Metabolic Alteration of Gut Microbiota Is Associated With Host Non-alcoholic Fatty Liver Disease Induced by Pyrethroid in *Xenopus Laevis*

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Research

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Abstract

Background: There is increasing awareness of the significance of the gut microbiome to host health, and a clear relationship has been established between the perturbed gut microbiome and multiple diseases. *Cis*-bifenthrin, a widely used agricultural pyrethroid insecticide, has been implicated as a cause of hepatotoxicity due to the oxidative stress produced during its metabolism by the liver. Studies have demonstrated the role of gut microbiota in gut-liver axis, it is possible that the perturbation of gut microbiota may also contribute to the toxicity of *cis*-bifenthrin on the liver.

Results: 16S rRNA gene sequencing suggested that *cis*-bifenthrin exposure significantly perturbed the gut microbiota composition, and metabolomics analysis showed signature metabolic shifts arising from exposure. Moreover, we also found altered functional regulation of lipids in the liver after *cis*-bifenthrin exposure, and the accumulation of lipid droplets in hepatocytes was observed.

Conclusions: Our results suggested *cis*-bifenthrin exposure disturbed the gut microbiota community and metabolite profile in frogs. Specifically, changes in bile acid metabolites altered bile acid hepatoenteral circulation, which affected lipid metabolism in the liver and ultimately caused the development of fatty liver disease. Our findings reveal novel insights into gut microbiota-host axis in frogs, and the perturbed microbial function provides novel mechanism contributing to *cis*-bifenthrin-induced toxicity.

Background

A growing body of research has demonstrated the essential role of the gut microbiome in the normal development and overall health of the host organism [1, 2]. The gut microbiota control physiology beyond the intestinal lumen as they can signal to cells throughout the host to regulate their activity through the production of metabolically active microbial metabolites [3, 4]. Intestinal dysbiosis can contribute to hepatic metabolic diseases via various obesity-associated mechanisms, including abnormalities in lipid metabolism [5]. Current reports on the chemical disruption of host-associated microbiota have generally been concerned with persistent organic pollutants (POPs) or antibiotics [6, 7], and fewer studies have well characterized the microbiome-associated challenges to healthy hosts from pesticide exposure.

Pesticides are indispensable for modern agricultural production. In recent decades, synthetic pyrethroid (SP) use has increased continuously due to its insecticidal potency and shorter breakdown time than organophosphate pesticides [8]. Today, SPs have extensive indoor and outdoor applications, including in agriculture, public health, and residential usage [9]. SP contamination is extensively observed in soil, water, streambed sediments and indoor dust. For instance, a maximum value of 901 ng/L of SP has been detected in the surface water samples of California coastal watersheds, with bifenthrin being the most commonly detected SP [10, 11]. The maximum concentration of SP pesticides found in the surface waters of the Pearl River Basin (China) was 29.72 g/L [12]. Additionally, SPs were detected in wild fish from Iberian river basins (Spain), with the highest concentration detected being 4938 ng/g lipid weight [13]. Moreover, it is worth noting that a considerable amount of research has shown that SPs ultimately

pose a health threat to wildlife and humans. Mounting laboratory studies have reported the toxic effects of SPs, including developmental toxicity, neurotoxicity, and reproductive toxicity, in different nontarget organisms [14-17]. In addition, in recent years, considering the indispensable and crucial role of the gut microbiome, specific attention has also been given to the influence of SPs on the abundance, composition and metabolic function of the gut microbiome. A previous study indicated that low-concentration exposure to permethrin significantly changed the abundance of the gut microbiota in rats but also altered the metabolic profiles, including the acetic and propionic acid contents [18]. However, the adverse influences of SPs on gut microbiota community structures and metabolite-mediated host metabolism are largely unknown. Mechanistic insight into the interplay of gut microbiota, host metabolism, and metabolic health has gained significant attention over the past decade [19]. The gut microbiota has been considered a “microbial organ” within the intestine that modulates the health phenotype of the host via a range of microbial metabolites. Bile acids are classical examples of metabolites that are metabolized in the intestine by the gut microbiota into secondary bile acids and therefore regulate host metabolic pathways [19].

Since the gut microbiota have been shown to play indispensable roles in the maintenance of normal hepatic functions and the pathogenesis of disease, greater insights into the mechanisms driving the interplay between SPs and the gut microbiome are needed to reveal SP-induced adverse effects and related susceptibilities. Therefore, the present study was conducted to address whether SP exposure would alter the gut microbiota community structure as well as the gut microbiota-related metabolic functions, and whether SP-induced intestinal dysbiosis would further impact the metabolic process of the host liver. We applied 16S rRNA gene sequencing, liquid chromatography-mass spectrometry (LC-MS) metabolomics profiling, and transcriptomics to probe functional interactions between liver function and the gut microbiome after exposure to pyrethroids. *Cis*-bifenthrin (*cis*-BF) is among the most commonly used SPs and is detected the most frequently [10, 11]. Amphibians are suitable for studying the effects of stressors from environmental toxicants since they can provide more comprehensive toxicological endpoints [20]. The African clawed frog (*Xenopus laevis*, *X. laevis*) is a widely used amphibian model organism and is regarded as an invaluable tool for ecotoxicological assessment [21, 22]. Therefore, *cis*-bifenthrin was chosen as the representative SP, and African clawed frogs were used as the bio model. Our study provides the first evidence of the functional interactions between liver function and the gut microbiome after pyrethroid exposure.

Methods

Animals and exposure

X. laevis adults were obtained from Nasco (Fort Atkinson, WI, USA). To alleviate suffering, animals were maintained humanely in accordance with the guidelines of the American Society for Testing and Materials [23]. Adult frogs (2 years old) were exposed to the environmentally relevant concentrations of 0.06 and 0.3 µg/L *cis*-BF (Sigma, St. Louis, MO, USA). Stock solutions were prepared with alcohol, and the alcohol concentrations were identical for all treatment groups. Each treatment consisted of 27 male frogs

that were randomly assigned to three replicates. Experiments were carried out at the same photoperiod (12 h light: 12 h dark) and temperature (22°C). During the 3 months of exposure, the exposure solutions were replaced daily to maintain the concentration of *cis*-BF. After exposure, the frogs were anesthetized in MS-222 and frozen in liquid nitrogen for subsequent analysis. All studies were approved by the Experimental Animal Ethics Committee of Zhejiang University.

Extraction and quantification of *cis*-BF

The *cis*-BF in the exposure solutions and intestines was extracted and detected according to a previous study with slight modifications [24]. For exposure solutions, 200 mL of exposure solution was mixed with sodium chloride (5.0 g) and dichloromethane (200 mL) in a 500 mL separatory funnel. The mixture was shaken for 5 min and left at room temperature for 5 min to separate the organic and aqueous phases. Then, the organic phase was collected, and the extraction procedure was repeated once again. The combined organic phase was evaporated to near dryness on a rotatory evaporator at 40°C. Frog intestine contents (1 g) were homogenized and transferred into a 50 mL polypropylene tube preloaded with 0.25 g NaCl and 1 g MgSO₄. Then, 5 mL acetonitrile was added to extract *cis*-BF, followed by sonication for 20 min. The mixture was centrifuged at 4500 rpm for 10 min, and the organic phase was transferred into a glass flask. The extraction procedure was repeated once again, the organic phase was combined and subsequently evaporated almost to dryness on a rotatory evaporator at 40°C. The residues from the exposure solution and tissues were reconstituted with 1 mL acetonitrile and transferred into a 1.5 mL polypropylene tube that contained 200 mg PSA. After vortexing and centrifugation (12000 g, 10 min), the supernatant was filtered with a 0.22-μm polytetrafluoroethylene filter and transferred into vials for quantitative analysis.

Oil red O staining

Fresh hepatic tissues were fixed in 4% (v/v) formaldehyde in phosphate buffer for 24 hours. The hepatic tissues were transferred into 15% and 30% sucrose solutions in turn at 4°C for dehydration. After dehydration, the hepatic tissues were embedded in optimal cutting temperature medium and subsequently cut into 8-μm-thick sections. The sections were stained with Oil Red O solution (Servicebio, Wuhan, China) for 10 min in the dark. The background differentiation procedure was performed in 60% isopropanol (Servicebio, Wuhan, China). Then, the sections were washed in water for 10 s and stained with hematoxylin. Glycerin gelatin (Servicebio, Wuhan, China) was used to seal the slices. The slices were observed and photographed on a light microscope (Olympus, Tokyo, Japan).

16S rRNA gene sequencing

Total genomic DNA was extracted from the intestinal contents of frogs using the PowerSoil® DNA isolation kit according to the manufacturer's instructions. Extracted DNA was amplified to target the V3-V4 regions of the 16S rRNA gene of bacteria using the primer pair 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT). The amplicons were assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and sequenced on an Ion S5™ XL platform. To obtain high-quality clean reads, the

raw reads were filtered on Cutadapt (Martin M., 2011). In addition, chimera sequences were removed with the UCHIME algorithm (UCHIME Algorithm). Uparse software (Uparse v7.0.1001) was used to choose operational taxonomic units (OTUs) with a threshold of 97% sequence similarity. A representative sequence from each OTU was selected for taxonomic annotation using the Silva Database (Version 132). The taxonomic information of the 16S rRNA gene sequences was obtained at different classification levels, including the phylum, class, order, family and genus levels. Alpha diversity (observed species, Chao1, Shannon, Simpson, ACE, and good coverage indices) and beta diversity were calculated with QIIME (Version 1.7.0). Linear discriminant analysis (LDA) effect size (LEfSe) was performed using the LEfSe tool.

Metabolomics analysis

The intestinal contents (100 mg) were ground with liquid nitrogen, and the homogenate was resuspended in prechilled 80% methanol (-20°C). After vortexing, the samples were incubated for 1 h at -20°C and then centrifuged at 14000 g for 20 min at 4°C. The supernatants were transferred to a new tube, dried in a vacuum concentrator, and then reconstituted in 60% methanol for LC-MS/MS analysis. LC-MS/MS analysis was performed on a Vanquish UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher). Chromatographic separation was performed using a Hyperil Gold column (100×2.1 mm, 1.9 µm). For the positive polarity mode (pos), the eluents were 0.1% FA in water (A) and methanol (B). For the negative polarity mode (neg), the eluents were 5 mM ammonium acetate (A, pH 9.0) and methanol (B). The solvent gradient was programmed as follows: 98% A, 1.5 min; 98-0% A, 12.0 min; 0% A, 14.0 min; 0-98% A, 14.1 min; 98% A, 17 min. The flow rate was 0.2 mL/min. The other optimized parameters were as follows: spray voltage, 3.2 kV; capillary temperature, 320°C; sheath gas flow rate, 35 arb; and aux gas flow rate, 10 arb. The raw data were processed with Compound Discoverer 3.0 (CD 3.0, Thermo Fisher) for peak alignment, peak picking, and quantitation for each metabolite. Then, the molecular features were matched with the mzCloud (<https://www.mzcloud.org/>) and ChemSpider (<http://www.chemspider.com/>) databases.

Transcriptomics analysis

The mRNA-Seq experiment was completed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Total RNA was isolated from frog liver using TRIzol reagent (Takara Bio Inc., Kusatsu, Japan). The RNA purity and integrity were assessed by using the A260/A280 ratio and agarose–formaldehyde gel electrophoresis, and the RNA concentration was also measured. A total amount of 3 µg of RNA was used for RNA sample preparation, and the sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA). The quality of the sequencing library was assessed on the Agilent Bioanalyzer 2100 system. Then, the library was sequenced on an Illumina HiSeq platform to generate 125 bp/150 bp paired-end reads. Reads containing adapters and poly-N and low-quality reads were removed to obtain clean reads. FeatureCounts v1.5.0-p3 was employed to calculate the clean read numbers mapped to each gene, and the FPKM value of each gene was also calculated based on the length of the gene and the read counts mapped to this gene. The DESeq2 R package (1.16.1) was used to

assess the differential expression analysis results, and genes with an adjusted p -value < 0.05 were considered differentially expressed. The statistical enrichment of differentially expressed genes in KEGG pathways was performed using the clusterProfiler R package.

Quantitative real-time PCR (qPCR) validation

Frog liver tissues (n = 3 replicates; each replicate contained three tissues) were homogenized with RNAiso plus (Takara Bio Inc, Kusatsu, Japan) to extract the total RNA. The total RNA contents were measured on an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The quality of the extracted total RNA was also examined via the 260/280 nm ratios and 1% agarose gel electrophoresis. The Prime Script™ RT reagent Kit (Takara Bio Inc, Kusatsu, Japan) was used to synthesize the cDNA. The real-time PCR was carried out on an ABI 7300 system (Applied Biosystems, CA, USA) using the SYBR Green kits (Takara Bio Inc, Kusatsu, Japan). Specific primers of the target genes were identified by the NCBI Primer-BLAST and showed in the SI (Table S1). The transcriptional stability of the reference gene was accessed using the GeNorm, and finally the 18s was chosen as the housekeeping gene. The gene transcription levels were calculated using $2^{-\Delta\Delta C_t}$ method.

Statistics

Data were presented as the mean $\pm$ standard error (SEM). SPSS 20.0 (SPSS, Chicago, IL, USA) was used to perform statistical analyses, and the comparison between the exposure groups and control group was performed using one-way analysis of variance (ANOVA) followed by Tukey's test. A p < 0.05 was considered to be significant.

Results

Cis-BF contents

The *cis*-BF concentrations were measured at 0 h and 24 h after renewing the exposure solutions, and the measured *cis*-BF concentrations were similar to the nominal exposure concentrations (Table S2). The *cis*-BF concentrations in the intestinal contents of *X. laevis* are shown in Fig. 1a. In the 0.06 and 0.3 $\mu\text{g/L}$ exposure groups, the *cis*-BF concentrations in the intestinal contents were 170.35 ± 10.95 and 266.60 ± 30.15 $\mu\text{g/kg}$, respectively (Fig. 1a). *Cis*-BF was not detected in the intestinal contents of the control group.

Lipid accumulation in the liver

Exposure to 0.3 $\mu\text{g/L}$ *cis*-BF increased the hepatosomatic index (HSI) of *X. laevis* (Fig. 1b). Compared with the control group (Fig. 1c), the hepatic lipid droplets in the liver of *X. laevis* in the 0.06 (Fig. 1d) and 0.3 $\mu\text{g/L}$ (Fig. 1e) exposure groups were obviously increased (Fig. 1f).

Gut microbiome changes

Cis-BF exposure significantly altered the composition, abundance and diversity of the gut microbiome community. In terms of the gut microbial structure, obvious differences were observed between the control group and all *cis*-BF exposure groups. The 0.06 and 0.3 µg/L *cis*-BF exposure groups were more similar (ANOSIM; $R = 0.07$, $p = 0.17$), with all *cis*-BF exposure groups showing obvious differences from the control group (0.06: $R = 0.18$, $p = 0.018$; 0.3: $R = 0.25$, $p = 0.013$; Table 1). Compared with the control group, 0.3 µg/L *cis*-BF exposure significantly decreased the diversity of the microbiome (Simpson: $p = 0.0379$ and Shannon: $p = 0.0312$; Fig. 2a, b and Table 2). A network based on the top 50 OTUs was also constructed to visualize the different bacteria in the intestinal contents of *X. laevis* in the control and *cis*-BF exposure groups. The result showed that 11 and 20 OTUs exclusively emerged in 0.06 and 0.3 µg/L exposure groups, respectively (Fig. 2c). Fig. 2d illustrates the relative abundance of the top 10 microbiota at the phylum level in *X. laevis*. At the phylum level, Firmicutes and Fusobacteria were the most abundant phyla, followed by Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria, Synergistetes, Chloroflexi, Tenericutes and Armatimonadetes. Obvious increases in the abundances of Chloroflexi and Armatimonadetes were observed in all *cis*-BF exposure groups. However, 0.3 µg/L *cis*-BF exposure significantly decreased the abundance of Bacteroidetes. At the genus level, several significantly changed genera are shown in Fig. 3a. Exposure to *cis*-BF (0.06 and 0.3 µg/L) also increased the abundances of the pathogenic bacteria *Plesiomonas* (Fig. 3b) and *Aeromonas* (Fig. 3c). LEfSe was performed to identify the specific bacteria that served as biomarkers in the control group and *cis*-BF treatment groups. The LEfSe results illustrated that 14 biomarkers with an LDA score > 4 were differentially abundant between the control group and the *cis*-BF treatment groups (Fig. 3d).

The metabolic profile of the gut microbiome

Three months of *cis*-BF exposure significantly disturbed the metabolic profiles of the *X. laevis* intestinal contents. Specifically, 60 (pos:36 and neg:24) and 73 (pos:36 and neg:37) metabolites with a fold change > 1.5 and $p < 0.05$ were observed in *X. laevis* treated with 0.06 (Figure S1a; Table S3) and 0.3 µg/L (Fig. 4a; Table S3) *cis*-BF, respectively.

In the 0.3 µg/L *cis*-BF treatment group, the contents of taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA) and glycocholic acid (GCA) were lower than those in the control group. However, the deoxycholic acid (DCA), taurochenodeoxycholic acid (TCDCA) and cholic acid (CA) levels were unaffected by *cis*-BF (Fig. 4b). In the 0.06 µg/L *cis*-BF treatment group, there were no significant changes in terms of bile acids (Fig. 4b).

All metabolites that changed significantly upon *cis*-BF exposure were subjected to KEGG pathway enrichment analysis, and 7 pathways were significantly enriched in the 0.3 µg/L *cis*-BF exposure group (Fig. 4c). The significantly enriched metabolic pathways included primary bile acid biosynthesis, secondary bile acid biosynthesis, bile secretion and cholesterol metabolism. In the 0.06 µg/L *cis*-BF treatment group, pyrimidine metabolism pathway was significantly enriched (Figure S1b; Table S4). Correlation analysis based on the top 20 OTUs and bile acids (Fig. 4d) indicated that *g_Clostridium_sensu_stricto_1*, *f_Lachnospiraceae*, *f_Porphryomonadaceae*, *g_Desulfovibrio*,

f_Erysipelotrichaceae, *f_Peptostreptococcaceae*, *p_Firmicutes*, *f_Family_XIII*, *g_Lachnoclostridium* and *s_Fusobacterium_varium* were obviously positively correlated with bile acids. However, *g_Romboutsia* showed a negative correlation with taurochenodeoxycholic acid.

Differentially expressed genes

We identified 5055 (2620 downregulated and 2435 upregulated genes; Figure S2a) and 5188 (2570 downregulated and 2618 upregulated genes; Figure S2b) differentially expressed genes in the 0.06 and 0.3 µg/L *cis*-BF treatment groups, respectively. All differentially expressed genes were assigned for KEGG pathway analysis, with the altered pathways shown in Table S5. The KEGG results showed that 34 and 17 pathways were obviously enriched in the 0.06 (Table S5) and 0.3 µg/L (Fig. 5a) *cis*-BF treatment groups, respectively. The enriched signaling pathways associated with metabolic signaling pathways, including fatty acid degradation, fatty acid metabolism and the PPAR signaling pathway, indicated that *cis*-BF exposure could influence fatty acid metabolism in the livers of frogs (Fig. 5b). Heatmaps of the differentially expressed genes involved in PPAR signaling pathway and fatty acid metabolism pathway are shown in Fig. 5c and 5d, respectively. The pathways of primary bile acid biosynthesis and fatty acid degradation were significantly enriched for both differentially expressed genes and differentially changed metabolites (Fig. 5e). The gene expression levels obtained from the transcriptomics were validated by using qPCR, and the fold-changes of selected genes were in agreement with transcriptomic results (Table S6).

Discussion

In this study, we clearly showed that environmentally relevant levels of *cis*-BF exposure induced a significant change in the frog gut microbiome and its metabolic profiles, leading to disorders of lipid metabolism in the liver. In addition, lipid metabolism disorders were strongly related to changes in bile acid production in the gut. Our results suggested that gut microbiota dysbiosis is involved in the abnormal lipid metabolism caused by SP.

High deposition of *cis*-BF in the gut contents of frogs indicates bioaccumulation and bioavailability of these compounds in the intestinal system, which suggests that *cis*-BF may interact with interstitial cells and the intestinal microbiome. The microbiome is relatively plastic and can be rapidly altered through factors such as diet, drugs, probiotics, and xenobiotic exposure [25]. Our 16S rRNA gene sequencing results (ANOSIM) showed significantly different community structures between groups, which indicates that *cis*-BF exposure significantly altered the community structures of the frog gut microbiome. A reduction in bacterial diversity was observed in *cis*-BF-treated *X. laevis*, suggesting that *cis*-BF altered the patterns of the gut microbial ecosystems, which may alter the mutually beneficial interaction toward another stable but harmful balance [26]. We identified a number of bacterial genera perturbed in *X. laevis* after *cis*-BF exposure. It is remarkable that several pathogenic bacteria of the *Plesiomonas* and *Aeromonas* genera were only observed in treated frogs. *Plesiomonas* is a microorganism of aquatic origin and plays a role as a pathogen of both humans and animals causing occasional opportunistic

infections and diarrhea [27]. *Aeromonas* species that are known to be pathogenic in humans are also pathogenic in fish [28]. Thus, our results showed that SP exposure induced the prevalence of several potentially pathogenic bacteria, which could increase the risk of disease development. In addition, several genera of the families *Erysipelotrichaceae*, *Ruminococcaceae* and *Lachnospiraceae*, which belong to the Firmicutes phylum, decreased in the intestinal tract of frogs. Previous studies have confirmed that the members of the family *Erysipelotrichaceae* are associated with lipidemic profiles within the host [29, 30]. The families *Lachnospiraceae* and *Ruminococcaceae*, which belong to *Clostridiales* within Firmicutes, have been associated with the maintenance of gut health [31]. A few members of the family *Lachnospiraceae* or *Ruminococcaceae* are known to convert conjugated primary bile acids to secondary bile acids. The loss of *Lachnospiraceae* and *Ruminococcaceae* in acute and chronic intestinal diseases has been linked to bile acid metabolic changes [32]. For instance, a previous study reported that in pouch patients who experience ulcerative colitis, members of the family *Ruminococcaceae* were significantly depleted, and this condition was associated with secondary bile acid deficiency [33]. Mice fed lard and bile acids showed changes in the dominant intestinal bacterial community, including a decline in the relative abundance of *Lachnospiraceae* linked to hepatic lipid rearrangements [34]. Therefore, our results suggested that the decrease in certain genera in the intestinal tracts of treated frogs may induce changes in the contents of microbial metabolites that can act both locally and systemically.

Consistent with the microbiome compositional changes, the metabolite profiles were also significantly altered, with a number of key pathways being disrupted by *cis*-BF exposure. In particular, a perturbation was found in cholesterol metabolism, primary bile acid biosynthesis, secondary bile acid biosynthesis and bile secretion in the intestinal microbiota of frogs treated at higher doses. Bile acids are metabolites of cholesterol that are synthesized in the liver and secreted into the intestine, and a portion of the total bile acids are dehydroxylated by intestinal bacteria to hydrophobic secondary bile acids [35]. Our results indicated that *cis*-BF possesses biological activities on the composition of the gut microbiota, which may cause changes in microbial metabolites. Specifically, decreased levels were found for bile acids, including glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), and sulfoglycolithocholic acid. An important finding in this study is that changes in bile acids were strongly positively associated with the abundance of the *Clostridium_sensu_stricto_1* bacterial family ($p \leq 0.01$). Linear discriminant analysis effect size also suggested that *Clostridium_sensu_stricto_1* was a typical biomarker found in the control group compared with the *cis*-BF exposure groups. In fact, a previous study reported that a small population of intestinal species in the genus *Clostridium* spp. are capable of deconjugating conjugated BAs and metabolizing bile acids [36]. Therefore, *Clostridium_sensu_stricto_1* is probably one of the most important genera that mediates BA profile changes induced by *cis*-BF. Obviously, future studies are needed to address the role of the gut microbiome in the changes in its metabolites after exposure to environmental toxicants. As a class of host-derived metabolites, BAs are signaling molecules through a variety of receptors to maintain metabolic homeostasis, such as hepatic lipid and energy homeostasis [37]. Disorders in bile acid metabolism could cause cholestatic liver diseases, dyslipidemia, fatty liver diseases, diabetes, etc [37]. In addition, a defect in the synthesis of the primary bile acids (such as cholic acid or chenodeoxycholic acid) in the liver would cause cholestasis and

the malabsorption of fat and fat-soluble vitamin [38]. It was also observed that bacterial dysbiosis observed in cirrhosis is linked to low bile acid levels entering the intestine [36]. Microbe-derived metabolites and the signaling pathways they affect might play important roles in the development of nonalcoholic fatty liver disease [39]. Therefore, the modification of BA metabolites after *cis*-BF exposure may perturb the normal physiological process of hepatic metabolism in frogs.

Transcriptomic analysis of the liver was performed in parallel, and differentially expressed genes were identified. KEGG analysis revealed that fatty acid metabolism-related terms were significantly enriched among these differentially expressed genes. For example, *cis*-BF treatment in frogs altered the activation of fatty acid metabolism, fatty acid degradation and the peroxisome proliferator activated receptor (PPAR) signaling pathway. PPARs are nuclear hormone receptors that modulate the expression of genes and are involved in adipogenesis and maintenance of metabolic homeostasis [40]. In particular, *xPPAR alpha* and *PPARG coactivator 1 alpha* (*ppara* and *ppargc1a*) gene were downregulated significantly, meanwhile, the mRNA of genes associated with fatty acid transport processes were significantly decrease. The inhibition of the expression levels of the receptors and these signaling pathway-related genes may lead to a decrease in disorders of fatty acid oxidation and lipid catabolism, resulting in increased lipid deposition in the liver. The association between the hepatic gene enriched pathways and the gut metabolic profile was evaluated, and significant enrichment was found in the primary bile acid biosynthesis pathway. Bile acids circulate between the liver and the intestine and back to the liver, which called enterohepatic circulation of the bile, play an essential role in the liver, biliary, and intestinal function and disease [37]. Clinical studies indicate that dysregulation of bile acid homeostasis and its related signaling pathways is associated with the occurrence of liver disease [39]. Our results further confirmed the important role of bile acids in liver metabolism, especially when challenged by environmental pollutants, and bile acid disorders have a part in inducing liver metabolism disorder. Although the regulatory mechanism remains unclear, as previous reports have suggested that bile acids can induce the expression of the human *PPAR alpha* gene and contribute to liver disease by altering nuclear bile acid receptor farnesoid X receptor (FXR) signaling [41, 42], indicating that the suppressed BA-FXR signaling pathway might participate in the regulation of fatty acid metabolism in liver. Further studies are needed to reveal the role of FXR in the regulation of lipid homeostasis and metabolic pathways upon *cis*-BF exposure. Our results suggest that changes in the metabolic capacities of intestinal microbes reduces the lipid metabolism function of the liver.

Lipids are stored within lipid droplets in cells [43]. To further investigate whether the hepatic metabolic pathway changes that were induced by *cis*-BF exposure led to alterations in lipid storage, we measured the formation of lipid droplets in hepatocytes. We observed that liver sections from exposed frogs showed increased lipid droplets by using oil red O staining. A previous study reported that lipid droplet accumulation is the hallmark of nonalcoholic fatty liver disease [43]. Our results indicated that the inhibition of metabolism-related molecular signaling pathways in the liver induces the accumulation of lipids, which leads to cellular dysfunction and liver disease, such as hepatic steatosis.

Conclusion

In summary, the present study demonstrates that environmentally relevant levels of *cis*-BF exposure significantly altered the structure and composition of intestinal microorganisms, resulting in changes in the intestinal metabolites involved in multiple metabolic pathways, especially bile acid metabolism, and ultimately interfered with lipid metabolism in the *X. laevis* liver. Our results indicated that gut microbes play a crucial role in regulating the toxicity of environmental toxicants. In particular, the interplay between gut microbes and xenobiotics may promote the unexpected development of toxicity, which is easily overlooked due to the lack of information on the direct-action sites of xenobiotic compounds in organisms. Therefore, our results highlight the capacity for physiological regulation by intestinal microorganisms, particularly in the potential amplification of chemical toxicity resulting from environmental toxicant exposure, which is important for the identification of potential ecological risks of pesticide use.

Abbreviations

POPs—Persistent organic pollutants; SP—Synthetic pyrethroid; LC-MS—Liquid chromatography-mass spectrometry; *cis*-BF—*Cis*-bifenthrin; OTUs—Operational taxonomic units; LEfSe—Linear discriminant analysis effect size; qPCR: Quantitative real-time PCR; HSI: hepatosomatic index; TCA: taurocholic acid; GCDCA: glycochenodeoxycholic acid; GCA: glycocholic acid; DCA: deoxycholic acid; TCDCA: taurochenodeoxycholic acid; CA: cholic acid; PPAR: peroxisome proliferator activated receptor; FXR: farnesoid X receptor.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw datasets supporting the results of this article has been deposited in the NCBI and the project numbers are PRJNA725147 and PRJNA725067.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ML, QW, and MW designed this study, ML, TL and JZ performed the exposure experiment and sample collection. ML, TL and JZ analyzed the data. ML and QW wrote the manuscript. All authors read and approved the manuscript.

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Not applicable.

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References

1. Ba Q, Li M, Chen P, Huang C, Duan X, Lu L, et al. Sex-Dependent Effects of Cadmium Exposure in Early Life on Gut Microbiota and Fat Accumulation in Mice. *Environ Health Persp.* 2017;125(3):437-46.
2. Gao B, Bian X, Mahbub R, Lu K. Sex-Specific Effects of Organophosphate Diazinon on the Gut Microbiome and Its Metabolic Functions. *Environ Health Persp.* 2017;125(2):198-206.
3. Ramírez-Pérez O, Cruz-Ramón V, Chinchilla-López P, Méndez-Sánchez N. The Role of the Gut Microbiota in Bile Acid Metabolism. *Ann Hepatol.* 2017;16(Suppl. 1):S21-S26.
4. Wang M, Cerna Va T. Overhauling the assessment of agrochemical-driven interferences with microbial communities for improved global ecosystem integrity. *Environ Sci Ecotechnol.* 2020;4:100061.
5. Amanda CS, Omar RL, Riezu-Boj JI, Milagro FI, Alfredo MJ. Diet, Gut Microbiota, and Obesity: Links with Host Genetics and Epigenetics and Potential Applications. *Adv Nutr.* 2019;10(1):S17-S30.
6. Zhang L, Nichols RG, Correll J, Murray IA, Tanaka N, Smith PB, et al. Persistent Organic Pollutants Modify Gut Microbiota–Host Metabolic Homeostasis in Mice Through Aryl Hydrocarbon Receptor Activation. *Environ Health Persp.* 2015;123(7):679-88.
7. Zhou L, Limbu SM, Shen M, Zhai W, Qiao F, He A, et al. Environmental concentrations of antibiotics impair zebrafish gut health. *Environ Pollut.* 2018;235:245-254.
8. Bertotto LB, Richards J, Gan J, Volz DC, Schlenk D. Effects of bifenthrin exposure on the estrogenic and dopaminergic pathways in zebrafish embryos and juveniles. *Environ Toxicol Chem.* 2017;37(1):236-46.

9. Saillenfait AM, Ndiaye D, Sabaté JP. Pyrethroids: Exposure and health effects – An update. *Int J Hyg Environ Health*. 2015;218(3):281-92.
10. Jiang W, Conkle JL, Luo Y, Li J, Xu K, Gan J. Occurrence, Distribution, and Accumulation of Pesticides in Exterior Residential Areas. *Environ Sci Technol*. 2016;50(23):12592-601.
11. Mimbs WH, Cusaac J, Smith LM, Mcmurry ST, Belden JB. Occurrence of current-use fungicides and bifenthrin in Rainwater Basin wetlands. *Chemosphere*. 2016;159(159):275-281.
12. Tang W, Wang D, Wang J, Wu Z, Li L, Huang M, et al. Pyrethroid pesticide residues in the global environment: An overview. *Chemosphere*. 2018;191:990-1007.
13. Corcellas C, Eljarrat E, Barceló D. First report of pyrethroid bioaccumulation in wild river fish: A case study in Iberian river basins (Spain). *Environ Int*. 2015;75:110-116.
14. Xiang D, Zhong L, Shen S, Song Z, Zhu G, Wang M, et al. Chronic exposure to environmental levels of cis-bifenthrin: Enantioselectivity and reproductive effects on zebrafish (*Danio rerio*). *Environ Pollut*. 2019;251:175-84.
15. Li M, Wu Q, Wang Q, Xiang D, Zhu G. Effect of titanium dioxide nanoparticles on the bioavailability and neurotoxicity of cypermethrin in zebrafish larvae. *Aquat Toxicol*. 2018;199:212-219.
16. Hu Y, Zhang Y, Vinturache A, Wang Y, Shi R, Chen L, et al. Effects of environmental pyrethroids exposure on semen quality in reproductive-age men in Shanghai, China. *Chemosphere*. 2020;245:125580.
17. Magnuson JT, Cryder Z, Andrzejczyk NE, Harraka G, Schlenk D. Metabolomic profiles in brains of juvenile steelhead (*Oncorhynchus mykiss*) following bifenthrin treatment. *Environ Sci Technol*. 2020;54(19):12245-53.
18. Nasuti C, Coman MM, Olek RA, Fiorini D, Verdenelli MC, Cecchini C, et al. Changes on fecal microbiota in rats exposed to permethrin during postnatal development. *Environ Sci Pollut Res*. 2016;23(11):10930-7.
19. Dabke K, Hendrick G, Devkota S. The gut microbiome and metabolic syndrome. *J Clin Invest*. 2019;129(10):4050-4057.
20. Li M, Zhu J, Fang H, Wang M, Wang Q, Zhou B. Coexposure to environmental concentrations of cis-bifenthrin and graphene oxide: Adverse effects on the nervous system during metamorphic development of *Xenopus laevis*. *J Hazard Mater*. 2020;381:120995.
21. Evariste L, Lagier L, Gonzalez P, Mottier A, Mouchet F, Cadarsi S, et al. Thermal Reduction of Graphene Oxide Mitigates Its In Vivo Genotoxicity Toward *Xenopus laevis* Nanomaterials-Basel. 2019;9(4):584.
22. Perez-Alvarez I, Islas-Flores H, Manuel Gomez-Oliván L, Barcelo D, Miren LDA, Perez Solsona S, et al. Determination of metals and pharmaceutical compounds released in hospital wastewater from Toluca, Mexico, and evaluation of their toxic impact. *Environ Pollut*. 2018;240:330-41.
23. American Society for Testing and Materials (A.S.T.M.) Standard guide for conducting the frog embryo teratogenesis assay—*Xenopus* (FETAX), E1439-91. In: Annual book of ASTM standards, vol 11.05. ASTM, Philadelphia;1998.p. 826–836.

24. Yang Y, Ye X, He B, Liu J. Cadmium potentiates toxicity of cypermethrin in zebrafish. *Environ Toxicol Chem.* 2016;35: 435-445.
25. Ursell LK, Haiser HJ, Treuren WV, Garg N, Reddivari L, Vanamala J, et al. The Intestinal Metabolome: An Intersection Between Microbiota and Host. *Gastroenterology.* 2014;146(6):1470-1476.
26. Alexis M, Marion L, Hugot JP. Gut Microbiota Diversity and Human Diseases: Should We Reintroduce Key Predators in Our Ecosystem? *Front Microbiol.* 2016;7:455.
27. Tang YW, Sussman M, Liu D, Poxton I, Merritt A. *Molecular Medical Microbiology* 2nd edition. Academic Press, Salt Lake City; 2015.
28. Fernández-Bravo A, Figueras MJ. An Update on the Genus *Aeromonas*: Taxonomy, Epidemiology, and Pathogenicity. *Microorganisms.* 2020;8(1):129.
29. Kaakoush NO. Insights into the Role of Erysipelotrichaceae in the Human Host. *Front Cell Infect Microbiol.* 2015;5:84.
30. Martínez I, Perdicaro DJ, Brown AW, Hammons S, Carden TJ, Carr TP, et al. Diet-Induced Alterations of Host Cholesterol Metabolism Are Likely To Affect the Gut Microbiota Composition in Hamsters. *Appl Environ Microb.* 2012;79(2):516-24.
31. Biddle A, Stewart L, Blanchard J, Leschine S. Untangling the Genetic Basis of Fibrolytic Specialization by Lachnospiraceae and Ruminococcaceae in Diverse Gut Communities. *Diversity.* 2013;5(3):627-40.
32. Suchodolski JS. Diagnosis and interpretation of intestinal dysbiosis in dogs and cats. *The Veterinary Journal.* 2016;215:30-37.
33. Sinha SR, Haileselassie Y, Nguyen LP, Tropini C, Wang M, Becker LS, et al. Dysbiosis-Induced Secondary Bile Acid Deficiency Promotes Intestinal Inflammation. *Cell Host Microbe.* 2020;27(4):659-760.
34. Just S, Mondot S, Ecker J, Wegner K, Rath E, Gau L, et al. The gut microbiota drives the impact of bile acids and fat source in diet on mouse metabolism. *Microbiome.* 2018;6:134.
35. Wang Y, Gao X, Zhang X, Xiao Y, Huang J, Yu D, et al. Gut Microbiota Dysbiosis Is Associated with Altered Bile Acid Metabolism in Infantile Cholestasis. *Msystems.* 2019;4(6).
36. Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS. Bile acids and the gut microbiome. *Curr Opin Gastroen.* 2014;30(3):332-338.
37. Chiang J. Bile acid metabolism and signaling. *Compr Physiol.* 2013;3(3):1191-212.
38. Engelking LR. Bile Acids. In: *Textbook of Veterinary Physiological Chemistry (Third Edition)*. Engelking LR. Salt Lake City: Academic Press; 2015.p. 397-405.
39. Chu H, Duan Y, Yang L, Schnabl B. Small metabolites, possible big changes: a microbiota-centered view of non-alcoholic fatty liver disease. *Gut.* 2019;68(2):359-370.
40. Fanale D, Amodeo V, Caruso S. The Interplay between Metabolism, PPAR Signaling Pathway, and Cancer. *Ppar Res.* 2017;2017:1-2.

41. Torra IP, Claudel T, Duval C, Kosykh V, Fruchart JC, Staels B. Bile Acids Induce the Expression of the Human Peroxisome Proliferator-Activated Receptor Gene via Activation of the Farnesoid X Receptor. *Mol Endocrinol.* 2003;17(2):259-72.
42. Chiang JYL. Bile acid metabolism and signaling in liver disease and therapy. *Liver Research.* 2017;1(1):3-9.
43. Seebacher F, Zeigerer A, Kory N, Krahmer N. Hepatic lipid droplet homeostasis and fatty liver disease. *Semin Cell Dev Biol.* 2020;108:72-81.

Tables

Table 1. The effects of *cis*-BF on gut microbial structure.

Group	R-value	P-value
Control-BF.0.06	0.18	0.018
Control-BF.0.3	0.25	0.013
BF.0.06-BF.0.3	0.07	0.17

Table 2. Alpha diversity in *Xenopus laevis* intestines after exposure to *cis*-BF for 3 months.

Group	Control	0.06 µg/L	0.3 µg/L
Observed Species	175.75 ± 62.14	187.75 ± 66.38	140.50 ± 49.67
Shannon	3.18 ± 1.12	2.68 ± 0.95	2.03 ± 0.72*
Simpson	0.77 ± 0.27	0.61 ± 0.22	0.57 ± 0.20*
Chao1	211.56 ± 74.80	223.00 ± 78.84	173.22 ± 61.24
ACE	216.64 ± 76.59	228.96 ± 80.95	178.73 ± 63.19
Goods coverage	0.999 ± 0.35	0.999 ± 0.35	0.999 ± 0.35
PD	17.63 ± 6.23	18.89 ± 6.68	15.98 ± 5.65

Figures

Figure 1

Effects of cis-BF exposure on the intestine and liver of *X. laevis*. a, Cis-BF concentrations in the intestine of *X. laevis* (n = 3). b, Hepatosomatic indices of *X. laevis* in the control and exposure groups (n = 9). Oil red O staining of livers of *X. laevis* treated with DMSO (c), 0.06 µg/L cis-BF (d) and 0.3 µg/L cis-BF (e). Magnification, ×200. f, Relative Oil Red O area in the livers of *X. laevis* in the control and exposure groups

(n = 3). Data are presented as mean $\pm$ SEM, *p < 0.05 and **p < 0.01 indicate significant differences compared with the control group.

Figure 2

Cis-BF exposure changed the gut microbiota community structure of *X. laevis*. Simpson (a) and Shannon (b) diversity of gut microbiota in *X. laevis* (n = 8). c, Network-based analysis of top 50 OTUs of gut microbiota in *X. laevis*. d, Bacterial community compositions at phylum level (top 10) of gut microbiota in

X. laevis (n = 8). Data are presented as mean ± SEM, *p < 0.05 indicates a significant difference compared with the control group.

Figure 3

Effects of cis-BF exposure on the gut microbiota composition at the genus level. a, The significant changed genera of gut microbiota in X. laevis after exposure to cis-BF (n = 8). The relative abundances of the pathogenic bacteria Plesiomonas (b) and Aeromonas (c) of gut microbiota in X. laevis (n = 8). d, The most differentially abundant taxa between control and cis-BF exposure groups. Data are presented as

mean ± SEM, **p < 0.01 indicates a significant difference compared with the control group. NS means there is no significant difference compared with the control group.

Figure 4

Cis-BF-induced the changes in metabolic profiles of the intestinal contents. a, Changes in the levels of metabolites (Fold change > 1.5 and p < 0.05) in the intestinal contents of *X. laevis* in 0.3 µg/L cis-BF exposure group (n = 6). b, The bile acids concentrations of the intestinal contents in *X. laevis* (n = 6). Data

are presented as mean ± SEM, *p < 0.05 and **p < 0.01 indicate significant differences compared with the control group. c, Enrichment ratio of pathways by KEGG pathway analysis in 0.3 µg/L cis-BF exposure group. d, Correlations between bile acids concentrations and the relative abundance of top 20 genera. Significantly negative or positive correlation (R > 0.5 or < -0.5; p < 0.05) is indicated by stars.

Figure 5

Hepatic gene expression profile in *X. laevis* after exposure to 0.3 µg/L cis-BF. a, Significant enriched pathways obtained by KEGG pathway analysis. b, Network-based analysis of the differentially expressed genes involved in the fatty acid metabolism pathway, PPAR signaling pathway and fatty acid degradation pathway. c, Heatmap of differentially expressed genes of PPAR signaling pathway. d, Heatmap of differentially expressed genes of fatty acid metabolism pathway. e, Significant enriched pathways for both differentially expressed genes and differentially changed metabolites. Data are presented as mean ± SEM, NS means there is no significant difference compared with the control group.

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