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Phthalate exposure alters gut microbiota composition and IgM vaccine response in human newborns

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ABSTRACT

Postnatal exposure to di-(2-ethylhexyl) phthalate (DEHP), a common plasticizer, is associated with allergy development in childhood, suggesting that DEHP exposure may dysregulate immune response in infants. We investigated whether DEHP exposure in newborns through medical treatment affected the gut microbiota pattern and vaccine response, which are both related to immune development. In this prospective cohort study from May 1, 2016 through July 31, 2017, newborns with respiratory distress who were given intravenous infusions (IVs) were enrolled as the DEHP group, and newborns who did not receive IVs were enrolled as the control group. We excluded patients with perinatal maternal probiotics, vaginal delivery, antibiotic treatment, and exclusive human milk or formula feeding. Of 118 infants, urinary phthalate metabolite analysis revealed that the calculated DEHP concentrations of the newborns treated with IVs ($n = 15$) were higher than those in the control group ($n = 10$) ($p = 0.0001$). DEHP exposure altered bacterial communities both in composition and diversity, particularly decreases in *Rothia* sp. and *Bifidobacterium longum* in the DEHP group. Furthermore, DEHP exposure significantly enhanced anti-HBsAg-IgM responses in the DEHP group ($p = 0.013$). Early-life DEHP exposure alter gut microbiota of newborns and may change their immune responses in later life.

1. Introduction

Di-(2-ethylhexyl) phthalate (DEHP), a ubiquitously distributed environmental endocrine disruptor (EED), is mainly used as plasticizers to increase the flexibility, transparency, durability, and longevity of substances (Hauser and Calafat, 2005). Due to advances in medical devices and medical treatment, newborn babies in neonatal intensive care units (NICUs) can be exposed to polyvinyl chloride (PVC)-made plastic medical devices that often contain DEHP, including endotracheal tubes, blood transfusion bags, nasogastric tubes, orogastric tubes, and intravenous infusions (IVs) (Loff et al., 2000). It has been shown that exposure to DEHP in NICUs, and especially in critical preterm infants, is much higher than that in adults (Calafat et al., 2004). Although there are no reports of adverse effects, an investigation on the impact of such iatrogenic DEHP exposure in this vulnerable population is needed.

In addition to developmental and reproductive systems (Lyche et al., 2009), DEHP may affect immune development and immune responses *in vitro* and *in vivo*. Its exposure may potentially alter the

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Abbreviations: Anti-HBsAg-IgM, anti-hepatitis B surface antigen IgM; DEHP, di-(2-ethylhexyl) phthalate; EED, environmental endocrine disruptor; HBV, hepatitis B virus; IVs, intravenous infusions; PND, postnatal day; rRNA, ribosomal RNA gene; TDI, tolerable daily intake

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function of neutrophils and stimulate the proinflammatory activity of non-immune cells in neonates *in vitro* (Vetrano et al., 2010). In addition, using an established asthma model, DEHP was shown to enhance allergen-specific airway inflammation via increases in Th2 and Th17 activity (Alfardan et al., 2018). Moreover, DEHP exposure has been shown to affect macrophage polarization in the context of melanoma tumor development (Lee et al., 2018). Taken together, these studies suggest that DEHP exposure via medical treatment may modify immune responses in newborns; however, direct evidence and the underlying mechanism are still lacking.

The development of the immune system in infants is largely shaped by the intestinal microbiome (Ahern et al., 2014). Melli et al. reviewed 21 studies in an investigation on the relationship between the intestinal microbiota and allergic diseases, and the results showed that an altered pattern of early-life microbiota was associated with the development of allergic diseases later in life (Melli et al., 2016). Recently, the abundance of the bacterial species *Faecalibacterium* sp., *Lachnospira* sp., *Veillonella* sp., and *Rothia* sp. (collectively abbreviated as FLVR) has been reported to be decreased in children with asthma (Arrieta et al., 2015). In addition, inoculating germ-free mice with FLVR resulted in obvious improvements in airway inflammation in their adult progeny, indicating the important role of these four bacterial taxa in the development of asthma later in life (Arrieta et al., 2015). However, the relationships between DEHP exposure, allergic disease and gut microbiome are still unknown.

Increasing evidence suggests that interactions between the intestinal microbiome and the immune system are particularly important in early life, as they both develop concurrently during early infancy (Zimmermann and Curtis, 2018). One previous study in infants reported consistent observation (Huda et al., 2014), in that a higher relative abundance of *Actinobacteria* and *Firmicutes* phyla was associated with higher levels of antigen-specific IgA or IgG in response to oral or parenteral vaccines; while a higher relative abundance of *Proteobacteria* and *Bacteroidetes* phyla were associated with lower responses. This suggests that the interplay between the microbiome and immune system may significantly contribute to vaccine effectiveness during infancy. However, the effects of DEHP exposure on developing microbiome and vaccine response in newborns are largely unknown.

The design of the present study included a prospective cohort, environmental exposure assessment, and hepatitis B virus (HBV) vaccine at birth. The results showed that the newborns exposed to DEHP through IVs developed an altered gut microbiota composition and enhanced plasma anti-hepatitis B surface antigen (HBsAg) IgM titers compared to controls. This study provides evidence supporting a direct link between early-life phthalate exposure and aberrant responses in the microbiome and against vaccines.

2. Materials and methods

2.1. Study subject enrollment

We enrolled newborns who were born at E-Da Hospital in southern Taiwan from May 1, 2016 through July 31, 2017 and who were diagnosed with respiratory distress by neonatologists requiring admission to a ward. The enrolled newborns who needed oxygen (O2) supplementation and supplied with IV fluids (Nako No. 5 inj., Tainan, Taiwan) starting from postnatal day 1 (PND1) to PND5 were classified as the DEHP exposure group, and those who needed O2 supply but without IV fluids were classified as the control group. The main components of the IV fluid are sodium chloride (0.82 mg/mL), sodium acetate anhydrous (1.8 mg/mL), potassium acetate (0.59 mg/mL), magnesium chloride $6H_2O$ (0.3 mg/mL), potassium phosphate monobasic (1.63 mg/mL), and dextrose monohydrate (100 mg/mL). All newborns were inoculated with recombinant hepatitis B vaccine (ENGERIX-B, GlaxoSmithKline Biologicals s.a., Rixensart, Belgium) at PND1. Urine samples were collected at PND3 for phthalate metabolite analysis, stool samples were collected at PND1 (meconium, T1), PND5 (T2), and PND10 (T3) for 16S ribosomal RNA gene (rRNA)-based next-generation sequencing (NGS) analysis, and blood samples were collected at PND8. We strictly controlled the factors which are known to significantly affect the gut microbiota in infants.

The clinical parameters of study subjects were recorded, including maternal gestation age, probiotics used by the mothers and newborns, mode of delivery and feeding, newborn gender, birth body weight, and perinatal antibiotics used. In order to observe the effect of transient DEHP exposure in the neonates, we strictly controlled the factors which are known to significantly affect the gut microbiota in infants. Therefore, the exclusion criteria included perinatal maternal probiotic consumption (Grzeskowiak et al., 2012), vaginal delivery (Huurre et al., 2008), newborns who received antibiotic treatment (Koenig et al., 2011), and newborns who exclusively received human milk or formula feeding during the hospital course (Pannaraj et al., 2017). The infant formula used to feed the newborns was from the same brand. The newborns of mothers who underwent caesarean sections and those with gestational ages between 35–40 weeks were included. At our hospital, all mothers who deliver newborns through caesarean section are treated with antibiotics (cephalosporin, Standard Chem & Pharm Co., Ltd, Taiwan) for medical purposes. The newborns with incomplete data were excluded. In total 25 newborns were enrolled, of whom 10 did not receive IV fluids (the control group), and 15 received IV fluids (the exposure group). All of the mothers of the enrolled newborns were HBsAg negative and HBeAg negative.

All eligible subjects were enrolled in the study after signing the informed consent approved by the respective recruitment hospitals. The study protocol was approved by the Institutional Review Boards of E-Da hospital (EMRP47104N). The study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki. Written informed consents were obtained from all enrolled mothers.

2.2. Detection of urinary phthalate metabolites

Nine phthalate monoester metabolites and their parent chemicals are summarized and abbreviated in Supplementary Table 1. Analysis of spot urine samples from the newborns at PND3 was performed according to a previous study (Wu et al., 2013). In brief, urine samples (1 mL) were thawed, transferred to a glass tube, added to a$^{13}C_4$-labelled internal standard of each phthalate metabolite (Cambridge Isotope Laboratories, Andover, MA, USA), and then buffered with 250 μL of ammonium acetate (1M, pH 6.5) and β-glucuronidase enzyme (from *E. coli*, K12, Roche Biomedical, Mannheim, Germany) (Huang et al., 2015a, 2015b). The samples were incubated in a 37 °C water bath for 90 min. After hydrolysis, each sample was acidified by adding 2 mL of phosphate buffer (0.14 M NaH2PO4 in 0.85% H3PO4), vortex-mixed, and centrifuged at 3500 rpm for 10 min. The supernatant was loaded into a solid-phase extraction cartridge (NEXUS, Agilent, Inc., Palo Alto, CA, USA). Formic acid (2 mL) and water (1 mL) were added to the cartridge to remove hydrophilic compounds, and then acetonitrile (2 mL) and ethyl acetate (2 mL) were added to elute the phthalate metabolites. The combined elutes were concentrated under a stream of dry nitrogen at 55 °C. Finally, the residues were reconstituted with 200 μL water and subjected to LC-MS/MS for analysis. For all detection and quantification analyses of the analytes, a Waters ACQUITY UPLC system (Waters Corporation, Milford, MA) coupled with a tandem MS (Finnigan TSQ Quantum Ultra triple-quadrupole MS, Thermo Electron, San Jose, CA, USA) in combination with Xcalibur software (Thermo-Finnigan, Bellefonte, PA, USA) was used.

2.3. Daily DEHP exposure

We calculated daily DEHP exposure from single urine samples at age 0 based on the equation according to a previous study (Kohn et al., 2000), where the creatinine excretion rate for age 0 was measured

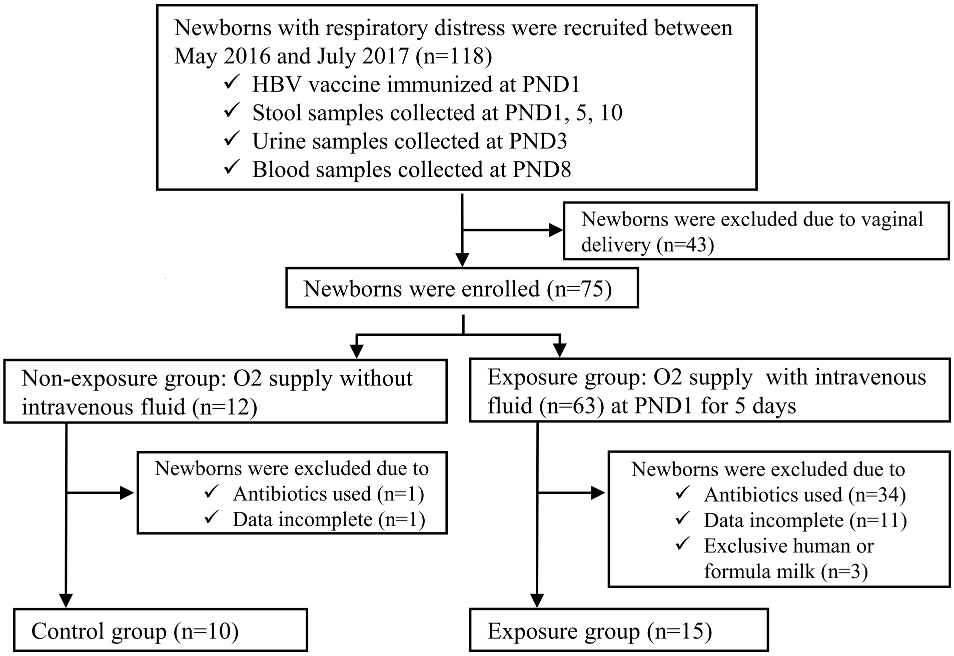

Fig. 1. Flowchart of the recruitment process of newborns. Mothers were invited to participate in the study if their newborns had clinical symptoms of respiratory distress. During the hospital course, urine and stool samples were collected for phthalate metabolite analysis and 16S rRNA gene-based NGS analysis, respectively. Blood samples were collected for detecting anti-hepatitis B surface antigen IgM. HBV: hepatitis B virus; PND: postnatal day.

according to a previous report (Mage et al., 2008) and normalized by body weight.

2.4. *Stool DNA extraction, library construction and sequencing*

DNA was extracted from approximately 200 mg of stool, adjusting the amount of buffers in proportion to the amount of stool being isolated using an EZNA Stool DNA kit (D4015; OMEGA Bio-Tek Inc., Norcross, GA, USA) according to the manufacturer's instructions. 16S rRNA gene amplification and library construction were performed according to the protocols provided by Illumina (https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html). Briefly, the V3–V4 region of bacterial 16S rRNA genes were amplified with the universal bacterial primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) containing Illumina overhang adapter sequences in the forward (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′) and reverse (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′) primers using a limited cycle PCR. Illumina sequencing adapters and dual-index barcodes were added to the amplicon target using a Nextera XT Index kit. Quantification and quality of the libraries were checked using a QSep100 Analyzer (BiOptic Inc., Taiwan). Finally, the libraries were normalized and pooled in an equimolar ratio and sequenced with an Illumina Miseq sequencer. Sequence reads have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB27307.

2.5. *Blood anti-HBsAg-IgM*

Human anti-HBsAg-IgM was measured and calculated according to the manufacturer's protocol (ADI cat#4205, Alpha Diagnostic Intl. Inc., San Antonio, TX, USA). In brief, plasma was first diluted with Working Sample Diluent along with a positive control and a calibrator and then incubated in an HBsAg-coated plate, followed by incubation with horseradish peroxidase conjugated anti-human IgM. After substrate incubation, absorbance was measured and represented as total activity units (net optical density (OD) unit multiplied by a dilution factor), and the net OD unit was defined as A450 OD - A630 OD.

2.6. *Sequence data analysis and statistical analysis*

We used the chi-square test to analyze categorical data and the Mann-Whitney *U* test for numerical data. The 16S rRNA gene sequencing data were analyzed as follows. The universal primers were removed from the demultiplexed paired reads using Cutadapt (v1.12; https://github.com/marcelm/cutadapt). The sequences were then processed using the DADA2 workflow (v1.6; http://bioconductor.org/packages/dada2/) in the R environment. Briefly, filtering, trimming and dereplication were performed on the forward and reverse reads independently, then reads were subjected to the denoise algorithm. Finally, the paired reads were merged and required a minimum 20 bp overlap, and chimeras were subsequently removed. The inferred ribosomal sequence variants (SVs) were subjected to taxonomy assignment using the SILVA database (v128; http://www.arb-silva.de) as the reference with a minimum bootstrap confidence of 80. Multiple sequence alignment of the SVs was performed using DECIPHER (v2.6.0; http://bioconductor.org/packages/DECIPHER/), and a phylogenetic tree was constructed from the alignment using phangorn (v2.3.1; https://cran.r-project.org/package=phangorn). A phyloseq object was created using the frequency table, taxonomy assignment and phylogenetic tree information, and community analyses were performed using phyloseq (v1.19.1; http://bioconductor.org/packages/phyloseq/). Raw abundance was converted to normalized abundance using the getVarianceStabilizedData() function of DESeq2 (v1.18.1; http://bioconductor.org/packages/DESeq2/) after converting the phyloseq data into a DESeq2 object using the phyloseq_to_deseq2() function. The exact Wilcoxon rank-sum test (Mann-Whitney *U* test) was performed to detect differentially abundant taxonomic ranks between the control and exposure groups. Unsupervised consensus hierarchical clustering was performed using ConsensusClusterPlus (v1.38.0; https://bioconductor.org/packages/ConsensusClusterPlus/), and the results were visualized as a heatmap representation generated using ComplexHeatmap (v1.12; http://bioconductor.org/packages/ComplexHeatmap/). We analyzed sample relatedness by performing principal coordinate analysis (PCoA), based on the weighted Unifrac metric and Jaccard distance.

Table 1
Characteristics between the control and DEHP exposure groups.

Variables	Control (n = 10)	Exposure (n = 15)	p value
	n (%) or mean ± standard deviation		
Sex, Male (%)	4 (16%)	9 (36%)	0.33
Birth weight (g)	2642.0 ± 361.0	2785.0 ± 400.2	0.20
Body weight at PND9 (g)	2642.0 ± 320.7	2764.0 ± 374.5	0.51
Gestational age (weeks)	37.5 ± 0.7	37.5 ± 1.4	0.46
Volume of human milk (mL)[a]	758.0 ± 520.7	812.8 ± 831.8	0.43
Volume of formula (mL)[a]	2724.0 ± 635.9	2209.2 ± 945.6	0.08
Volume of IV fluid (mL)[a]	–	457.9 ± 211.5	NA[b]
Total fluid intake (mL)[a]	3482.0 ± 292.7	3479.9 ± 430.0	0.50

[a] Total fluid volume on the first 9 days after birth.

[b] NA: not applicable.

3. Results

3.1. Newborns who received IV fluid treatment had high urine levels of DEHP metabolites

As several factors can significantly affect the development of an infant's gut microbiota (Ahern et al., 2014), the mother-infant pairs were selected under stringent conditions as shown in Fig. 1. Newborns that were delivered by caesarean section, did not receive antibiotics or probiotics, and received mixed feeding were selected. As shown in Table 1, except for the administration of IV fluid, there were no significantly differences in gestational age, newborn birth weight, and volume of milk consumed between the control and exposure groups. To identify the possible intragenic phthalate components from IV fluid treatment in the newborns, we initially analyzed the distributions and concentrations of nine phthalate metabolites (list and abbreviations shown in Supplementary Table 1) in the urine collected at 3 days after IV fluid treatment. In the exposure group, four urinary creatinine-adjusted DEHP metabolites were significantly higher than those in the control group, including MEHP, MEOHP, MEHHP, and MECPP (Fig. 2A). Thus, the level of daily DEHP exposure (ΣDEHP) calculated according to these four metabolites was significantly higher in the exposure group than in the control group (Fig. 2B). The exposure level of DEHP in the newborns with IV fluid treatment ranged from 1.26 to 27.25 μg/kg body weight/day, which is still below the human tolerable daily intake (TDI) dose of 50 μg/kg body weight/day as determined by the European Food Safety Authority (EFSA) (SCENIHR., 2015). In contrast, the metabolites for the other five phthalates were non-detectable in all samples (data not shown). These data suggested that IV fluid was the major source of phthalate chemicals in the newborns who were admitted to hospital, and that DEHP was the main phthalate chemical involved.

3.2. Intravenous DEHP exposure altered the gut microbiota in the newborns

We further investigated the effect of DEHP on the developing gut microbiome. To assess the dissimilarity (β diversity) among bacterial communities, the unweighted (qualitative) and weighted (quantitative) UniFrac distances were visualized with PCoA plots (Fig. 3A and B), and Jaccard's similarities using NMDS plot (Fig. 3C). Time-dependent differences were detected where microbiota from the meconium samples (T1) were distinct from those in later feces samples (T2 and T3). Samples from both the control and DEHP groups did not form well-defined clusters, suggesting the structure of microbiota following DEHP exposure were not significant changed. The *Firmicutes*-to-*Bacteroidetes* ratios were significantly changed over time in both groups, but not between the two groups (Fig. 5A). Nonetheless, when comparing the weighted UniFrac and Jaccard measures between time points, there were significant differences between the control and exposure groups at T1-T2 (Fig. 3B and C). In both measures, the differences in β diversity between T1 and T2 were lower in the exposure group. This suggests that the bacterial composition in the infants from the exposure group had a significantly lower variability than that in the controls during the first 5 days. In addition, the significant differences in the unweighted measures between T1 and T3 suggested that the infants from the exposure group attained more bacterial taxa exclusive to T3 than the controls over time (Fig. 3A). The DEHP-exposed microbiota also showed higher dispersion (Fig. 3C), indicating higher variability in the abundant bacteria taxa with respect to the controls.

The DESeq2 statistical testing framework was used to identify differential abundant ribosomal sequence variants (RSVs) in the control and exposure groups. Consensus clustering was used to cluster the 48 differentially abundant RSVs and 75 samples (see Fig. 4 for the heatmap). All meconium samples (T1) had similar abundance profiles of the 48 RSVs (Cluster 1). The majority of the control samples were in a single cluster (Cluster 2), whereas the exposed samples had more variations in the abundance of RSVs and formed three clusters (Cluster 3, 4 and 5). The heatmap showed that the control microbiota had higher abundances of *Bifidobacterium longum* (SV011, SV017), *Klebsiella* (SV014 and SV027), *Streptococcus* (SV015, SV022, SV025) and *Rothia* (SV054), and that some controls had higher abundances of *Bifidobacterium breve* (SV018), *Enterococcus faecium* (SV070), and *Veillonella* (SV031, SV049, and SV077). These bacteria are generally considered to be normal gut commensals and/or beneficial bacteria (Arrieta et al., 2015; Matamoros et al., 2013). On the other hand, Cluster 3 of the DEHP-exposed microbiota lacked these bacterial taxa and were enriched with bacteria due to extrinsic influences and/or opportunity pathogens such as *Haemophilus parainfluenzae* (SV065) and *Staphylococcus* (SV052, SV078, SV085) (Castano-Rodriguez et al., 2018; Dominguez-Bello et al., 2010; Matamoros et al., 2013). In addition, Cluster 5 of the DEHP-exposed microbiota was enriched with several unclassified *Enterobacteriaceae* bacteria (SV012, SV020, SV045). Cluster

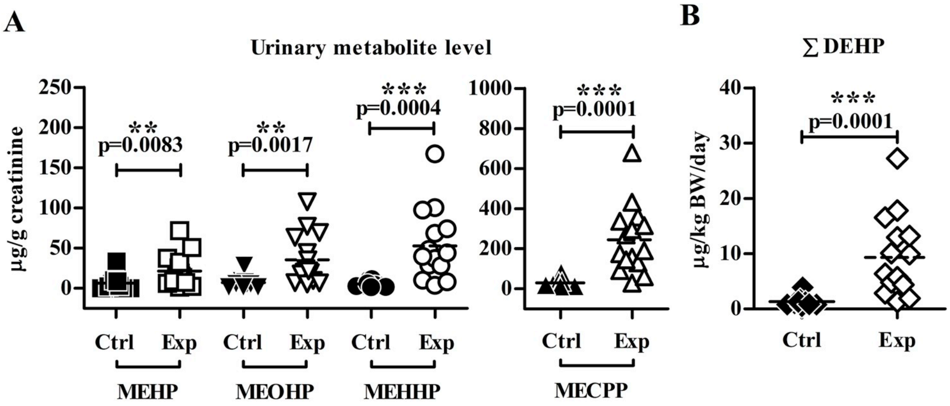

Fig. 2. Newborns who received IVs showed higher DEHP exposure than controls. Analysis of urinary phthalate metabolites in control (Ctrl) and exposure (Exp) groups. A, The levels of DEHP metabolites were normalized to urinary creatinine levels of the corresponding samples from the newborns at PND3. B, The calculated daily DEHP exposure level (ΣDEHP) in the newborns at PND3. $^{**}p < 0.01$ and $^{***}p < 0.001$ by the Mann-Whitney *U* test. BW = body weight; 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.

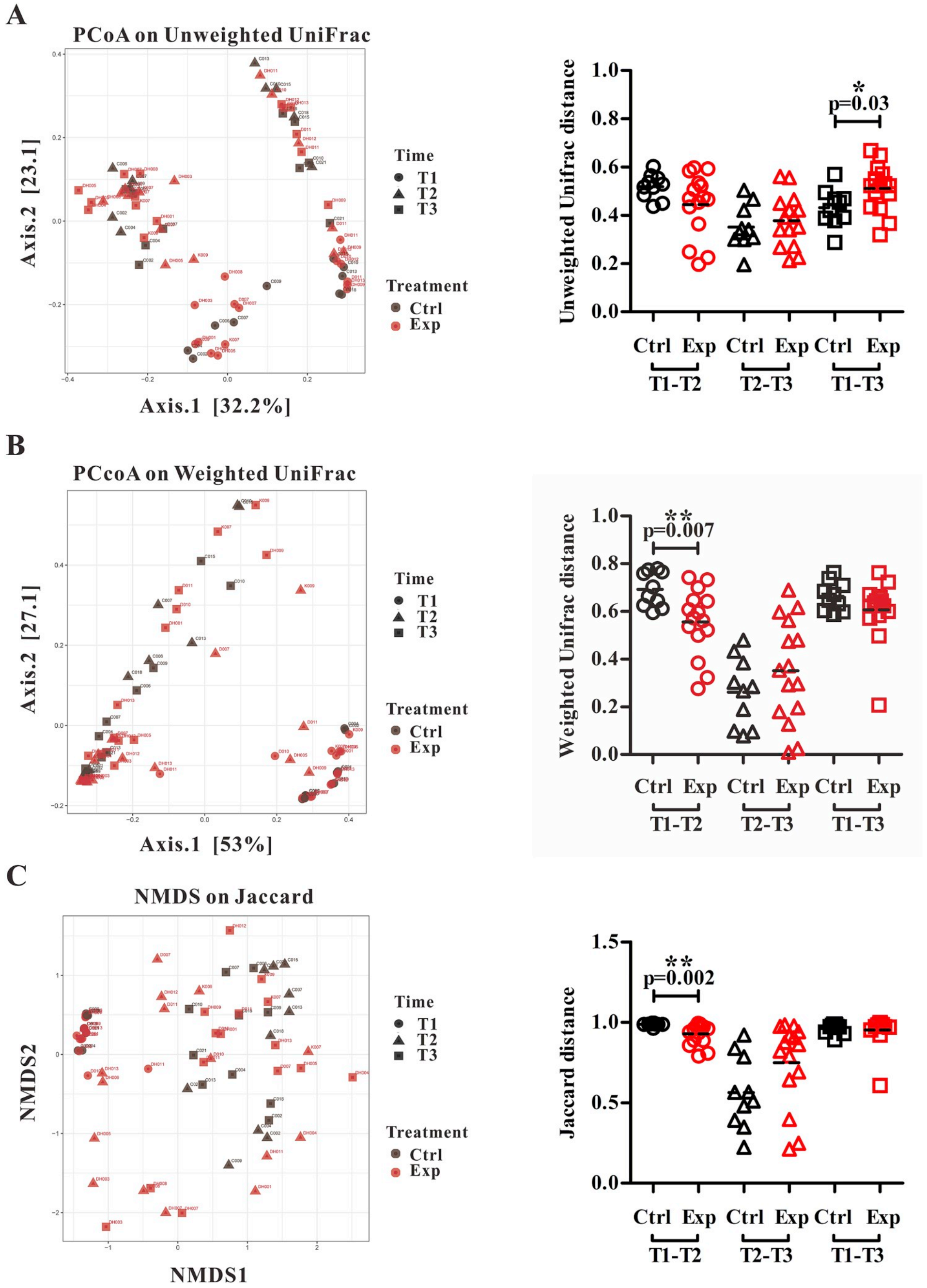

Fig. 3. 16S rRNA gene analysis revealed alterations of the infant gut microbiota after DEHP exposure. Fecal samples at PND1 (T1, before exposure), PND5 (T2, during exposure), and PND10 (T3, after exposure) were collected to perform 16S rRNA gene-based NGS analysis. Non-metrical multidimensional scaling (NMDS) and principal coordinates analysis (PCoA) were used to investigate β diversity among bacterial communities. The experimental groups are indicated as follows: control (Ctrl), DEHP group (Exp). The unweighted (qualitative) (A) and weighted (quantitative) UniFrac distances (B) were analyzed and plotted using the classical multidimensional scaling PCoA approach. (C) Jaccard's distances were ordinated by NMDS. *p < 0.05 and **p < 0.01 by the Mann-Whitney *U* test.

4 of the DEHP-exposed microbiota was more similar to the control microbiota with respect to the abundance of the 48 RSVs, but lacked key bacteria such as *Bifidobacterium* and *Veillonella* and had a high abundance of *Escherichia/Shigella* (SV004).

3.3. A low abundance of allergy-associated gut microbiota in the newborns with intravenous DEHP exposure

A previous study reported that the transiently decreased FLVR levels in 3-month-old infants were at a high risk of developing asthma (Arrieta et al., 2015). In our study, *Rothia* increased rapidly and was detectable in fecal samples from PND5 newborns; however, it was significantly lower in the DEHP-exposed newborns (T2; p = 0.003), but reached similar level in DEHP groups compared to the controls (T3) (Fig. 5B left). Although *Veillonella* started to appear after birth and was consistently elevated between PND5 and PND10 in two infants in the control group, DEHP exposure did not significantly affect its level in our study (Fig. 5B right). Neither *Faecalibacterium* nor *Lachnospira* colonized the infant guts at the first 10 days. In addition, the level of *Bifidobacterium longum* was significantly decreased after DEHP exposure compared to the controls (T3, p = 0.0319) (Fig. 5C). It has been reported that *Bifidobacterium* colonization is associated with normal gut development (Matamoros et al., 2013). Furthermore, we observed transient increase of *Staphylococcus* and decrease of *Streptococcus* in the newborns exposed to DEHP (Fig. 5D). Taken together, the fecal microbiota from controls were comprised of more normal commensals than the DEHP-exposed group, and the DEHP-exposed microbiota had more opportunistic pathogens, a warning sign of an unhealthy gut.

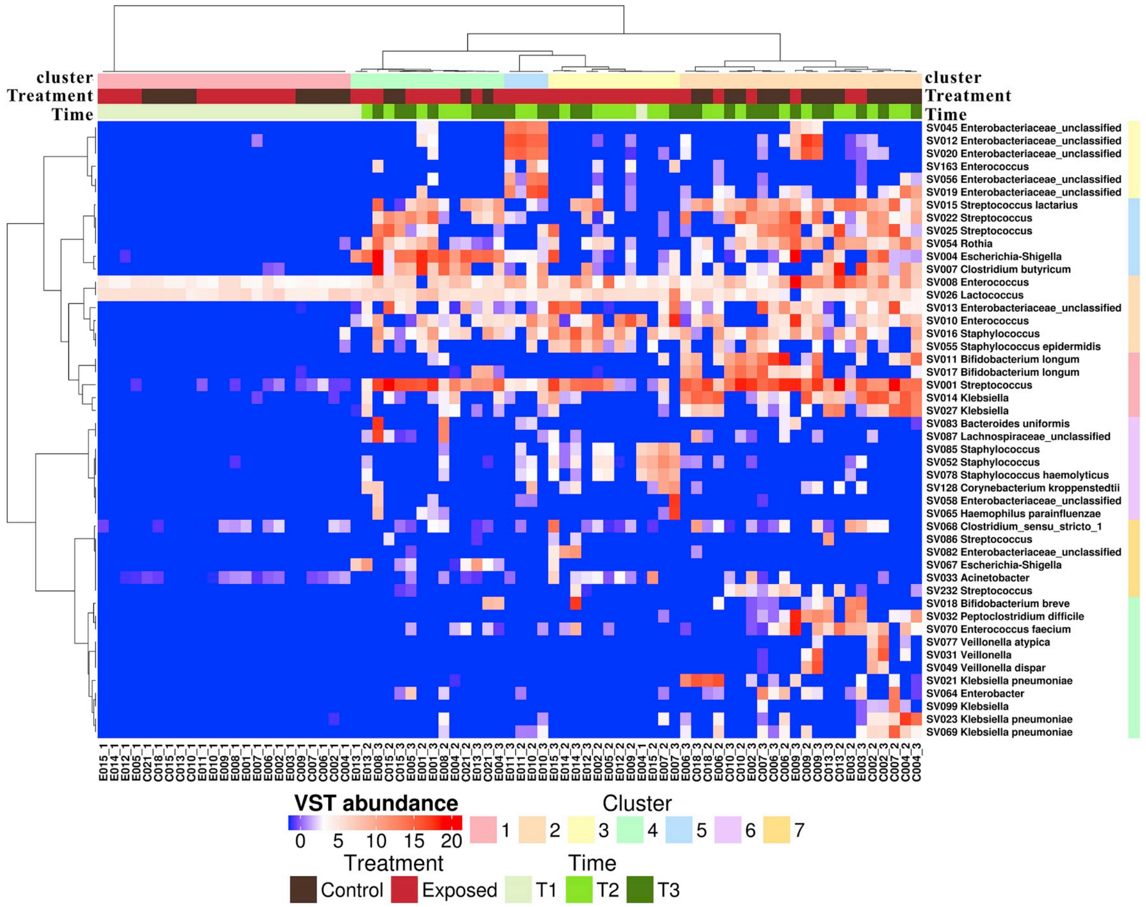

Fig. 4. Heatmap revealed changes in infant gut microbiota composition after DEHP exposure. Fecal samples collected from unexposed infants (control group) were labelled as C1 to C3 and DEHP exposed infants as E1 to E3 at PND1 (T1), PND5 (T2), and PND10 (T3), respectively. The heatmap shows the changes in the abundance of 34 ribosomal sequence variants (SVs) after DEHP exposure.

3.4. Elevated level of anti-HBsAg-IgM in the newborns with intravenous DEHP exposure

We then examined the effect of postnatal DEHP exposure through IVs on primary immune responses against hepatitis B vaccine. The hepatitis B vaccine has been included in vaccine immunization schedules for newborns within 24 h of birth in Taiwan since 1986. None of the mothers of the enrolled infants had active HBV infection in the present study, and IgM cannot cross the placenta during pregnancy. Therefore, anti-HBsAg-IgM could only be produced by the newborns themselves, and consequently we used the level of anti-HBsAg-IgM at PND8 to examine the effect of intravenous DEHP exposure. As shown in Fig. 6, although the titers of anti-HBsAg-IgM were still low 1 week after immunization, intravenous DEHP exposure significantly enhanced the titers of antigen-specific IgM compared to the controls, suggesting an aberrant primary antibody response in response to DEHP exposure in these newborns.

4. Discussion

Epidemiologic studies have suggested associations between chronic exposure to phthalates, and particularly DEHP, and the risk of allergies and asthma in children (Kolarik et al., 2008); however the mechanisms of action remain unclear and the causal relationship has not been established. The present human study suggests that exposure to low doses of DEHP through medical treatment in newborns may result in transient gut microbial dysbiosis and an aberrant primary immune response against vaccines. The finding of microbial dysbiosis is consistent, at least in part, with a previous report in which the transient reduction in gut FLVR in early infancy significantly increased the risk of developing asthma in children (Arrieta et al., 2015). However, the link between DEHP-mediated early-life gut microbial dysbiosis and altered immune development still needs to be investigated in future studies.

DEHP, a ubiquitously distributed EED, seems to have a more profound effect on fetuses or neonates than adults even at environmentally relevant doses (Jahreis et al., 2018). This study provides evidence that medical procedures such as IVs are also an important source of DEHP exposure in newborns (Fig. 2). Development of the gut microbiome in newborns can be affected by various factors including delivery mode, type of feeding, and the use of probiotics and antibiotics (Matamoros et al., 2013; Pannaraj et al., 2017). In order to observe the likely transient and altered microbiota pattern, we applied several criteria to limit variations in composition in the newborns at the start of the study (T1). After controlling for confounding factors, the result showed that short-term exposure to DEHP promoted transient gut microbial dysbiosis in the newborns, suggesting that long-term exposure to DEHP may persistently alter the gut microbiota pattern as newborns and children are continually exposed to DEHP through breast milk (Lin et al., 2011), food (Ji et al., 2014), PVC-made toys, and house dust in their daily life (Kolarik et al., 2008).

As hypothesized, we found intravenous DEHP exposure transiently altered the gut microbiota composition in the newborns, even though the estimated exposure level of DEHP was less than the human TDI of 50 μg DEHP/kg body weight/day (SCENIHR, 2015). Although this level of exposure did not affect the dominant bacterial phyla composition, the *Firmicutes*-to-*Bacteroidetes* ratios were significantly changed with time in both groups (Fig. 5A). This suggests that the intestinal microbiota undergoes maturation starting from birth (Mariat et al., 2009). On

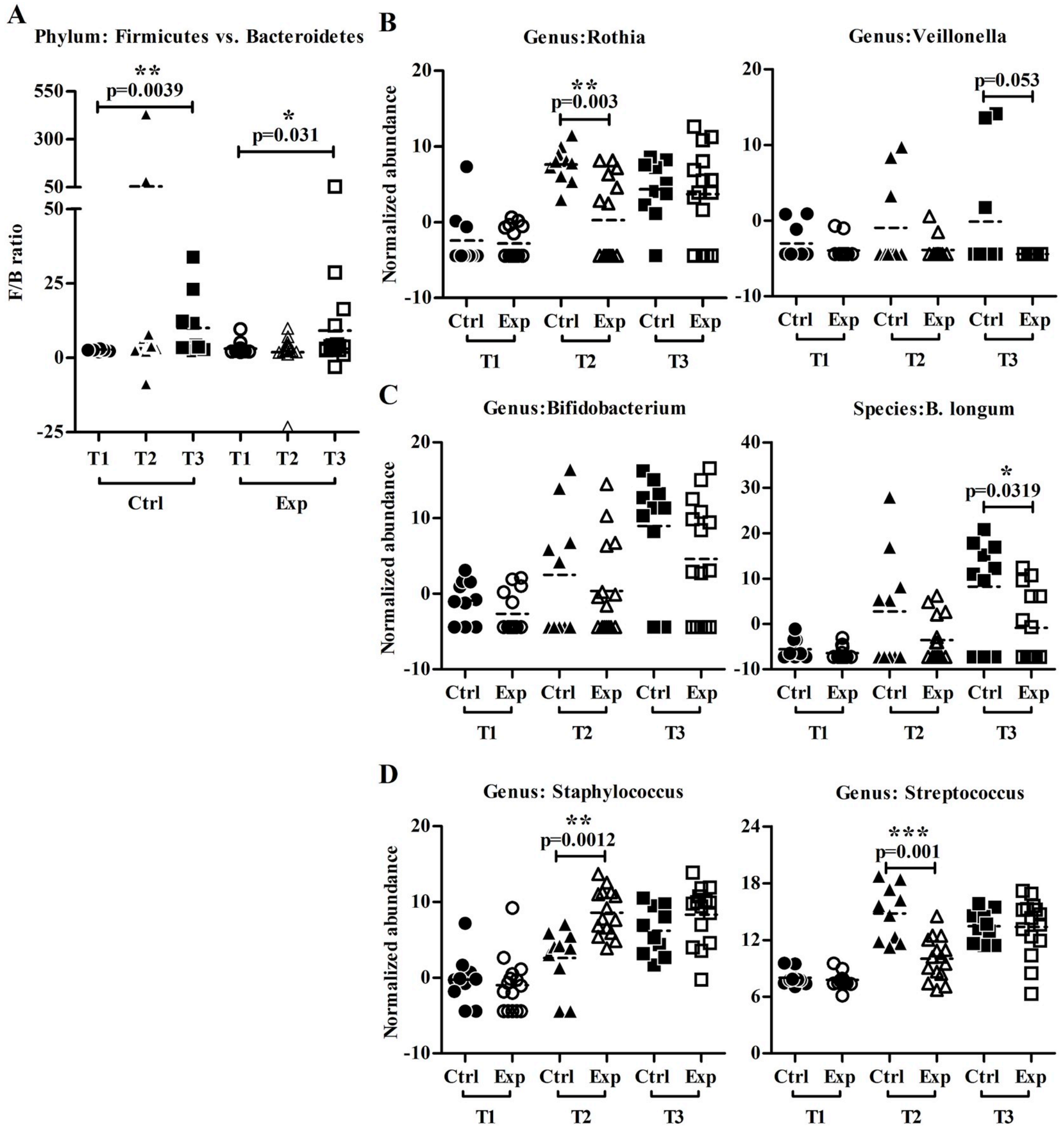

Fig. 5. The abundance of selected gut bacterial genera and species associated with childhood allergy. (A) *Firmicutes*-to-*Bacteroidetes* ratios in the control (Ctrl) and DEHP-exposed groups (Exp). (B–D) Normalized abundance of selected gut bacterial genera and species as indicated. X-axis represents different time points in the two groups. The horizontal line marks the mean value. Ctrl, n = 10; Exp, n = 15. $^*p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ by the Mann-Whitney *U* test.

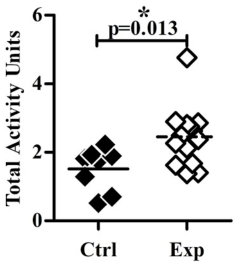

Fig. 6. The level of anti-HBsAg-IgM in the newborns. Analysis of plasma anti-HBsAg-IgM in the control (Ctrl) and exposure (Exp) groups. $^*p < 0.05$ by the Mann-Whitney *U* test.

the other hand, DEHP exposure promoted changes in several bacterial taxa as shown in the heatmap (Fig. 4). A previous study demonstrated that decreased levels of gut FLVR significantly promoted the development of asthma in children and a murine model (Arrieta et al., 2015). Our results showed that *Rothia* was significantly decreased during DEHP exposure (T2, Fig. 5B), and that *Veillonella* tended to decrease after DEHP exposure (T3, Fig. 5B, p = 0.053), whereas *Lachnospira* and *Faecalibacterium* were not yet observed in the newborns. Interestingly, the presence of *Rothia* and *Veillonella* in human milk has been associated with a lower incidence of asthma (Pannaraj et al., 2017). In addition to these four allergy-associated bacterial genera, we also observed a significant reduction in the abundance of *B. longum* after DEHP exposure through IVs (Fig. 5C). *B. longum* is generally considered to be probiotic. It has been shown to have beneficial effects on infant health to prevent or treat several disorders, including infection-induced behavior changes, obesity, allergic disease and celiac disease (Arboleya et al., 2016; Di Gioia et al., 2014). Furthermore, we also found changes in the abundance of *Staphylococcus* and *Streptococcus* during DEHP exposure (Fig. 5D), although the role of these two genera in the development of allergies or neonatal immunity is still controversial (Melli et al., 2016). Taken together, this study suggests that postnatal DEHP exposure, even below the TDI dose, may alter the gut microbiota and consequently affect the development of immunity later in life.

This study also showed that postnatal DEHP exposure through medical treatment, even below the TDI dose, promoted antigen-specific primary IgM responses in the newborns. Consistent with our observations, previous animal studies have shown that DEHP can act as an adjuvant to aberrantly enhance antigen-specific humoral immunity through oral (Guo et al., 2012) or inhalation routes (Larsen et al., 2007) at a relatively high dose. In asthma model, oral exposure to DEHP promoted IgE and IgG1 humoral immunity, at least in part by altering the function of T follicular helper cells, which play an important role in regulating B cell function (Han et al., 2014). In terms of DEHP exposure and IgM response in humans, this study is the first to show that intravenous DEHP exposure can alter IgM responses to vaccinations in newborns. However, it is difficult to analyze the anti-HBsAg-IgG affected by iatrogenic DEHP exposure in the newborns in this design setting due to the following two reasons. One is the fact that maternal anti-HBsAg-IgG is transported across placenta and not easily distinguished from endogenous anti-HBsAg-IgG in newborns (Wang et al., 2011). The other reason is that environmental DEHP through ingestion or inhalation would be significantly disturb the effect of iatrogenic DEHP exposure on endogenous anti-HBsAg-IgG after the newborns were discharged. Taken together, chronic exposure to DEHP at an environmental dose may affect not only primary immunity but also secondary responses. Further studies are needed to investigate the underlying mechanisms.

As the establishment of bacterial communities and development of the immune system is highly interactive during the early years of life (Ahern et al., 2014), intravenous DEHP-mediated alterations in IgM vaccine responses may, at least in part, be due to gut microbiota dysbiosis. Another possible reason is that DEHP and its metabolites may directly affect B cell homeostasis during infancy. This is supported by Bissonnette et al. who showed that MEHP at environmentally relevant doses can suppress the proliferation and induce the apoptosis of pro/pre-B cells *in vitro* (Bissonnette et al., 2008). Although *in vivo* evidence is still lacking, the impact of long-term exposure to DEHP on the development of microbiome and neonatal immunity requires further investigation.

There are two limitations to the present study. First, in order to control for confounding factors that affect gut microbiota, we excluded many subjects, resulting in a small number of cases. Interestingly, even with few participants, the results still showed that DEHP exposure significantly affected the composition of the microbiota and IgM responses to vaccinations in the newborns. Second, it is important to establish a neonatal animal model and/or to follow a birth cohort over a long period to elucidate the long-term impact of DEHP exposure on immune system. Our findings showed that IV-mediated DEHP exposure promoted IgM responses against the HBV vaccination in our newborns; however, it still cannot suggest that DEHP exposure may have a beneficial impact on vaccinations. This is because that immune responses and mechanisms are significantly different in the context of recombinant protein or attenuated live vaccine. The long-term impact of DEHP exposure on different types of vaccines requires further in-depth investigations.

5. Conclusions

Accumulating evidence shows that gut microbial dysbiosis is associated with an increasing number of diseases, suggesting that the development of a “healthy” microbiome in newborns may lead to a “good” start for a healthy adult life, including well-developed immune responses as well as a well-regulated metabolic machinery. This study provides evidence of the impact of ubiquitously distributed EEDs, such as DEHP, on the pattern of gut microbiota and its potential impact on of the health of the newborns.

Conflicts of interest

The authors declare that they have no competing interests.

Declaration of interests

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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Appendix A. Supplementary data

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.110700.

Authors’ contributions

YNY and JLS designed the study. YNY, YJY and SNY organized the cohort. YNY, CYW and YTS collected samples. YNY, YCY, IHL, YYC, and HYL conducted experiments and analyzed data. YNY, IHL, and JLS wrote the final manuscript. All authors read and approved the final manuscript.

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