Maternal Probiotic Therapy Protects Against DE-71-Induced Neurodevelopmental Delays in a Sex-Dependent Manner

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1 Introduction

Polybrominated diphenyl ethers (PBDEs) are anthropogenic environmental toxicants that form 209 theoretical congeners. PBDEs have been added commercially to a wide range of applications ranging from electronics, foams and carpets, construction, transportation, and textiles due to their flame-resistant chemical properties[1]. Despite legislative bans in Europe and a voluntary phase-out of production in the US starting in 2005, PBDE exposure remains an ongoing problem. The emission of PBDEs is predicted to continue until 2050; it's estimated that of the 70 kilotons of Penta BDEs and BDE 209 recycled since 1970, 45 kilotons are expected to reappear in new products due to increasing recycling rates; thus re-exposing the general population[2].

Human and animal studies have identified that the major health effects associated with PBDE exposures are endocrine disruption, reproductive and developmental toxicity, and neurotoxicity[3]. However, current knowledge of their effects on developmental benchmarks are limited. According to the Barker hypothesis, fetal growth is a determinant of perinatal and postnatal health, and low birth weight is associated with chronic disease later in life[4]; thus whether PBDEs may impact fetal growth is a public health concern. However, results of several studies on the relationship between PBDE body burden and growth measures are incongruent, reporting no association, positive, negative, or both depending on the congener studied[5]. In rodent studies, Kodavanti and colleagues found that developmental DE-71 exposure altered female offspring body weight from postnatal days (PNDs) 35-60[6]. Lower body weight was also found by our group in female mice maternally exposed to 0.1 mg/kg/d DE-71[7]. Therefore, these reports from humans and animals may indicate that PBDE effects on developing offspring depend on the congener and dose studied.
Growth is a complex process involving the interplay between genetic, nutritional, and hormonal factors including growth hormone (GH), insulin-like growth factors (IGF), and thyroid hormone (TH). PBDEs are well known disruptors of (THs) and insulin-like growth factors that are critical for fetal growth and development in humans and rodents. The hypothalamic-pituitary-thyroid (HPT) axis is particularly vulnerable to disruption by PBDEs,[6],[8] which bear structural homology to the circulating THs. Human studies examining the association between PBDE body burdens and plasma THs have found both positive[9] and negative correlations,[1] while most animal studies have shown that DE-71 reduces serum Thyroxine (T4) concentrations,[6][10]. Similarly, pregnant women show a negative correlation between PBDE body burden, especially BDE-100, -153, and circulating thyroid stimulating hormone (TSH)[10]. We previously reported perinatal exposure to low DE-71 reduces TH species in the female and male postnatal brain (Kozlova et al., 2021 *Organohalogen Compd*). *Therefore, based on these previous findings, we speculate that hypothyroid effects of early-life exposure to DE-71 are detrimental to the growth of developing offspring and reversal of these thyroid alterations warrants further study.*

An emerging influencer of life-long health is the gut microbiome[11]. Early life disruption of the microbiome by xenobiotics has been linked to onset of human disease later in life[12]. Developing infants who have a less complex microbiome may be particularly vulnerable to xenobiotic influence over susceptibility to disease later in life[13]. PBDEs have been associated with altered adult, maternal, and childhood microbiome profiles in humans and animals. Consumption of lactic acid bacteria has been shown to overcome GH resistance in children, while mice which consume the probiotic *Lactobacillus reuteri* (LR) resist age-associated atrophic changes of the thyroid gland and increase plasma levels of T4[14]. In this study, we examined the effects of maternal LR supplementation on offspring developmental milestones. We tested the hypothesis that LR protects offspring from developmental deficits induced by PBDEs. Mouse dams were exposed to DE-71 and LR concurrently and offspring were examined on postnatal days (PND) 2-30.

2 Materials and Methods

2.1 Developmental exposure to DE-71

Animals were housed in a controlled environment: temperature (21.1–22.8°C), light (12:12 light–dark; lights on at 0800 h). Humidity range was 20-70%. Mice had access to food and water *ad libitum*. Care and treatment of animals was performed in compliance with guidelines from NIH and approved by the University of California Riverside Institutional Animal Care and Use Committee. In brief, C57Bl6/N mouse dams (F0) were exposed to the penta-PBDE mixture, DE-71, via oral administration of 0 (VEH/CON) or 0.1 mg/kg/d (L-DE71) from 3 wk prior to gestation (GD) through end of lactation on postnatal day (PND) 21 as described[7]. Litters averaged 4-9 pups/dam and were not culled.

2.2 Culture and probiotic treatment

Lactobacillus reuteri MM4-1A ATCC-PTA-6475 (gift of BioGaia, Sweden) was cultured anaerobically with deoxygenated MRS media at 37 °C. Strain identity was confirmed by 16S amplification and Sanger 16S sequencing prior to experimental use. OD_{600} measurements were performed on a spectrophotometer and bacterial colony counting was performed to quantify bacteria cultured on LB agar. Regression analysis of the correlation between colony-forming units (CFU) and OD_{600} indicated that the experimental groups received ~$1x10^8$ CFU/mouse/day. The daily inoculation volume was normalized to the equivalent of 300 µL of OD_{600}= 0.4 culture. Dams were orally gavaged with LR daily beginning on the first night of mating until weaning of pups at PND 22.

2.3 Fecal DNA isolation and bacterial quantification by quantitative PCR (qPCR)

Fecal pellets were obtained from dams before (basal), after 11 and 25 daily LR doses, and from offspring after weaning at PND 22 and 30. DNA was isolated from mechanically disrupted pellets using a commercially available kit (ZymoBiomics DNA Miniprep). Purity and quantity of DNA was assessed by determining the optical density (OD) photometrically using 260/280 nm and 260/230 nm ratios (NanoDrop ND-2000). *L.reuteri* and general bacteria DNA oligonucleotide PCR primers (Integrated DNA Technologies, Inc., USA) were designed using previously published sequences and validated to be 90-110% efficient. *RT-qPCR:* Ten ng of DNA was used per reaction using a CFX Connect thermocycler and the Luna Universal PCR Master Mix (New England Biolabs, USA). Fold gene expression was measured relative to the reference gene (general bacteria primer) *16S*, and differential gene expression was determined by comparison to saline control mice using the Pfaffl method.

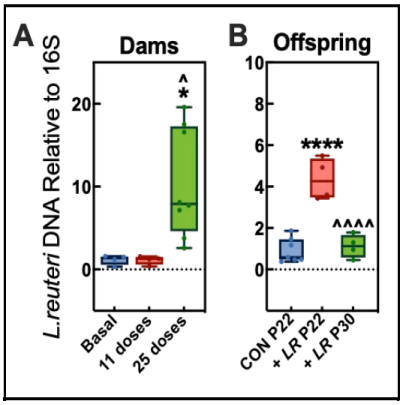

Figure 1: Maternal probiotic treatment with *L.reuteri* increases gut colonization in dams and their offspring. *indicates significantly different from baseline (basal) (**A**) or saline-treated offspring (CON) (**B**), *p<.05, ****p<.0001. ^indicates significantly different from 11 doses (A) or from LR treated offspring at P22 saline treated offspring (B), ^p<.05, ^^^^p<.0001. At each time point, samples represent VEH/CON and L-DE-71 treated dams (A) male and female offspring (B).

2.4 Plasma thyroid immunoassay

Plasma total T4 was quantified by colorimetric enzymatic kit (K050-H1, Arbor Assays) following manufacturer instructions. Samples were prepared with supplied dissociation reagent and diluted with assay buffer. Sensitivity and dynamic range were 0.3 ng/mL, 20-0.63 ng/mL, respectively. Of note, the thyroxine antibody in the kit has an 89% reactivity with rT3. Plasma total thyroxine was quantified by interpolating absorbance values at 450 nm using a 4-parameter-logarithmic standard curve of known concentrations.

2.5 Offspring early developmental milestones

Pups were transferred to a cage filled with clean bedding placed over a heating pad to maintain the external temperature at 35 °C. Somatic growth parameters were measured every other day: body weight (PND 2-30), body and tail lengths (PND 2-14), eyelid opening and incisor eruption (PND 6-14). The following criteria were used for eye opening: 0 = eyes closed, 1 = one eye open, 2 = both eyes open. The following criteria were used for incisor eruption: 0 = no apparent growth, 1 = lower jaw eruption, 2 = both lower and upper jaw eruption

2.5.1 Righting reflex

The righting reflex is a measure of latency to turn from a supine to prone position conducted PND2-14. Observer 1 placed the pup on its back and held the position for 5 seconds. Observer 2 recorded the time until all 4 limbs touched the surface in a prone position using a stopwatch. A maximum of one minute was given for each trial for a total of 3 trials per mouse. Pups were allowed to rest in between trials.

2.6 Statistical Analyses

Statistical analysis was performed using GraphPad Prism (version 8.4.3). Within groups comparison was performed using Student's t-test or One-way or repeated measures Two-way ANOVA if more than two groups were compared.

3 Results

3.1 Gut colonization of LR in offspring after maternal transfer

Dams received LR by oral gavage daily beginning on the day they were mated. Fecal pellets were subjected to qPCR analysis before (basal) and after 11 and 25 LR doses. qPCR analysis of LR DNA relative to general 16S bacterial DNA showed increased expression of LR in supplemented dams compared to saline controls (CON) after 25 but not 11 doses (Fig. 1A). LR treatment of dams ceased at pup weaning. Feces were collected from LR offspring at PND 22 and 30 and compared to saline controls at PND 22. LR DNA expression was significantly augmented in offspring at PND 22 and levels normalized at PND 30, nine days after discontinuation of LR treatment (Fig. 1B).

3.2 Maternal LR supplementation does not protect against reduced postnatal body weight in male offspring exposed to L-DE-71

Analysis of markers of somatic growth revealed no effect of L-DE-71 exposure in female offspring but produced a decrease in body weight gain in male offspring at PND 28. In females, LR supplementation of L-DE-71 group improved body weight gain as compared to L-DE-71 at PND 4, 6, 8, 20 and 22 (Fig. 2A). In L-DE-71 males, LR treatment did not improve reduced body weight at PND 28. In comparison to VEH/CON, LR supplementation in VEH/CON worsened body weight gain in males at PND 28, 30 but improved in females at PND 4 (Fig. 2A, B). However, LR supplementation improved tail growth in VEH/CON and L-DE-71 females at PND 2, 6, 10 (Fig. 2C). No exposure effects were observed in body length (Fig. 2E, F).

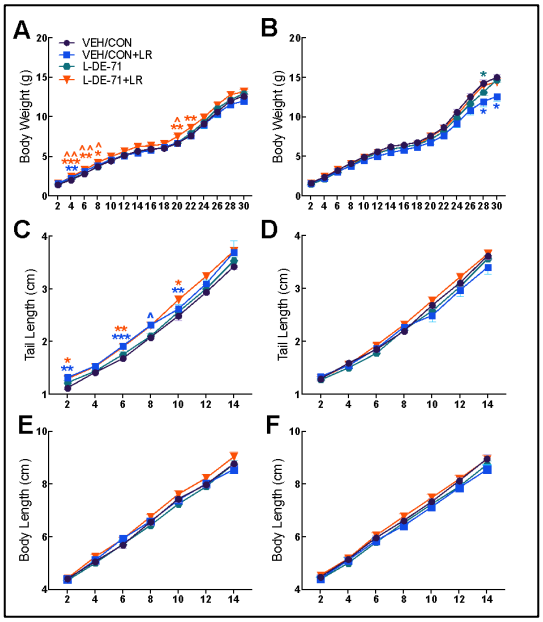

Figure 2: Maternal LR supplementation alters indices of somatic growth in offspring exposed to DE-71. (**A**) Body weight trajectory females. (**B**) Body weight trajectory males. (**C**) Tail length females. (**D**) Tail length males. (**E**) Body length females. (**F**) Body length males. Values represented are mean ± SEM. Repeated measures Two-Way ANOVA. The differently colored asterisk indicates the group being compared, *indicates significantly different vs VEH/CON, *p<.05, **p<.01 ***p<.001. ^indicates significantly different from L-DE-71, ^p<.05, ^^p<.01. *n*, 8-19 pups/group. X-axis values indicate pup postnatal day.

3.3 Maternal LR supplementation protects against deficient righting reflex in males exposed to L-DE-71

We also measured sensorimotor ability and other developmental benchmarks in offspring. There was no effect of L-DE-71 exposure on the latency to surface righting in male or female offspring compared to VEH/CON (**Fig. 3A, B**). However, LR supplementation improved righting reflex since reduced latency scores were seen in L-DE-71 + LR at PND 6 as compared to L-DE-71 male offspring. For eye opening, L-DE-71 + LR females showed significantly greater mean scores at PND 14 as compared to VEH/CON, which contained the most delayed conspecifics (**Fig. 3C**). Males showed no effect of exposure or treatment (**Fig. 3D**). Incisor eruption was delayed in L-DE-71 females but normalized in L-DE-71 + LR females **(Fig. 3E).** In VEH/CON females, LR supplementation improved eruption scores at PND 10 and worsened them at PND 12.

3.4 LR supplementation increases plasma T4 levels in dams

Because LR supplementation normalized deficient surface righting scores in L-DE-71 male offspring, we tested the hypothesis that these effects were associated with changes in TH levels. LR supplementation produced elevated plasma levels of T4 in L-DE-71 as well as VEH/CON dams as compared to unsupplemented controls (**Fig. 4**). There was no effect of L-DE-71 exposure on dam T4 levels. In light of other studies showing decreased TH measures in human cord blood, rodent, and lamb plasma T4 in association with PBDE burden[3], the unchanged dam T4 at PND 22 may be explained by the lower doses given and time point examined in the current study. Other animal studies used higher doses and examined times during gestation[15].

4 Discussion

Our results show that chronic DE-71 exposure at an environmentally relevant concentration (0.1 mg/kg/d) reduces body weight gain and the ability of surface righting in developing males and incisor eruption in developing females. Our findings support those found in a study of 20 Taiwanese infants reporting an association between elevated PBDE levels in breast milk and decreased birth weight, length, and chest circumference[5], particularly for the congeners BDE-47, 99, 100, and 209. Other human studies have found an inverse association with PBDEs and birth weight[5], body length[5], or adverse birth outcomes. In contrast, other studies found no association between maternal PBDEs and offspring birth weight head circumference[5] and body length or both positive and negative associations depending on the congener studied[16]. In rodent studies, developmental DE-71 exposure lowers female offspring body weights from postnatal days (PNDs) 35-60[6] at 0.1 mg/kg/d)[7]. Examining developmental milestones, Costa and colleagues found no effect of BDE-99 on righting reflex, eye opening or incisor eruption[17] and Gee and others, found no effects on other developmental milestones[18]. Several studies report different effects of PBDEs on body weight with increases[18] decreases[19] and no changes[20]. For example, low dose BDE-47 exposure increased body weight and length in male and female rat pups from low dose pre- and postnatal PBDE-47 exposure[21]. Adverse effects on dentition have been reported for polychlorinated biphenyls (PCBs), structurally similar chemicals to PBDEs[22]. Thus, deficient growth, dentition, and righting reflex found in our study may indicate that the congener composition in DE-71 may be more detrimental even at low doses (0.1 mg/kg/d). In support of poor righting reflex in L-DE-71 males, PBDE body burden in children has also been associated with worse performance on neurodevelopmental testing at ages 1-4 and 6 years in a longitudinal cohort of approximately 100 children in NYC[1].

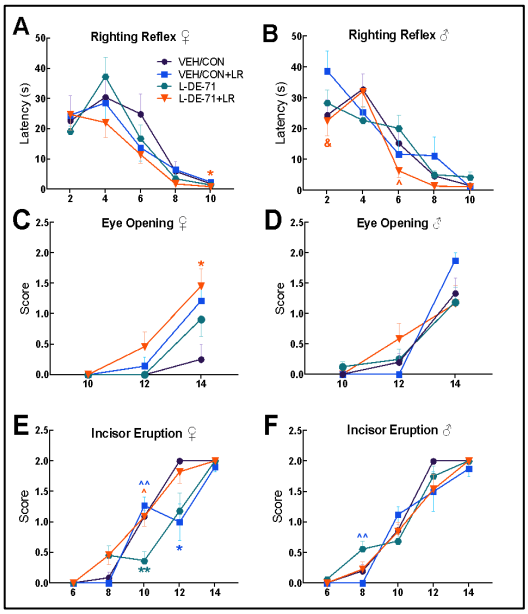

Figure 3: Effects of perinatal L-DE-71 exposure and maternal supplementation with *L. reuteri* (LR) on developmental benchmarks. (**A**) Righting reflex females. (**B**) Righting reflex males. (**C**) Eye opening females. (**D**) Eye opening males. (**E**) Incisor eruption females. (**F**) Incisor eruption males. Values represent mean ± SEM. Repeated measures Two-Way ANOVA. Colored asterisks indicate the group being compared, *indicates significantly different vs VEH/CON, *p<.05, **p<.01. ^indicates significantly different from L-DE-71, ^p<.05, ^^p<.01, &indicates significant different from VEH/CON+LR, &p<.01. *n*, 8-16pups/group. X-axis values indicate pup post-natal day.

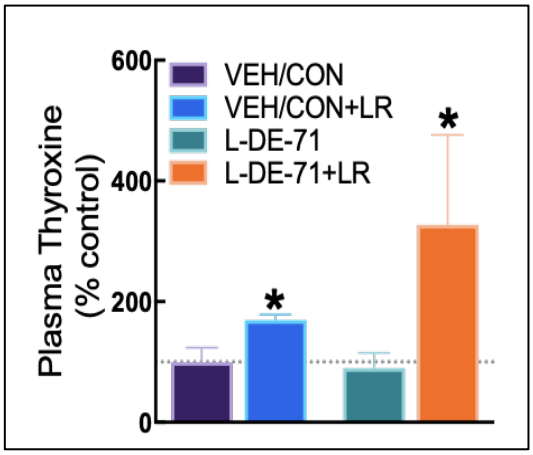

Figure 4: Dam plasma thyroxine levels are increased in LR supplemented dams. Plasma T4 levels were measured with EIA and normalized to percent VEH/CON. Mean plasma levels were evaluated for L-DE-71 exposure and LR treatment effects using Student's t-test. *indicates significantly different from corresponding supplemented group. *p<.05. n, 3-8 dams/group. Values represent mean ± SEM

We also tested the hypothesis that probiotic therapy could offset detrimental developmental effects of PBDEs. Maternal transfer of LR to offspring was confirmed through significantly elevated *L. reuteri* DNA expression in fecal samples collected from offspring receiving maternal LR transfer until PND 21. LR supplementation of L-DE-71 exposed dams during gestation and lactation normalized latency to righting reflex in male offspring and incisor eruption in female offspring. However, LR therapy did not reverse body weight deficit in males. Our findings identify a novel approach to protecting offspring against harmful neuro/developmental toxicity produced by perinatal PBDE exposure. No previous studies have explored maternal transfer of probiotics as a potential therapy against xenobiotic contamination or as a therapy to reverse developmental deficits. It has been speculated that probiotic therapy may provide xenobiotic biotransformation by the gut microbiome[12]. Supplementation with *L.reuteri* may also work to normalize developmental benchmarks in other ways via bacterial effects on the TH system. For example, our findings show that *L.reuteri* treatment increases maternal T4 levels which are required for normal growth and development of the fetus and offspring. Indeed, up to 30% (human) and 60% (mice) of T4 required at birth is provided by maternal T4[23]. The improvement in body weight and eye opening deficits produced by L-DE-71 seen in LR supplemented females may be mediated through a T4-dependent mechanism as one previous study shows that 3 ppm PTU, a thyroid synthesis inhibitor, decreased body weight gain at PND 21, and delayed eye opening at PND 16 in rat pups[24]

5 Conclusions

Our study provides support for the possibility that modulating the gut microbiota via maternal probiotic therapy may provide therapeutic benefit for CNS toxicity caused by infiltration of environmental pollutants during early development.

6 Acknowledgments

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