



Long-term developmental alterations in intestinal microbiota, metabolism, and T-cell homeostasis in offspring rats following maternal tetrabromobisphenol A exposure

Shuyang Huang^a, Chongxiang Zeng^a, Jiao Yang^a, Zenghua Qi^{b,c,d}, Yinfeng Zhou^{c,d}, Guoxia Zhang^{a,*,1}, Yingxin Yu^{b,c,d,*,1}

^a Department of Environmental Health, Guangdong Provincial Key Laboratory of Tropical Disease Research, School of Public Health, Southern Medical University, Guangzhou 510515, PR China

^b Guangdong-Hong Kong-Macao Joint Laboratory for Contaminants Exposure and Health, Center for Disease Prevention and Control of Guangdong Province, Guangzhou 510430, PR China

^c Guangdong-Hong Kong-Macao Joint Laboratory for Contaminants Exposure and Health, Guangdong Key Laboratory of Environmental Catalysis and Health Risk Control, Institute of Environmental Health and Pollution Control, Guangdong University of Technology, Guangzhou 510006, PR China

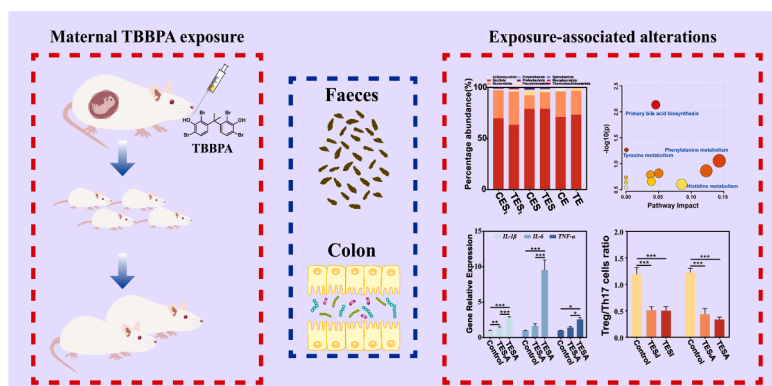
^d Guangzhou Key Laboratory of Environmental Catalysis and Pollution Control, Key Laboratory of City Cluster Environmental Safety and Green Development, School of Environmental Science and Engineering, Guangdong University of Technology, Guangzhou 510006, PR China

HIGHLIGHTS

- Altered Treg/Th17 balance indicated disturbed T-cell homeostasis in offspring.
- Maternal TBBPA exposure was associated with colonic inflammation in F1 offspring.
- Maternal TBBPA exposure was associated with altered gut microbiota and SCFA profiles.
- Exploratory metabolomics identified bile acid biosynthesis as a top-ranked pathway.

GRAPHICAL ABSTRACT

Tetrabromobisphenol A (TBBPA) is a widely used brominated flame retardant that is now ubiquitously detected in environmental media, wildlife, and humans. Due to its persistence, widespread use, and potential for maternal transfer, TBBPA may pose sustained risks during sensitive developmental periods. Increasing evidence links TBBPA exposure to developmental, metabolic, and immune-related adverse effects, raising concern over its long-term health implications. Its environmental ubiquity and potential developmental toxicity underscore the need for continued monitoring, exposure control, and strengthened environmental risk assessment.



* Corresponding author.

** Corresponding author at: Guangdong-Hong Kong-Macao Joint Laboratory for Contaminants Exposure and Health, Center for Disease Prevention and Control of Guangdong Province, Guangzhou 510430, PR China.

E-mail addresses: guoxiazhang@smu.edu.cn (G. Zhang), yuyingxin@gdut.edu.cn (Y. Yu).

¹ The study is an interdisciplinary research. The two corresponding authors have equal contributions to the article.

ARTICLE INFO

Keywords:

Brominated flame retardant
Microbiota
Short-chain fatty acids
Untargeted metabolomics
T cell homeostasis

ABSTRACT

Tetrabromobisphenol A (TBBPA), a widely used brominated flame retardant, has been associated with adverse health effects. An exploratory NHANES analysis of urinary bisphenol A (BPA) was performed to provide general population-level context for bisphenol-related metabolic and inflammatory associations because urinary TBBPA measurements were unavailable. We investigated whether maternal TBBPA exposure ($170 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) was associated with developmental alterations in offspring intestinal homeostasis. Maternal TBBPA exposure was associated with colonic inflammatory features, increased pro-inflammatory cytokine expression, and reduced Treg/Th17 ratios in F1 offspring. Gut microbiota composition was altered, including phylum- and genus-level differences, accompanied by altered fecal short-chain fatty acid composition and concentrations. Exploratory untargeted metabolomics identified nominally altered MS/MS-supported putatively annotated metabolite features. Primary bile acid biosynthesis had the lowest raw pathway-level p value and was therefore considered the top-ranked exploratory pathway, although no pathway remained significant after pathway-level FDR correction. Collectively, maternal TBBPA exposure was associated with developmental alterations in gut microbiota composition, microbial metabolites, fecal metabolic profiles, and intestinal T-cell homeostasis in F1 offspring, providing insight into the potential developmental intestinal immunotoxicity of TBBPA.

1. Introduction

Tetrabromobisphenol A (TBBPA), one of the most widely used brominated flame retardants, is extensively applied in textiles, electronics, plastics, and furniture. Due to its release during production, use, recycling, and disposal, TBBPA has been widely detected in environmental media and human biological samples, raising concerns about chronic exposure and related health risks [1,2]. Increasing evidence indicates that TBBPA induces multi-organ toxicity, including hepatotoxic, neurotoxic, developmental, reproductive, and endocrine-disrupting effects [3,4]. Recent studies further suggest that gut microorganisms can biotransform TBBPA and its analogs, while chronic exposure may also disturb gut microbial composition and metabolism [5]. Of particular concern, TBBPA has been detected in maternal and cord blood as well as human breast milk, indicating maternal-fetal and lactational transfer and thus the possibility of prenatal and neonatal exposure [6]. Given its environmental ubiquity and potential developmental risks, systematic investigation of the developmental effects of maternal TBBPA exposure in F1 offspring is essential for a more comprehensive assessment of its long-term risks to human populations.

The gut microbiota is a key regulator of host metabolic homeostasis, immune function, barrier integrity, and gut-brain signaling. Environmental stressors can disturb this microbial ecosystem and promote dysbiosis, thereby contributing to downstream metabolic and inflammatory abnormalities [7]. Growing evidence links gut dysbiosis to liver disease, endocrine dysfunction, and autoimmune disorders through complex host-microbe interactions [8–10]. These bidirectional effects are mediated through interconnected gut-liver and gut-brain axes that influence intestinal, hepatic, immune, and neural functions [11]. In addition, microbiota imbalance can impair intestinal barrier integrity and contribute to the onset and progression of inflammatory bowel disease, particularly Crohn's disease and ulcerative colitis [12]. Accordingly, gut microbial signatures are increasingly regarded as promising noninvasive biomarkers for evaluating intestinal health and disease susceptibility [13].

Environmental pollutants can substantially reshape gut microbiota composition and function, thereby altering microbial metabolism and inflammatory homeostasis [14]. Recent evidence indicates that micro- and nanoplastics, including polystyrene particles, disrupt gut microbial structure and aggravate intestinal and systemic toxicity through microbiota-dependent mechanisms [15]. Persistent organic pollutants (POPs), especially brominated flame retardants and Polychlorinated biphenyls (PCBs), are likewise increasingly recognized as microbiota-disrupting contaminants. In maternal exposure models, DE-71 and PCB 126 altered offspring gut microbiota and were accompanied by disturbed gut-liver metabolic and inflammatory signaling [16,

17]. Importantly, recent studies show that gut bacteria can biotransform TBBPA and its analogs into rhamnosylated and debrominated metabolites, while chronic TBBPA exposure can also disturb gut microbial homeostasis and intestinal metabolism [5,18]. These findings support the hypothesis that TBBPA may contribute to host metabolic and immune dysfunction, at least in part, through microbiota-mediated mechanisms.

Environmental contaminants can disrupt intestinal immune homeostasis by altering gut microbiota, epithelial barrier integrity, and mucosal inflammatory signaling [19]. Recent studies show that microplastics and pollutant-loaded microplastics aggravate colonic inflammation and barrier injury, accompanied by increased pro-inflammatory responses [20,21]. Emerging evidence further indicates that per- and polyfluoroalkyl substances (PFAS) mixtures impair intestinal barrier function through microbiota-dependent metabolic dysregulation, while maternal exposure to pollutants such as PFOS and tris(n-butyl) phosphate (TnBP) can induce persistent microbiome-related alterations in offspring [22–24]. Collectively, these findings suggest that early-life pollutant exposure may reprogram intestinal and immune homeostasis in F1 offspring. However, whether maternal TBBPA exposure is associated with intestinal inflammatory features and altered immune homeostasis in F1 offspring has not been systematically characterized and remains an important knowledge gap.

Therefore, the present study hypothesized that maternal TBBPA exposure may be associated with offspring gut microbiota and metabolic alterations, which may in turn relate to changes in intestinal immune homeostasis and colonic inflammatory features. To characterize maternal exposure-associated alterations in microbiota and metabolic networks in offspring, we employed an integrated multi-omics approach combining 16S rRNA gene sequencing and untargeted metabolomics. Complementary flow-cytometric analysis of offspring colonic tissues enabled quantitative assessment of Treg and Th17 cell populations. The present findings provide new insights into microbiota-metabolite-immune crosstalk associated with susceptibility to intestinal inflammatory conditions in F1 offspring, thereby advancing our understanding of environmental toxicant-mediated gut pathogenesis.

2. Materials and methods

2.1. Study population

This cross-sectional analysis utilized data from six consecutive cycles of the National Health and Nutrition Examination Survey (NHANES) (2005–2006, 2007–2008, 2009–2010, 2011–2012, 2013–2014, and 2015–2016), a nationally representative survey conducted by the Centers for Disease Control and Prevention (CDC) in 2-year cycles. The study was approved by the National Center for Health Statistics Institutional Review Board, and all participants signed written informed consent

forms. Because urinary TBBPA measurements were not available in NHANES, urinary BPA was analyzed only as an ancillary exploratory measure of general bisphenol-related outcomes and was not used to represent TBBPA exposure.

For the present analysis, adults aged 20 years or older with available urinary BPA measurements were eligible. Participants were further excluded if outcome data, urinary creatinine, survey design variables, or covariate information were missing. After applying these exclusion criteria, 7673 participants were included in the gut function analysis and 6178 participants were included in the metabolic analysis. For the immune/inflammatory analysis, the final sample size varied according to the availability of specific laboratory measurements. A weighted regression framework was used to assess the associations of urinary BPA with gut-related, metabolic, and immune/inflammatory outcomes, adjusting for age, sex, race, alcohol use, and urinary creatinine. In addition, quartile-based analyses, sex-stratified analyses, sex-interaction analyses, and sensitivity analyses were performed.

2.2. Standards and reagents

Tetrabromobisphenol A (TBBPA, purity = 98%) was purchased from Shanghai Aladdin Biochemical Technology Co, Ltd. (Shanghai, China). The short-chain fatty acid (SCFA) standards, including acetic acid, propionic acid, butyric acid, isobutyric acid, and isovaleric acid, were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). All other chemicals and reagents, including ether, sulfuric acid, acetone, and anhydrous calcium chloride, were purchased from Guangzhou Chemical Reagent Factory. Corn oil was purchased from Gold Arowana (Shenzhen, China). More reagents are provided in the [Supplementary material](#) (Text S1).

2.3. Animal treatment

In the present study, 48 female and 24 male SD rats (weighing 200 ± 30 g, approximately 8 weeks old) from the Southern Medical University's Laboratory Animal Center in Guangzhou, China were acquired, and none of these animals had ever engaged in mating or reproduced. The experiments were approved by the Ethics Review Committee of Southern Medical University (SCXK 2021-0041 and SMUL2022053).

Before the experiment, SD rats were housed in a specific pathogen-free (SPF) animal facility under controlled environmental conditions, with the temperature maintained at $22\text{--}24^\circ\text{C}$, relative humidity at $50 \pm 10\%$, and a 12-h light/dark cycle. Animals were provided ad libitum access to water and a commercial standard laboratory animal compound diet manufactured by the Guangdong Medical Laboratory Animal Center, China. According to the product label, the diet complied with the Chinese national standard GB14924.3-2010 for laboratory animal feed and met the relevant feed hygiene requirements. The diet was not a purified phytoestrogen-free formulation, and phytoestrogen contents were not independently quantified in the present study. All control and TBBPA-exposed rats received the same type of standard chow throughout the experiment to minimize diet-related variation among groups.

After a 7-day acclimatization period, three female and one male rat were randomly selected and co-housed overnight. Pregnancy was confirmed the following morning in female rats through either vaginal plug observation or microscopic identification of spermatozoa in vaginal smears. Successful mating was designated as gestational day 0 (GD0).

After mating, 48 females with confirmed or suspected pregnancy were initially assigned to six experimental groups, with eight females allocated to each group to allow for possible pseudopregnancy. Six confirmed pregnant dams were ultimately retained in each group for the experimental study, corresponding to six independent litters per group. On PND4, each litter was standardized to eight offspring. Candidate offspring were then selected based on median body weight, with

individuals deviating by more than $\pm 25\%$ from the litter median excluded from subsequent selection. For offspring analyses, sampling was performed on an endpoint-specific basis. Depending on the endpoint, one or more offspring could be selected from a contributing litter. The number of offspring analyzed, sex distribution, number of contributing litters, and number of offspring sampled per litter are summarized in [Table S1](#). The experimental design comprised TE (gestational TBBPA exposure GD0-20, sacrificed GD20), CE (gestational corn oil control GD0-20, sacrificed GD20), TES_i (continuous TBBPA exposure GD0-PND21 with offspring TES_iI sacrificed PND28 and TES_iA sacrificed PND56), CES_i (continuous corn oil control GD0-PND21 with offspring CES_iI sacrificed PND28 and CES_iA sacrificed PND56), TES (gestational-only TBBPA exposure GD0-21 with offspring TES_iI sacrificed PND28 and TES_iA sacrificed PND56), and CES (gestational-only corn oil control GD0-21 with offspring CES_iI sacrificed PND28 and CES_iA sacrificed PND56). All offspring were breastfed until weaning at PND21 before standard chow administration. The group nomenclature used T for TBBPA treatment, C for control, E for gestational exposure, S for no lactational exposure, S_i for lactational exposure, I for immature offspring (PND28), and A for adult offspring (PND56), with the complete experimental design illustrated in [Fig. 1a](#). The initial dose calculation used the EPA body surface area method (K_m factor: $37/6 = 6.2$). After adjustment for pregnant body weights (65 kg for humans and 0.3 kg for rats), an effective scaling factor of 4.5 was obtained. Using the reported maximum human serum concentration of $310\text{ ng}\cdot\text{g}^{-1}$ lipid [25], this calculation yielded a nominal value of $1.7\text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$. However, because serum concentrations cannot be directly converted to oral doses without toxicokinetic information, this value was not considered a human-equivalent dose or an estimate of environmentally relevant exposure. Previous studies reported no significant developmental toxicity in SD rats following oral TBBPA administration at doses up to $1000\text{ mg}\cdot\text{kg}^{-1}$ [26]. A 100-fold factor was subsequently applied during the original dose-selection process, resulting in the experimental dose of $170\text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$. This factor was used for experimental dose selection rather than for quantitative human-to-animal extrapolation. Accordingly, the $170\text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ dose used in the present study was treated as a high-dose hazard-identification regimen rather than a quantitatively human-equivalent exposure.

2.4. Sample collection of SD rat feces and tissue

Fecal samples from dams and offspring were collected in 2 or 50 mL centrifuge tubes three days prior to sacrifice and stored at -80°C . Rats were euthanized under chloral hydrate anesthesia via cardiac or abdominal aortic exsanguination. For prenatal dissection groups, maternal liver, lungs, kidneys, colon, and small intestine were harvested immediately post-necropsy. In other groups, cardiac perfusion was performed with 50 mL saline for immature offspring and 100 mL for adults and dams prior to organ collection. Intestinal morphometry included measurements of colon and small intestine length. All organs were weighed and cryopreserved at -80°C .

2.5. 16S rRNA gene sequence amplification analysis

The V3-V4 hypervariable regions were amplified using primer sequences consistent with previously published methodology in our laboratory [27], with additional methodological details provided in Text S2. Data for the 16S rRNA genes have been submitted and are maintained at the National Center for Biotechnology Information (NCBI).

2.6. Bioinformatics analysis

Microbiome analysis was performed following the Atacama soil microbiome tutorial" in the QIIME2 documentation (<https://docs.qiime2.org/2019.1/>). Raw FASTQ sequences were imported into QIIME2-compatible format using the q2-tools import plugin. Sequence

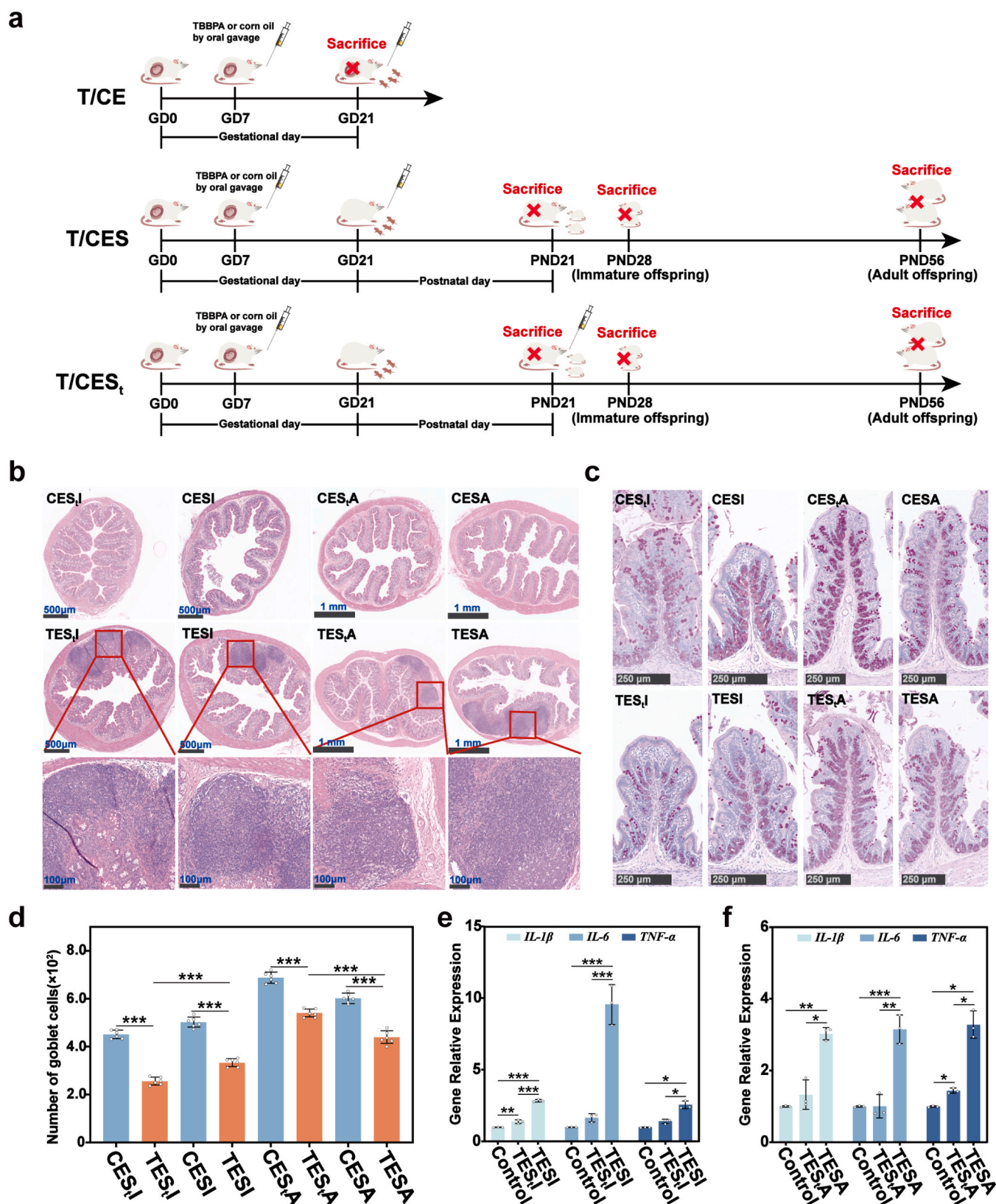


Fig. 1. Effect of TBBPA on pathological changes and goblet cells in offspring colonic tissues ($n = 3$). (a) Experimental design of TBBPA exposure. (b) Representative images of colonic H&E staining ($2.5 \times, 5 \times, 15 \times$); (c) Representative images of colonic PAS staining ($10 \times$); (d) Colonic PAS staining goblet cell count; (e-f) *TNF- α* , *IL-6*, and *IL-1 β* relative mRNA expression in offspring colonic tissues. (Data are expressed as means $\pm$ SD. The p -values $*$ < 0.05 , $**$ < 0.01 and $***$ < 0.001 are significantly different. Endpoint-specific offspring sample sizes, sex distributions, numbers of contributing litters, and offspring sampled per litter are provided in Table S1.).

processing was conducted with the q2-dada2 plugin to perform quality filtering, read trimming, error correction, read merging, and chimera removal, generating amplicon sequence variant (ASV) feature tables [28]. Additional methodological details are available in Text S3.

2.7. Sample treatment

The pretreatment method for fecal short-chain fatty acid extraction has been described in detail in a previous study [27]. More details are provided in Text S4. Colonic tissue samples were fixed with paraformaldehyde for 16–24 h. Following fixation, tissues were dehydrated, embedded in paraffin, and sectioned at 4 μ m thickness. After deparaffinization, H&E staining and PAS staining were performed. The colonic paraffin sections were observed and photographed by optical biological microscope and scored according to a standard procedure. In addition, the morphology of colonic goblet cells was observed and counted. Colonic tissues from each group were used for total RNA extraction and reverse transcription following established protocols [29]. 30 mg of colon was collected from each rat into 2 mL RNase-free centrifuge tubes. Additional methodological details are provided in Text S5. Primer sequences and specifications are listed in Table S2.

For each offspring group, colonic segments (approximately 2 cm) were aseptically collected from three randomly selected SD rats and immediately placed in ice-cold PBS (4 °C) for adipose/mesenteric tissue removal. Following longitudinal incision, luminal contents were cleared through sequential PBS washes (4 °C). Lamina propria T lymphocyte isolation and flow cytometry staining procedures are detailed in Text S6.

Fecal samples were lyophilized for 48 h, precisely aliquoted (50 mg), and homogenized with 1 mL of extraction solvent. Complete protocols for fecal sample preparation in untargeted metabolomics are detailed in Text S7.

2.8. Instrumental analysis

The quantification of SCFAs was performed by gas chromatography (GC) using an HP-FFAP fused-silica capillary column with helium carrier gas (1.5 mL·min⁻¹ flow rate). Samples were injected in 1 μ L aliquots. Complete GC analytical parameters for SCFA detection are provided in Text S4.

Untargeted metabolomic profiling was conducted using a Thermo Scientific Ultimate 3000 liquid phase system equipped with a Waters HSS T3 analytical column (2.1 $\times$ 100 mm, 1.8 μ m particle size). Complete chromatographic conditions and mass spectrometry parameters for the global metabolomics analysis are detailed in Text S7.

Flow cytometry analysis was performed using a BD LSRFortessa system (BD Biosciences, San Jose, CA, USA), with subsequent data processing conducted in FlowJo software (v10.0; FlowJo LLC, Ashland, OR, USA).

2.9. Statistical analysis

For the NHANES analysis, statistical analyses were performed in R using survey-weighted regression models that accounted for strata, primary sampling units, and subsample weights. Logistic regression was used for binary outcomes and linear regression for continuous outcomes. Main models were adjusted for age, sex, race, alcohol use, and urinary creatinine, and additional quartile, trend, sex-stratified, interaction, and sensitivity analyses were performed. A two-sided $p < 0.05$ was considered statistically significant.

All statistical analyses for the animal experiments were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Intergroup comparisons were conducted using one-way ANOVA followed by LSD or Dunnett's post hoc tests, while two-group comparisons were performed using independent-samples t -tests. The litter structure differed among offspring endpoints, as detailed in Table S1. Histopathology, qPCR, and flow cytometry included one offspring per contributing litter, whereas

16S rRNA sequencing, SCFA analysis, and metabolomics included six offspring from three contributing litters, with two offspring sampled per litter. Because litter clustering was not explicitly modeled for these latter analyses and only three contributing litters were available, the corresponding findings were interpreted as exploratory. Residual within-litter dependence cannot be excluded and is acknowledged as a limitation. Sex-specific statistical analyses were not performed because of the limited sample sizes. Multivariate analysis of metabolomic data was performed using SIMCA (v14.1; Umetrics, Sweden). PCA-X was performed using both biological and pooled QC samples to evaluate overall sample distribution and QC clustering. Supervised OPLS-DA was then used to visualize group separation. Model performance was assessed using $R^2X(\text{cum})$, $R^2Y(\text{cum})$, $Q^2(\text{cum})$, CV-ANOVA, and permutation testing. Negative Q^2 intercepts in permutation testing were interpreted as evidence against obvious overfitting rather than as proof of strong predictive validity. Metabolite features were screened based on nominal $p < 0.05$ from univariate statistical analysis. Fold change was calculated as the mean abundance ratio between exposed and control groups. VIP predictive scores from the exploratory OPLS-DA model were calculated for feature prioritization and were not used as a formal inclusion criterion. Fold change, VIP predictive score, nominal p value, and FDR-adjusted q value were reported in the supplementary differential metabolite table. No fixed fold-change threshold or FDR-adjusted q -value threshold was applied as an additional inclusion criterion. Metabolic pathway analysis was conducted using MetaboAnalyst 5.0, a web-based analytical platform. Pathway-level multiple-testing correction was performed using the false discovery rate (FDR) method, with FDR-adjusted $q < 0.05$ considered statistically significant. Spearman correlation analysis was performed to evaluate associations between selected bacterial genera and MS/MS-supported putatively annotated fecal metabolite features based on six offspring samples. Because multiple taxa-metabolite pairs were tested, nominal p values were adjusted using the Benjamini-Hochberg false discovery rate method. Correlation coefficients, nominal p values, and FDR-adjusted q values were reported in the supplementary correlation table. Associations with FDR-adjusted $q < 0.05$ were considered FDR-supported, whereas associations with nominal $p < 0.05$ but $q \geq 0.05$ were considered exploratory. Correlation results were interpreted as associative and were not used to infer causal relationships.

3. Results

3.1. Exploratory NHANES analysis of urinary BPA

To provide broad population-level context for general bisphenol-related metabolic and immune-associated outcomes, we analyzed urinary BPA in NHANES because urinary TBBPA measurements were not available. Weighted baseline characteristics are presented in Table S3. In the main adjusted models, urinary BPA was positively associated with triglyceride-related traits and white blood cell count, whereas no significant associations were observed for self-reported gastrointestinal disorder, bowel movement frequency, C-reactive protein, lymphocyte percentage, or neutrophil percentage (Table 1). Detailed quartile-based, sex-stratified, sensitivity, and graphical analyses are provided in the Supplementary Materials (Tables S4–S6; Fig. S1). Overall, these findings provide limited exploratory context regarding general bisphenol-related metabolic and immune-associated outcomes and were not incorporated into the mechanistic interpretation of the maternal TBBPA animal study.

3.2. Effects of TBBPA exposure on phenotype, body weight, and organ indices

General health status encompassing fur condition, food and water intake, and behavioral patterns remained unaltered across all TBBPA-exposed dams and offspring. Fetal counts (Fig. S2a) and neonatal body weight (Fig. S2b) exhibited no intergroup differences. A significant

Table 1

Main associations of urinary BPA with gut function, metabolic traits, and immune/inflammatory markers in the NHANES-based complementary human analysis.

Module	Outcome	Main model	
		Estimate (95% CI)	p-value
Gut function	Gastrointestinal disorder	OR 0.992 (0.861, 1.143)	0.911
	Bowel movement frequency	β -0.066 (-0.393, 0.260)	0.683
Metabolism	Body weight	β 0.046 (-0.584, 0.676)	0.884
	BMI	β 0.112 (-0.106, 0.330)	0.308
	Waist circumference	β 0.037 (-0.445, 0.519)	0.878
	Triglycerides (mg/dL)	β 4.906 (1.223, 8.588)	0.010
	Triglycerides (mmol/L)	β 0.055 (0.014, 0.096)	0.010
	Glucose (mg/dL)	β 0.347 (-0.936, 1.630)	0.591
	Glucose (mmol/L)	β 0.015 (-0.055, 0.085)	0.668
	Log triglycerides	β 0.034 (0.010, 0.058)	0.006
	Log glucose	β 0.001 (-0.007, 0.010)	0.739
Immune/inflammation	White blood cell count	β 0.087 (0.018, 0.155)	0.014
	Lymphocyte percentage	β 0.022 (-0.217, 0.262)	0.853
	Neutrophil percentage	β -0.014 (-0.300, 0.273)	0.924
	CRP	β -0.029 (-0.063, 0.006)	0.100
	Log CRP	β -0.040 (-0.094, 0.015)	0.146

Estimates are presented as odds ratios (ORs) with 95% confidence intervals (CIs) for gastrointestinal disorder and β coefficients with 95% CIs for continuous outcomes. Main models were adjusted for age, sex, race, alcohol use, and urinary creatinine, as appropriate. BPA was natural log-transformed in all regression models. Because urinary TBBPA measurements were not available in NHANES, urinary BPA was analyzed only as an ancillary exploratory measure of general bisphenol-related outcomes and was not used to represent TBBPA exposure.

reduction in body weight was observed only in TES_I offspring relative to controls (Fig. S2c), whereas TES_A and TESA offspring had body weights comparable to their respective controls ($p > 0.05$; Fig. S2d). Analyses of organ indices disclosed reduced hepatic and renal indices in CES_I versus CES_I offspring ($p < 0.05$; Fig. S2e), TES_A adults displayed diminished kidney indices relative to TESA ($p < 0.05$; Fig. S2f). TES_I offspring exhibited increased small intestinal and colonic length indices relative to CES_I (Figs. S2g, i). These morphological perturbations were most pronounced in immature offspring; continuous lactational exposure (TES_I) was associated with greater intestinal length indices during development.

3.3. Pathological changes and goblet cells in offspring colonic tissues

Histopathological examination of offspring colonic tissues identified inflammatory cell infiltration and lymphoid hyperplasia across all exposure groups (Fig. 1b). Quantitative scoring confirmed elevated histopathological scores in TES_I, TES_I, TES_A, and TESA groups relative to controls (Fig. S3a). H&E staining revealed intact colonic architecture, orderly glandular arrangement, and minimal inflammatory infiltration in control offspring. Exposed groups displayed marked inflammatory responses, consistent with exposure-associated mucosal inflammatory changes. PAS staining further showed goblet cell atrophy, reduced cell density (Fig. 1d), and diminished mucus production in exposed

offspring, whereas control goblet cells retained normal rounded morphology with abundant mucin secretions and intact mucosal coverage (Fig. 1c). Goblet cell depletion further indicated mucosal alterations in exposed offspring.

3.4. Expression of IL-1 β , IL-6, and TNF- α in Offspring colonic tissues

Quantitative PCR analysis disclosed significantly elevated mRNA expression of pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α in colonic tissues of TES_I and TESA offspring relative to controls (Fig. 1e, f). TESA offspring exhibited the most pronounced IL-6 expression ($p < 0.05$). Significant increases were also observed in TNF- α expression in TES_I offspring and IL-1 β expression in TES_A offspring ($p < 0.05$). Moreover, TES_I and TESA groups displayed markedly higher cytokine mRNA levels than their TES_I and TES_A counterparts, respectively ($p < 0.05$). These expression patterns indicate colonic pro-inflammatory activation in offspring, with the TES groups showing a stronger cytokine response than the corresponding TES_I groups.

3.5. Immunological homeostasis in the offspring colonic tissues

Lymph node hyperplasia observed in both immature and adult offspring colons prompted examination of Treg/Th17 balance in colonic lamina propria lymphocytes. Flow cytometry identified Th17 cells as CD4⁺IL-17A⁺ and Treg cells as CD4⁺CD25⁺Foxp3⁺, with frequencies expressed as percentages of total CD4⁺ T cells. Exposed offspring showed significantly higher Th17 cell frequencies (Fig. 2c, d) and lower Treg cell frequencies ($p < 0.05$; Fig. 2a, b), resulting in reduced Treg/Th17 ratios (Fig. S3b). Overall, these flow cytometry results indicate that maternal TBBPA exposure was associated with altered colonic T-cell homeostasis in offspring.

3.6. Diversity of gut microbiota

Microbial α -diversity was assessed via Chao1 (richness) and Shannon (evenness) indices. In the exploratory microbiota analysis, the Chao1 index showed nominally lower bacterial richness in both TES_I and TE dams relative to controls ($p < 0.05$), with TE exhibiting the most pronounced decrease (Fig. 3a). This reduction persisted in offspring, though intergroup differences among exposed offspring failed to reach statistical significance ($p > 0.05$; Fig. 3b). The Shannon index showed significantly lower microbial evenness in both dams and offspring (Fig. 3c, d). β -Diversity analysis (PCoA) demonstrated distinct clustering patterns among exposure groups, with clear intergroup separation and tight intragroup aggregation (Fig. 3e-g), indicating substantial exposure-associated differences in microbial community composition. Integration of α -diversity, β -diversity, and LefSe results (Figs. S4a-c) indicates that maternal TBBPA exposure was associated with persistent alterations in gut microbiota composition in F1 offspring, with differences in community structure observed across exposure durations.

3.7. Phylum and genus level gut microbiota composition

Exploratory phylum-level analysis showed compositional differences following TBBPA exposure (Fig. 4a-c). The fecal microbiome was predominantly constituted by Bacillota, Bacteroidota, Pseudomonadota, and Actinomycetota. In the TES_I group, Bacteroidota abundance decreased from 70.0% to 63.5% ($p > 0.05$), while Bacillota increased from 27.3% to 32.4% ($p > 0.05$). Similar reciprocal patterns manifested in TES_I and TES_A offspring. The largest phylum-level differences were observed in TES_I offspring: Bacillota decreasing from 60.9% to 34.2% ($p < 0.001$), Bacteroidota from 35.8% to 16.3% ($p < 0.01$), and Pseudomonadota from 0.5% to 48.1% ($p < 0.001$) (Fig. 4b). These phylum-level differences were less pronounced in adult offspring, with profiles approaching those of the corresponding controls.

At the genus level, TES_I dams displayed reduced abundances of

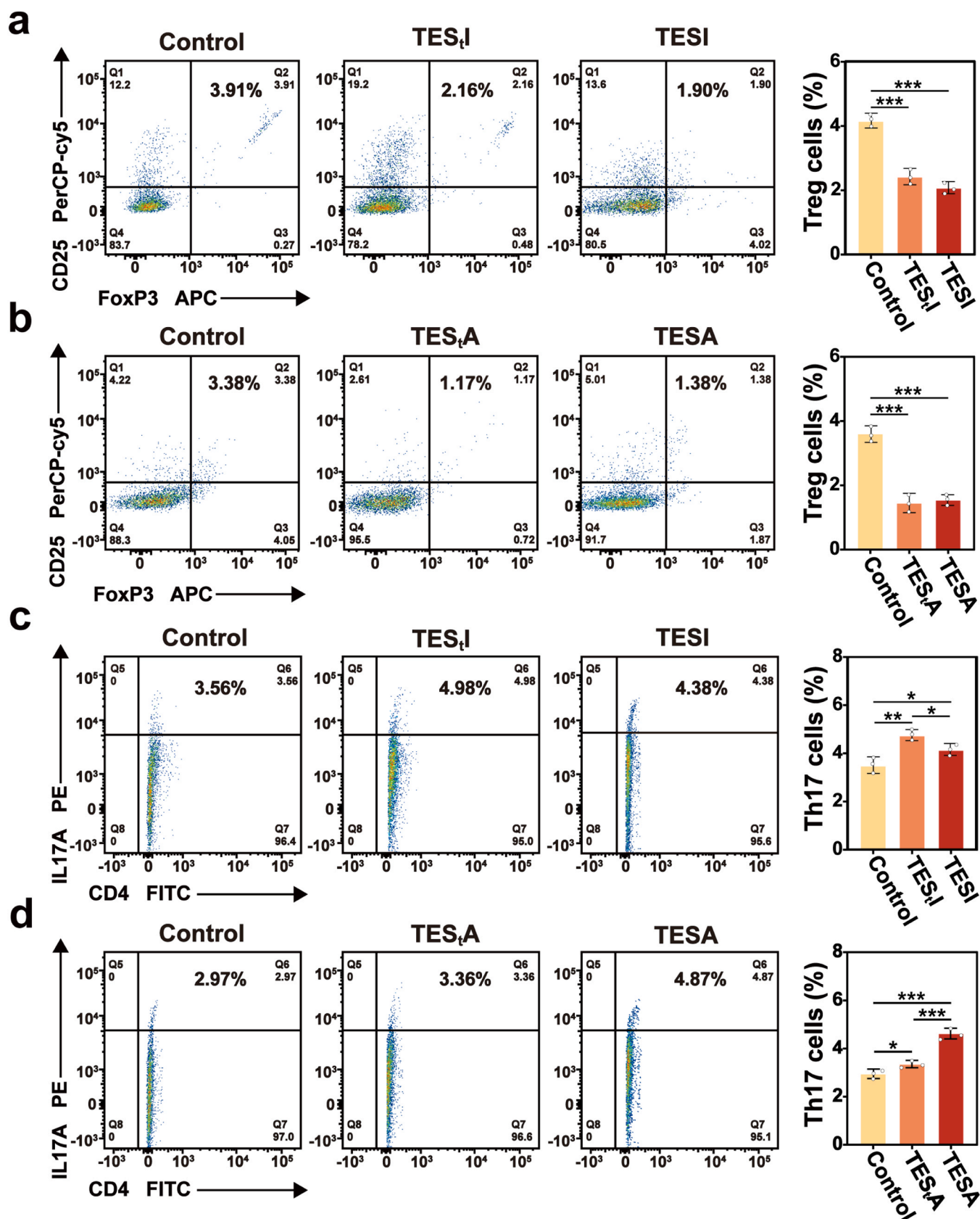


Fig. 2. Effect of TBBPA on the immunological homeostasis in the offspring colonic tissues ($n = 3$). (a-b) Percentage of Treg cells in offspring colonic tissues; (c-d) Percentage of Th17 cells in offspring colonic tissues. (Data are expressed as means $\pm$ SD. The p -values * < 0.05 , ** < 0.01 and *** < 0.001 are significantly different. Endpoint-specific offspring sample sizes, sex distributions, numbers of contributing litters, and offspring sampled per litter are provided in Table S1.).

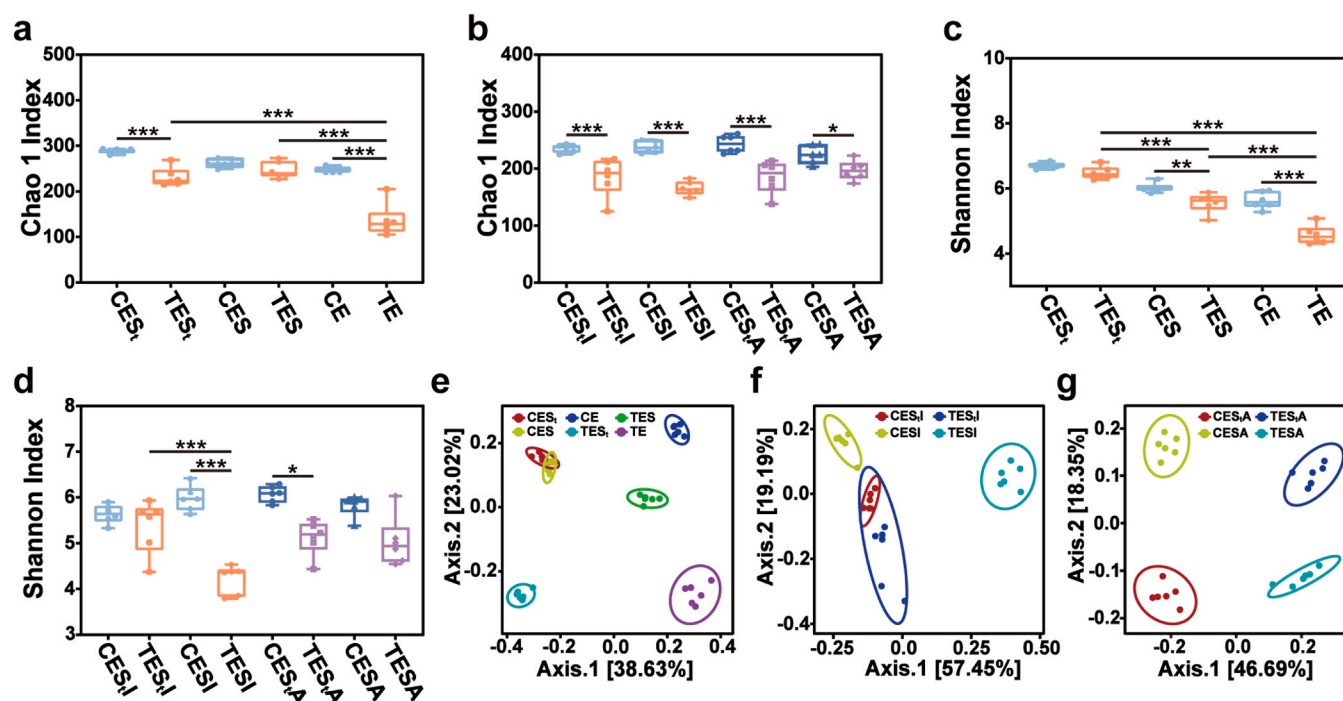


Fig. 3. Effect of TBBPA on the diversity of gut microbiota ($n = 6$). (a-b) Chao 1 index of α -diversities of gut microbiota; (c-d) Shannon index of α -diversities of gut microbiota; (e-g) PCoA of microbial β -diversities. (Data are expressed as means $\pm$ SD. The p -values * < 0.05 , ** < 0.01 and *** < 0.001 are significantly different. Endpoint-specific offspring sample sizes, sex distributions, numbers of contributing litters, and offspring sampled per litter are provided in Table S1. Because these analyses included two offspring from each of three contributing litters and litter dependence was not explicitly modeled, the findings are presented as exploratory.).

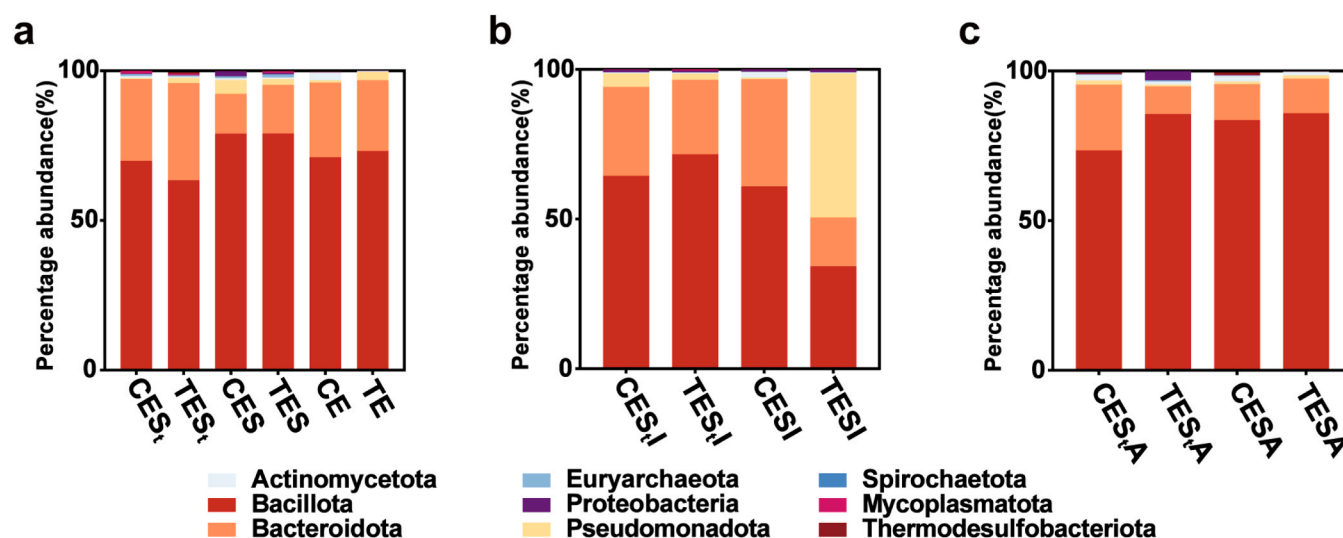


Fig. 4. Relative abundance at the phylum level (a-c) ($n = 6$). (Data are expressed as means $\pm$ SD. The p -values * < 0.05 , ** < 0.01 and *** < 0.001 are significantly different. Endpoint-specific offspring sample sizes, sex distributions, numbers of contributing litters, and offspring sampled per litter are provided in Table S1. Because these analyses included two offspring from each of three contributing litters and litter dependence was not explicitly modeled, the findings are presented as exploratory.).

Massiliimalia, *Mogibacterium*, and *Petrocella*, whereas TES dams exhibited marked increases in *Butyrivibrio*, *Monoglobus*, and *Rodentibacter* (Figs. S5a, b). Differences in immature offspring microbiota composition were observed across exposure durations. TESI manifested reduced abundances of *Butyrivibrio*, *Monoglobus*, and *Rodentibacter* relative to TES_I, alongside decreased *Eubacterium* and *Clostridium* versus controls. Conversely, TES_A showed higher relative abundances of *Eubacterium*, *Clostridium*, *Vallitalea*, and *Guopingia*, while both TES_A and TESA displayed substantial depletion of *Desulfovibrio* and *Bifidobacterium*. Together, these phylum- and genus-level compositional shifts indicate

developmental-stage-dependent microbial remodeling following maternal TBBPA exposure, with the most pronounced microbiota alterations observed in immature offspring and partial normalization by adulthood.

3.8. Short-chain fatty acid metabolism

Method validation showed good system stability (consistent retention times and peak intensities, Fig. S6), strong linearity ($R^2 > 0.998$ for all standard curves, Table S7), high accuracy (recovery: 92.6%–110.8%),

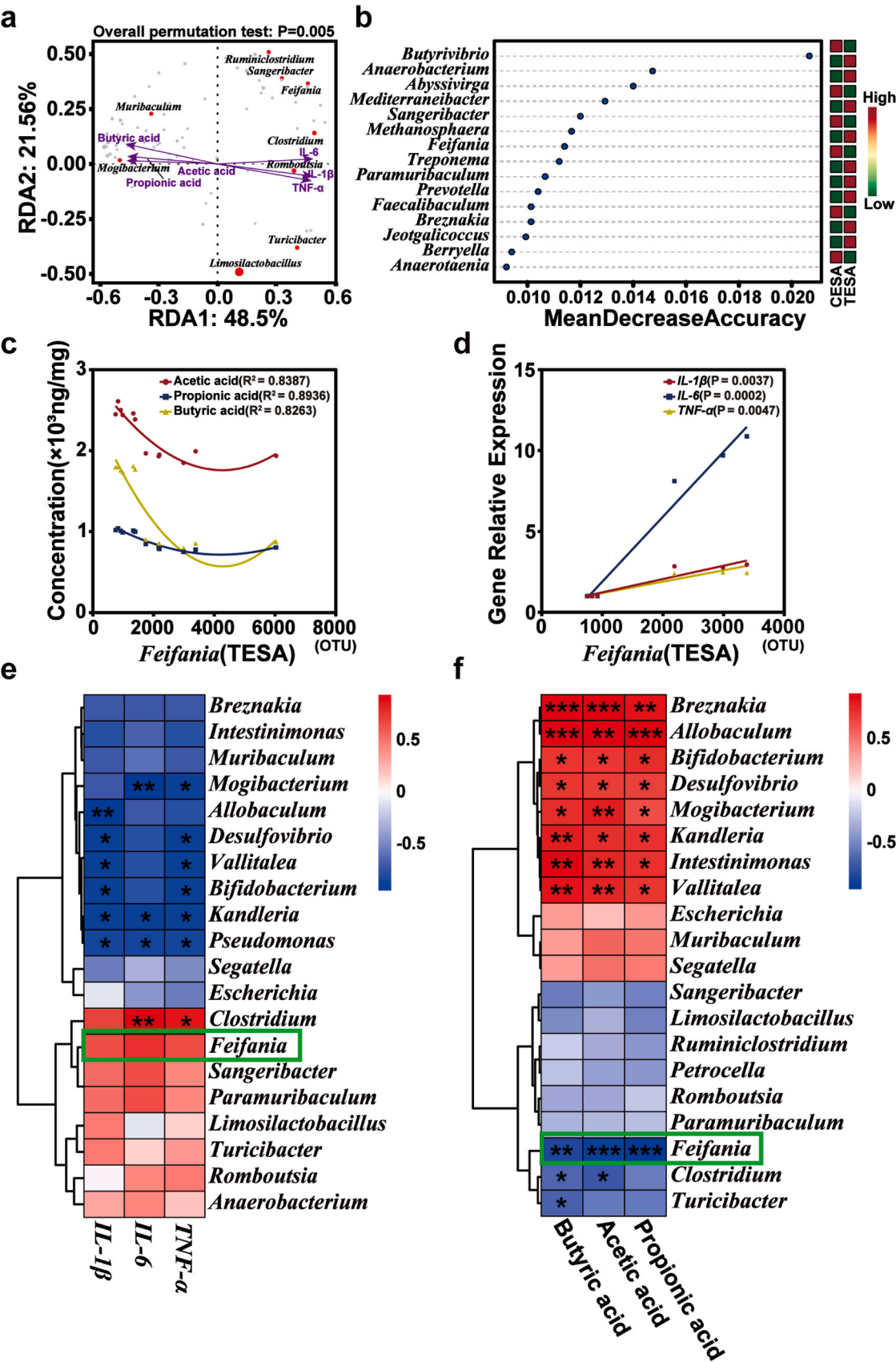


Fig. 6. Association of gut microbiota with inflammatory factors and SCFAs in TESA group under TBBPA exposure. (a) RDA analysis of gut microbiota with inflammatory factors and SCFAs; (b) Random forest analysis; (c) Scatter plot of correlation between *Feifania* abundance and SCFAs content; (d) Scatter plot of correlation between *Feifania* abundance and inflammatory factors relative expression; (e) Correlation heat map of gut microbiota and SCFAs; (f) Correlation heat map of gut microbiota and inflammatory factors. (The p -values $* < 0.05$, $** < 0.01$ and $*** < 0.001$ are significantly different.).

adjusted q value for primary bile acid biosynthesis was 0.5687. Therefore, these pathway-level findings were considered exploratory. Enrichment analysis further suggested potential perturbations in starch and sucrose metabolism, steroid hormone biosynthesis, galactose metabolism, and porphyrin metabolism (Fig. S7e). Overall, these results suggest exploratory fecal metabolic alterations in adult offspring after maternal TBBPA exposure.

3.11. Exploratory microbiota-metabolite association analysis

Spearman correlation analysis revealed exploratory associations between altered bacterial genera and MS/MS-supported putatively annotated fecal metabolites (Fig. S8; Table S13). A total of 1824 genus-metabolite pairs were tested. Among them, 135 pairs showed nominal significance at $p < 0.05$, but none remained significant after Benjamini-Hochberg FDR correction at $q < 0.05$. Therefore, these nominal associations were interpreted as exploratory only. At the nominal level, *Limosilactobacillus*, *Mogibacterium*, *Ruminiclostridium*, *Vallitalea*, *Romboutsia*, *Turicibacter*, *Clostridium*, *Anaerobacterium*, and *Bifidobacterium* were correlated with multiple metabolites. Notably, bile acid-related metabolites, including cholic acid, glycocholic acid, chenodeoxycholic acid glycine conjugate, and lithocholic acid glycine conjugate, showed nominal associations with altered bacterial taxa, consistent with the exploratory pathway analysis, in which primary bile acid biosynthesis had the lowest raw pathway-level p value. Correlations involving propionic acid and other microbial-associated metabolites were also observed, which may be relevant to the reduced fecal SCFA concentrations in offspring. When considered alongside the observed Treg/Th17 alteration, cytokine changes, and mucosal abnormalities, these exploratory associations suggest concurrent microbial, metabolic, and immune-related changes. However, given the small sample size and the absence of FDR-supported associations, these correlations remain exploratory and do not establish causality or directionality among microbiota dysbiosis, metabolic disturbance, and mucosal immune changes.

4. Discussion

The NHANES analysis was included only as an ancillary exploratory population-level analysis of general bisphenol-related metabolic and immune-associated outcomes. Urinary BPA was positively associated with triglyceride-related traits and white blood cell count, consistent with previous reports of associations between BPA exposure and cardiometabolic or inflammatory outcomes [30–32]. However, because the NHANES analysis differed substantially from the present animal study in exposure compound, biological matrix, population, life stage, design, and outcomes, and because urinary BPA does not represent TBBPA exposure, these findings were not used to infer TBBPA-specific developmental toxicity or to support the mechanistic interpretation of the animal findings.

Except for reduced body weight in TES_I offspring, no overt phenotypic abnormalities were observed in TBBPA-exposed offspring. Organ to body weight ratios did not differ significantly between exposed and control groups. These observations are consistent with recent evaluations showing that TBBPA exposure generally does not markedly affect body or organ weights in rats across different dosing paradigms [33,34]. The multigenerational oral administration at 1000 mg·kg⁻¹·bw⁻¹ produces no significant effects on mortality, body weight, or tissue weights [26]. Collectively, the weight reduction in TES_I offspring may not be directly attributable to maternal TBBPA exposure.

In contrast, TBBPA exposure was associated with pronounced reductions in microbial diversity and alterations in community structure. This finding is consistent with recent evidence that maternal or early-life exposure to flame retardants can perturb gut microbiota homeostasis in F1 offspring. Maternal exposure to resorcinol bis(diphenyl) phosphate

decreased gut microbial abundance and diversity in dams and offspring, whereas maternal TnBP exposure caused significant shifts in α -diversity and β -diversity in both generations [24,35]; developmental DBDPE exposure likewise disrupted intestinal microbiota and promoted intestinal inflammation [36]. Clinically, reduced α -diversity and altered community structure are consistently observed in Crohn's disease and ulcerative colitis, including at disease onset [37]. In addition, chronic PFOS exposure has recently been shown to trigger colitis through a dysbiosis-driven gut microbiota-bile acid axis, further supporting the pathological relevance of pollutant-induced microbial disruption [38]. Therefore, the alterations in α - and β -diversity observed here are consistent with substantial changes in gut microbial community structure and may be relevant to susceptibility to intestinal inflammatory disorders.

At both the phylum and genus levels, TBBPA exposure was associated with alterations in gut microbiota composition. TESI offspring exhibited substantial reductions in Bacteroidota and Bacillota together with increased Proteobacteria, a pattern resembling recent offspring microbiome disturbances reported after maternal flame-retardant exposure and broadly consistent with IBD-associated dysbiosis [25, 39]. In addition, recent toxicological studies indicate that TBBPA or related brominated flame retardants can promote intestinal dysbiosis, inflammation, and loss of beneficial commensals, further supporting the pathological relevance of the compositional shifts observed here [36,38, 40]. These findings therefore suggest that TBBPA exposure-associated microbiota alterations may favor a pro-inflammatory microbial state and may be relevant to an intestinal environment associated with inflammatory susceptibility.

Fecal SCFA analysis revealed a marked contrast between exposed dams and their offspring: exposed dams showed increased acetic and propionic acid levels, whereas all offspring groups exhibited generalized reductions in acetic, propionic, and butyric acids, with adult offspring (TES_IA and TESA) showing the most pronounced decreases. This pattern is broadly consistent with recent meta-analytic evidence showing that fecal SCFA levels are reduced in inflammatory bowel disease, particularly during active disease [41]. Acetic acid, propionic acid, and butyric acid are the major microbiota-derived SCFAs and play important roles in maintaining epithelial barrier integrity, regulating mucosal immunity and inflammation, and shaping host metabolic homeostasis [42,43]. Recent toxicological studies further suggest that bisphenols and other environmental pollutants can alter microbial SCFA production or lower acetate availability, supporting the existence of a pollutant-microbiota-SCFA axis [44,45]. Although SCFA-based supplementation has been explored as a potential adjunctive strategy for ulcerative colitis, current evidence remains limited [46]. Therefore, the marked SCFA depletion in offspring feces may be relevant to reduced availability of microbiota-derived metabolites involved in intestinal barrier and immune regulation.

Maternal TBBPA exposure was associated with significantly higher colonic mRNA expression of pro-inflammatory cytokines (*TNF- α* , *IL-6*, and *IL-1 β*) in offspring. This aligns with recent evidence that bisphenol exposure promotes intestinal inflammation and cytokine production. In mice, BPA increased colonic immune-cell infiltration and macrophage GHSR-dependent expression of pro-inflammatory mediators [47], whereas in rats BPA induced small-intestinal injury accompanied by oxidative stress, inflammation, and microbiota alteration [48]; similarly, TBBPA was recently shown to trigger intestinal injury with elevated *IL-6*, *TNF- α* , and *IL-1 β* through ROS/NF- κ B signaling [49]. A recent study further showed that another brominated flame retardant, Decabromodiphenyl ethane, induced intestinal structural damage and inflammation in zebrafish, supporting the broader relevance of flame-retardant-induced gut inflammation across species [36]. Collectively, these results suggest that maternal TBBPA exposure is associated with long-term colonic pro-inflammatory activation in offspring. A key finding of this study was the altered Treg/Th17 balance in offspring colonic tissues following maternal TBBPA exposure. This result is

consistent with the concept that intestinal microbiota, epithelial barrier status, and mucosal T-cell responses are closely interconnected. However, the present study does not establish the directionality among gut dysbiosis, mucosal injury, and T-cell alteration. Microbial dysbiosis and impaired barrier integrity may increase microbial antigen exposure and promote Th17-related inflammatory responses, whereas altered Treg/Th17 balance may also aggravate mucosal injury and secondarily reshape the microbial community [50]. Therefore, the observed microbiota, histological, cytokine, and T-cell changes should be interpreted as associated features rather than as evidence of a fully validated causal pathway. This cautious interpretation is also supported by related toxicological studies showing that bisphenol exposure can induce intestinal inflammation and microbiota alteration [48,49], TBBPA can promote intestinal injury with increased inflammatory cytokine expression [51], and developmental exposure to environmental pollutants can disrupt intestinal barrier function and Th17/Treg balance [52, 53]. Future studies using fecal microbiota transplantation, antibiotic depletion, barrier-function assays, and targeted immune or metabolite interventions are needed to clarify the causal links among microbiota disruption, mucosal injury, and T-cell homeostasis after maternal TBBPA exposure.

Exploratory pathway analysis showed that primary bile acid biosynthesis had the lowest raw pathway-level *p* value and was therefore described as the top-ranked putatively perturbed pathway, although it did not remain significant after pathway-level FDR correction. Several bile acid-related metabolites, including cholic acid, showed nominal associations with altered bacterial taxa in the exploratory microbiota-metabolite correlation analysis. This finding is noteworthy because gut microbiota dysbiosis is closely linked to bile acid metabolic disturbance in intestinal inflammation. Recent evidence indicates that inflammatory bowel disease is commonly associated with increased primary bile acids, reduced secondary bile acids [54], and impaired microbiota-bile acid crosstalk, while disruption of bile acid-related microbial metabolites has also been implicated in colitis-associated carcinogenesis [55]. Phenylalanine, tyrosine, and histidine are likewise important substrates for gut microbial metabolism and can be converted into bioactive compounds that influence epithelial function, immune responses, and host metabolic homeostasis [56,57]. Moreover, recent toxicological studies show that environmental pollutants can perturb both bile acid balance and microbiota-related amino acid metabolism, supporting the biological plausibility of the metabolic disturbances observed here [58]. Collectively, these exploratory findings suggest potential alterations in bile acid and aromatic amino acid metabolism following maternal TBBPA exposure, although these pathway-level observations require validation in larger studies.

5. Advantages and limitations of the research

This study provides a systematic investigation of the developmental effects of maternal TBBPA exposure on gut microbiota composition and associated metabolic profiles in offspring using an SD rat model with defined gestational and/or lactational exposure windows. Integrative analysis of multiple endpoints, including elevated pro-inflammatory cytokine expression, altered Treg/Th17 balance, and exploratory metabolic pathway changes, suggests that maternal TBBPA exposure is associated with colonic inflammatory and immune-related alterations in offspring. These findings provide baseline data for evaluating potential intestinal effects of maternal TBBPA exposure and may inform future mechanistic studies.

Several limitations warrant consideration. First, the single-dose design precludes evaluation of a dose-response relationship. The administered dose of 170 mg·kg⁻¹·day⁻¹ represents a high-dose hazard-identification regimen. The original dose-selection approach did not account for route-specific toxicokinetics and therefore should not be interpreted as establishing a human-equivalent or environmentally relevant exposure level. Extrapolation of the present findings to real-

world human exposure should accordingly be made with caution. Second, urinary BPA was analyzed in NHANES because urinary TBBPA data were unavailable. Accordingly, the NHANES analysis should be interpreted only as ancillary exploratory context rather than as direct epidemiological evidence for TBBPA exposure. Third, although this study integrated microbiome, SCFA, metabolomic, cytokine, and T-cell endpoints, it did not include causal validation experiments, such as antibiotic depletion, fecal microbiota transplantation, germ-free animal models, SCFA or bile acid intervention, or targeted pathway inhibition. Therefore, the observed microbiota-metabolite-immune associations cannot establish a fully causal mechanistic chain. Fourth, litter-level independence was not uniform across offspring analyses. In particular, the 16S rRNA sequencing, SCFA, and metabolomic analyses included two offspring from each of three contributing litters, and litter-level dependence was not explicitly modeled. Because only three contributing litters were available for these endpoints, additional litter-level inferential modeling was considered statistically unreliable; therefore, these findings were interpreted as exploratory. Residual within-litter dependence cannot be excluded where clustering was not explicitly modeled. The modest number of contributing litters and small sample sizes limited statistical power and precluded sex-specific analyses. Future studies with larger sample sizes are needed to further evaluate potential litter- and sex-related effects. Fifth, this study evaluated only F1 offspring after maternal TBBPA exposure. Because unexposed F2 or F3 generations were not examined, these findings should be restricted to developmental or early-life exposure-associated effects in F1 offspring and should not be extrapolated to unexposed later generations. Future studies should incorporate environmentally relevant dose gradients, litter-aware statistical designs, and targeted mechanistic experiments to clarify the causal pathways through which maternal TBBPA exposure affects offspring intestinal and immune homeostasis.

6. Conclusion

The environmental persistence and health risks of flame retardants remain a major public health concern. In this study, maternal TBBPA exposure was associated with colonic inflammatory features, altered Treg/Th17 balance, gut microbiota alterations, and metabolic alterations in offspring, suggesting long-term alterations in intestinal microbial, metabolic, and immune homeostasis. These findings provide further insight into the potential developmental intestinal immunotoxicity associated with maternal TBBPA exposure. However, the observed microbiota-metabolite-immune relationships remain associative, and further studies are needed to clarify the underlying causal pathways.

Environmental implication

Tetrabromobisphenol A (TBBPA) is a widely used brominated flame retardant that is now ubiquitously detected in environmental media, wildlife, and humans. Due to its persistence, widespread use, and potential for maternal transfer, TBBPA may pose sustained risks during sensitive developmental periods. Increasing evidence links TBBPA exposure to developmental, metabolic, and immune-related adverse effects, raising concern over its long-term health implications. Its environmental ubiquity and potential intergenerational toxicity underscore the need for continued monitoring, exposure control, and strengthened environmental risk assessment.

CRediT authorship contribution statement

Chongxiang Zeng: Data curation. **Jiao Yang:** Methodology. **Zenghua Qi:** Data curation. **Yinfeng Zhou:** Data curation. **Shuyang Huang:** Writing – original draft, Methodology, Data curation. **Yingxin Yu:** Supervision, Funding acquisition, Conceptualization. **Guoxia Zhang:** Writing – review & editing, Supervision, Data curation.

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.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (82173476 and 41991314), the Talent Project of Center for Disease Prevention and Control of Guangdong Province (2024D344), and the Guangdong-Hong Kong-Macao Joint Laboratory for Contaminants Exposure and Health (2020B1212030008).

Supplementary material

Reagents (Text S1); 16S rRNA gene sequence amplification analysis (Text S2); Bioinformatics analysis of the gut microbiota (Text S3); Extraction and instrumental analysis of SCFAs from fecal samples (Text S4); Colon tissue total RNA extraction, reverse transcription step and qPCR assay (Text S5); Steps for T-lymphocyte extraction from colon tissue (Text S6); Non-targeted metabolomics fecal sample pre-treatment and instrumental analysis (Text S7); Number of contributing litters, offspring sample size, sex distribution, and offspring sampled per litter for each experimental endpoint. (Table S1); The primers for real-time q-PCR. (Table S2); Weighted baseline characteristics of participants according to quartiles of urinary BPA in the NHANES complementary analysis. (Table S3); Adjusted quartile-based associations of urinary BPA with gut function, metabolic traits, and immune/inflammatory markers in the exploratory NHANES analysis. (Table S4); Sex-stratified and sex-interaction analyses for the associations of urinary BPA with metabolic and immune/inflammatory outcomes in the exploratory NHANES analysis. (Table S5); Sensitivity analyses for the associations of urinary BPA with gut function and inflammatory outcomes in the exploratory NHANES analysis. (Table S6); Linear characteristics of SCFAs, detection limits and quantitative limits of methods. (Table S7); Stability and recovery rate of SCFAs detection. (Table S8); Fecal SCFAs content in each group ($\mu\text{g}\cdot\text{mL}^{-1}$) (Table S9); Quality-control and model-validation parameters for fecal untargeted metabolomics analysis. (Table S10); Nominally significant MS/MS-supported putatively annotated fecal metabolite features in TES_A offspring compared with CES_A offspring. (Table S11); KEGG pathway analysis of fecal metabolomic features in TES_A offspring compared with CES_A offspring. (Table S12); Spearman correlation coefficients, nominal p values, and FDR-adjusted q values for associations between bacterial genera and fecal metabolites. (Table S13); Associations of urinary BPA with triglyceride-related metabolism and white blood cell count in the exploratory NHANES analysis. (Figure S1); Effects of TBBPA exposure on phenotype, body weight, and organs. (Figure S2); H&E staining score and Treg/Th17 ratio in offspring colonic tissues. (Figure S3); LEfSe analysis of each group. (Figure S4); Heat maps of relative abundance at the genus level. (Figure S5); Total ion flow chart of mixed standard solution. (Figure S6); Metabolomic results of adult offspring. (Figure S7); Spearman's rank correlation between 76 fecal metabolites and 24 bacterial genera. (Figure S8).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2026.143706.

Data availability

Data will be made available on request.

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