivity analyses in which exposure biomarkers were standardized for specific gravity (Mortamais et al., 2012) before the characterization of their association with gut microbiota parameters. We also repeated the analyses by adjusting for the existence of an hospitalization of the child during the first year of life (yes vs. no), which may be associated with phthalates exposure and gut microbiota dysbiosis. Finally, we added gestational duration, weight and length at birth and at one year in the covariates of the regression models relative to prenatal exposures; and we further adjusted for weight and length at one year to the regression models relative to postnatal exposures although these adjustments were not required by our a priori DAGs.

All statistical analyses were performed with R version 4.1.0.

3. Results

3.1. Study population

Out of 484 families participating in SEPAGES cohort, 356 children had available gut microbiota data (Fig. S2). The maternal and child characteristics are presented in Tables S2 and S3 and have been described in detail in Davias et al. (2024). Pre- and early post-natal exposure to phthalates and DINCH in the SEPAGES cohort have been described in Philippat et al. (2021). Table S4 shows comparisons of biomarkers of exposure to phthalates between the 356 SEPAGES mothers and children included and those excluded from our study. Maternal exposure to MiBP during the third trimester of pregnancy was significantly higher in excluded mothers compared to included ones ($p = 0.02$). Fig. S3 shows Pearson correlations between concentrations of exposure biomarkers. Pearson's correlations coefficients were 0.5 between Σ DEHP metabolites and MEP, 0.6 between Σ DEHP and ohMPPH, and 0.3 between MEP and ohMPPH concentrations at one year (Fig. S3). Urinary biomarker concentrations were not strongly correlated with maternal and child characteristics (Fig. S4). Fig. S5 shows Pearson correlations between gut microbiota α -diversity indices and the 4 most abundant phyla. Distribution of the α -diversity indices and the taxonomy of the gut microbiota of children at 12 months of age are given Table S5; their relation with maternal and child characteristics have been described in Davias et al. (2024).

3.2. Adjusted associations between biomarkers of exposure to phthalates and DINCH and gut microbiota diversity

The numbers of children included were 349 and 344 for the analyses with exposures assessed during the second and third trimesters of

pregnancy, and 345 for the 12-month postnatal exposures.

3.2.1. α -diversity

In the single-chemical models, after correction for multiple testing, child urinary concentrations of DEHP metabolites (molar sum of the metabolites), of MEP (a metabolite of DEP) and of ohMPPH (a metabolite of DPHP) at 12 months of age were associated with higher Shannon α -diversity (p -values: 0.001, 0.0006 and 0.001, respectively, Table 1 and Fig. 1).

Among the associations that did not pass the significance threshold corrected for multiple testing ($p < 0.0012$) in the single-chemical models, those with the lowest p -values included the following suggestive associations: child urinary concentrations at 12 months of MnBP, a metabolite of DnBP, and of MiBP, a metabolite of DiBP, with higher α -diversity indices (p -values ≤ 0.03 for both indices); child concentration at 12 months of MBzP, a metabolite of BBzP, with higher Shannon diversity (p -value: 0.03); child concentrations at 12 months of the following compounds with higher richness: Σ DEHP metabolites, Σ DiNP metabolites, MEP (metabolite of DEP) and ohMPPH (metabolite of DPHP) (p -values ≤ 0.03 , Table 1 and Fig. 1).

When we investigated a possible mixture effect of exposure biomarkers on the α -diversity indices using BKMR model (Fig. 2 and Fig. S6), increased concentration of the mixture at 12 months was associated with higher specific richness and Shannon α -diversity relative to when all concentrations were fixed at 10th percentile (Fig. 2). Posterior inclusion probabilities of the BKMR models considering the 12 month-exposure window were in favor of Σ DEHP metabolites, MEP and ohMPPH as the main contributors of the mixture (Table S6). There were no other indications of an overall mixture effect associated for the other exposure windows. Increasing MnBP concentration during the second trimester and MEP at 12 months from the 25th to the 75th percentile, when other exposure biomarkers were fixed at the 50th or the 75th percentiles was associated with higher Shannon α -diversity. Conversely, increasing Σ DiNCH concentration during the third trimester of pregnancy from the 25th to the 75th percentile, with other biomarkers concentrations at the 25th or 50th, was associated with α -diversity decrease (Fig. S6).

3.2.2. β -diversity

For each exposure biomarker, we tested whether children gut microbiota exposed to "low" (1st tertile), "medium" (2nd tertile) or "high" (3rd tertile) concentrations differed in terms of β -diversity distance (Bray-Curtis dissimilarity index, Table S7). There were differences in community composition in relation with Σ DiNCH concentration at 12 months (p -value: 0.0010). No other association passed the threshold corrected for multiple testing (p -value < 0.0012). Without correction for multiple testing, there were differences in community composition in relation with MEP concentration during the second trimester of pregnancy (p -value: 0.01) and with MEP, Σ DiNP, ohMPPH concentrations at 12 months (p -values: 0.02, 0.03 and 0.01, respectively).

3.3. Adjusted associations between biomarkers of exposure to phthalates and DINCH and gut microbiota taxonomy

The number of children included was 355 and 350 for analyses implying exposures during the second and third trimesters of pregnancy respectively, and 351 for the 12-month postnatal exposures.

3.3.1. Phylum level

The associations between concentrations of exposure biomarkers and the 4 most abundant phyla in 12-month children gut microbiota are presented in Table S8. None of the associations between the 24 exposure variables and the 4 most abundant phyla passed the threshold corrected for multiple testing (p -value < 0.00011). Among the strongest associations, Σ DEHP metabolite concentrations (molar sum) and ohMPPH (a metabolite of DPHP) concentration at 12 months were associated with

Table 1

Adjusted associations between biomarkers of exposure to phthalates and DINCH and α -diversity indices of the child gut microbiota at 12 months (single chemical models, sample sizes between 344 and 349).

Exposure biomarker and time window	Specific richness				Shannon diversity		
	N ^a	Beta ^b	95% CI ^c	p-value ^d	Beta ^b	95% CI ^c	p-value ^d
ΣDEHP metabolites^e							
Mother, pregnancy trim. 2	349	-1.0	-6.1, 4.1	0.71	-0.06	-0.17, 0.05	0.27
Mother, pregnancy trim. 3	344	-2.0	-7.4, 3.4	0.47	0.00	-0.11, 0.11	0.98
Child, 12 months ^f	345	4.8	1.0, 8.6	0.01	0.13	0.05, 0.21	0.001
MnBP							
Mother, pregnancy trim. 2	349	1.5	-3.6, 6.6	0.56	0.04	-0.06, 0.15	0.42
Mother, pregnancy trim. 3	344	2.1	-2.7, 6.8	0.39	0.08	-0.01, 0.18	0.09
Child, 12 months	345	4.2	0.4, 8.0	0.03	0.10	0.02, 0.18	0.01
ΣDiNP metabolites^e							
Mother, pregnancy trim. 2	349	-1.6	-5.1, 1.9	0.37	-0.06	-0.14, 0.01	0.08
Mother, pregnancy trim. 3	344	-0.5	-4.5, 3.5	0.82	-0.04	-0.13, 0.04	0.29
Child, 12 months	345	5.1	0.6, 9.6	0.03	0.08	-0.02, 0.17	0.12
MiBP							
Mother, pregnancy trim. 2	349	0.5	-4.4, 5.4	0.85	-0.01	-0.11, 0.09	0.84
Mother, pregnancy trim. 3	344	0.9	-3.5, 5.4	0.68	0.01	-0.08, 0.10	0.77
Child, 12 months	345	4.4	1.0, 7.8	0.01	0.09	0.02, 0.17	0.009
MBzP							
Mother, pregnancy trim. 2	349	-0.3	-4.5, 3.9	0.90	-0.03	-0.12, 0.05	0.45
Mother, pregnancy trim. 3	344	0.7	-3.1, 4.6	0.70	0.01	-0.06, 0.09	0.71
Child, 12 months	345	2.4	-0.1, 4.8	0.06	0.06	0.01, 0.11	0.03
MEP							
Mother, pregnancy trim. 2	349	-0.7	-3.7, 2.4	0.66	0.00	-0.07, 0.06	0.95
Mother, pregnancy trim. 3	344	0.5	-2.4, 3.4	0.73	0.04	-0.02, 0.10	0.20
Child, 12 months	345	4.1	0.8, 7.4	0.01	0.12	0.05, 0.19	<0.001
ohMPHP							
Mother, pregnancy trim. 2	349	0.5	-4.6, 5.5	0.86	-0.03	-0.14, 0.07	0.57
Mother, pregnancy trim. 3	344	1.3	-4.0, 6.7	0.62	-0.05	-0.16, 0.06	0.41
Child, 12 months	345	5.5	1.1, 9.9	0.01	0.15	0.06, 0.24	0.001
ΣDINCH metabolites^e							
Mother, pregnancy trim. 2	349	-0.3	-3.4, 2.8	0.86	-0.02	-0.09, 0.04	0.47
Mother, pregnancy trim. 3	344	-1.9	-5.0, 1.2	0.22	-0.04	-0.11, 0.02	0.17
Child, 12 months	345	0.9	-2.2, 4.0	0.57	0.05	-0.01, 0.12	0.10

^a Number of observations.

^b Average changes in α -diversity indices when the biomarker concentration is multiplied by Euler number e, or about 2.72; adjusted for MiSeq analytic batch effect, child age at feces collection, delivery mode, interpregnancy interval, child sex, period of introduction of solid food, breast-feeding status up to 48 weeks, antibiotics use during the first year of life, presence of pets at home, passive smoking during the first year of life, and maternal characteristics (parity, age, and BMI before pregnancy, education, and smoking status during pregnancy). Associations with postnatal exposures are also adjusted for gestational duration and weight and length at birth.

^c CI: Confidence Interval.

^d After correction for multiple testing, the significance threshold is 0.0012.

^e Formulas for the calculation of molar sums ($\mu\text{M/L}$): DEHP = MEOHP/292 + MECPP/308 + MEHP/278 + MEHHP/294 + MMCHP/308; DiNP = ohMiNP/308.2 + oxoMiNP/306.2 + cxMiNP/322.2; DINCH = ohMINCH/314.42 + oxoMINCH/312.4.

^f MMCHP concentration was not included in the calculation of the molar sum of DEHP metabolites at 12 months.

higher relative abundance of Firmicutes (p -values: 0.0006 and 0.007, respectively).

As an additional analysis aiming to document if Firmicutes abundance could underly part of the association between these exposure biomarkers and gut microbiota diversity, we investigated to which extent Firmicutes relative abundance was associated with Shannon α -diversity index; an increase by 10% of the Firmicutes relative abundance was associated with higher Shannon α -diversity at 12 months of age (Beta: 0.12; 95% CI, 0.09, 0.15; p -value<0.001) when adjusted for the same covariates as the main analysis.

When we investigated a possible mixture effect of exposure biomarkers on the most abundant phyla using BKMR model (Fig. 3), increased concentrations of the mixture during the second trimester of pregnancy was associated with lower relative abundance of Bacteroidetes, while increased concentrations of the mixture during the third trimester of pregnancy and at 12 months were associated with lower relative abundance of Proteobacteria relative to when all concentrations were fixed at 10th percentile. The most important contributors of the mixture effects were MEP during the second and third trimesters of pregnancy and Σ DEHP metabolites at 12 months for the effect on Firmicutes phylum; and Σ DEHP during the third trimester of pregnancy and Σ DINCH during the second trimester of pregnancy and at 12 months for the mixture effect on Proteobacteria phylum (Table S9). Increasing

Σ DINCH concentration during the third trimester of pregnancy from the 25th to the 75th percentile when other exposure biomarkers were fixed at the 25th or 50th percentile, and increasing Σ DiNP and MBzP concentrations at 12 months from the 25th to the 75th percentile when other exposure biomarkers were fixed at the 25th percentile were associated with decreased Firmicutes phylum. Conversely, increasing Σ DEHP and MBzP concentrations at 12 months from the 25th to the 75th percentile, when other exposure biomarkers were fixed at the 75th percentile, were associated with higher Firmicutes phylum. Increasing Σ DINCH concentrations at 12 months from the 25th to the 75th percentile, when other exposure biomarkers were fixed at either the 25th, the 50th or the 75th percentiles, were associated with higher Actinobacteria phylum. Increasing MBzP concentration during the third trimester from the 25th to the 75th percentile, with others exposure biomarkers at the 50th or 75th percentile, was associated with lower Bacteroidetes phylum. Finally, increasing Σ DiNP concentration during the third trimester and Σ DINCH concentration at 12 months from the 25th to the 75th percentile, with others exposure biomarkers at the 25th or 50th percentile, were associated with lower Proteobacteria phylum (Fig. S7).

3.3.2. Genus level

Forty-six of the 192 genera present in the children gut microbiota at 12 months of age were detected in at least 30% of children. Child Σ DEHP

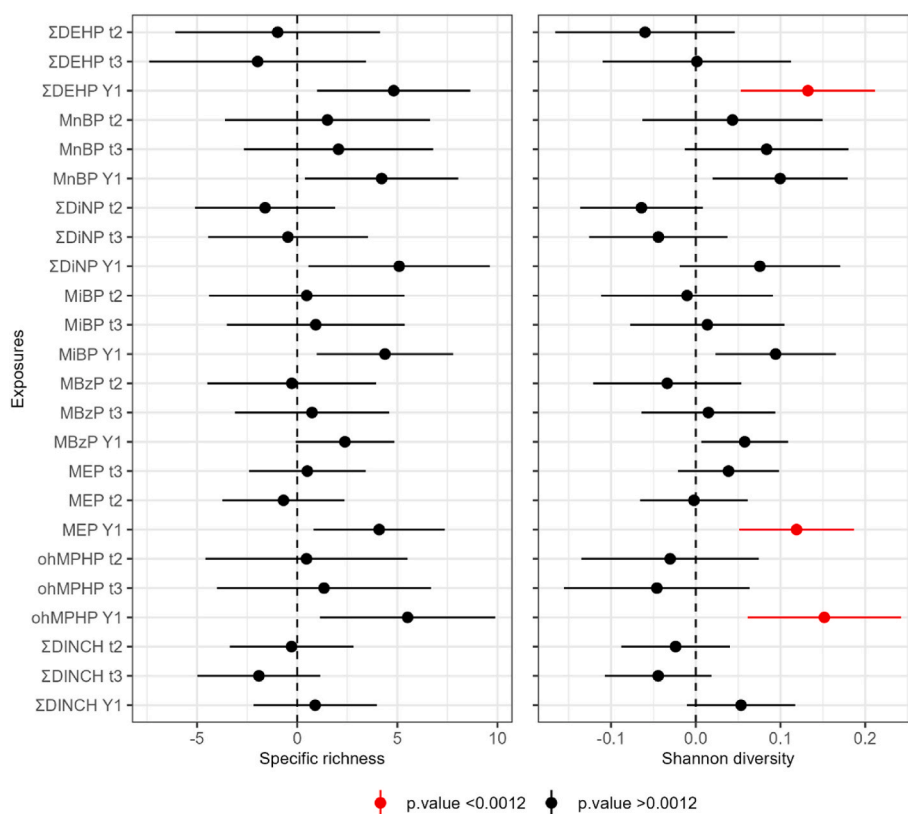


Fig. 1. Adjusted associations between biomarkers of exposure to phthalates and DINCH and α -diversity indices of the child gut microbiota at 12-month-old (single-chemical models, sample sizes between 344 and 349).

Average changes in α -diversity indices when the level of exposure to the biomarker is multiplied by Euler number e , or about 2.72; adjusted for MiSeq analytic batch effect, child age at feces collection, delivery mode, interpregnancy interval, child sex, period of introduction of solid food, breast-feeding status up to 48 weeks, antibiotics use during the first year of life, presence of pets at home, passive smoking during the first year of life, and maternal characteristics (parity, age, and BMI before pregnancy, education, and smoking status during pregnancy). Associations with postnatal exposures are also adjusted for gestational duration and weight and length at birth.

After correction for multiple testing, the significance threshold was 0.0012.

All the chemicals are continuous variables with a Neperian logarithm transformation. Formulas for the calculation of molar sums ($\mu\text{M/L}$): DEHP = MEOHP/292 + MECPP/308 + MEHP/278 + MEHHP/294 + MMCHP/308; DiNP = ohMiNP/308.2 + oxoMiNP/306.2 + cxMiNP/322.2; DINCH = ohMINCH/314.42 + oxoMINCH/312.4.

MMCHP concentration was not included in the calculation of the molar sum of DEHP metabolites at 12 months.

t2: second trimester exposure, t3: third trimester exposure, Y1: 12-month exposure.

metabolite concentration at 12 months of age was associated with higher relative abundance of *Coprococcus* genus (p -value: 7×10^{-6}). This was the sole significant association among the 24 exposure variables and the 46 genera after adjusting for multiple tests, with a significance threshold of $p < 0.00011$. Among the strongest associations (p -value < 0.0035 , which is above the threshold corrected for multiple testing), MEP, MiBP and MnBP urinary concentrations at 12 months tended to be associated with higher *Coprococcus* genus. Σ DEHP metabolites and MiBP urinary concentrations at 12 months also tended to be associated with higher *Clostridium sensu stricto*. Σ DEHP urinary concentrations at 12 months tended to be associated with higher *Lachnospiraceae incertae sedis*, *Fusicatenibacter* and *Intestinibacter* genera, Σ DINCH at 12 months with lower *Saccharibacteria genera incertae sedis*, and MBzP at 12 months with higher *Terrisporobacter* genus. During the second trimester of pregnancy, Σ DiNP tended to be associated with lower *Flavonifractor* genus, while MiBP tended to be associated with higher *Enterobacter* genus. Finally, Σ DEHP metabolites concentration during the third trimester of pregnancy tended to be associated with lower *Streptococcus* genus. The p -values and the direction of the associations according to bacterial genus and exposure are shown Fig. 4, and complete results of the genus analyses are shown in Table S10.

3.4. Sensitivity analyses

Using another threshold (10,000 instead of 5000) of rarefaction to define α -diversity indices did not strongly influence our results (Table S11). Using the biomarker urinary concentrations standardized for specific gravity led to increases in p -values without modifications of the beta coefficients towards the null by 10–25% (Table S12). Adjusting for hospitalization during the first year of life in addition to the other covariates did not strongly change the results (Table S13). Finally, further adjusting associations with prenatal exposures for gestational duration, weight and length at birth and at one year, and further adjusting models with postnatal exposures for weight and length at one year in addition to the other covariates did not strongly influence the results (Table S14).

4. Discussion

4.1. Overview

In our couple-child cohort of 356 families, Σ DEHP metabolites, DEP metabolite (MEP), and DPHP metabolite (ohMPPH) at 12 months were associated with higher α -diversity of the children's gut microbiota at 12 months of age after correction for multiple comparison. The overall

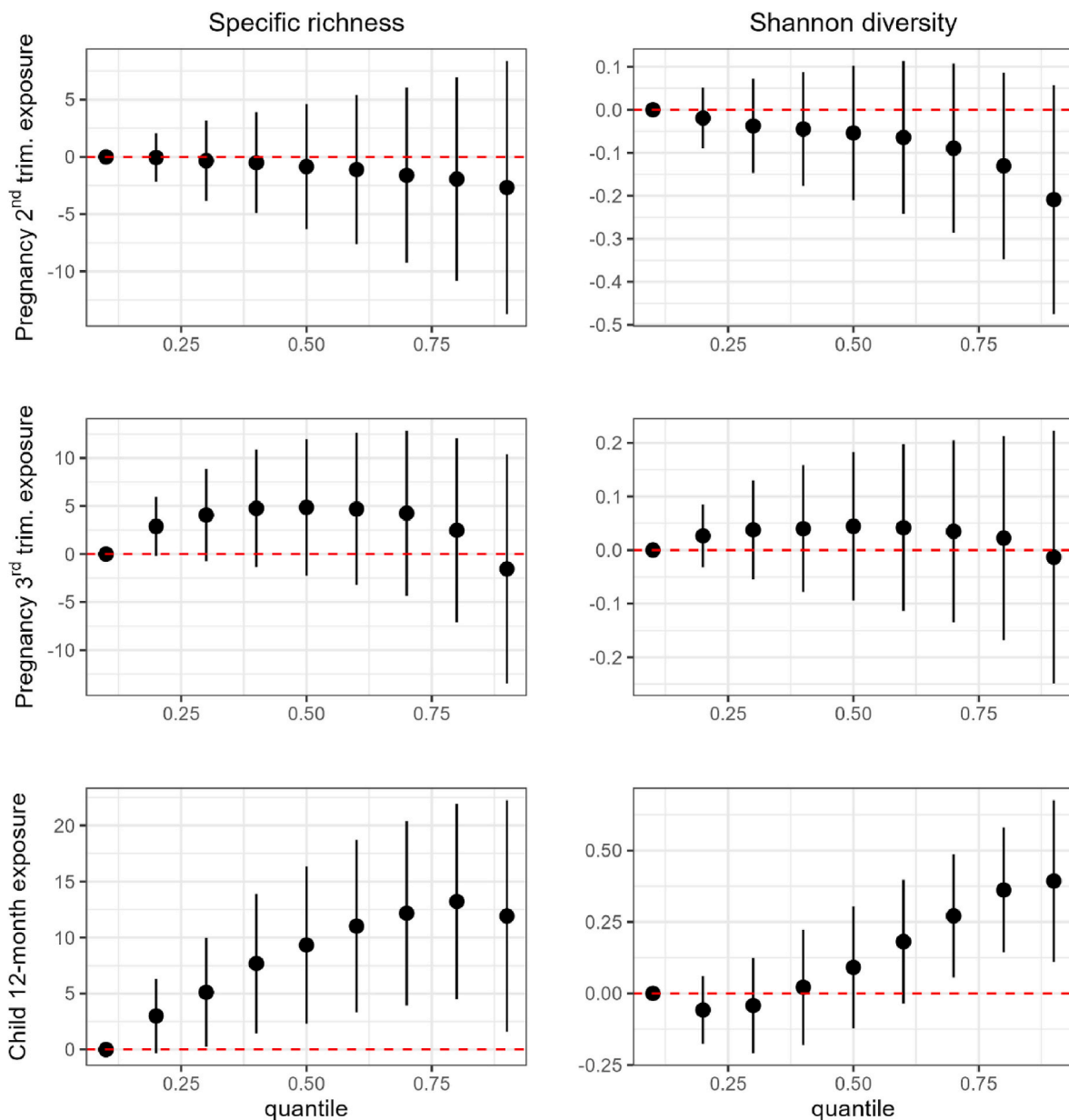


Fig. 2. Expected changes in α -diversity indices associated with concurrently increasing quantiles of all biomarker exposures (continuous variables), relative to when all biomarker exposures are fixed at their 10th percentile (mixture models, sample sizes between 344 and 349).

Adjusted for MiSeq analytic batch effect, child age at feces collection, delivery mode, interpregnancy interval, child sex, weight, period of introduction of solid food, breast-feeding status up to 48 weeks, antibiotics use during the first year of life, presence of pets at home, passive smoking during the first year of life, and maternal characteristics (parity, age, and BMI before pregnancy, education, and smoking status during pregnancy). Associations with postnatal exposures are also adjusted for gestational duration and weight and length at birth.

All the chemicals are continuous variables with a Neperian logarithm transformation. Formulas for the calculation of molar sums ($\mu\text{M/L}$): $\text{DEHP} = \text{MEOHP}/292 + \text{MECPP}/308 + \text{MEHP}/278 + \text{MEHHP}/294 + \text{MMCHP}/308$; $\text{DiNP} = \text{ohMiNP}/308.2 + \text{oxoMiNP}/306.2 + \text{cxMiNP}/322.2$; $\text{DINCH} = \text{ohMINCH}/314.42 + \text{oxoMINCH}/312.4$.

MMCHP concentration was not included in the calculation of the molar sum of DEHP metabolites at 12 months.

Posterior inclusion probabilities of the BKMR mixture models are available in [Table S6](#).

mixture effect at 12 months was also associated with higher α -diversity, with the ΣDEHP , DEP and DPHP metabolites being the most important contributors to the mixture effect. ΣDEHP metabolites urinary concentration was associated with higher *Coprococcus* genus. Those associations were cross-sectional, with exposure biomarker concentrations and gut microbiota being assessed at 12 months of age, and should thus be interpreted with caution due to the risk of reverse causality.

4.2. Comparison with the literature and biological plausibility

The median urinary concentration of phthalate metabolites in the mothers from our study was similar to that from other European cohorts ([Warembourg et al., 2019](#)), whereas concentrations at 12 months of age were lower than those reported in studies involving children aged between 12 and 24 months ([Fromme et al., 2013](#); [Navaranjan et al., 2020](#); [Watkins et al., 2014](#)), with the exception of MEHP, a metabolite of DEHP.

Phthalates can cross the placenta-blood barrier, leading to fetal

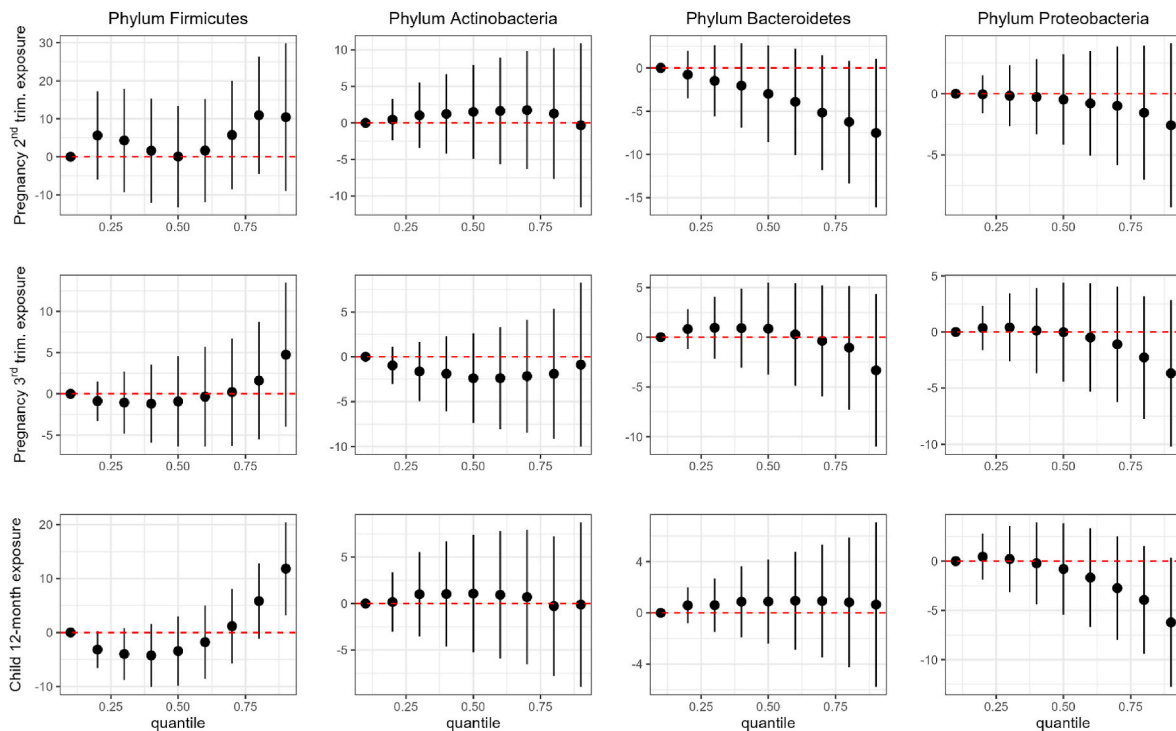


Fig. 3. Expected changes in phyla relative abundances associated with concurrently increasing quantiles of all biomarker exposures (continuous variables), relative to when all biomarker exposures are fixed at their 10th percentile (mixture models, sample sizes between 350 and 355).

Adjusted for MiSeq analytic batch effect, child age at feces collection, delivery mode, interpregnancy interval, child sex, period of introduction of solid food, breastfeeding status up to 48 weeks, antibiotics use during the first year of life, presence of pets at home, passive smoking during the first year of life, and maternal characteristics (parity, age, and BMI before pregnancy, education, and smoking status during pregnancy). Associations with postnatal exposures are also adjusted for gestational duration and weight and length at birth.

All the chemicals are continuous variables with a Neperian logarithm transformation. Formulas for the calculation of molar sums ($\mu\text{M/L}$): $\text{DEHP} = \text{MEOHP}/292 + \text{MECPP}/308 + \text{MEHP}/278 + \text{MEHHP}/294 + \text{MMCHP}/308$; $\text{DiNP} = \text{ohMiNP}/308.2 + \text{oxoMiNP}/306.2 + \text{cxMiNP}/322.2$; $\text{DINCH} = \text{ohMINCH}/314.42 + \text{oxoMINCH}/312.4$.

MMCHP concentration was not included in the calculation of the molar sum of DEHP metabolites at 12 months.

Posterior inclusion probabilities of the BKMR mixture models are available in [Table S9](#).

exposure (Dutta et al., 2020). One toxicological study reported that prenatal low dose DEHP exposure in mice dams modestly increased the α -diversity at 4 weeks and 6 weeks of age in their offsprings and caused alterations in the gut microbial community at 6 weeks of age (as indicated by weighted UniFac β -diversity); this change was still seen in male offspring at 12 weeks of age (Fan et al., 2020). In our study, none of the associations between prenatal exposure biomarkers and gut microbiota diversity at 12 months were significant after correction for multiple testing. Toxicological studies investigating the effects of postnatal phthalate exposure on the gut microbiota in animal models reported higher α -diversity after DEHP exposure (Chiu et al., 2021; Wang et al., 2020), which is consistent with our study. Previous epidemiological studies investigating the effects of phthalate exposure on the human gut microbiota diversity are scarce. In a small prospective study, Yang et al. did not investigate α -diversity but found that the gut microbiota β -diversity of newborns, assessed from meconium and stool samples collected on days 1, 5, and 10, was not statistically different across DEHP exposure groups ($n = 25$) (Yang et al., 2019).

Single- and multi-chemical model (BKMR) results were consistent regarding associations with α -diversity, identifying the Σ DEHP metabolites, MEP and ohMHP urinary concentrations at 12 months as being associated with higher Shannon α -diversity.

The notion that high α -diversity is always beneficial is questioned in children (Shade, 2017), especially in relation to neurodevelopmental disorders; indeed several studies reported higher α -diversity in children with autism spectrum disorders aged between 2 and 12 years compared to neurotypical children (Chen et al., 2020; Ding et al., 2020; Li et al.,

2019; Zhai et al., 2019; Zou et al., 2020; Zurita et al., 2020). It has also been shown that specific phthalates can impact brain development and behavior in toxicological studies on animal models (Bakoyiannis et al., 2021; Gore et al., 2019), and epidemiological evidence in this regard is growing (Radke et al., 2020). Whether an effect of phthalates on child neurodevelopment could be partly mediated by alterations in the gut microbiota warrants further investigation.

Our results must be interpreted with caution. A possible source of bias relates to the fact that diet is a possible source of phthalates, specifically of high molecular weight phthalates such as DEHP, DiNP, BBzP, and DPHP; the introduction of solid food is also a well-documented source of increase of the gut microbiota diversity (Kumbhare et al., 2019); consequently, the association that we observed between the concentrations of some phthalates and gut microbiota diversity may be confounded by the introduction of solid food. For this reason, and although the period of introduction of solid food was not associated with α -diversity indices (Davias et al., 2024), we adjusted all our analyses for the period of introduction of solid food, assessed from questionnaires. In addition, we cannot discount the possibility that part of the associations (which implied exposures assessed at the same age as the gut microbiota) was due to reverse causality. Indeed, phthalate metabolism begins in the gastrointestinal tract, where it has been shown that DnBP and DEHP are initially hydrolyzed by intestinal esterase to monoesters, which are further transformed to their respective oxidative metabolites by intestinal cytochrome P450 enzymes (Choi et al., 2013; Ozaki et al., 2017). The gut microbiota may also be involved in xenobiotic metabolism (Abdelsalam et al., 2020). For example, Chiu et al. demonstrated

children. We assessed biomarkers of exposure to phthalates across 3 perinatal time windows, which allowed a unique and precise evaluation of phthalates exposure during crucial developmental stages. For exposure biomarkers with short half-life and high intra-individual variability such as the phthalate metabolites analyzed in this work, the within-subject biospecimen pooling method on which we relied has demonstrated, for a given sample size, to reduce bias in the effect estimates and to enhance power, in comparison with studies of similar sample size that depend on a single urine sample per participant (Perrier et al., 2016; Vernet et al., 2019).

A potential limitation is that we studied the impact of perinatal exposure to a large number of chemicals measured during three different exposure windows, resulting in multiple tests of associations, which is why we corrected for multiple testing. Note that we only corrected for tests related to phthalate and DINCH families, ignoring previous tests on phenols and per- and poly-fluoroalkyl substances (Davias et al., 2024). In spite of the relatively large sample size compared to the existing literature, the power of our study was consequently probably low. In debates concerning the necessity of adjustment for multiple comparisons in environmental epidemiology and toxicology, some authors argue that corrections for multiple comparisons may not be essential if the comparisons are all pre-planned, selected, reported, and considered in the interpretation of the results (Garofalo et al., 2022; Perneger, 1998; Rothman, 2014, 1990), as was the approach in our study. Although the analyses accounted for potential confounders, the possibility of unmeasured and residual confounding remains. Notably, a more precise adjustment of the associations for diet than what the SEPAGES cohort data allowed could have improved the models, as the child diet is likely a significant source of bias, as mentioned earlier; and this study did not consider exposure to chemicals other than phthalates. Cross-sectional associations between biomarkers of exposure to phthalates at 12 months and child gut microbiota at the same age should be interpreted with caution due to the risk of reverse causality, as discussed above. Lastly, while 16S rRNA gene sequencing provided valuable insights, future studies using whole genome sequencing could assess functional changes and species-level impacts, which may be crucial for understanding the specific effects of phthalate exposure on the child gut microbiota.

5. Conclusion

Diversity of the gut microbiota in early life may possibly be influenced by exposure to some phthalates (DEHP, DEP, DPHP) or their metabolites. The relative abundance of certain taxa may also be influenced by DEHP or its metabolites. Our study is the first to examine the impact of perinatal exposure to phthalates and DINCH metabolites on the gut microbiota of 12-month-old children. The relationships observed were cross-sectional, meaning that reverse causality cannot be ruled out.

CRediT authorship contribution statement

Aline Davias: Writing – original draft, Methodology, Formal analysis. **Sarah Lyon-Caen:** Writing – review & editing, Supervision, Project administration, Methodology, Data curation, Conceptualization. **Mathieu Rolland:** Writing – review & editing, Methodology. **Nina Iszatt:** Writing – review & editing. **Cathrine Thomsen:** Writing – review & editing, Methodology. **Azemira Sabaredzovic:** Writing – review & editing, Conceptualization. **Amrit Kaur Sakhi:** Writing – review & editing, Methodology. **Celine Monot:** Writing – review & editing. **Yamina Rayah:** Writing – review & editing. **Zehra Esra Ilhan:** Writing – review & editing. **Claire Philippat:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Merete Eggesbø:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Patricia Lepage:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis,

Conceptualization. **Rémy Slama:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Ethics approval and consent to participate

The SEPAGES study was approved by an ethics committee (CPP: Comité de Protection des Personnes Sud-Est V) and the French data privacy committee (CNIL: Comité National Informatique et Liberté). Both mother and father of the expected child signed an informed consent form prior to inclusion.

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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.

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Abbreviations

ASVs	Amplicon Sequence Variants
BBzP	benzylbutyl phthalate
BMI	Body Mass Index
CNIL:	Commission Nationale de l'Informatique et des Libertés
CPP	Comité de Protection des Personnes
cxMiNP	mono-4-methyl-7-carboxyethyl phthalate
DnBP	di-n-butyl phthalate
DEHP	di(2-ethylhexyl) phthalate
DEP	diethyl phthalate
DiBP	di-iso-butyl phthalate
DINCH	di(isononyl) cyclohexane-1,2-dicarboxylate
DiNP	di-isononyl phthalate
DNA	DeoxyriboNucleic Acid
DPHP	bis(2-propylheptyl) phthalate
FOSAs	fluorooctane sulfonamides
GUMME	Gut Microbiota in early childhood and Maternal Environmental exposures
MnBP	mono-n-butyl phthalate
MBzP	monobenzyl phthalate
MEP	monoethyl phthalate
MiBP	mono-isobutyl phthalate
MECPP	mono(2-ethyl-5-carboxypentyl) phthalate
MEHHP	mono(2-ethyl-5-hydroxyhexyl) phthalate
MEHP	mono(2-ethylhexyl) phthalate
MEOHP	mono(2-ethyl-5-oxohexyl) phthalate
MMCHP	mono-2-carboxymethyl hexyl phthalate
NIPH	Norwegian Institute of Public Health
ohMINCH	2-(((hydroxy-4-methyloctyl)oxy)carbonyl) cyclohexanecarboxylic acid
ohMiNP	mono-4-methyl-7-hydroxyoctyl phthalate
ohMPPH	6-hydroxy monopropylheptyl phthalate
OTU	Operational Taxonomic Unit
oxoMINCH	2-(((4-methyl-7-oxooctyl)oxy)carbonyl) cyclohexanecarboxylic acid
oxoMiNP	mono-4-methyl-7-oxooctyl phthalate
SEPAGES cohort	Suivi de l'Exposition à la Pollution Atmosphérique durant la Grossesse et Effet sur la Santé

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2024.125204>.

Data availability

The authors do not have permission to share data.

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